UNIVERSIDAD NACIONAL AUTONOMA DE MEXICO

UNIDAD ACADEMICA DE LOS CICLOS PROFESIONAL Y DE POSGRADO
COLEGIO DE CIENCIAS Y HUMANIDADES

INSTITUTO DE FISIOLOGIA CELULAR

MUERTE CELULAR POR EXCITOTOXICIDAD IN VIVO: POSIBLES MECANISMOS Y ESTRATEGIAS DE PROTECCION

TESIS PARA OBTENER EL GRADO DE DOCTOR EN
INVESTIGACION BIOMEDICA BASICA

PRESENTA

Ma. de Lourdes Massieu Trigo

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RESUMEN

La exposición de las neuronas a altas concentraciones de glutamato o aminoácidos relacionados con éste da lugar a su degeneración y muerte. El glutamato puede producir daño celular a través de la activación de sus dos tipos de receptores ionotrópicos (acoplados a un canal iónico): los NMDA, y los no-NMDA (que incluyen a los receptores AMPA y kainato) tanto *in vivo* como *in vitro*. A Este tipo de daño celular se le ha denominado como excitotóxico ya que está ligado a la sobreactivación de los receptores glutamatérgicos, y por ende a la sobrexcitación de las neuronas. Debido al descubrimiento de que los antagonistas de los receptores NMDA son muy eficientes como neuroprotectores en modelos de isquemia, la mayoría de las investigaciones se han avocado al estudio de la participación de los receptores NMDA en procesos de neurodegeneración. Muchos de estos estudios se han enfocado sobre bloqueadores no-competitivos de este receptor como el MK-801 dada su actividad sistémica. Además, consideraciones teóricas argumentan que este tipo de bloqueadores son más eficientes que los competitivos ya que su actividad no depende de la concentración de glutamato extracelular (que sería la responsable del daño).

En el primer trabajo que se presenta en la tesis se hizo un estudio comparativo de las propiedades neuroprotectoras de dos bloqueadores de los receptores NMDA, uno de tipo no-competitivo, el MK-801 y uno competitivo, el CGP 37849. Los compuestos se probaron en un modelo de excitotoxicidad *in vivo* que consiste en la administración del ácido quinolínico, un agonista endógeno de los receptores NMDA, en el estriado de la rata. El daño se evaluó 7 días después de la cirugía siguiendo la degeneración de las neuronas colinérgicas y GABAérgicas del estriado al determinar la actividad de las enzimas acetil colintransferasa (ChAT) y glutamato descarboxilasa (GAD), marcadores de estos dos tipos celulares, respectivamente. Los antagonistas fueron administrados tanto intraperitonealmente como coinyectados en el estriado junto con el ácido quinolínico, ya sea inmediatamente ó 1-6 h después de la administración del agonista. Los resultados muestran que es posible prevenir la muerte de las neuronas colinérgicas aún administrando sistémicamante los

antagonistas 6 h después del ácido quinolínico y las GABAérgicas si se administran de 1-2 h después. Ambos antagonistas fueron igualmente eficientes en este paradigma experimental. Cuando los antagonistas se administraron directamente en el estriado, los dos tipos celulares se protegieron del daño aún inyectando los bloqueadores 1-2 h después del ácido quinolínico, siendo el CGP 37849 más eficiente, que el MK-801. Los resultados indican que los antagonistas competitivos y no competitivos del receptor NMDA fueron igualmente eficientes neuroprotectores en este modelo de excitotoxicidad, y que el CGP 37849 pudiera ser incluso más eficiente ya que su vida media en el cerebro es más prolongada que la del MK-801.

El papel de los receptores no-NMDA en el desarrolllo de procesos neurodegenerativos no se ha estudiado tan extensamente como el de los receptores NMDA, debido a la falta de antagonistas selectivos. En el segundo trabajo que se presenta en la tesis se estudió el efecto neuroprotector del NBQX (uno de los antagonistas más selectivos de los receptores no-NMDA sintetizado recientemente), sobre el daño excitotóxico producido por la inyección de algunos agonistas glutamatérgicos en el estriado de la rata *in vivo*. El daño neuronal se evaluó de la misma manera que en el trabajo anterior, pero también se hizo un análisis histológico cuantitativo de las células picnóticas en cortes teñidos con violeta de cresilo. El NBQX se coinyectó intraestriatalmente junto con cada uno de los agonistas glutamatérgicos estudiados (AMPA, kainato, NMDA y ácido quinolínico). Este antagonista protegió contra el daño neuronal inducido por la administración de AMPA, kainato y NMDA, pero no así contra el inducido por el ácido quinolínico. El NBQX fue igualmente eficiente neuroprotector contra el daño producido tanto por AMPA como por kainato. Los resultados apoyan la potencialidad terapéutica del NBQX contra el daño neuronal producido por la sobreactivación de los receptores glutamatérgicos.

La remoción del glutamato extracelular del espacio sináptico está a cargo de sus sistemas de recaptura de alta afinidad. Se ha argumentado que la falta de toxicidad del glutamato administrado intracerebralmente, se debe a su rápida remoción del espacio extracellular por su sistema de recaptura. Teniendo esto en

mente, en el tercer trabajo que se presenta en la tesis decidimos administrar dos inhibidores de la recaptura de glutamato, el dihidrokainato (DHK) y el L-*trans*-pirrolidin-2,4-dicarboxilato (PDC) en el estriado de la rata *in vivo*, y al mismo tiempo se midieron por HPLC los niveles extracelulares de glutamato colectados mediante una membrana de microdiálisis. También se hicieron inyecciones agudas de concentraciones altas de estos compuestos en el estriado. El daño celular se evaluó bioquímicamante, a través de la determinación de la ChAT y la GAD, e histológicamante en cortes teñidos con violeta de cresilo. Los resultados indican que el DHK (50 mM) y el PDC (25 mM) producen incrementos idénticos en las concentraciones extracelulares de glutamato y aspartato. Sin embargo, el DHK también produjo elevaciones en la concentración de otros aminoácidos. Se observó daño celular sólo después de la administración de DHK, el cual se previno parcialmente tanto por MK-801 como por NBQX. No se observó daño neuronal después de la administración de PDC, ni de otros dos inhibidores que fueron estudiados: el DL-treo-ß-hidroxiaspartato y el L-aspartato-ß-hidroxamato. Los resultados indican que la acumulación de glutamato a través de la inhibición de sus sistema de recaptura no es suficiente para producir muerte neuronal en el estriado de la rata *in vivo.* El efecto neurotóxico del DHK se discute en téminos de su posible interacción directa con los receptores glutamatérgicos postsinápticos.

LISTA DE ABREVIATURAS

***Trans*-ACPD**	*Trans*-DL-1-amino-1,3-ciclopentano-*trans*-dicarboxilato=*cis*-DL-1-amino-1,3-ciclopentano dicarboxilato.
AMPA	α- amino-3-hidroxi-5-metil-isoxasol-4-propionato
L-AP-4	L-2-amino-4-fosfonobutirato
AP-5	2- amino-5-fosfonovalerato
AP-7	2-amino-5-fosfonoheptanoato
ALS	Esclerosis amiotrófica lateral
BMAA	ß- N-metilamino-L-alanina
BOAA	ß-N-oxalilamino-L-alanina
BW1003C87	5-(2,3,5,-triclorofenil)-piridin-2,4-diamino 1.1 etanosulfonato.
CGP 37849	Acido 2-amino-4-metil-5-fosfono-3-pentóico
CGP 40116	D-enantiómero del CGP 37849
CGS 19755	*cis*-4-fosfonometil-2-piperidin-dicarboxilato-1-(cis-2-carboxi-piperidin-4-il)-propil-1-fosfonato.
ChAT	Acetil colintransferasa
CNQX	6-ciano-7-nitroquinoxalin-2,3-diona
CPPeno	3-(2-carboxipiperazin-4-il)-propenil-1-fosfonato
DHK	Acido dihidrokaínico
GAD	Descarboxilasa glutámica
GYKI 52466	1-(4-aminofenil)4-metil-7,8-metilendioxi-5H-2,3-benzodizepina
HPLC	Cromatogafía líquida de alta presión
NBQX	2,3-dihidroxi-6-nitro-7-sulfamoil-benzo(f)quinoxalina
NMDA	N-metil-D-aspartato
MCPG	α-metil-4-carboxifenilglicina
MK-801	Maleato de dizocilpina
MPTP	1-metil-4-fenil-1,2,3,6-tetrahidropiridina
PCP	Fenciclidina
PDC	L-trans-Pirrolidin-2,4-dicarboxilato
SIDA	Síndrome de inmunodeficiencia adquirida

PREFACIO

La presente tesis consta de tres partes. La primera parte incluye una revisión de la literatura más relevante acerca de lo que se conoce como daño neuronal mediado por excitotoxicidad, y de algunos ejemplos de neuropatologías en humanos en donde el daño cerebral aparentemente está ligado a este mecanismo de muerte neuronal. Los ejemplos que se mencionan pueden dividirse en dos clases: la primera incluye aquellos casos en los que se produce un daño cerebral agudo, como son la isquemia o el trauma cerebral, y aquellos que son consecuencia de una enfermedad neurodegenerativa progresiva como son la enfermedad de Alzheimer y la Corea de Huntington. La finalidad de esta primera fase de la tesis es la de situar el trabajo experimental dentro de un contexto general que lo liga a situaciones reales dentro de la clínica, y a su vez destaca su posible relevancia terapéutica.

La segunda parte de la tesis está compuesta del trabajo experimental en sí, que se presenta a través de tres trabajos independientes (dos de ellos publicados, y uno envíado para publicación) pero que forman parte de un mismo tema: la investigación acerca de los posibles mecanismos de la muerte neuronal por excitotoxicidad en la rata *in vivo* (el tercer trabajo aborda esta pregunta), y acerca de cuales podrían ser las posibles estrategias para prevenir esta neurodegeneración (en los dos primeros trabajos se proponen algunas). El primer trabajo se titula **"A comparative analysis of the neuroprotective properties of competitive and uncompetitive N-methyl-D-aspartate receptor antagonists *in vivo*: implications for the process of excitotoxic degeneration and its therapy"**, y fue realizado en el laboratorio del Dr. Graham Fagg en Ciba-Geigy, Basilea, Suiza

durante una estancia autorizada como parte del doctorado; el segundo trabajo que lleva como título **"2,3-Dihydroxy-6-nitro-7-sulfamoyl-Benzo(f)quinoxaline protects against both AMPA and kainate-induced lesions in the *rat* striatum"**, y el tercer trabajo titulado **"Accumulation of extracellular glutamate by inhibition of its uptake is not sufficient for inducing neuronal damage: an *in vivo* microdialysis study"**, se llevaron a cabo en el laboratorio del Dr. Ricardo Tapia en el Instituto de Fisiología Celular de la UNAM, México.

La tercera parte de la tesis está compuesta de una discusión general de los tres trabajos, en donde se destacan las contribuciones de éstos al conocimiento del proceso excitotóxico y a la posible intervención terapéutica para prevenirlo.

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ABSTRACT

Exposure of neuronal cells to high concentrations of glutamate or related amino acids leads to their degeneration and death. Glutamate can produce neuronal damage through the activation of its two types of ionotropic (coupled to ionic channels) receptors: the NMDA and the non-NMDA (which include AMPA and kainate receptor subtypes) both *in vivo* and *in vitro.* The term excitotoxicity has been proposed for this type of neuronal damage since it is related to the overactivation of glutamate receptors and consequent overexcitation of neurons. Due to the discovery that NMDA antagonists are efficient neuroprotectans in ischemia models, most of the studies have focused on the role of NMDA receptors in neurodegenerative processes. Many studies have used non-competitive NMDA antagonists, such as, MK-801 due to its systemic activity. Besides theoretical considerations argue against competitive antagonists relative to non-competitive since the action of the later does not depend on the extracellular glutamate concentration (which is implicated in neuronal damage). In the first work that conforms the thesis we have done a comparative study of the neuroprotective properties of two blockers of the NMDA receptors, a non-competitive, Mk-801, and a competitive, CGP 37849. Both compounds were tested in a model of excitotoxicity *in vivo* through the administration of quinolinic acid, an endogenous agonist of the NMDA receptors, in the rat striatum. Neuronal damage was evaluated through the degeneration of GABAergic and cholinergic cells7 days after the surgery as determined by the activity of the enzymes cholin actetyltransferase (ChAT) and glutamate decarboxylase (GAD), markers of these two types of cells, respectively. Antagonists were administrated intraperitoneally as well as coinjected with quinolinic acid in the striatum, either inmediately or 1-6 h after agonist administration. Our results show that cholinergic cells can be rescued from death by the administration of the antagonists even 6 h after quinolinic acid injection, while GABAergic cells only if NMDA antagonists are administered 1-2 h after the agonist. Both antagonists were equally efficient in our experimental paradigm. When NMDA antagonists were administered directly in the striatum, cholinergic and GABAergic cells were protected even when the antagonists were injected 1-2 h post-quinolinic acid, resultiing CGP

37849 more efficient than MK-801. Our results show that both competitive and non-competitive antagonists of the NMDA receptors were equally efficient neuroprotectants in this model of excitotoxicity, and that CGP 37849 could be even more efficient due to its longer half life in brain as compared to MK-801.

The role of non-NMDA receptors in the development of neurodegenerative disorders has not been as extensively studied as NMDA receptors due to the lack of selective antagonists. In the second study of the present thesis, we have studied the neuroprotective properties of NBQX, one of the most selective antagonists of non-NMDA receptors recently sinthetized, on the excitotoxic damage induced by the administration of some glutamatergic agonists in the rat striatum *in vivo*. Neuronal damage was evaluated as described in the previous study but a quantitative histological analysis of pycnotic cells was also performed in cresyl violet stained sections. NBQX was intrastriatally coinjected with each of the glutamate agonists studied (AMPA, kainate, NMDA and quinolinic acid). NBQX protected neuronal damage induced by AMPA, kainate and NMDA but not by quinolinic acid. This antagonist was equally efficient neuroprotectant against neuronal damage induced both by AMPA and kainate. The results favor the possible therapeutic potentiality of NBQX against cell damage induced by overactivation of glutamate receptors.

The clearence of extracellular glutamate from synaptic cleft depends on the activity of its high affinity glutamate uptake system. It has been suggested that the lack of toxicity of glutamic acid *in vivo* is due to its rapid clearence from the synaptic cleft through its uptake system. Having this in mind, in the third study that conforms the thesis, we tested the effcet of the administration of two glutamate uptake inhibitors, dihydrokainate (DHK) and L-trans-pyrrolidin-2,4-dicarboxilate (PDC), on the extracellular accumulation of glutamate and cell injury. Extracellular levels of glutamate were measured from samples colected through a microdialysis membrane and by means of HPLC. High concentrations of glutamate uptake inhibitors were also acutely injected in the rat striatum and cell damage was assessed by biochemical determination of ChAT and GAD activities and by histological examination of cresyl violet stained sections. According to the results DHK (50 mM) and PDC (25 mM)

Agradecimientos

Agradezco especialmente al Dr. Graham E. Fagg por haberme introducido en el área de investigación acerca de la degeneración neuronal mediada por excitotoxicidad y por todo el apoyo que me brindó para trabajar en su laboratorio durante mi estancia en Basilea, Suiza, y al Dr. Ricardo Tapia por todo su apoyo para realizar el trabajo de tesis en su laboratorio en el Instituto de Fisiología Celular de la UNAM. Agradezco también al Dr. Francisco Fernández de Miguel por el interés que siempre a manifestado acerca de mi trabajo, así como el apoyo que siempre me ha brindado para llevarlo a cabo. Agradezco también a todos mis compañeros y amigos del laboratorio de quienes siempre recibí ayuda a lo largo de la realización de las investigaciones que conforman la tesis, y a mi madre y hermana cuyo valiosao apoyo siempre tengo presente.

INTRODUCCION

El ácido glutámico es un aminoácido que se ha reconocido como uno de los neurotransmisores excitadores más abundantes en el sistema nervioso central de los mamíferos (Fagg y Foster, 1983; Fonnum, 1984). Durante la última década un gran cúmulo de evidencias señalan que además de su función como neurotransmisor el glutamato desempeña un papel importante en los procesos de neurodegeneración asociados a desórdenes neurológicos, tanto de tipo agudo como progresivo. Dentro del primer caso, se ha encontrado una asociación entre los aminoácidos excitadores, glutamato y aspartato, y el daño celular que se observa después del trauma cerebral, la hipoglicemia y la isquemia cerebral. Entre los desórdenes neurológicos progresivos en los que se ha sugerido la participación de estos aminoácidos, se encuentran la enfermedad conocida como Corea de Huntington, la enfermedad de Alzheimer, la esclerosis amiotrófica lateral, la enfermedad de Parkinson y la más recientemente clasificada como demencia asociada al síndrome de inmunodeficiencia adquirida (SIDA). También se ha propuesto que el glutamato participa en el proceso de neurodegeneración asociado a la epilepsia.

Las primeras observaciones que sugirieron que el glutamato podía desempeñar un papel como neurotransmisor y a su vez producir muerte celular, datan de estudios de hace más de 30 años. Curtis et al. en 1959 descubrieron que el glutamato y sus análogos al ser superfundidos en células de la médula espinal del gato, producían un aumento en la tasa de disparo de éstas; en 1954, Hayashi descubrió las propiedades convulsivantes de la inyección intracerebral de glutamato en el perro (ver Watkins, 1978); y Lucas y Newhouse en 1957 observaron por primera

vez que la inyección intraperitoneal de glutamato en ratas inmaduras produce degeneración de las células de la retina. Más de una década después Olney y colaboradores observaron que la inyección de glutamato y sus análogos en ratones inmaduros producía neurodegeneración en algunas zonas del sistema nervioso central, como el hipotálamo, y en otras regiones cuya barrera hematoencefálica no estaba totalmente desarrollada (Olney, 1969, 1971; Olney et al. 1971). En estudios subsecuentes, el mismo autor demostró la correlación entre las propiedades neurotóxicas del glutamato y sus análogos y su capacidad para despolarizar a las células nerviosas. De esta manera surge el término excitotoxicidad para definir el daño celular producido por la activación prolongada de los receptores glutamatérgicos (ver Olney, 1978). En la actualidad el concepto de excitotoxicidad ha cambiado pues se vislumbra como un fenómeno global, que si bien se inicia por la sobreactivación de los receptores glutamatérgicos, no sólo se debe a la sobreexcitación y por tanto a la falla energética de la células, sino a la activación de una serie de cadenas metabólicas que incluyen la activación de múltiples enzimas, que a su vez conlleva a la destrucción de las células nerviosas.

A lo largo de esta revisión se discutirán los trabajos más relevantes y recientes acerca de los distintos tipos de receptores a glutamato, los posibles mecanismos que dan lugar al proceso excitotóxico, así como la posible participación de los aminoácidos excitadores en el desarrollo de los procesos neurodegenerativos, tanto agudos como progresivos.

1. AMINOACIDOS EXCITADORES Y SUS RECEPTORES

Los aminoácidos excitadores glutamato y aspartato interactúan por lo menos con cuatro tipos distintos de receptores denominados de acuerdo a sus agonistas. Estos son el receptor al N-metil-D-aspartato (NMDA), el receptor al kainato, el receptor al α-amino-3-hidroxi-5-metilisoxasolpropionato (AMPA) y el receptor metabotrópico. Los tres primeros tipos de receptores están acoplados a canales iónicos y por tanto se denominan como receptores ionotrópicos, mientras que el receptor metabotrópico se denomina así por estar acoplado a una proteína G a través de la cual activa a la fosfolipasa C para producir los segundos mensajeros diacilglicerol e inositol trifosfato. Los receptores AMPA y kainato, por compartir entre sí algunas características y para diferenciarlos de los NMDA, también se denominan en su conjunto como receptores no-NMDA. Se ha descrito un quinto tipo de receptor a glutamato, denominado receptor L-AP-4 por ser sensible al L-2-amino-4-fosfonobutirato, un análogo del glutamato que inhibe sinapsis excitadoras en diversas regiones del sistema nervioso (Koerner y Cotman, 1981; Collins, 1982; Davis y Watkins, 1982; Lanthorn et al. 1984). Este receptor podría estar también localizado en la retina en donde el L-AP4 parece mimetizar la acción del transmisor natural en las células bipolares (Slaughter y Miller, 1981). En el hipocampo aparentemente tiene una localización presináptica y podría modular la liberación de glutamato (Cotman et al. 1986).

Receptores No-NMDA. Dentro de los receptores no-NMDA se encuentran los receptores AMPA y los receptores kainato. El conocimiento de las propiedades farmacológicas y electrofisiológicas de estos receptores se retrasó con respecto al de los receptores NMDA debido a la falta de antagonistas selectivos. No fue sino hasta

1988 que se sintetizaron las quinoxalindionas, como el CNQX y el NBQX, que fueron los primeros antagonistas con selectividad para estos receptores (Honoré et al. 1988; Sheardown et al. 1990). Los receptores AMPA presentan una cinética de apertura muy rápida, son permeables a Na^+ y K^+ pero no a calcio (pero ver más adelante), su apertura presenta poca dependencia de voltaje (Mayer y Weestbrook, 1987a; Ascher y Nowak, 1988) y se desensibilizan rápidamente (Tang et al. 1989). Los principales agonistas de estos receptores son el quiscualato y el AMPA. Su distribución coincide con la de los receptores NMDA, por lo que se ha sugerido que ambos subtipos de receptores actúan en concierto para activar a la neurona postsináptica. Además, dado que las respuestas sinápticas rápidas son bloqueadas por antagonistas no-NMDA (Mayer y Westbrook, 1987a; Collingridge y Lester, 1989), se ha sugerido que la transmisión sináptica rápida está mediada por los receptores no-NMDA y que su activación precede la de los NMDA (los cuales para activarse requieren de la despolarización de la membrana, ver siguiente inciso). Además de los agonistas y antagonistas de los receptores AMPA descritos hasta la fecha, se ha encontrado que otros agentes modulan su actividad. Por ejemplo, los barbituratos antagonizan (Simmonds y Horne, 1988) y el zinc potencia (Koh y Choi, 1988) las respuestas del receptor AMPA.

Al igual que los receptores AMPA, en la mayoría de las neuronas los receptores a kainato son permeables a Na^+ y K^+ pero no a calcio (Mayer y Westbrook 1987a, pero ver más adelante), y su permeabilidad no depende del voltaje (Mayer y Westbrook, 1987a,b; Ascher y Nowak, 1988). Sin embargo, las respuestas producidas

por el kainato son variadas y complejas.

La diversidad de las respuestas al glutamato quizás refleja la existencia de múltiples receptores con diferentes combinaciones de subunidades y distribución en el sistema nervioso. Durante los últimos tres años estudios de clonación han demostrado la existencia de por lo menos 9 subunidades capaces de formar receptores a glutamato activados por AMPA, kainato y quisqualato pero no por NMDA o L-AP4. La primera subunidad de receptores a glutamato de tipo no-NMDA fue clonada por primera vez por Hollman et al. en 1989. Esta subunidad se denominó GluR1 (o GluR-A), es una proteína de 889 aminoácidos y presenta cuatro posibles segmentos transmembranales. Esta proteína, al expresarse en ovocitos de *Xenopus* da lugar a un receptor que puede activarse tanto por AMPA como por kainato. Este estudio demostró que homómeros de esta proteína pueden dar lugar a un receptor funcional aunque su activación no produce respuestas idénticas a las observadas naturalmente en las neuronas. En estudios posteriores, los mismos autores y otros aislaron otras tres subunidades activadas por AMPA y kainato, que se designaron como GluR2-GluR4 (o GluR-B - GluR-D) (Nakanishi et al. 1990; Boulter et al. 1990; Keinänen et al. 1990; Sommer et al. 1990; Sakimura et al. 1990; Hollman et al. 1991). Estos estudios han demostrado que la combinación de heterómeros (pero no de homómeros) de estas subunidades produce receptores con características muy similares a las de los receptores observados naturalmente en las neuronas. Es interesante mencionar que la combinación de estas subunidades da lugar a receptores con diferente permeabilidad al calcio. El receptor homomérico compuesto por subunidades GluR2 y los heteroméricos que contienen esta subunidad, presentan

poca permeabilidad al calcio, mientras que los heterómeros que no la contienen muestran alta permeabilidad a este catión. Esto se debe a que la subunidad GluR2 contiene en la secuencia de su segundo segmento transmembranal (TM2) una arginina, mientras que las otras tres subunidades contienen un residuo de glutamina en la misma posición. Experimentos de mutagénesis dirigida indican que la presencia de arginina en este segmento determina la baja permeabilidad al calcio, mientras que la substitución de arginina por glutamina resulta en una alta permeabilidad al catión (Verdoorn et al. 1991; Köhler et al. 1993). Este hallazgo es muy importante ya que como se ha mencionado anteriormente se pensaba que los receptores AMPA/kainato eran permeables sólo a cationes monovalentes.

Se han clonado otras 5 subunidades que son activadas preferencialmente por kainato; tres de ellas se han denominado GluR5-GluR7 (Bettler et al. 1990; Bettler et al. 1992; Egebjerg et al. 1991; Sommer et al. 1992), y dos de ellas KA1-KA2 (Werner et al. 1991; Herb et al. 1992). Todas estas subunidades presentan la estructura básica de los receptores AMPA/kainato, incluyendo los 4 segmentos transmembranales. Es interesante mencionar que aproximadamente el 30% del mRNA de la subunidad GluR5 y el 75% de la GluR6 presentan una secuencia que codifica para arginina en vez de una glutamina, mientras que el mRNA de la subunidad GluR7 presenta predominantemente una secuencia para un residuo de glutamina en el segmento TM2 (para mayor información sobre el tema ver, Nakanishi, 1992; Sommer et al. 1992; Seeburg, 1993).

Receptores NMDA. Como se mencionó anteriormente, el receptor NMDA está acoplado a un canal iónico. Este es permeable a sodio y a calcio (MacDermott et

al. 1986; Mayer y Westbrook, 1987b; Ascher y Nowak, 1988) y su apertura depende de voltaje, ya que el ión Mg^{2+} que se encuentra normalmente bloqueando el poro del canal es liberado al depolarizarse la membrana (Nowak et al. 1984; Mayer y Westbrook, 1987b). Una característica interesante de las corrientes sinápticas mediadas por este receptor, es su lenta iniciación y su larga duración (el tiempo de decaimiento de la corriente es de varios cientos de milisegundos). Se ha sugerido que la larga duración de las corrientes es consecuencia más bien de la acción prolongada del glutamato sobre el receptor y no de la reactivación de éste por el glutamato residual en el medio extracelular (Lester et al. 1990).

Se han descrito hasta la fecha por lo menos seis sitios diferentes en este receptor: el sitio del ligando endógeno, es decir, el sitio al que se une el glutamato y también el D-aspartato; el sitio de unión a la glicina cuya ocupación facilita la acción del glutamato sobre el receptor (Johnson y Ascher, 1987); el sitio de unión a las poliaminas, cuya ocupación puede tener efectos potenciadores o inhibidores sobre la acción del glutamato dependiendo del tipo de poliamina que se una (Ranson y Stec, 1988; Williams et al. 1989); el sitio al que se une el Mg^{2+} que está en el propio canal iónico y al cual se une el MK-801, un potente antagonista no competitivo del receptor (Wong et al. 1986); y un sitio donde se une zinc (Weestbrook y Meyer, 1987; Peters et al. 1987). Recientemente se descubrió un sitio modulador de óxido reducción al que se une el glutatión (Sucher y Lipton, 1991).

Actualmente se han clonado 6 subunidades con las propiedades correspondientes al receptor NMDA. La primera subunidad, denominada NMDAR1,

fue clonada por primera vez por Moriyoshi et al. en 1991. Esta subunidad es un péptido de 938 aminoácidos con 4 segmentos transmembranales y un dominio extracelular largo. Este receptor presenta un alto grado de homología en su secuencia y perfil de hidrofobicidad con los receptores AMPA/kainato. Utilizando el sistema de expresión en ovocitos de *Xenopus* y técnicas electrofisiológicas, Moriyoshi et al. demostraron que el receptor clonado contiene las propiedades farmacológicas y electrofisiológicas del receptor natural, incluyendo la selectividad a agonistas y antagonistas, modulación por glicina, permeabilidad al calcio, bloqueo por magnesio, e inhibición por zinc. Se ha demostrado la existencia de 6 isoformas adicionales de la subunidad NMDAR1 generadas por cortes alternativos en el mismo gen. Estas se diferencían unas de otras en la secuencia de sus extremos amino y carboxilo terminal, y se denominan NMDAR1A (que corresponde a la subunidad NMDAR1 previamente clonada) y NMDAR1B-NMDAR1G (Sugihara et al. 1992).

Varios laboratorios al mismo tiempo reportaron la clonación de otras 4 subunidades del receptor NMDA, denominadas NMDAR2A-NMDAR2D provenientes de diferentes genes (Kutsuwada et al. 1992; Monyer et al. 1992). En contraste con la subunidad NMDAR1, la expresión de homómeros de cualquiera de las subunidades NMDAR2 en ovocitos de *Xenopus* no produce ninguna respuesta electrofisiológica. Sin embargo, la combinación de heterómeros de la subunidad NMDAR1 con cualquiera de las NMDAR2 potencia la respuesta al glutamato o al NMDA. Las características farmacológicas y electrofisiológicas del receptor expresado en ovocitos varían de acuerdo a la combinación de las subunidades. Estos resultados sugieren que en la naturaleza el receptor al NMDA posiblemente está formado por

heterómeros de diferentes subunidades, y que la subunidad NMDAR1 es fundamental para la formación de un receptor funcional. Es interesante mencionar que el mRNA de la subunidad NMDAR1 tiene una distribución muy amplia en el sistema nervioso central de la rata, mientras que la distribución del mRNA de las otras subunidades es más restringida (Moriyoshi et al. 1991; Monyer et al. 1992). De manera similar a lo que ocurre con los receptores AMPA/kainato, estudios de mutagénesis dirigida han mostrado que la subunidad NMDAR1 presenta un residuo de asparagina en el segmento TM2 en una posición equivalente al sitio glutamina/arginina de los receptores AMPA/kainato. La presencia de este residuo le confiere al receptor permeabilidad al calcio, inhibición por Mg^{++} y Zn^{++} y antagonismo por MK-801.

Receptores metabotrópicos. En 1985 se observó por primera vez que la exposición de las células nerviosas a glutamato o a sus análogos estimulaba la hidrólisis de los fosfolípidos de inositol (Sladeczek et al. 1985), y en 1987 se demostró que esta respuesta está mediada por la activación directa de receptores a amino-ácidos excitadores (Sugiyama et al. 1987). La hidrólisis de los fosfolípidos de inositol producida por la activación de estos receptores está mediada por una proteína G, y resulta en la formación de los segundos mensajeros diacilglicerol e inositol trifosfato, el cual moviliza calcio de pozas intracelulares.

Farmacológicamante este receptor se caracteriza por tener al glutamato, al quiscualato, al ibotenato y el *trans*-DL-1-amino-1,3-ciclopentano-*trans*-dicarboxilato =*cis*-DL-1-amino-1,3-ciclopentanodicarboxilato (*trans*-ACPD) como principales

agonistas, y al α–metil–4–carboxifenilglicina (MCPG) como antagonista. El NMDA, el AMPA y el kainato no activan a este receptor, y los antagonistas de estos receptores tienen efectos sobre la activación de los receptores metabotrópicos.

Estudios de clonación hasta la fecha revelan la existencia de 6 subunidades diferentes de receptores metabotrópicos, denominadas mGLUR1-mGLUR6. La primera de ellas fue clonada por dos grupos independientes (Masu et al. 1991; Houamed et al. 1991) al mismo tiempo. Estos estudios revelaron que es una proteína de 1199 aminoácidos que no presenta homología en su secuencia con ninguno de los receptores acoplados a proteínas G que se han clonado hasta el momento. Presenta 7 segmentos transmembranales y dos secuencias hidrofílicas muy largas en el amino y carboxilo terminal. Estudios posteriores revelaron la existencia de 5 subunidades más (Tanabe et al. 1992; Abe et. al. 1992) con estructuras moleculares muy parecidas a la del mGluR1. El papel funcional de estos receptores parece estar más bien relacionado con funciones reguladoras. Por ejemplo, estos receptores aparentemente participan el la depresión a largo plazo que ocurre en el cerebelo; en el hipocampo controlan la excitabilidad celular a través de la inhibición de una corriente de potasio; y también se ha observado que pueden suprimir la actividad sináptica a través de la inhibición de la liberación de glutamato (ver Miller, 1991; Nakanishi, 1992).

2. EXCITOTOXICIDAD

Características. Como se mencionó anteriormente, el término excitotoxicidad se refiere a la capacidad del glutamato y otros aminoácidos excitadores de destruir a las neuronas. El daño por excitotoxicidad presenta características citopatológicas bien definidas. Por ejemplo, después de 30 min de la inyección sistémica de glutamato en roedores jóvenes, las células del núcleo arcuato del hipotálamo muestran un evidente hinchamiento de las dendritas y los somas celulares, el cual es seguido de la degeneración de organelos intracelulares y picnosis nuclear. Después de varias horas las células se vuleven necróticas y son fagocitadas. El daño es predominantemente postsináptico, ya que las terminales nerviosas y los axones no se ven afectados (Olney, 1969; Olney, 1972). Este tipo de daño celular se observa también en animales adultos cuando se les administra glutamato o sus análogos directamente en el cerebro (McGeer y McGeer, 1976; Schwarcz et al. 1983) .

Los posibles mecanismos de excitotoxicidad han sido estudiados extensamente por varios grupos en neuronas cultivadas y en rebanadas de tejido nervioso. En las neuronas cultivadas de la corteza cerebral, Choi ha descrito dos fases principales del daño celular producido por la exposición a glutamato. Durante la primera, denominada fase aguda, hay un hinchamiento marcado del soma y las dendritas, el cual depende de la presencia de Na^+ y Cl^- externo (Rothman, 1985; Choi, 1987). Este se presenta después de algunos minutos de la exposición a glutamato, es reversible y puede ser producida por agentes despolarizantes tales

como la concentración alta de potasio y la veratridina. Este fenómeno se debe posiblemente a la entrada de sodio, seguida del flujo pasivo de cloro y agua. La mayoría de las células recuperan su volumen original después de algunas horas y en ciertas circunstancias sobreviven (en ausencia de calcio externo, ver abajo). La segunda fase se caracteriza por la desintegración tardía de las células nerviosas que ocurre algunas horas después de la exposición inicial a glutamato y depende de la presencia de calcio externo. Esta degeneración retardada o tardía puede ser mimetizada por agentes capaces de aumentar la entrada de calcio a las células, como el ionóforo A23187 (Choi, 1985, 1987). Otros grupos también han observado la dependencia del daño excitotóxico de la presencia de calcio externo en diferentes tipos de neuronas cultivadas o en rebanadas de tejido nervioso (Garthwaite y Garthwaite 1986; Rothman et al. 1987; Eliren y Lehman, 1989; Manev et al. 1989). Además, varios trabajos han demostrado que la muerte neuronal parcial que se observa con concentraciones bajas de glutamato se potencia al elevar la concentración de calcio externo (Choi, 1987; Hahn et al. 1988; Ogura et al. 1988). En células corticales cultivadas el mecanismo excitotóxico predominante es el retardado (dependiente de calcio), sobre todo si la concentración de glutamato es alta y la exposición es corta. Sin embargo, es posible que el componente agudo (dependeinte de sodio) *per se*, sea suficiente para producir daño excitotóxico (por lisis) si es lo suficientemente intenso (Rothman, 1985; Choi, 1987; Price, 1985).

Farmacología. La activación de los tres tipos de receptores ionotrópicos a glutamato (NMDA, AMPA y kainato) da lugar a la degeneración de las células nerviosas tanto *in vivo* como *in vitro*. Sin embargo, experimentos en neuronas

corticales cultivadas muestran que las condiciones en las que se produce daño celular por estimulación de los receptores NMDA no son las mismas que aquellas en las que se produce daño por activación de los receptores no-NMDA. Utilizando antagonistas específicos de los receptores NMDA (AP5), se ha observado que la muerte neuronal producida por un exposición corta a altas concentraciones de glutamato (5 min, 500 μM), está mediada primordialmente por la acción de los receptores NMDA (Choi et al. 1988; Michaels y Rothman, 1990). Por el contrario, los antagonistas de los receptores no-NMDA (CNQX) reducen sólo parcialmente la neurodegeneración (Koh y Choi, 1991). Además, la fase aguda o de hinchamiento se previene cuando se antagonizan tanto los receptores no-NMDA como los NMDA (Koh y Choi, 1991). A su vez, tanto la fase aguda como la retardada producidas por la activación directa del receptor a NMDA (usando NMDA), son bloqueadas por antagonistas de éste (Choi et al. 1988).

Una diferencia fundamental entre el daño producido por NMDA y el producido por AMPA o kainato en células corticales cultivadas, es el tiempo de exposición necesario para ejercer sus efectos. Mientras que una exposición corta (5 min) al NMDA produce muerte celular generalizada (Kim et al. 1987), el kainato produce hinchamiento pero no muerte, y el AMPA ni una ni otra (Koh et al. 1990a). Sin embargo, tanto el kainato como el AMPA son potentes excitotoxinas si se incuban a lo largo de 24 horas (Koh et al. 1990a). A diferencia de lo que ocurre en las células corticales cultivadas, estudios en sistemas más íntegros, como son rebanadas de hipocampo y de cerebelo muestran que tanto el AMPA como el kainato pueden producir neurodegeneración con incubaciones cortas (30 min); además, en

rebanadas de cerebelo se ha identificado también una fase aguda, que produce necrosis edematosa, y una retardada que produce necrosis obscura no edematosa, cuando el tejido es expuesto a altas concentraciones de AMPA (Garthwaite y Garthwaite, 1991a y 1991b).

Los estudios citados anteriormente sugieren fuertemente que por lo menos en las neuronas corticales cultivadas, la fase retardada del daño excitotóxico producido por el glutamato depende de la presencia de calcio externo y de la activación de los receptores NMDA. La dependencia de calcio externo en la excitotoxicidad inducida por la activación de los receptores no-NMDA se ha demostrado en diferentes tipos de células cultivadas, como las del hipocampo (Rothman et al. 1987); las del cerebelo (Garthwaite and Garthwaite, 1986), y las del estriado (Weiss y Choi, 1992). En las neuronas corticales ésta dependencia no parece ser tan clara.

Participación del calcio. La correlación cuantitativa entre la degeneración subsecuente a la exposición rápida de las células al glutamato, y la cantidad de calcio acumulada intracelularmente (Hartley et al. 1993; Randall y Thayer, 1992; Michaels y Rothman, 1990), ha permitido postular la participación de la entrada de calcio al interior celular en el proceso excitotóxico. La entrada de calcio a las células puede ocurrir a través de diversas vías: los receptores glutamatérgicos, los canales de calcio dependientes de voltaje, y el intercambiador Na^{+}/Ca^{2+}. Probablemente en las neuronas corticales cultivadas la entrada de calcio inducida por glutamato al igual que el daño celular, está mediada principalmente por los receptores NMDA, ya que puede bloquearse casi totalmente con antagonistas de este receptor (Hartley et al.

1993). En contraposición, la entrada de calcio subsequente a la estimulación de los receptores AMPA/kainato en esta preparación, puede ocurrir también a través de canales de calcio dependientes de voltaje, ya que antagonistas de estos canales atenúan el daño celular producido por AMPA y kainato pero no por NMDA (Weiss et al. 1990). Si bien estos resultados están de acuerdo con la baja permeabilidad al calcio de los receptores no-NMDA, recientemente Borson et al. 1994, demostraron en células cultivadas del cerebelo que la activación de los receptores no-NMDA promueve la entrada de calcio a través de estos receptores, y que éste influjo de calcio está correlacionado con el efecto tóxico del AMPA y el kainato. La discrepancia entre los resultados obtenidos en neuronas corticales y del hipocampo o del cerebelo probablemente se debe a las diferentes proporciones de receptores NMDA y no-NMDA presentes en estos tipos celulares, así como a la diversidad en su composición (combinación de subunidades). Por otra parte, además de la participación de los receptores glutamatérgicos en el daño excitotóxico, es posible que el intercambiador Na^{+}/Ca^{2+} responsable de la extrusión de calcio del interior celular pueda contribuir a este proceso, ya que inhibidores de éste incrementan la muerte celular inducida por glutamato (Andreeva et al. 1991).

De lo expuesto anteriormente se sugiere que hay una estrecha relación entre la acumulación de calcio intracelular y el daño excitotóxico. Esta acumulación no ocurre necesariamente por el influjo de calcio sino también a través de su liberación de pozas internas, mecanismo que puede ser mediado por el inositol trifosfato. En este sentido es interesante mencionar que el compuesto denominado dantroleno,

capaz de reducir la liberación de calcio de organelos celulares, atenúa la toxicidad del glutamato en cultivos de neuronas corticales y de retina (Frandsen y Schousboe, 1992; Lei et al. 1992). En concordancia con esto, es de esperarse que la estimulación de los receptores metabotrópicos glutamatérgicos dé lugar a la neurodegeneración, ya que al estar acoplados a la hidrólisis de los lípidos de inositol producen inositol trifosfato y subsecuentemente la acumulación de calcio interno. Sin embargo, el ACPD, que es el agonista más selectivo de los receptores metabotrópicos, no induce daño, sino que por el contrario reduce el daño celular mediado por NMDA (Koh et al. 1991b) y por condiciones de hipoxia (Opitz y Reymann, 1993) (aunque McDonald y Schoepp (1992) reportaron que este mismo compuesto potencia el daño cerebral producido por NMDA).

Otra de las características importantes de la muerte neuronal mediada por glutamato es que ésta no ocurre de manera inmediata sino que requiere de varias horas para desarrollarse. En esta particularidad se asemeja mucho a la muerte celular como consecuencia de la isquemia y de la hipoxia cerebral (ver más adelante). Experimentos *in vivo* han demostrado que las neuronas pueden rescatarse de la muerte celular, mediante la administración de antagonistas de los receptores NMDA, aún después de varias horas de la inyección inicial del agonista (Foster et al. 1988; Massieu et al. 1993, trabajo que se presenta en esta tesis). Los mecanismos de la muerte celular retardada *in vivo* no están claros. Una posibilidad es que después de la exposición de un grupo de neuronas a altas concentraciones de algún agonista glutamatérgico, la sobreactivación de los receptores NMDA y no-NMDA se extienda hacia poblaciones celulares más alejadas del grupo inicial, ya

sea por transmisión sináptica excitadora o por fuga de glutamato proveniente de las células dañadas. De esta manera la protección por los antagonistas observada después de la administración inicial del agonista reflejaría unicamente el bloqueo de la expansión de la excitación y del daño. Una segunda posibilidad es que la hiperactivación de los receptores glutamatérgicos sea limitada por mecanismos intrínsecos (amortiguamiento del calcio interno) o extrínsecos, como la inhibición sináptica. La muerte retardada también se ha observado *in vitro* (Rothman et al. 1987; Hartley y Choi, 1989; Manev et al. 1989) y se ha sugerido que está dada por la amplificación del proceso inicial, debido a la liberación de glutamato endógeno de las células inicialmente estimuladas (ver Choi, 1988; Choi et al. 1988).

Posibles mecanismos. Aunque parece claro que el incremento de la concentración interna de calcio es un requerimiento para la muerte celular por excitotoxicidad, los pasos siguientes no están bien dilucidados. Una posibilidad es que el incremento de los iones calcio en el interior celular dé lugar a la activación de proteasas, las cuales contribuirían al daño celular al degradar proteínas estructurales. De acuerdo con esta hipótesis se ha observado que la administración de leu-peptina, un inhibidor de la proteasa calpaina I, previene de la muerte celular asociada a la isquemia (Lee et al. 1991). Otro posible mecanismo de daño es a través de la producción de radicales libres. Al elevarse el calcio intracelular se activan fosfolipasas que dan lugar al rompimiento de la membrana celular y a liberación de ácido araquidónico, cuyo metabolismo favorece la producción de radicales libres (Chan et al. 1985). La liberación de ácido araquidónico por activación de la fosfolipasa A_2 inducida por NMDA se ha descrito en células de cerebelo (Lazarewicz

et al. 1988) y de estriado (Dumuis et al. 1988). La inhibición de la fosfolipasa A_2 o del subsecuente metabolismo del ácido araquidónico protege contra el efecto tóxico del glutamato (Dumuis et al. 1988; Lazarewicz et al.1988). Otra fuente de radicales libres es la producción de óxido nítrico por activación de la óxido nítrico sintasa inducida por NMDA (Dawson et al. 1991). El óxido nítrico puede reaccionar con el superóxido y dar lugar a peroxinitrito y a la formación de radicales hidroxilo altamente reactivos, los cuales favorecen la lipoperoxidación. Una observación que favorece esta hipótesis es que la inhibición farmacológica de la síntesis de óxido nítrico o la ausencia de su precursor, protegen contra el daño celular excitotóxico (Wallis et al. 1992). A su vez, inhibidores de la lipoperoxidación protegen contra el daño inducido por glutamato (Monyer et al. 1990). Se ha observado también que proteasas activadas por calcio, como la calpaina I, catalizan la conversión de la xantina deshidrogenasa a xantina oxidasa, la cual al intervenir en el catabolismo las bases púricas favorece la producción de radicales libres.

Otro mecanismo a través del cual el glutamato puede dar lugar a un estrés oxidativo es a través de la inhibición del transportador de cisteína, por el cual presenta afinidad. La cisteína forma parte del tripéptido glutatión el cual es un agente reductor intracelular que funciona como un mecanismo de rescate del estrés oxidativo. Este mecanismo de excitotoxicidad se ha reportado en la línea celular N18-RE-105, que es un hibridoma (retino-glioma) (Murphy et al. 1989) y la adición de antioxidantes como la idebenona y el α-tocoferol protegen contra la toxicidad del glutamato en estas células (Miyamoto et al. 1989). Experimentos *in vivo* también han

demostrado que la administración de antioxidantes protege contra la toxicidad inducida por kainato y quiscualato pero no por quinolinato en el estriado de la rata (Miyamoto y Coyle, 1990).

Por último, se ha sugerido que la activación de la proteína cinasa C inducida por el glutamato y sus análogos está involucrada en el daño excitotóxico, ya que la administración de gangliósidos tanto *in vivo* como *in vitro*, que inhiben la translocación de la proteína cinasa C del citoplasma a la membrana plasmática, protegen contra el daño inducido por excitotoxicidad (Manev et al. 1989; Skaper et al. 1989; Lombardi et al. 1989).

En la fig. 1 (pag 29) se esquematizan las posibles cascadas metabólicas que pueden iniciarse por la entrada de calcio y dar lugar a la muerte celular.

En conclusión, el proceso de excitotoxicidad originalmente utilizado para referirse a la capacidad del glutamato y sus análogos para destruir a la células nerviosas ahora se concibe como un proceso amplio, que si bien es iniciado por la estimulación prolongada de los receptores glutamatérgicos, incluye la expresión de diversas cascadas metabólicas posiblemente iniciadas por un aumento en la entrada de calcio, que dan lugar a la destrucción de las células nerviosas.

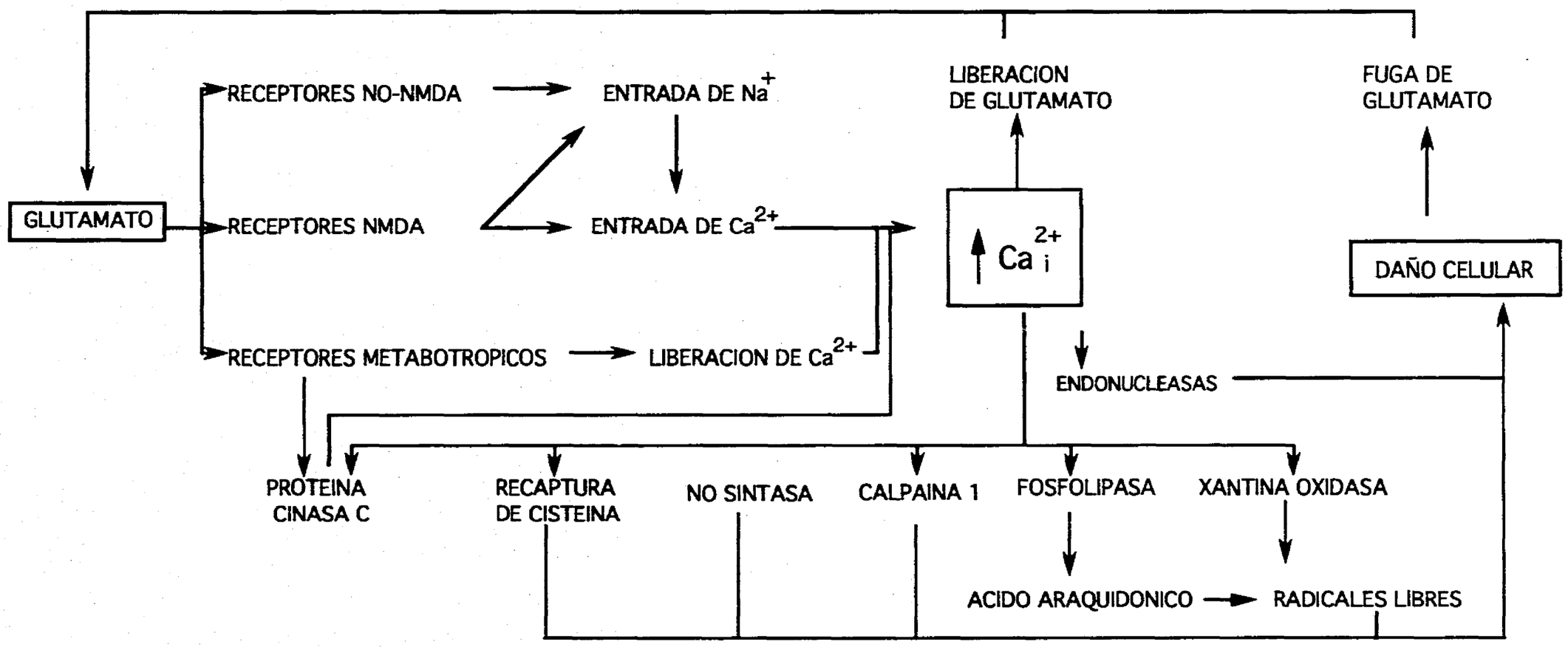

Fig 1 Posibles mecanismos involucrados en la muerte celular por excitotoxicidad
Tomado de Choi, D.W. (1988), *Neuron* 1, 623. Parcialmente modificado.

3. EXCITOTOXICIDAD Y DAÑO CEREBRAL AGUDO

Excitotoxicidad en la isquemia, el trauma cerebral y la hipoglicemia

La isquemia cerebral es la condición neuropatológica en la que hay datos más claros que indican una posible relación entre la activación de los receptores glutamatérgicos y la neurodegeneración subsecuente. Por isquemia cerebral se entiende la disminución de la circulación sanguínea cerebral, y puede ocurrir como consecuencia de una variedad de situaciones tales como: trauma cerebral (por un golpe), hipoxia perinatal, hemorragia subaracnoidea (consecuencia del rompimiento de un aneurisma de una arteria cerebral principal), hemorragia cerebral (consecuencia del daño vascular debido a hipertensión crónica), paro cardíaco, cirugía cardiovascular, o de un accidente vascular cerebral. Un accidente vascular cerebral produce una isquemia focal (ver más adelante) y resulta de la reducción del flujo sanguíneo cerebral a causa de la oclusión de una arteria (trombosis) o de una embolia (formación de un coágulo en alguna arteria principal). En algunos pacientes se produce la isquemia por una combinación entre el adelgazamiento de un vaso proximal y la reducción del flujo sanguíneo cerebral total, debido, por ejemplo, a un paro cardíaco. Independientemente de la causa, las consecuencias de la reducción sostenida de la circulación cerebral son las mismas: el daño neuronal irreversible, y la infartación cerebral que involucra todos los elementos celulares. El grado de reducción del flujo sanguíneo que es crítico para producir un episodio isquémico depende de la duración del accidente vascular. Se conoce que durante la isquemia sostenida, la circulación cerebral disminuye de 50 a 17 ml/min por 100 g de tejido

cerebral, es decir, el flujo cerebral se mantiene sólo en un 35% del normal (Ver McCullogh et al. 1991).

La isquemia cerebal puede clasificarse en dos tipos. (1) La isquemia cerebral focal y aguda, y (2) la isquemia cerebral difusa y retardada (Meldrum y Garthwaite, 1990). Estos tipos de isquemia dependen de la manera en que ocurren, y en animales experimentales pueden reproducirse con modelos de isquemia focal y global, respectivamente (Ginsber y Busto, 1989). La isquemia focal es un modelo de isquemia humana y se produce por la oclusión transitoria o permanente de la arteria cerebral media (ACM). El daño resultante de este tipo de isquemia se restringe a un área de infartación en aquellas regiones suplementadas por la arteria ocluída. La región del infarto comprende una zona central de tejido en donde el flujo sanguíneo es mínimo y las reservas energéticas se agotan, por lo cual las células situadas ahí están destinadas a morir. La región que rodea a esta zona central se denomina zona de "penumbra", y en ella la reducción del aporte sanguíneo (dependiendo de la cantidad de vascularización colateral) y la depleción energética son parciales (Nedergaard et al. 1986; Nagasawa y Kogure, 1989), y por lo tanto las células localizadas ahí son potencialmente rescatables (Ginsberg y Busto, 1989; MacCulloch et al. 1991). La isquemia global (es un modelo de paro cardíaco e hipotensión), ocurre por la oclusión temporal de las arterias que irrigan la totalidad cerebral, las carótidas y las vertebrales. El daño resultante de este tipo de isquemia es difuso, y afecta a neuronas que están en regiones más susceptibles al daño, como son la región CA1 del hipocampo, el estriado, las capas 3, 5 y 6 de la corteza cerebral, y el cerebelo. Además el daño no ocurre de manera inmediata sino que requiere de 1 a 2

días para desarrollarse (Kirino et al. 1982; Pulsinelli et al. 1982). En algunas situaciones, como en el caso del trauma cerebral, puede presentarse daño focal y retardado (ver McCulloch et al. 1991).

Los principales datos que señalan la participación de la activación de los receptores glutamatérgicos en el daño cerebral asociado a la isquemia son las siguientes:

(1) Las características citopatológicas del daño subsecuente a la isquemia cerebral y a la hipoglicemia son muy similares a las que se producen por la exposición directa de las neuronas a altas concentraciones de glutamato o sus análogos (Brown y Brierly, 1972; Van Reempts, 1984; Simon et al. 1984; Wieloch, 1985) .

(2) Estudios de microdiálisis *in vivo* indican que hay una gran acumulación de glutamato y aspartato extracelular en aquellas regiones que exhiben daño celular como consecuencia de períodos de isquemia focal y global (Benveniste et al. 1984; Globus et al. 1988; Hillered et al. 1989; Butcher et al. 1990; Graham et al.1990; Baker et al. 1991), de hipoglicemia (Sandberg et al. 1986) o de trauma cerebral (Fanden et al. 1989). El origen de la elevación de estos aminoácidos en el espacio extracelular se ignora, pero se ha propuesto que se deba a un aumento en su liberación presináptica inducida por despolarización, ya que se ha observado que como consecuencia de la isquemia hay un aumento en la concentración de potasio externo (Astrup et al. 1977; Attwell et al. 1993). Otra posibilidad es que estos aminoácidos se acumulen extracelularmente debido al mal funcionamiento de sus sistemas de recaptura, tanto en neuronas como en glia, que normalmente se encargan de

eliminarlos del espacio sináptico (ver Atwell et al. 1993). Además se ha demostrado que el glutamato, entre otros aminoácidos puede liberarse como consecuencia del hinchamiento celular (Sánchez-Olea y Pasantes-Morales, 1990), lo cual podría ocurrir durante la isquemia dado que se presenta edema. La magnitud en el incremento de las concentraciones extracelulares de glutamato subsecuente a la isquemia cerebral focal y global y al trauma cerebral, varían de 3.5 a 10 veces sobre los niveles basales, alcanzando concentraciones de 5 a 80 μM (Benveniste et al. 1984; Globus et al. 1988; Baker et al. 1991; Fanden et al. 1989; Hillered et al. 1989; McCulloch et al. 1991). Estas concentraciones de glutamato tienen efectos neurotóxicos *in vitro* (Choi, et al. 1987; Garthwaite y Garthwaite 1991a y 1991b).

(3) Se ha encontrado que tanto durante la isquemia focal como global hay un aumento en la concentración intracelular de calcio (Desphande et al. 1987;Dux et al. 1987; Siesjö y Bengston, 1989).

(4) La remoción de la inervación excitadora atenúa el daño producido por isquemia cerebral. Wieloch et al. (1985) mostró por primera vez que la destrucción de la vía perforante (inervación excitadora glutamatérgica de las células piramidales de la región CA1 del hipocampo) reduce el daño neuronal observado en CA1 subsecuente a la oclusión temporal bilateral de las carótidas. Benveniste et al. (1989), demostraron que la destrucción de las células piramidales de CA3 (cuyas fibras dan lugar a las colaterales de Schaffer que inervan a la región CA1) no sólo protege a las células piramidales de CA1 contra el daño isquémico, sino que evita la elevación de los niveles extracelulares de glutamato y aspartato (pero no de GABA) que se observa durante y después del período isquémico.

(5) Quizás la evidencia más fuerte que a colocado a los aminoácidos excitadores como protagonistas del daño neuronal producido por la isquemia, es la demostración de muchos laboratorios de que tanto antagonistas de los receptores NMDA como no-NMDA protegen contra la neurodegeneración inducida por la isquemia. El primer trabajo que utilizó esta estrategia de neuroprotección fue el de Jorgensen y Diemer (1982). A pesar de su falta de éxito (probablemente debida a la inestabilidad del antagonista utilizado, el dietilester del ácido glutámico, GDEE), este trabajo abrió la brecha para numerosos estudios subsecuentes con antagonistas más selectivos de los receptores NMDA, que han mostrado distintos grados de éxito.

En 1984 Simon et al. encontraron que la administración intrahipocampal de AP7, un antagonista competitivo del receptor al NMDA, protege contra el daño inducido en la región CA1 del hipocampo después de la oclusión bilateral de las carótidas. En general, efectos neuroprotectores claros y consistentes de los bloqueadores del receptor NMDA se han observado en experimentos realizados con modelos de isquemia focal. La administración, tanto de antagonistas competitivos de este receptor (AP7, CGS 19755, D-CPPeno y el CGP 4116) (Roman et al. 1989; Simon y Shiraishi, 1990; Bullock et al. 1990; Swan y Meldrum,m 1990; Sauer et al. 1993), como no competitivos (MK-801, PCP y dextrometorfan (Gill et al. 1988; Ozyurt et al. 1988; Park et al. 1988; Steinberg et al. 1989; Tamura et al. 1988; Bielenberg et al. 1989), resulta en una reducción del volumen del infarto (en aproximadamante un 60%) provocado en la corteza cerebral por la oclusión de la arteria cerebral media. Sin embargo, en estos estudios el efecto protector de los antagonistas sobre el daño neuronal producido en el estriado es muy pequeño. Esto se ha atribuído a que en

este modelo de isquemia el flujo sanguíneo cerebral se reduce principalmente en el estriado (representaría la zona central del infarto), mientras que en la corteza la isquemia es incompleta (representaría la zona de penumbra); de aquí que las células rescatables por los antagonistas del receptor NMDA sean las situadas en la corteza. En concordancia con esto, la neuroprotección por antagonistas del los receptores NMDA ha sido mucho menos exitosa en los modelos de isquemia global (Wieloch et al. 1989; Buchan y Pulsinelli, 1990). Además de las razones mencionadas anteriormente, se ha sugerido que diferencias metodológicas en los distintos laboratorios, como el control de la temperatura cerebral, la severidad de la oclusión (por ejemplo si es más proximal o más distal), y el metabolismo energético del tejido isquémico, pueden dar lugar a estas discrepancias (para discusión ver McCulloch et al. 1991). El concenso general en este respecto es que los antagonistas del receptor NMDA protegen contra el daño isquémico en aquellos modelos o en aquellas regiones donde el metabolismo energético está preservado parcialmente, pero que son ineficientes cuando hay una depleción total de energía (Wieloch et al. 1989; Siesjö y Bengtsson, 1989: McCulloch et al. 1991).

Además del efecto protector de los antagonistas de los receptores NMDA contra el daño producido por la isquemia, éstos también reducen el daño neuronal tanto *in vivo* como *in vitro* provocado por la hipoglicemia (Wieloch et al. 1985; Monyer et al. 1989), o por daño traumático (Fanden et al. 1989; Tecoma et al. 1989).

A diferencia de los antagonistas del receptor NMDA, el NBQX, que es un antagonista de los receptores no-NMDA, protege contra el daño producido tanto por la isquemia cerebral global (Sheardown et al. 1990; Diemer et al. 1992; LePelliet et al.

1992) como focal (Gill et al. 1992).

4. EXCITOTOXICIDAD POR EPILEPSIA Y DAÑO CEREBRAL CRONICO

La participación de los procesos excitotóxicos en la neurodegeneración que se presenta como consecuencia de estados epilépticos y de enfermedades crónicas degenerativas, no es tan clara como en el caso de la isquemia. La falta de concordancia entre la cinética de activación de los receptores glutamatérgicos (del orden de milisegundos) y la evolución gradual de un desorden degenerativo progresivo (del orden de años), ha sido el principal problema para ligar estos dos eventos. Por estas razones se revisarán sólo brevemente los padecimientos más importantes.

Excitotoxicidad en la epilepsia. Una enorme cantidad de literatura respalda la potencialidad antiepiléptica de los antagonistas de los receptores NMDA y no-NMDA en diversos modelos de epilepsia (para revisión ver Chapman, 1991) y no será revisada aquí. Sólo nos enfocaremos a los principales datos que relacionan la muerte celular subsecuente al "status epilepticus" (una manifestación de epilepsia que se caracteriza por una actividad epiléptica sostenida), con el proceso de excitotoxicidad. Un estatus epiléptico puede producirse en animales por la administración de ciertos compuestos químicos convulsivantes, como el ácido kaínico, el cual produce neurodegeneración de las células piramidales de la región CA3 y CA4 del hipocampo. En esto se asemeja a la degeneración que se observa en cerebros de pacientes que fallecieron con estatus epilépticus (Nadler et al. 1978; Ben-Ari, 1985; McGeer y McGeer, 1976: DeGiorgio, 1992). Las observaciones principales

que ligan este tipo de daño con un proceso excitotóxico son las siguientes: (1) Compuestos que producen estatus epiléptico, como el ácido kaínico, inducen un aumento en los niveles de glutamato extracelular (Young y Bradford, 1986; Young et al. 1988). (2) La inyección intracerebral de glutamato o ácido kaínico, o la estimulación sostenida de de las fibras de la vía perforante (aferencias excitadoras glutamatérgicas de la región CA3 del hipocampo), produce degeneración de las células piramidales de CA3 y CA4 (McGeer y McGeer, 1976; Sloviter, 1983). (3) Las células dañadas presentan características morfológicas similares a las que se presentan como consecuencia de la excitotoxicidad (Evans et al. 1984; Sloviter, 1983). (4) El calcio se acumula en las neuronas dañadas (Griffiths et al. 1984). (5) Antagonistas del receptor NMDA protegen contra la neurodegeneración producida por crisis límbicas prolongadas (Clifford et al. 1990). Sin embargo, la relación entre la generación de crisis epilépticas y la presencia de daño celular no parece ser tan clara. Por ejemplo, las crisis producidas por la inyección intrahipocámpica de ácido quinolínico (que es un agonista endógeno débil del receptor al NMDA) pueden contrarestarse con antiepiépticos, pero no así el daño celular producido por las mismas (Vezzani et al. 1986).

Excitotoxicidad en enfermedades crónicas neurodegenerativas.

Corea de Huntington. La posible relación entre la neurodegeneración que se observa en los pacientes con corea de Huntington y la excitotoxicidad proviene de la observación de que la inyección de excitotoxinas como el ácido kaínico y el ácido quinolínico en el cuerpo estriado de la rata, reproduce muchas de las características neuroquímicas e histológicas del daño observado en esta patología (McGeer y

McGeer, 1976; Coyle y Schwarcz, 1976; Schwarcz et al. 1983; Beal et al. 1986, pero ver también Davies y Roberts, 1987). Por ejemplo, estas lesiones producen un decremento en el número de células colinérgicas y GABAérgicas del cuerpo estriado (que también se dañan en la enfermedad de Huntington), pero no de las que presentan reacción positiva a la tinción de la NADPH-diaforasa y de la acetilcolinesterasa, las cuales también se preservan en la enfermedad de Huntington (para revisión ver DiFiglia, 1990). Además, Young et al. (1989) encontraron una pérdida selectiva de los receptores NMDA en cerebros de pacientes con esta enfermedad. Sin embargo, no se ha demostrado que exista un aumento considerable de la concentración de ácido quinolínico en pacientes con corea de Huntington (Reynolds et al. 1988; Heyes et al. 1991), aunque sí una disminución del ácido kinurénico, que es un antagonista endógeno de los receptores NMDA (Beal et al. 1990).

Enfermedad de Alzheimer. Existen algunas evidencias de que mecanismos excitotóxicos podrían estar involucrados en la neurodegeneración asociada a la enfermedad de Alzheimer, aunque éstas no son de ninguna manera conclusivas. (1) Algunas de las alteraciones del citoesqueleto que se observan en el tejido cerebral proveniente de pacientes con Alzheimer, pueden reproducirse experimentalmente en neuronas cultivadas del hipocampo de la rata (Mattson 1990) y de la corteza cerebral de humano (Mattson et al. 1991), al ser incubadas en presencia de aminoácidos excitadores, o en condiciones en las que se eleva el calcio intracelular. Por ejemplo en neuronas embrionarias cultivadas de la corteza cerebral de humano, se ha observado que al incubarse en presencia del ionóforo de

calcio A23187, hay una pérdida de los microtúbulos y una acumulación de filamantos de 8-15 nm tanto en el soma como en los axones. Aunque no se observan filamentos helicoidales pareados como los que son característicos de las marañas neurofibrilares de la enfermendad de Alzheimer, en ocasiones se observan filamentos torcidos, aunque de periodicidad y diámetro variable, que recuerdan a aquéllos. Por otra parte, las neuronas incubadas con A23187 presentan una mayor inmunoreactividad contra dos anticuerpos (Alz-50 y 5E2) que reconocen epítopes de la proteína Tau, que es una proteína del citoesqueleto cuya inmunoreactividad se encuentra incrementada en las marañas neurofibrilares presentes en los cerebros de pacientes con Alzheimer (Mattson et al. 1991). Resultados muy similares se encontraron en células de hipocampo cultivadas al incubarse en presencia de glutamato, de concentraciones altas de potasio y de A23187. En todas estas condiciones la concentración de calcio interna se incrementa, y si el calcio es eliminado del medio extracelular, no se producen los cambios antigénicos mencionados en ninguna de estas condiciones. Es interesante mencionar, que el aumento en la inmunoreactividad al Alz-50 y al 5E2 ocurre incluso cuando las células son expuestas a concentraciones subtóxicas de glutamto o KCl, lo cual indica que las alteraciones del citoesqueleto no están forzosamante ligadas a la neurodegeneración. (2) Las células corticales cultivadas, al ser expuestas por períodos prolongados (2 días) a la presencia de la proteína ß- amiloide, la cual se acumula en grandes cantidades en las placas seniles que son características de los cerebros de los pacientes con Alzheimer, potencia el efecto neurotóxico del glutamato, el NMDA y el kainato (Koh et al. 1990b). Este mismo resultado se encontró

en neuronas corticales de humano, y además se mostró que la presencia de la proteína ß-amiloide en el cultivo potencia la entrada de calcio inducida por glutamato, así como la entrada de calcio y la neurotoxicidad inducida por el ionóforo de calcio A23187. Más aún, en neuronas cultivadas la adición simultánea de ß-amiloide y glutamato resultó en cambios antigénicos de las proteínas del citoesqueleto similares a los que se observan en el tejido cerebral de los pacientes con Alzheimer (Mattson et al. 1992). Sin embargo, en ninguno de estos trabajos se encontró un efecto neurotóxico *per se* de la proteína ß-amiloide. Además los experimentos *in vivo* que han intentado demostrar un efecto neurotóxico de esta proteína han arrojado datos contradictorios, pues algunos han encontrado dicho efecto tóxico (Kowall et al. 1991) y otros no (Stein-Behrens et al. 1992).

Enfermedad de Parkinson. Existe una sola evidencia que sugiere que la neurodegeneración de la substancia nigra que tiene lugar como consecuencia de la enfermedad de Parkinson puede deberse a un proceso excitotóxico mediado por receptores glutamatérgicos. El primer estudio que sugirió esta posibilidad es el de Turski et al. (1991) quienes describieron que antagonistas del receptor NMDA, tanto competitivos (MK-801) como no competitivos (AP7) son capaces de proteger a las células dopaminérgicas de la substancia nigra contra la neurodegeneración inducida por la toxina denominada 1-metil-4-fenil-1,2,3,6-tetrahidropiridina (MPTP), que frecuentemente se usa en animales como un modelo experimental de la enfermedad de Parkinson. Aunque este resultado fue refutado más tarde por Sonsalla et al. (1992), quienes fracasaron en demostrar un efecto protector del MK-801 en el mismo modelo en roedores. Storey et al. (1992) encontraron que la inyección directa del

MPP+ (metabolito activo del MPTP) en el estríado de la rata, produce una lesión cuyas características concuerdan con una lesión excitotóxica, que además se previene con un tratamiento de MK-801 durante 24 hrs. Por otra parte, recientemente Klockgether et al. (1991) encontraron que el antagonista de los receptores no-NMDA, NBQX, protege a las células dopaminérgicas de la substancia nigra de la degeneración producida por la administración de MPTP.

Esclerosis amiotrófica lateral. Recientemente se encontró que el gene que codifica para la subunidad GluR5 se localiza en el cromosoma 21 humano en la vecindad del gene que determina la esclerosis amiotrófica lateral familiar, o enfermedad de la neurona motora, que se caracteriza por la degeneración de las neuronas motoras de la raíz ventral de la médula espinal (Eubanks et al. 1993). Se ha sugerido que la degeneración de estas neuronas está ligada a un proceso excitotóxico, ya que, además de poseer los receptores glutamatérgicos, se ha observado que éstas células carecen de proteínas reguladoras de la homeostasis de calcio, como la parvoalbúmina y la calbindina 28. Además, éstas células tienen reducido el transporte de glutamato (Rothstein et al. 1992), y degeneran al ser expuestas a inhibidores de la recaptura de glutamato (Rothstein, 1993).

Se ha sugerido que procesos excitotóxicos participen también en la generación de desórdenes neurológicos crónicos ligados al consumo de toxinas análogas de aminoácidos excitadores. El consumo de la ß-N-oxalilamino-L-alanina (BOAA), toxina que deriva de la leguminosa *Lathyrus sativus*, forma parte de la dieta de los habitantes de algunas regiones de Africa del este y del sur de Asia y se ha asociado al latirismo que se caracteriza por paraplejia espástica (Spencer et al.

1986). Esta toxina presenta propiedades excitadoras en neuronas de la médula espinal que al parecer estan ligadas a la activación de receptores AMPA/kainato (Bridges et al. 1989).

Por otra parte, la ß-N-metilamino-L-alanina (BMAA) , deriva de la semilla de la cicadácea *Cycas circinalis*, y forma parte de la dieta de los habitantes de la isla de Guam en el pacífico. El consumo de esta toxina se ha implicado en la generación de la demencia tipo Parkinson derivada de la esclerósis amiotrófica lateral (ALS), que frecuentemente se asocia con síntomas clínicos de parkinsonismo y demencia senil (Spencer et al. 1987). Estudios *in vitro* revelan que la BMAA no es directamente tóxica pero que en presencia de bicarbonato probablemente forma algún derivado (posiblemente un α–metil carbamato) con propiedades tóxicas (Weiss y Choi, 1988), cuyos efectos son bloqueados por antagonistas de los receptores NMDA (Ross et al. 1987).

Demencia asociada al SIDA. En algunos pacientes infectados con el virus de inmunodeficiencia humano, HIV-1, se produce secundariamente a la infección, demencia y alteración de las funciones cognocitivas. El examen postmortem de los cerebros de estos pacientes revela la presencia de neuritas distróficas, infiltración de macrófagos, y pérdida de neuronas. Los mecanismos que dan lugar a esta neuropatía no están claros. Sin embargo, existen fundamentalmente dos evidencias en favor de que este daño neurológico esté mediado por un proceso excitotóxico: (1) Se ha encontrado una mayor concentración de ácido quinolínico en el líquido cefaloraquídeo de pacientes infectados con el virus del SIDA, en

comparación con pacientes normales de la misma edad (Heyes et al. 1989). (2) La proteína de la cápside del virus HIV-1 (denominada gp120) da lugar a un incremento en el flujo intracelular de calcio y a la muerte de las células ganglionares de la retina *in vitro* (Lipton et al. 1991), efecto que aparentemente está mediado a través de receptores NMDA. La presencia de la proteína gp120 hace más susceptibles a las neuronas a la toxicidad por glutamato, ya que concentraciones de este aminoácido que normalmente no son neurotóxicas en presencia de ésta, sí lo son. Esta observación, aunada a la de Susel et al. (1991), quienes encontraron que la perfusión prolongada de concentraciones bajas de ácido quinolínico en el estriado de la rata *in vivo*, da lugar a la neurodegeneración de las células estriatales, y a la de Whetsell y Schwarcz (1989), quienes encontraron el mismo resultado en células cultivadas, favorece la hipótesis de que un proceso excitotóxico puede contribuir a la neuropatología asociada al SIDA.

II OBJETIVOS GENERALES

Dado que en cada uno de los trabajos presentados en la tesis se plantean sus objetivos particulares, en esta sección se puntualizan los objetivos generales de los tres trabajos en su conjunto.

1) Conocer si los antagonistas tanto de los receptores NMDA como no-NMDA protegen contra lesiones excitotóxicas producidas en el estriado de la rata *in vivo* .

2) Conocer si es posible proteger a las neuronas estriatales del daño excitotóxico con antagonistas de los receptores NMDA, administrándose a diferentes tiempos después de la lesión.

3) Conocer si la elevación de glutamato endógeno por medio de la administración de inhibidores de su sistema de recaptura en el estriado de la rata, da lugar a la degeneración neuronal.

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A COMPARATIVE ANALYSIS OF THE NEUROPROTECTIVE PROPERTIES OF COMPETITIVE AND UNCOMPETITIVE *N*-METHYL-D-ASPARTATE RECEPTOR ANTAGONISTS *IN VIVO*: IMPLICATIONS FOR THE PROCESS OF EXCITOTOXIC DEGENERATION AND ITS THERAPY

L. MASSIEU,* K. H. THEDINGA, M. MCVEY and G. E. FAGG
CIBA GEIGY Ltd, Pharmaceutical Research Division, CH-4002 Basel, Switzerland

Abstract – Injection of the *N*-methyl-D-aspartate receptor agonist, quinolinic acid, into the rat striatum *in vivo* results in the degeneration of cholinergic and GABAergic neurons, as determined seven days later using the marker enzymes, choline acetyltransferase and glutamate decarboxylase, respectively. Such damage was dose-dependently prevented by CGP 37849 or MK-801 (competitive and uncompetitive *N*-methyl-D-aspartate receptor antagonists, respectively) administered systemically or intrastriatally at the same time as quinolinic acid.

The neuroprotective activity of CGP 37849 was associated with the D-enantiomer, CGP 40116 (ED_{50} 7.5 mg/kg i.p.), which was approximately 1.5-fold and 3.5-fold more potent than the related compounds, D-CPPene and CGS 19755, respectively. CGP 37849 was a weaker neuroprotectant than MK-801 (ED_{50} 0.8 mg/kg i.p) when administered systemically, but was dramatically more potent following coinjection with quinolinic acid (ED_{50}'s 0.2 and 117 nmol, respectively). When injected intrastriatally 0.5–2 h post-quinolinic acid, CGP 37849 was protective over the entire period studied, whereas MK-801 was less effective at all post-quinolinic acid injection times. The finding that CGP 37849 is neuroprotective when administered intrastriatally 1–2 h post-quinolinic acid supports the hypothesis that a period exists following excitotoxic insult in which neurons are not committed to die, and can be rescued by blockade of ongoing *N*-methyl-D-aspartate receptor activation.

Competition studies indicated that, when coinjected with 100–400 nmol quinolinic acid into the striatum, CGP 37849 exhibited kinetics predicted of a competitive *N*-methyl-D-aspartate receptor antagonist (declining neuroprotective potency with increasing doses of agonist), whereas MK-801 displayed a complex picture, with weak protective activity at low doses of quinolinic acid. Following systemic administration, neither antagonist was markedly affected by the dose of excitotoxin. When given i.p. at up to 6 h post-quinolinic acid, CGP 37849 and MK-801 showed essentially identical profiles of post-insult protection: degeneration of cholinergic neurons was reduced significantly throughout the entire post-insult period, whereas GABAergic neurons were protected only when drugs were administered 2 h or earlier post-quinolinic acid.

The data indicate that competitive and uncompetitive *N*-methyl-D-aspartate receptor antagonists are effective neuroprotectants *in vivo*, and that parameters such as drug lipophilicity or mechanism of action at the receptor do not impinge upon their properties as systemically active cerebroprotectants.

Prolonged exposure of cerebral neurons to high concentrations of L-glutamate or related amino acids leads to their death and degeneration.[7,23,24] In early studies of this phenomenon, Olney showed that the neurotoxic properties of acidic amino acids were related to their ability to depolarize and excite central neurons, and the term "excitotoxicity" was coined to describe this form of activity-dependent neuronal damage.[24] Although agonists at each of the three excitatory amino acid receptor subtypes can elicit neurodegenerative changes,[8] the discovery that *N*-methyl-D-aspartate (NMDA) receptor antagonists are highly effective neuroprotectants in animal models of cerebral ischaemia and hypoglycaemia[35,38] resulted in a focus on the role of NMDA receptor-mediated cellular events in neurodegenerative disease processes. In recent years, the involvement of excitatory amino acid mechanisms in ischaemic cerebral damage has gained support from a variety of convergent data: (i) the cytopathological nature of the lesion; (ii) increased extracellular levels of

*To whom correspondence should be addressed at: Departamento de Neurociencias, Instituto de Fisiologia Celular, Universidad Nacional Autonoma de Mexico, Apartado Postal 70600, 04510-Mexico D. F., Mexico (present address).

Abbreviations: AMPA, α-amino-3-hydroxy-5-methyl-isoxazole-4-proprionate; CGP 37849, 2-amino-4-methyl-5-phosphono-3-pentenoic acid; CGP 39551, 2-amino-4-methyl-5-phosphono-3-pentenoato-1-ethyl ester; CGP 39653, 2-amino-4-propyl-5-phosphono-3-pentenoato; CGP 40116, D-enantiomer of CGP 37849; CGP 40117, L-isomer of CGP 37849; CGS 19755, *cis*-4-phosphonomethyl-2-piperidine carboxylic acid-1-(*cis*-2-carboxypiperidine-4-yl-)-propyl-1-phosphonic acid; ChAT, choline acetyltransferase; CPPene, 3-(2-carboxypiperazin-4-yl)-propenyl-1-phosphonic acid; GAD, glutamate decarboxylase; HA966, 3-amino-1-hydroxypyrrolid-2-one; MK-801, dizocipine maleate; NMDA, *N*-methyl-D-aspartate.

glutamate and aspartate during ischaemia; (iii) augmented neuronal survival following excitatory deafferentation; and (iv) neuroprotection by NMDA receptor antagonists of distinct mechanistic classes.[21,22,30]

The characteristics of NMDA receptor-mediated neuronal death have been investigated *in vitro* and *in vivo*. In cortical cultures, exposure to high concentrations of NMDA receptor agonists leads to a delayed (24 h) Ca^{2+}-dependent neurodegeneration, which can be prevented by NMDA receptor antagonists applied during or shortly after agonist application.[6,16,18,29] Delayed degeneration also is a characteristic of NMDA receptor-induced neuronal insult *in vivo*,[20,26,28] moreover, neuroprotection by uncompetitive NMDA receptor blockers has been reported even when administered systemically several hours post-insult.[14,17] These observations have led to a hypothesis in which excitotoxic neuronal death is proposed to be a protracted event, requiring NMDA receptor activation and Ca^{2+} influx over an extended time-period before irreversible neuronal death ensues.[14,15] A corollary of this hypothesis, of major clinical significance, is that it may be possible therapeutically to interrupt the sequence of neurodegenerative events using NMDA receptor antagonists after the initial insult.

Most studies of the cerebroprotective properties of NMDA receptor antagonists *in vivo* have focused on uncompetitive blockers (e.g. MK-801, phencyclidine, ketamine), largely owing to the systemic activity of such compounds.[15] Competitive NMDA receptor antagonists have been less comprehensively studied. Indeed, it has been theorized that such compounds may be weaker neuroprotectants, since the presence of high extracellular levels of glutamate or other NMDA receptor agonists following cerebral insult may critically reduce their efficacy.[2] The goal of the present study was to compare and contrast the properties of competitive and uncompetitive NMDA receptor antagonists with regard to their abilities to prevent the degeneration of rat striatal neurons *in vivo* induced by direct injection of the NMDA receptor agonist, quinolinic acid.[33,36] Our aims were (i) to extend previous studies[14] on the mechanisms of quinolinic acid-induced delayed degeneration *in vivo*, and (ii) to determine the relative merits of these two types of antagonists as neuroprotectants. Our investigations focused primarily on the uncompetitive NMDA receptor antagonist, MK-801[12,39] and the competitive antagonist, CGP 37849[9,10] which shows high systemic anticonvulsant activity in rodents.[5,31] We report a comprehensive analysis of the neuroprotective effects of these compounds, covering intracerebral and systemic drug administration, delayed neuroprotection, and the kinetics of antagonist action *in vivo*. Some of these data have been presented in conference format.[11]

EXPERIMENTAL PROCEDURES

Surgical and stereotaxic procedures

Male albino rats (Tif:RAIf[SPF], 200–300 g weight) were anaesthetized with Equithesin (25 ml Vetanarcol (pentobarbital sodium 162 mg/ml, benzyl alcohol 20 mg/ml, 21.25 g chloral hydrate, 10.63 g $MgSO_4$, 198 ml propan-1,2-diol and 50 ml ethanol in 500 ml distilled water; dosage 3.75 ml/kg i.p.[34]) and secured in a Kopf stereotaxic frame with the nose-bar positioned at −3.3 mm. An incision was made in the skin overlying the cranium, and a 1–2-mm diameter hole was drilled in the skull to permit injection of quinolinic acid into the right striatum (coordinates: A 0.7 mm from bregma, L 3.0 mm from midline, V 5.1 mm from dura). Quinolinic acid (200 nmol unless stated otherwise; dissolved in 1 M NaOH, adjusted to pH 7.4 with 1 M HCl and brought to the appropriate volume using 0.1 M phosphate buffer, pH 7.4) was injected (1 μl over a 2 min period) via a 26-gauge stainless steel needle connected to a Hamilton syringe, using a CMA/100 microinjection pump (Carnegie). After injection, the needle was left in place for a further 2 min before removal and suture of the skin. Animals were allowed to recover and were maintained at 22°C on a 12-h light/dark cycle, with food and water *ad libitum*. In some experiments, α-amino-3-hydroxy-5-methyl-isoxazole-4-propionate (AMPA; 50 nmol in 1 μl) or kainic acid (10 nmol in 1 μl) were injected into the right striatum instead of quinolinic acid.

Drug treatments

Potential neuroprotective agents were administered intrastriatally (either by coinjection with quinolinic acid or by later injection at the same coordinates in 0.1 M phosphate buffer, pH 7.4) or intraperitoneally (i.p.; 1 ml/kg body weight; solution in 0.1 M phosphate buffer, pH 7.4) at the indicated times following quinolinic acid injection (see Results). Since the effect of the anaesthesia lasted from 2 to 3 h there was no need to give a second injection during the post-insult intrastriatal injection of antagonists.

Evaluation of neuronal degeneration

Quinolinic acid-induced degeneration of cholinergic and GABAergic neurons in the striatum was quantitatively determined by means of the marker enzymes, choline acetyltransferase (ChAT) and glutamate decarboxylase (GAD), respectively.[14] This method has been used in various studies involving the neuroprotective properties of several compounds[3,14] and the loss of these enzyme activities has been shown to be related to histological determined neuronal death.[32a] Seven days after surgery, rats were sacrificed and their striata were dissected, weighed and frozen on dry ice. Each striatum was sonicated (Branson Sonifier 250, setting 5) for 20 s in 25 vol (wt/vol) of 50 mM Tris–HCl (pH 7.4) containing 0.2% Triton X-100, and aliquots were used for the determination of ChAT and GAD activities as described previously by Foster *et al.*[14] All assays were conducted in duplicate.

Data analysis and statistics. Neuronal loss induced by quinolinic acid was determined as the percentage reduction of ChAT and GAD activities in the injected striatum relative to the contralateral uninjected striatum, after subtraction of blank values.[14] For most experiments, only data on ChAT activity are graphically illustrated, since similar results were obtained for both enzymes. Data for both neuronal markers are shown when differences were apparent. In all cases, values reported are means ± S.E.M. of data from the indicated number of animals. Statistical comparisons were made using Dunnett's *t*-test and Mann–Whitney's *U*-test as indicated in figures.

Tissue decay of N-*methyl*-D-*aspartate receptor antagonists following striatal injection*

[^{3}H]MK-801 (140 nmol in 1 μl) or [^{3}H]CGP 39653

(0.2 nmol in 1 μl) diluted in 0.1 M phosphate buffer (pH 7.4) were injected into the right striata of anaesthetized rats as described above for quinolinic acid injections. Animals were killed 5, 30, 60 and 120 min later and their striata rapidly dissected and weighed. Radioactivity in the tissue samples was determined after sonication in 50 mM Tris HCl/0.2% Triton X-100 by liquid scintillation spectrometry. Striatal half-lives ($t_{1/2}$) were determined by linear regression analysis of the slopes of semi-logarithmic plots (in tissue radioactivity vs time).

Materials

Quinolinic acid was purchased from Aldrich (Steinheim, West Germany) and MK-801 from Research Biochemicals Inc. (Natick, U.S.A.). D-CPPene was generously provided by Dr P. L. Herrling (Sandoz Ltd, Basle, Switzerland). CGP 37849, CGP 40116, CGP 40117, CGP 39551, CGP 39653 and CGS 19755 were synthesized in the laboratories of Ciba Geigy (Basel, Switzerland and Summit, U.S.A.). L-[1-^{14}C]Glutamate (52.6 mCi/mmol), [1-^{14}C]acetyl coenzyme A (59.3 mCi/mmol), [^{3}H]MK-801 (17.8 Ci/mmol) and [^{3}H]CGP 39653 (37.4 Ci/mmol) were obtained from Du Pont New England Nuclear (Boston, U.S.A.). All other reagents were of the highest analytical grade available from Fluka (Buchs, Switzerland) or from Sigma (St Louis, U.S.A.).

RESULTS

Dose relationship for quinolinic acid-induced neurodegeneration

Intrastriatal injection of quinolinic acid resulted in a dose-dependent decrease of ChAT and GAD activities, the half-maximal dose being approximately 100 nmol. At the dose of 200 nmol selected for most experiments reported here, enzyme activities were reduced routinely by 75–90%; thus, 200 nmol quinolinic acid was a near-maximal, but not a supramaximal dose.

The ChAT and GAD activities measured in striatal tissue were in good agreement with those reported previously.[14,32] For example, for six animals injected with 200 nmol of quinolinic acid, ChAT and GAD activities were, respectively, 13.5 ± 0.5 and 17.9 ± 0.8 nmol/mg wet tissue per h for the unlesioned left striatum and 2.1 ± 0.4 and 2.3 ± 0.4 nmol/mg wet tissue per h for the lesioned right striatum. Injection of buffer alone (no quinolinic acid) elicited no significant change of either ChAT (injected striatum, 12.7 ± 0.8 nmol/mg per h; contralateral striatum, 12.9 ± 0.5 nmol/mg per h; $n = 5$) or GAD activities (injected, 18.7 ± 1.8 nmol/mg per h; contralateral, 21.4 ± 2.2 nmol/mg per h; $n = 5$).

Blockade of quinolinic acid-induced neurodegeneration by intraperitoneal CGP 37849 and MK-801

Injection of antagonist immediately after 200 nmol quinolinic acid. Systemic (i.p.) administration of the competitive NMDA receptor antagonist, CGP 37849, immediately after striatal injection of quinolinic acid dose-dependently antagonized the excitotoxin-induced reduction of ChAT and GAD activities (Fig. 1a). Similar observations were made using the uncompetitive blocker, MK-801, although this compound was more potent than CGP 37849 (ED_{50}'s 0.7 and 15 mg/kg, respectively, for ChAT activity, and 0.8 and 19.0, respectively, for GAD activity). At sufficient dose, both compounds totally prevented quinolinic acid-induced reductions of striatal ChAT and GAD activities (Fig. 1A). Since preliminary experiments with other non-competitive antagonists (phencyclidine and ketamine, 1 mg/kg; and HA966, 30 and 100 mg/kg) showed poor protective effects in these

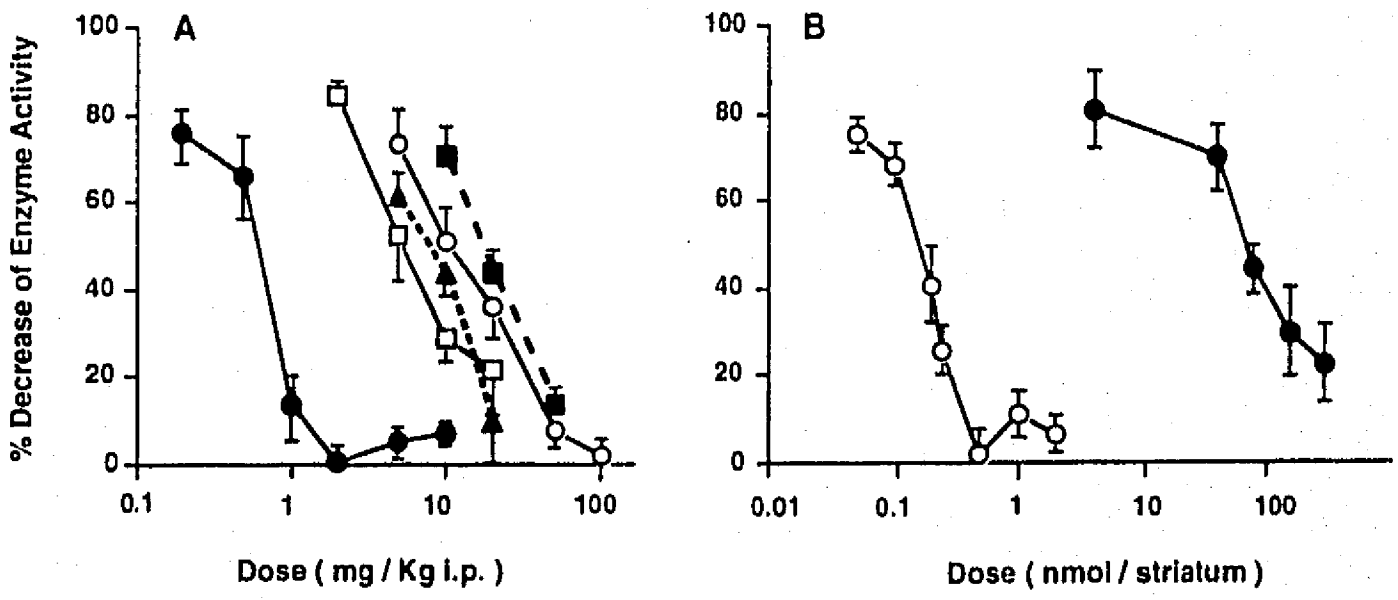

Fig. 1. Dose-response curves illustrating the neuroprotective properties of NMDA receptor antagonists in the rat *in vivo*. Quinolinic acid (200 nmol) was injected into the right striatum and the percentage loss of ChAT activity was determined seven days later by reference to the contralateral (uninjected) striatum. Antagonists were (A) administered i.p. immediately after quinolinic acid injection or (B) co-injected with quinolinic acid directly into the striatum. Values are means ± S.E.M. of data from four to 11 animals per dose group. The percentage decrease of ChAT activity in control animals was 86 ± 1%. Similar curves were obtained when GAD activity was employed as a marker of neuronal degeneration. ED_{50} (mg/kg) values are the following for ChAT and GAD determinations, respectively: A, MK-801, 0.7 and 0.8; CGP 40116, 7 and 8; CGP 37849, 15 and 19; CGS 19755, 22 and 31; D-CPPene, 9 and 16. B, (nmol/striatum) MK-801, 100 and 130; CGP 37849, 0.18 and 0.26. ●, MK-801; □, CGP 40116; ▲, D-CPPene; ○, CGP 37849; ■, CGS 19755.

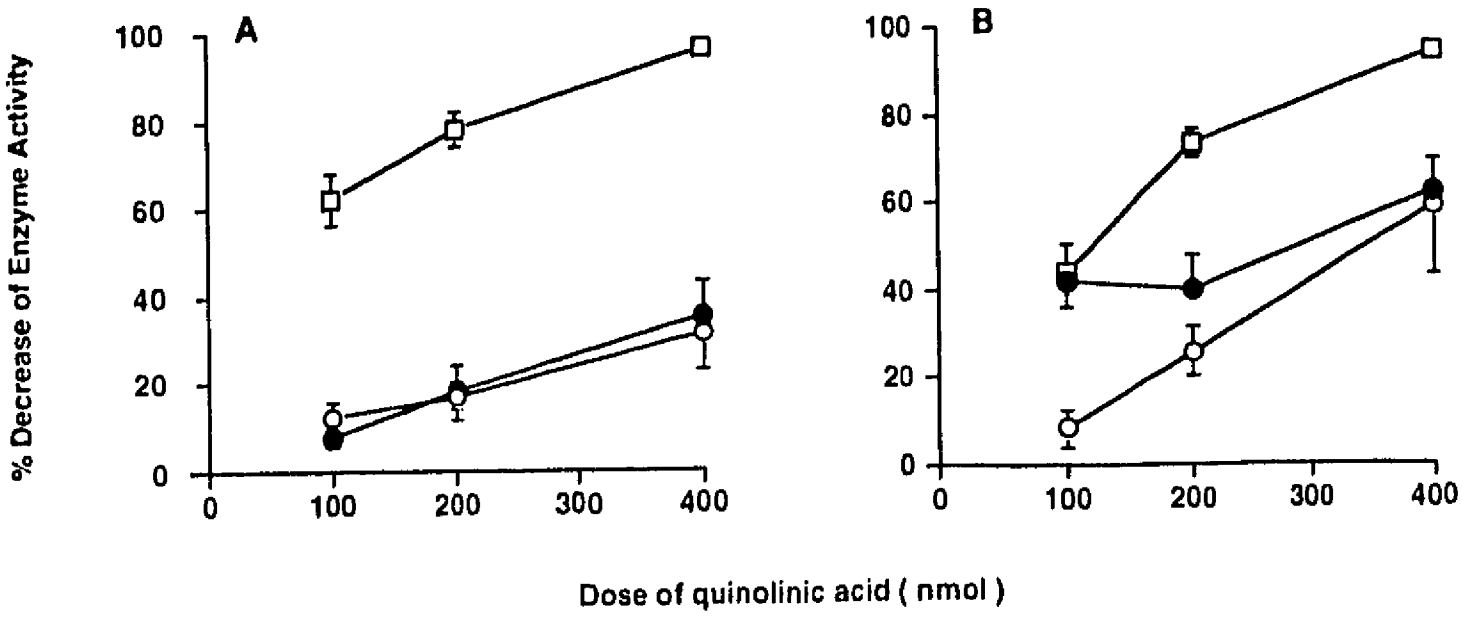

Fig. 2. Neuroprotective effects of CGP 37849 and MK-801 as a function of the dose of quinolinic acid injected into the rat striatum *in vivo*. Quinolinic acid-induced loss of ChAT activity was determined as described under Fig. 1. A and B show dose–response curves describing the loss of striatal ChAT activity induced by 100–400 nmol quinolinic acid alone (□) or in the presence of CGP 37849 (○) or MK 801(●). For experiments illustrated in A, CGP 37849 (30 mg/kg) and MK-801 (1 mg/kg) were administered i.p.; in B, CGP 37849 (0.25 nmol) and MK-801 (160 nmol) were co-injected into the striatum with quinolinic acid. Values are means ± S.E.M. of data from five to seven animals per group.

experimental conditions, the study was focused on MK-801.

Injection of antagonists immediately after varying doses of quinolinic acid. In a subsequent set of experiments, the protective effects of CGP 37849 or MK-801 on striatal damage induced by different doses of quinolinic acid (100, 200 and 400 nmol; a range selected to include submaximal and supramaximal doses) were investigated. Doses of CGP 37849 (30 mg/kg i.p.) and MK-801 (1 mg/kg i.p.) were chosen to lie on the linear but upper portions of their dose–response curves (Fig. 1A), in order that potential competition phenomena induced by changing the degree of excitotoxic insult might be observed. Similar results were obtained for both drugs (Fig. 2A). With increasing doses of quinolinic acid, competitive and uncompetitive NMDA receptor antagonists exhibited a slight trend towards decreased protective efficacy. In Fig. 2A the data for ChAT activity are shown. The same result was obtained when GAD activity was determined.

Blockade of quinolinic acid-induced neurodegeneration by intrastriatal CGP 37849 and MK-801

Co-injection of antagonist with 200 nmol quinolinic acid. When coinjected with 200 nmol quinolinic acid directly into the right striatum, the competitive NMDA receptor antagonist, CGP 37849, potently and dose-dependently prevented excitotoxic loss of ChAT and GAD activities (Fig. 1B). The ED_{50} calculated values were 0.18 and 0.26 nmol for ChAT and GAD activities, respectively. Complete prevention of striatal damage was achieved at doses of 0.5 nmol and above. In contrast, the uncompetitive blocker, MK-801, was two to three orders of magnitude less effective, with ED_{50} values of 100 nmol for ChAT and 130 nmol for GAD activities, respectively. Complete protection was not achieved at the highest dose tested (Fig. 1B: doses greater than 300 nmol were not examined owing to solubility problems). Since the difficulty in achieving maximum protection at these high doses of MK-801 potentially could be attributed to a neurotoxic effect of this agent,[25] the effect of MK-801 (160 nmol) alone on ChAT and GAD activities was evaluated in some experiments; however, no differences were observed between MK-801-injected and contralateral striata. Similarly, intrastriatal injection of CGP 37849 (2 nmol) alone did not alter ChAT or GAD activities (data not shown). Although no damage was found based on ChAT and GAD activity determinations, a lack of neurotoxicity of the antagonists at this high concentrations can not be ruled out without an accurate histological analysis.

Co-injection of antagonist with varying doses of quinolinic acid. Co-injection of 0.25 nmol CGP 37849 (as above, a dose chosen to lie on the linear portion of the antagonist dose–response curve; see Fig. 1B) into the striatum with 100, 200 or 400 nmol quinolinic acid yielded a pattern of neuroprotection consistent with previous studies showing that this compound is a competitive antagonist at the NMDA receptor recognition site.[10] Thus, the percentage protection by CGP 37849 progressively decreased as the dose of quinolinic acid was increased (Fig. 2B). A different pattern was seen in the case of MK-801. At the dose employed (160 nmol), the percentage reduction of excitotoxic damage was not significantly different from that of CGP 37849 at doses of 200 or 400 nmol quinolinic acid (Fig. 2B), but poor neuroprotection was observed at the lowest dose of quinolinic acid (100 nmol), whether considered in terms of ChAT (3 ± 7%; data shown in Fig. 3b) or GAD (26 ± 4%, $n = 7$) activities. Since this was an unexpected result,

this experiment was repeated on a separate occasion, with an identical outcome.

Post-insult blockade of quinolinic acid-induced neurodegeneration by CGP 37849 and MK-801

Intraperitoneal administration of antagonists. Previous studies have shown that MK-801 prevents excitotoxic damage when administered several hours after striatal injection of quinolinic acid,[14] and it was of interest to compare the post-insult protective efficacy of competitive and uncompetitive NMDA receptor antagonists in the present series of experiments. For this purpose, doses of each antagonist were selected that maximally blocked striatal damage when administered i.p. immediately after injection of 200 nmol quinolinic acid; these were 50 mg/kg CGP 37849 and 2 mg/kg MK 801 (Fig. 1A). A similar time-course for neuroprotection was obtained with both drugs (Fig. 3). Both compounds efficaciously reduced quinolinic acid-induced striatal damage when administered immediately or 1 h following excitotoxin injection, but were progressively less effective when given at later times. Both prevented quinolinic acid-induced degeneration of cholinergic neurons more effectively than of GABAergic neurons; thus, excitotoxic losses of ChAT activity were significantly reduced (by about 50%) by CGP 37849 or by MK 801 even when administered as long as 6 h post-insult (the longest time-point studied), whereas losses of GAD activity were prevented only when drugs were administered 2 h or earlier post-insult (Fig. 3). There were no statistically significant differences between the neuroprotective effects of 50 mg/kg CGP 37849 and 2 mg/kg MK-801 at any administration time.

Decreased neuroprotection by drugs administered post-insult potentially might result from (i) the presence of a population of cells which is irreversibly damaged and hence cannot be protected by the antagonists under study, or (ii) an apparent reduction in drug potency. The latter possibility is of particular concern in the case of competitive NMDA receptor antagonists, since their apparent potency would be predicted to be highly dependent on the concentrations of endogenous NMDA receptor agonists in the extracellular space. Additional experiments were therefore conducted to determine whether, when administered i.p. 2 h post-insult, 100 mg/kg CGP 37849 offered increased protection over the 50 mg/kg dose. No significant differences were observed. The percentages of striatal damage at doses of 50 mg/kg and 100 mg/kg CGP 37849 were, in the case of ChAT activity, 27 ± 9 and 20 ± 12, and for GAD activity, 46 ± 10 and 48 ± 8, respectively ($n = 9$). In the case of MK-801 (also administered 2 h post-insult), doses of 1 and 2 mg/kg yielded a similar level of neuroprotection by the drug (percentage loss of ChAT activity, 16 ± 6 and 30 ± 8; and of GAD activity, 55 ± 10 and 48 ± 8, respectively, $n = 9$). Thus, the doses of 50 mg/kg CGP 37849 and 2 mg/kg MK 801 utilized for the time-course experiments shown in Fig. 3 were measuring the maximum neuroprotective effects of these antagonists.

Intrastriatal administration of antagonists. One hypothesis to account for the unexpectedly large difference between the neuroprotective potencies of CGP 37849 and MK-801 when coinjected directly into the striatum with quinolinic acid (Fig. 1B) is that neuronal death is triggered by NMDA receptor activation at a critical time after the initial quinolinic acid injection[3,14] and that MK-801 is no longer present at sufficient concentration at that time. If so, then one might predict that MK-801 would apparently in-

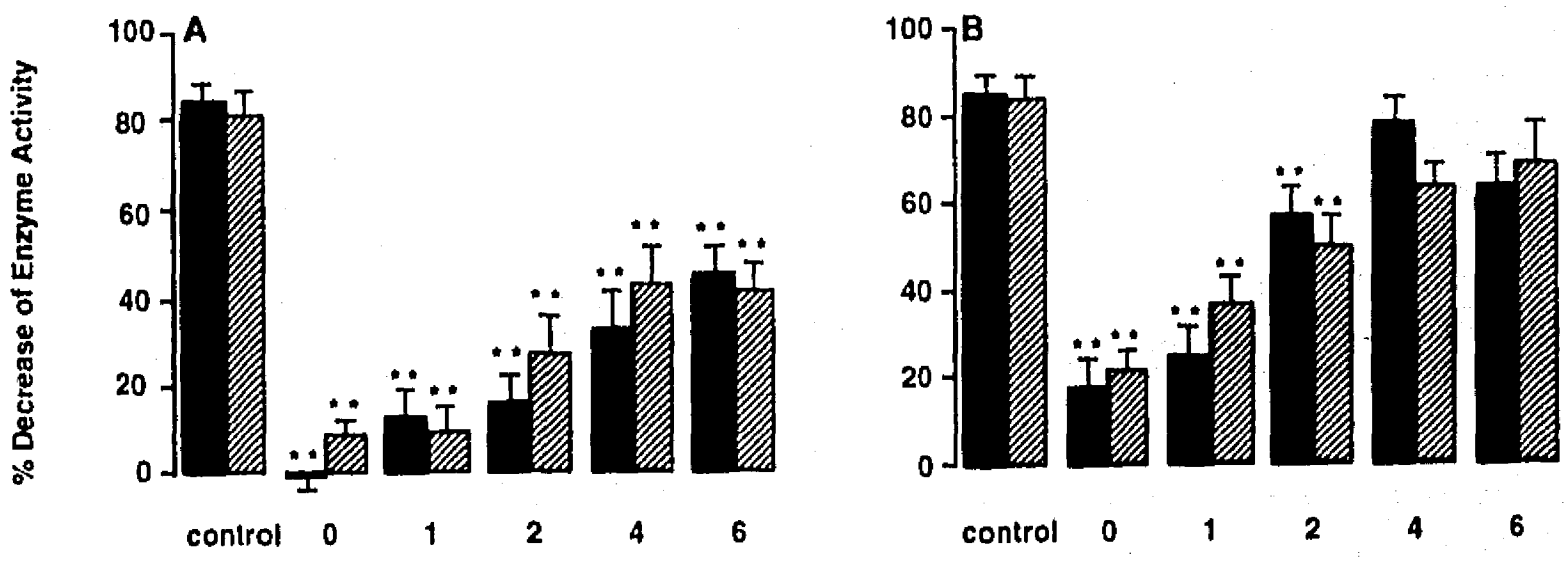

Fig. 3. Histograms illustrating the neuroprotective efficacy of CGP 37849 (50 mg/kg i.p., hatched bars) and MK-801 (2 mg/kg i.p., solid bars) administered 0–6 h after injection of 200 nmol quinolinic acid into the rat striatum *in vivo*, based on measurements of (A) ChAT and (B) GAD activities. Control animals received quinolinic acid alone; data from two series of control animals are shown, each being conducted in parallel with a drug treatment group. Values are means ± S.E.M. of data from five to nine animals per group. $^{*}P < 0.05$, $^{**}P < 0.01$ for significance of difference between indicated group and its respective control (Dunnett's *t*-test).

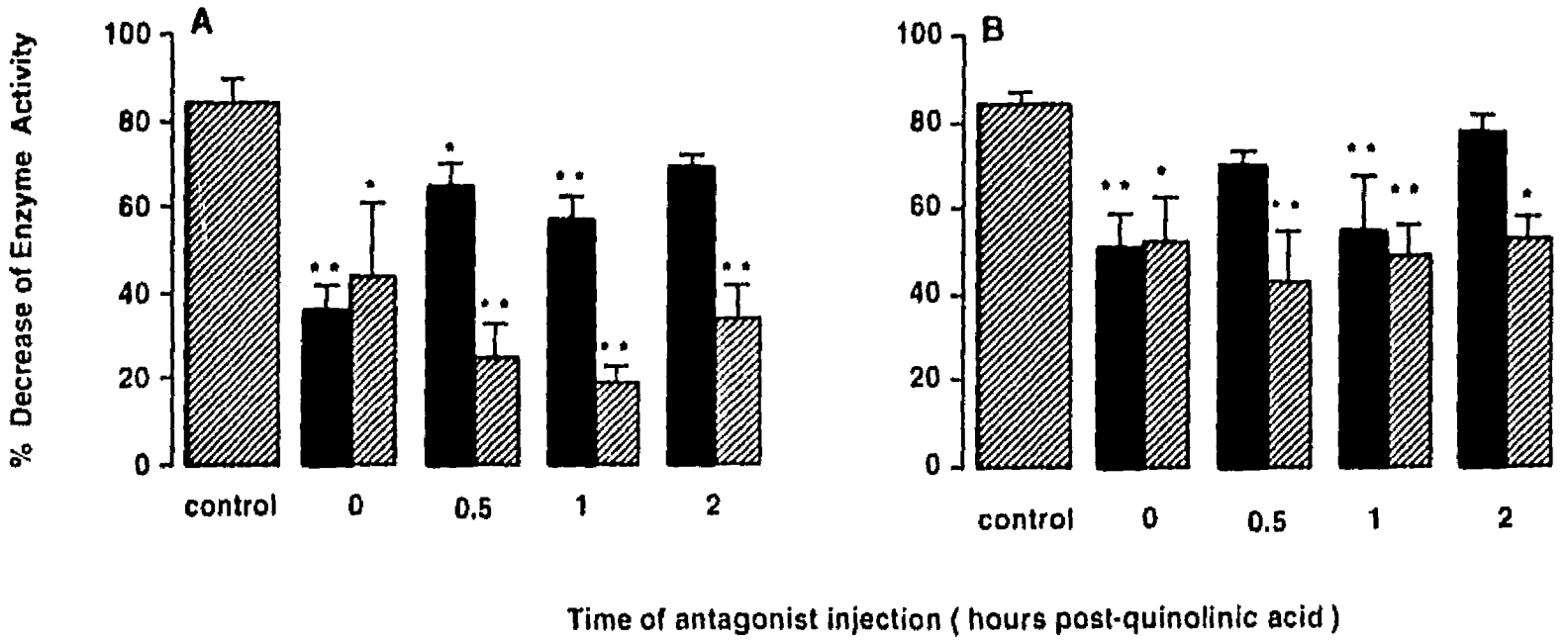

Fig. 4. Histograms illustrating the neuroprotective effects of CGP 37849 (0.2 nmol, hatched bars) and MK-801 (80 nmol, solid bars) administered intrastriatally 0–2 h after injection of 200 nmol quinolinic acid into the rat striatum *in vivo*, based on measurements of (A) ChAT and (B) GAD activities. Control animals received quinolinic acid alone. Values are means ± S.E.M. of data from four to seven animals per group. $^{*}P < 0.05$, $^{**}P < 0.01$ for significance of difference between indicated group and controls (Dunnett's *t*-test). $P < 0.05$ for significance of differences between MK-801 and CGP 37849 at the 0.5-, 1- and 2-h time-points for ChAT activitiy. No differences were found for GAD activity determinations (Mann–Whitney's *U*-test).

crease in neuroprotective potency when administered closer in time to the postulated critical post-insult period of NMDA receptor activation. This was tested by administering MK-801 and CGP 37849 intrastriatally at the same coordinates as quinolinic acid, at doses (80 and 0.2 nmol, respectively) close to their ED_{50} values when coinjected with quinolinic acid (Fig. 1B), and determining whether their protective efficacy was altered at times up to 2 h post-insult.

In direct contrast to the predicted result, MK-801 exhibited lower neuroprotection at all times post-insult than when coinjected with quinolinic acid (Fig. 4). CGP 37849, tested in parallel, showed stable or slightly increased neuroprotective properties over the same time-period. Similar results were obtained whether based on cholinergic or GABAergic marker enzymes, although the differential pattern of activities between competitive and uncompetitive NMDA receptor antagonists was clearer in the case of ChAT activity. The differences between MK-801 and CGP 37849 were statistically significant at the 0.5, 1 and 2 h post-insult time-points in the case of ChAT activity ($P < 0.05$). No statistically significant differences were obtained for GAD determinations.

Blockade of quinolinic acid-induced neurodegeneration by other N-*methyl*-D-*aspartate receptor antagonists*

The comparative neuroprotective properties of several antagonists of the NMDA receptor were evaluated following i.p. administration immediately after striatal injection of 200 nmol quinolinic acid. Construction of dose–response curves (Fig. 1A) revealed that CGP 40116 (the D-enantiomer of CGP 37849) was the most potent neuroprotectant of the competitive NMDA antagonists examined (ED_{50} 7.0 and 8.0 mg/kg for ChAT and GAD determinations, respectively), being approximately 1.5-fold and 3.5-fold more potent than D-CPPene (ED_{50} 9.0 and 16.0 mg/kg for ChAT and GAD determinations, respectively) and CGS 19755 (ED_{50}'s 22 and 31 mg/kg for ChAT and GAD determinations, respectively). The L-isomer of CGP 37849 (CGP 40117) was examined at a dose of 10 mg/kg and was found to be ineffective (data not shown). CGP 39551, the carboxyethylester of CGP 37849,[10] showed lower neuroprotection than CGP 37849 at a dose of 20 mg/kg (not shown) and was not further studied.

Effect of CGP 37849 *on AMPA- and kainate-induced neurodegeneration*

The selectivity of CGP 37849 as an antagonist of NMDA receptor-mediated neuronal degeneration was assessed by examining its effects on the loss of striatal ChAT and GAD activities induced by AMPA (50 nmol) and kainate (10 nmol). At a dose of 50 mg/kg administered i.p. immediately after excitotoxin injection, CGP 37849 had no protective effect on the neurodegeneration induced by either agent. In the case of AMPA, striatal ChAT and GAD activities were reduced, respectively, by 81 ± 8 and 80 ± 3% in controls, and by 63 ± 9 and 78 ± 6% in CGP 37849-treated animals. Kainate injection led to a reduction in striatal ChAT and GAD activities, respectively, of 87 ± 2 and 85 ± 2% in controls, and 79 ± 7 and 76 ± 8% in animals administered CGP 37849.

Tissue decay of N-*methyl*-D-*aspartate receptor antagonists following striatal injection*

The rate of elimination of NMDA antagonists following their injection directly into the striatum was estimated using [^{3}H]MK-801 and, since CGP 37849 is not available in tritiated form, the structural and

pharmacological analogue, [^{3}H]CGP 39653.[34] Semilogarithmic plots (not shown) describing the decay of striatal radioactivity following direct injection of these substances were linear (correlation coefficients: CGP 39653, −0.968; MK-801, −0.976), indicating mono-exponential elimination over the 5–120-min period sampled. The $t_{1/2}$ values calculated from these plots were 85 min in the case of CGP 39653 and 25 min for MK-801.

DISCUSSION

Comparative neuroprotective properties of N-*methyl*-D-*aspartate receptor antagonists*

MK-801, an uncompetitive antagonist which acts by blockade of the open NMDA receptor ion channel,[39,12,19] was initially shown by Foster *et al.*[13,14] to reduce excitotoxic neuronal death when systemically administered either before or some hours after excitotoxin injection. In the present study an ED_{50} of 0.8 mg/kg (i.p) (averaged from ChAT and GAD activities) was determined for MK-801, and maximal protection was achieved with 2 mg/kg when administered immediately after quinolinic acid. It was previously reported that the administration of 1 and 3 mg/kg MK-801 1 h before quinolinic acid leads to only partial protection while maximal protection was achieved with 10 mg/kg (no intermediate dose used).[14] These data might suggest that drug potency estimates may vary depending on the different times used relative to excitotoxic insult and might be influenced by drug pharmacokinetics.

A dose of 2 mg/kg MK-801 appears to be maximal for post-insult protection at least for cholinergic cells (60 and 45% protection was seen even when administered 4 and 6 h after quinolinic acid, respectively), since an increase from 2 to 10 mg/kg does not result in further protection when administered at the same post-insult times.[14] In contrast, GABAergic cells are better protected when 10 mg/kg of MK-801 is administered[14] instead of 2 mg/kg. As previously suggested,[14] the earlier degeneration of GABAergic cells might be due to the presence of a larger number of NMDA receptors in these cells compared to cholinergic. Our data are in good agreement with the earlier observations, even to the extent of showing a similar post-insult time-course of protection and differential rescue of striatal cholinergic and GABAergic neurons.

Like MK-801,[13,14] the competitive NMDA antagonist CGP 37849 efficaciously reduced quinolinic acid-induced striatal damage following systemic administration and selectively antagonized neurodegeneration induced by NMDA receptor activation (with no effect against AMPA- or kainate-elicited neuronal loss). Nevertheless, CGP 37489 was less potent than MK-801, presumably reflecting its greater polarity and limited blood–brain barrier permeability. Dose–response analyses indicated that the neuroprotective activity of CGP 37849 was associated with the D-stereoisomer (CGP 40116), and that this was approximately 1.5- and 3.5-fold more potent than the antagonists D-CPPene and CGS 19755, respectively. These data correlate well with the relative potencies of the same substances in competitive NMDA receptor binding assays,[1,9,10] indicating that neuroprotection is a direct function of NMDA receptor blockade.

Unexpectedly, CGP 37849 proved to be substantially (> 500-fold) more potent as a neuroprotectant than MK-801 following direct intrastriatal injection with quinolinic acid. A similar finding has been reported with regard to the anticonvulsant activity of these antagonists following intracerebroventricular injection, although the CGP 37849 was only 12-fold more potent than MK-801.[5] In contrast, CGP 37849 is 10–70-fold weaker than MK-801 at their respective membrane binding sites (depending whether K_i values for CGP 37849 at agonist or antagonist binding sites are considered[10,40]), and is somewhat weaker than MK-801 as an antagonist of NMDA-evoked neuronal depolarizations in brain slice preparations *in vitro*.[27,39] One hypothesis to account for the discrepancy between these predictions and the observed results is that there is a critical period during which NMDA receptor activation leads to neuronal death, that is extended beyond the initial excitotoxin injection, and that MK-801 is eliminated before such a critical period is completed. Thus, drug pharmacokinetics will be a critical determinant of neuroprotective potency. That this hypothesis might contribute to the relative protective potencies of intrastriatal CGP 37849 and MK-801 is supported by the striatal half-lives determined here (i.e. MK-801, 25 min; CGP 39653, 85 min), and by previous studies showing that, following systemic administration, the brain half life of MK-801[37] is shorter than that of CGP 37849 (Wiegand *et al.*, personal communication). Another possibility is that, as a lipophilic drug, MK-801 is rapidly sequestered in cerebral membranes[37] and removed from the extracellular space. Either way, the functional concentration of MK-801 may be rapidly depleted, and this may contribute to its low neuroprotective potency following local striatal injection.

Theoretical considerations indicate that competitive NMDA receptor antagonists may be less effective cerebroprotectants than uncompetitive blockers since, following brain ischaemia or trauma, their efficacy may be critically reduced by competition with toxic levels of excitatory amino acids.[2,7] This question was approached directly here by injecting different doses of quinolinic acid into the striatum and by determining the neuroprotective properties of fixed submaximal doses of CGP 37849 and MK-801 administered i.p. and intrastriatally. Following coinjection with quinolinic acid into the striatum, CGP 37849 displayed properties consistent with its competitive antagonist kinetics as determined from studies *in vitro*.[10] However, the results with regard to MK-801 were complex. It behaved similarly to CGP

37849 at high doses of quinolinic acid (200–400 nmol) and was weak at the lowest dose of quinolinic acid examined (100 nmol). The latter observation was sufficiently surprising that we repeated this experiment on a separate occasion, and obtained similar results. Currently, we have no good explanation for this anomalous behaviour. However, a similar result was obtained in a previous kinetics study,[3] using a supramaximal dose of MK-801 (10 mg/kg) administered intraperitoneally at different post-insult times with varying concentrations of quinolinic acid. No explanation was either provided by the authors. A histological analysis might be helpful to clarify this point, since the presence of normal-appearing neurons has been reported even in close vicinity to the injection site in MK-801-treated rats previously injected with 120 nmol quinolinic acid.[14]

Despite the clear changes in protective efficacy of CGP 37849 and MK-801 following coinjection with varying doses of quinolinic acid directly into the striatum, their neuroprotective potencies following i.p. administration remained similar at different quinolinic acid doses. It thus appears that, following systemic administration, pharmacokinetics or the large pool of drug in the body override the kinetics of agonist–antagonist–receptor interactions as determined at the cellular level. The result of this is that the mechanism of action of CGP 37849 and MK-801 at the receptor (competitive/uncompetitive) is not a critical factor affecting their systemic neuroprotective efficacy in the face of varying degrees of excitotoxic lesion.

Delayed excitotoxic neurodegeneration

Early studies showing that neurons exposed to excitotoxic insult can be rescued by post-insult administration of NMDA receptor antagonists involved the uncompetitive NMDA receptor antagonist, MK-801.[14] The present study indicates that the competitive antagonist, CGP 37849, shows an identical profile of systemic post-insult neuroprotective efficacy; at no time during the 6-h post-insult period examined was there any significant difference between the protective properties of MK-801 and CGP 37849. Since MK-801, being a highly lipophilic drug, traverses the blood–brain barrier more rapidly than CGP 37849 (as evidenced by their relative latencies to peak anticonvulsant activity[4,31]), one would predict that CGP 37849 would show lower post-insult protective efficacy than MK-801.[22] Thus, administration of higher doses of the competitive antagonist (here, 50 mg/kg i.p., compared with 2 mg/kg MK-801) may result in similar initial rates of brain entry, since (in a diffusion-limited system) the rate of blood–brain barrier permeation will be directly related to the concentration of drug in plasma.

Based on the finding that CGP 37849 was substantially more potent than MK-801 following intrastriatal co-injection with quinolinic acid, we hypothesized that the critical trigger for neuronal death may occur after excitotoxin injection[3,14] and that MK-801 might be a more potent protectant if administered intrastriatally closer in time to this postulated critical period. In direct contrast to this hypothesis, our results showed that MK-801 declined in neuroprotective potency at all post-insult administration times studied (0.5–2 h). These data indicate that striatal neurons are not commited to die even after 1–2 h exposure to quinolinic acid. Our observations support the postulate that excitotoxic degeneration in vivo requires extended post-insult activation of NMDA receptors,[3,14] and that neurons can be rescued by delayed administration of NMDA receptor antagonists.

CONCLUSION

In conclusion, the present results demonstrate that, in all parameters examined, CGP 37849 and MK-801 show similar neuroprotective properties following systemic administration. The competitive antagonist, CGP 37849, did not display inferior protective efficacy either in the presence of high levels of excitotoxin or when administered post-insult, as predicted on theoretical grounds.[2,7,22] Indeed, under some experimental conditions (intrastriatal drug administration), evidence was obtained to indicate that CGP 37849 potentially may have therapeutic advantages over MK-801 (possibly related to its slower elimination from the brain). Clinical evaluation of NMDA receptor antagonists for the prevention of excitotoxic cerebral damage, such as occurs following brain ischaemia or trauma, seems warranted.

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2,3-DIHYDROXY-6-NITRO-7-SULFAMOYL-BENZO(f)QUINOXALINE PROTECTS AGAINST BOTH AMPA AND KAINATE-INDUCED LESIONS IN RAT STRIATUM *IN VIVO*

L. MASSIEU* and R. TAPIA

Departamento de Neurociencias, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, AP 70-253, 04510-México, D.F., México

Abstract—In the present work we have tested the neuroprotective effect of 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(f)quinoxaline (NBQX) on the excitotoxic damage induced by the injection of several glutamate receptor agonists into the rat striatum. NBQX was co-injected with each of the agonists studied (1 μl) in the striatum and damage was assessed by the determination of both glutamate decarboxylase and choline acetyltransferase activities in striatal homogenates, five days after the lesion. Additionally, animals were transcardially perfused with 0.9% saline/4% paraformaldehyde and brain coronal sections were stained with Cresyl Violet for histological analysis. Our results show that NBQX (25 nmol) did not protect against the damage induced by the intrastriatal injection of 200 nmol quinolinic acid monitored by either choline acetyltransferase or glutamate decarboxylase activity. In contrast, the same concentration of NBQX partially protected against 200 nmol *N*-methyl-D-aspartate induced damage; this protection was more notable as detected by changes in choline acetyltransferase activity. When non-*N*-methyl-D-aspartate receptor agonists were used as excitotoxins, coinjection of NBQX (25 nmol) resulted in a notable protection against both α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA, 40 nmol) and kainate (10 nmol) induced neurodegeneration. At this concentration, protection was slightly better in AMPA-injected animals (71% protection averaged from choline acetyltransferase and glutamate decarboxylase enzyme activities) as compared to kainate-injected animals (47.5% protection). When a higher concentration of NBQX was tested (40 nmol) the protection against kainate improved to 65% while that against AMPA remained constant (64% protection). Quantitative analysis of damaged cells in Cresyl Violet-stained sections corroborated the protective effect of NBQX against neuronal damage mediated by non-*N*-methyl-D-aspartate receptors.

It is concluded that NBQX equally protects against AMPA- and kainate-induced lesions in the striatum *in vivo* and that non-*N*-methyl-D-aspartate receptor antagonists might be useful as protectors against neuronal damage produced by excess activation of glutamate receptors.

Excitatory amino acid receptors, particularly of the *N*-methyl-D-aspartate (NMDA) type, are known to participate in neuronal damage produced by a variety of neuronal insults, such as ischaemia, anoxia and epilepsy. The role of non-NMDA glutamatergic receptors in the development of excitotoxic cell damage associated to these processes has not been as extensively studied as the involvement of NMDA receptors, due to the lack of selective non-NMDA receptor antagonists. The discovery of the quinoxalinediones,[15] a series of potent inhibitors of the non-NMDA excitatory amino acid receptor subtypes, facilitated the study of the pharmacology of the α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA)/kainate receptors. One of such compounds, 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(f)quinoxaline (NBQX), in contrast to the previously described quinoxalinediones, shows 30-times more affinity for the AMPA than for the kainate receptor subtype, and is less potent in displacing [^{3}H]CPP [3(2-carboxypiperazine-4-yl)propyl-1-phosphonic acid] and [^{3}H]glycine binding to rat brain membranes.[25] However, *in vivo* the blocking action of NBQX appears to be less selective for AMPA, since in the hippocampus it shows better protection against kainate than against AMPA-induced cell damage.[23]

NBQX has been shown to also be a good neuroprotective drug against transient global[6,18,25] and focal ischaemia,[11] glutamate-mediated brain edema[30] and ischaemia-induced loss of cerebellar Purkinje cells.[2] Furthermore, NBQX has also been shown to protect against the associated postischaemic behavioral abnormalities in the gerbil.[16]

In the present work we have studied the neuroprotective effects of NBQX against striatal lesions induced by non-NMDA receptor and NMDA receptor agonists in the rat, *in vivo*. Cell damage was

*To whom correspondence should be addressed.

Abbreviations: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionate; CCP, 3(2-carboxypiperazine-4-yl)propyl-1-phosphonic acid; ChAT, choline acetyltransferase; GAD, glutamate decarboxylase; NBQX, 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(f)quinoxaline; NMDA, *N*-methyl-D-aspartate.

assessed by the determination of the enzyme activity of choline acetyltransferase (ChAT) and glutamate decarboxylase (GAD), markers of acetylcholine and GABA containing neurons, respectively, as well as by quantitative histological analysis of brain sections.

EXPERIMENTAL PROCEDURES

Intrastriatal microinjection

Male Wistar rats bred in our animal house (200–220 g weight) were anaesthetized with equithesin (pentobarbital sodium 162 mg/ml, benzyl alcohol 20 mg/ml, chloral hydrate 21.25 g, $MgSO_4$ 10.63 g, propan-1,2-diol 198 ml, ethanol 50 ml, in 500 ml distilled water; dosage 3.75 ml/kg, i.p.[10]) and placed in a stereotaxic frame with the nose bar positioned at −3.3 mm. The skin was removed to expose the skull and a 1–2-mm diameter hole was drilled (coordinates: A + 0.7 mm from bregma, L 2.8 mm from midline, V 4.6 mm from dura) to permit injection of the excitatory amino acid agonists in the left striatum. Glutamate receptor agonists were dissolved in 1 M NaOH, adjusted to pH 7.4 with 1 M HCl and brought to the desired volume with 0.1 M phosphate buffer, pH 7.4. Final concentrations were (nmol/μl): quinolinate (200), NMDA (200), AMPA (40) and kainate (10). NBQX was dissolved in 0.2 M NaOH, adjusted to pH 7.5–8.5 with HCl and brought to the appropriate volume with 0.1 M phosphate buffer, pH 7.4. Final concentration was 25 or 40 nmol/μl, as indicated in results. For co-injection, solutions of appropriate concentrations of the glutamate receptor agonists and of NBQX were mixed. In all cases a 1-μl vol was injected at a rate of 0.5 μl/min via a 27-gauge stainless-steel needle connected to a Hamilton syringe, using a microinjection pump (Carnegie). After completion of the injection, the needle was left in place for two additional minutes, then removed and the skin was sutured. Control animals were injected with vehicle solution (0.1 M phosphate buffer, pH 7.4). Animals were maintained for five days with food and water *ad libitum*, and then were either decapitated or perfused for biochemical or histological analysis, respectively.

Enzyme assays

Degeneration of cholinergic and GABAergic neurons in the striatum was quantitatively determined by means of the marker enzymes ChAT and GAD, respectively. Five days after surgery, rats were killed by decapitation and both striata were dissected and weighed. Each striatum was sonicated (Branson Sonifier 250, setting 2) for 12 s in 25 vol (wt/vol) of cold water. Aliquots were used for the determination of ChAT and GAD activities. ChAT activity was measured according to the radiometric method of Fonnum[9] with some modifications.[19] GAD activity was determined by the radiometric method of Albers and Brady[1] slightly modified.[19]

Neuronal loss induced by the excitatory amino acid receptor agonists was determined as the per cent decrease of ChAT and GAD activities in the injected striatum relative to the contralateral uninjected striatum, after subtraction of blank values.[10] Statistical comparisons were made using the non-parametric Mann–Whitney *U*-test, unless otherwise stated.

Histology

Five days after surgery, animals were anesthetized and transcardially perfused with 250 ml 0.9% saline followed by 250 ml 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. Brains were extracted and left in fixative for additional 24 h. Brains were transferred successively to 10, 20 and 30% sucrose (24 h each), and 30-μm coronal sections were obtained in a cryostat. They were collected on gelatine-coated slides and stained with Cresyl Violet. Sections were examined by light microscopy and the lesions quantitatively assessed with the aid of an image-analyser system (Macintosh Image 1.44). Sections were analysed both by the measurement of the damaged area and by counting the number of pycnotic cells. The area of damaged tissue was determined in nine sections per rat, three anterior to the needle tract, three in which the needle tract was visible, and three posterior to the needle tract. For the cell counting, three tissue sections per animal were analysed, one anterior and one posterior to the needle tract, and a middle section in which the needle tract was visible. The field chosen for the analysis in this middle section was not in close vicinity to the tract, in order to avoid the numerous glial cells present there. Pycnotic cells were counted in one single 10X field (area 2.3 mm^2).

Materials

Quinolinic acid, kainate and AMPA hydrobromide were purchased from RBI (Natick, MA., U.S.A.). NMDA from Sigma Chemical Co. (St Louis Mo., U.S.A.), and NBQX was generously provided by Dr L. Nordholm from Novo Nordisk A.S., Copenhagen, Denmark.

RESULTS

Behavioral observations

Intrastriatal administration of NMDA, quinolinic acid, AMPA and kainate reproducibly induced intense ipsilateral barrel rotation in all the animals as soon as they recovered from anesthesia (about 2 h after the injection). This behavior lasted for at least 3 h and was also present when NBQX was co-injected with any of the excitatory amino acid receptor agonists studied.

*Non-*N*-methyl-*D*-aspartate receptor-mediated striatal neurodegeneration*

AMPA-induced lesions. ChAT and GAD activities observed in striatal homogenates were in good agreement with those reported previously.[10] In control buffer-injected animals, ChAT and GAD activities were, respectively, 11.6 ± 0.33 and 17.32 ± 0.58 nmol/mg wet tissue per h in the injected striatum, and 12.9 ± 1.08 and 18.01 ± 0.76 nmol/mg wet tissue/h in the non-injected striatum ($n = 4$).

As shown in Fig. 1A, B, administration of 40 nmol AMPA reliably produced a notable decrease in both ChAT (79.4% decrease) and GAD (82.7% decrease) activities relative to the contralateral non-injected striatum. When 25 nmol/μl of NBQX was co-injected with AMPA, ChAT activity was diminished by only 19.7% and GAD activity by only 22.8%, indicating 72.0% ± 11.9 and 72.2 ± 11.7% protection for ChAT and GAD activities, respectively. In additional experiments, four animals were injected with AMPA and with a higher concentration of NBQX (40 nmol). No further protection was observed with this higher dose of NBQX, as compared to that of 25 nmol (Fig. 1A, B).

For histological analysis five animals were injected with AMPA (40 nmol) without antagonist and five were co-injected with AMPA (40 nmol) and NBQX (25 nmol). All animals that received AMPA alone

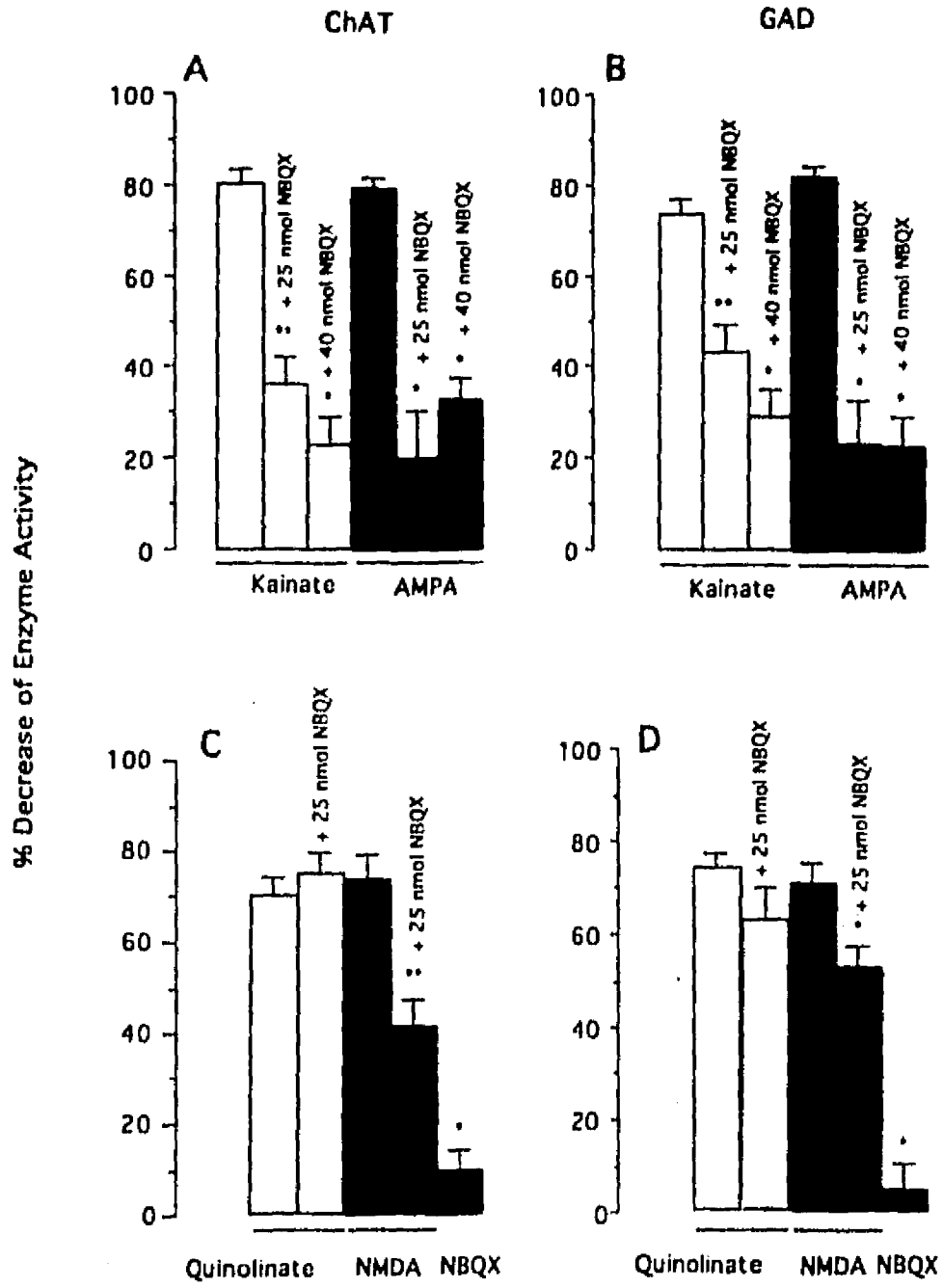

Fig. 1. Neuroprotective effect of NBQX against striatal neuronal damage induced by glutamate agonists, as detected by decrease of ChAT (A, C) and GAD (B, D) activities. Kainate (10 nmol) and AMPA (40 nmol) effects are shown in A and B, and quinolinic acid (200 nmol) and NMDA (200 nmol) effects are depicted in C and D. As indicated by the corresponding bar, AMPA- and kainate-treated animals were co-injected with two concentrations of NBQX, 25 and 40 nmol, whereas quinolinic acid and NMDA-treated animals were coinjected with a single concentration of NBQX (25 nmol). Additional animals were injected with NBQX (25 nmol) alone (last bar in C, D). Animals were killed five days after the surgery. Values represent means ± S.E.M. Number of animals in each group was: A and B, $n = 6$–8 except NBQX 40 nmol ($n = 4$); C and D, $n = 7$–10, except quinolinate alone ($n = 5$) and NBQX alone ($n = 4$). $^{*}P < 0.05$ and $^{**}P < 0.005$ for significance of difference between the indicated group and its respective control (Mann–Whitney U-test).

showed an extensive lesion in the injected striatum, characterized by numerous pycnotic cells present both close to the injection site and in the surrounding tissue. None or very few "normal appearing" cells were present, and the typical cell distribution of the striatum observed in Cresyl Violet-stained sections was completely disrupted (Fig. 2A). Small cells, probably representing glial cells, were seen along the needle tract. In buffer-injected animals, glial proliferation was also seen along the needle tract but none or a very small number of pycnotic cells were present either in close vicinity to the injection site or in the surrounding area (Fig. 2C). When NBQX was coinjected with AMPA, the typical histological appearance of the striatum was preserved and normal large cells were observed in close vicinity to the needle tract (Fig. 2B).

The quantitative analysis of the Cresyl Violet-stained sections revealed that the number of pycnotic cells in the AMPA-NBQX-treated striatum, was about 15% of that in the tissue injected only with AMPA (Table 1). A similarly notable protection (94%) was observed when the extent of the lesion was analysed by measurement of the damaged area (data not shown).

Kainate-induced lesions. As shown in Fig. 1A, B, intrastriatal injection of 10 nmol kainate produced 82% and 71.9% decreases of ChAT and GAD activities, respectively, relative to the non-injected striatum. When NBQX (25 nmol) was coinjected with kainate, ChAT and GAD activities diminished by only 36% and 43.0%, representing 57.05 ± 6.64% and 38.06 ± 8.7% protection, respectively. In contrast to AMPA-induced degeneration, further

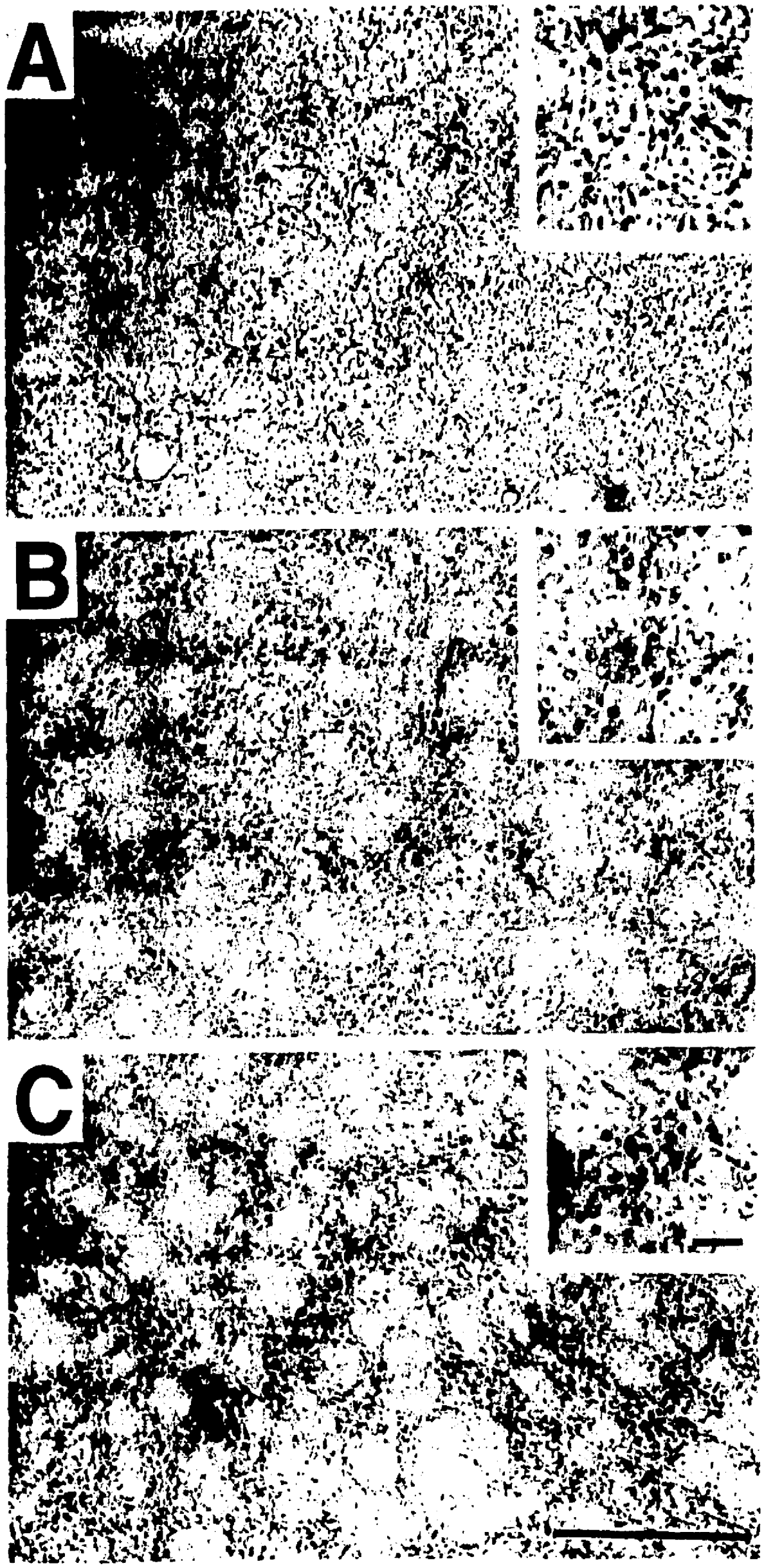

Fig. 2. Photomicrographs (10 ×) showing the protective effect of NBQX (25 nmol) against AMPA (40 nmol) induced neurodegeneration in the striatum, five days after surgery. (A) Animals were intrastriatally injected with AMPA alone; (B) co-injected with AMPA + NBQX; (C) injected with vehicle (buffer). Insets show 20 × magnification of the corresponding section. Scale bars = 300 μm and 100 μm (inset).

Table 1. Quantitative analysis of striatal histological sections of animals treated with NBQX

Treatment	Number of pycnotic cells/mm² Mean (*n*)	Range
AMPA	202 (5)	163–237
AMPA + NBQX	29 (5)	24–53
Kainate	159 (3)	161–182
Kainate + NBQX	42 (5)	30–57
Vehicle	14 (5)	11–16

Quantitative analysis of striatal histological sections of animals injected with AMPA or kainate either alone or with NBQX (25 nmol) as indicated in Figs 2 and 3. The number of pycnotic cells in tissue sections was counted as described in Experimental Procedures. Data are expressed as no. of pycnotic cells/mm².

protection against kainate was observed when the dose of NBQX was increased to 40 nmol (69.3 ± 8.0% and 62.8 ± 8.4% of protection for ChAT and GAD, respectively) (Fig. 1A, B).

When the per cent protection of NBQX (25 nmol) against AMPA was compared with that against kainate-induced neurodegeneration, statistically significant differences were found only for GAD activity (72.2 ± 11.7% protection for AMPA versus 38.1 ± 8.7% protection for kainate-induced damage). No differences were seen when the concentration of NBQX was increased to 40 nmol.

Histological analysis of kainate-injected animals revealed a widespread striatal damage around the injection site and surrounding tissue, similar to that observed with AMPA (Fig. 3A). In NBQX-treated animals pycnosis was observed only in the tissue adjacent to the injection tract but not in the surrounding tissue (Fig. 3B). Quantitative analysis showed 74% less pycnotic cells in kainate-NBQX-treated striatum as compared to the kainate-treated tissue (Table 1). A similar result (82% protection) was found when the damaged area was measured (data not shown). In agreement with the enzyme measurements, a slightly better protection of NBQX against AMPA than against kainate-induced damage was revealed by the cell counting analysis: 85% and 74% protection for AMPA and kainate, respectively.

Few pycnotic cells were seen in buffer-injected animals, close to the injection site, and this was different from NBQX-treated animals ($P < 0.05$) and from AMPA and kainate-injected animals ($P < 0.005$, Student's *t*-test, Table 1).

N-*Methyl*-D-*aspartate receptor-mediated striatal neurodegeneration*

In order to test the selectivity of NBQX, its neuroprotective effect against NMDA and quinolinic acid-induced neurodegeneration was tested (Fig. 1C, D).

Intrastriatal administration of 200 nmol quinolinic acid or NMDA reduced both ChAT and GAD activities by about 70%. In contrast to the results with AMPA and kainate, NBQX (25 nmol) coinjection did not protect against quinolinic acid-induced damage, as monitored either by ChAT or GAD activity. However, a significant protection was found when 25 nmol NBQX was co-injected with 200 nmol NMDA, which was less evident for GABAergic cells (44.0 ± 7.8% and 23.9 ± 6.23% protection for ChAT and GAD, respectively) (Fig. 1C, D).

A control group of four animals was injected with 25 nmol NBQX without agonist. A small reduction (6.5% averaged from ChAT and GAD) of enzyme activities was found (Fig. 1C, D).

DISCUSSION

*Protection against non-*N-*methyl-*D-*aspartate receptor agonists*

The decrease in ChAT and GAD activities induced by the intrastriatal administration of glutamate receptor agonists observed in the present study are in agreement with the data reported previously by other authors.[5,10,21] Our results show that NBQX, a non-NMDA receptor antagonist, protects similarly against both AMPA and kainate-induced neurodegeneration in the rat striatum *in vivo*. According to the enzyme assays, a tendency for better protection against AMPA than against kainate-induced lesions was observed when 25 nmol NBQX was co-injected with the glutamate receptor agonists studied. However, when the dosage of NBQX was increased to 40 nmol identical protection was found against both AMPA and kainate-induced neurodegeneration. This slightly better protection against AMPA-induced damage was also observed when the quantitative histological analysis was performed but was even less evident.

These findings indicate that in the striatum *in vivo* NBQX is equally effective against AMPA and kainate receptors. This is in contrast to the reported higher affinity of NBQX for AMPA than for kainate binding sites in rat cortical membranes.[25] Also in contrast to these findings *in vitro*, it has been reported that in the rat hippocampus *in vivo* NBQX (24 nmol), shows considerably better protection against kainate than against AMPA-induced lesions in spite of the low dose of AMPA used.[23] The discrepancy between the latter work and the present results can be due to the presence of different types of non-NMDA receptors in distinct brain regions. It is known that a great variety of non-NMDA receptor subtypes exist, depending on their subunit combination,[3,7,12,14,17,24,29] and that their distribution among brain regions differ.[13,17,20,22,26] Therefore, it is possible that the non-NMDA receptors more abundant in the hippocampus differ from those in the striatum, both in subunit composition and agonist and antagonist selectivity. Thus, the present finding that NBQX equally protects against AMPA and kainate-induced lesions suggests that, in the striatum, both AMPA and kainate-induced cell degeneration is mediated by the activation of the same receptor subtype.

Protection against N-*methyl*-D-*aspartate receptor agonists*

Surprisingly, 25 nmol NBQX partially protected against the lesion induced by the intrastriatal administration of 200 nmol NMDA, but not against the damage induced by the injection of the same amount of quinolinic acid. Since both compounds are agonists at the NMDA receptor, they would be expected to share the same mechanisms of excitotoxicity. Although a clear explanation of this discrepancy cannot be provided based on the present results, it can be speculated that the mechanisms of excitotoxicity of NMDA and quinolinic acid are in fact different. In agreement with this hypothesis, microdialysis experiments have shown that NMDA can induce the release of endogenous glutamate and aspartate when administered in the rat striatum *in vivo*, and thus activation of both NMDA and non-NMDA receptors can be predicted.[4,31] In contrast, in a similar *in vivo* experiment, quinolinic acid predominantly induced the release of endogenous aspartate while glutamate release was very poorly stimulated.[8] Furthermore, quinolinic acid-induced release of glutamate was not found in the hippocampus *in vivo*.[27] From these findings it might be hypothesized that quinolinic acid-induced neurodegeneration is mediated mainly by NMDA receptor activation, while NMDA-induced damage involves the activation of both non-NMDA and NMDA receptors.

In contrast to the good protective action of NBQX against non-NMDA mediated neurotoxicity, it did not prevent the behavioral alterations produced by the administration of any of the agonists studied. This result suggests that induction of neurotoxicity and behavioral changes respond to different mechanisms. This result agrees with the previous suggestion that induction of epileptic seizures and neuronal degeneration by quinolinic acid administration in the rat hippocampus occur by different mechanisms.[27,28]

Although a slightly better production was revealed by the histological cell-counting method compared to the determination of ChAT and GAD activities (15–25% more protection), in general there is good agreement between the two procedures. The difference can be due to the fact that enzyme activities were determined in homogenates containing the whole striatum while the cell-counting was performed only in sections close to the injection site, where prominent damage was observed. Therefore, the percentage of damage enzymatically determined could be an underestimation. On the other hand, when the damaged area was measured, the calculated per cent protection was 9% above that calculated by the cell-counting method, both for AMPA and kainate-injected animals.

CONCLUSION

NBQX is a good neuroprotectant against non-NMDA receptor-mediated excitotoxicity in the rat striatum *in vivo*, and this neuroprotection is similar against AMPA and kainate-induced neurodegeneration. These results show that activation of non-NMDA receptors contributes substantially to the development of glutamate-mediated excitotoxic damage and suggests a therapeutic potentiality of non-NMDA receptor antagonists in the treatment of neurological disorders associated with neural insults such as ischaemia, anoxia and epilepsy.

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ACCUMULATION OF EXTRACELLULAR GLUTAMATE BY INHIBITION OF ITS UPTAKE IS NOT SUFFICIENT FOR INDUCING NEURONAL DAMAGE: AN IN VIVO MICRODIALYSIS STUDY

Lourdes Massieu, *Alberto Morales-Villagrán and Ricardo Tapia

Departamento de Neurociencias. Instituto de Fisiología Celular, Universidad Nacional Autónoma de México. AP 70-253, 04510-México, D.F., México, and *Facultad de Ciencias Biológicas, Universidad de Guadalajara, 44420-Guadalajara, Jal.

Tel: 525-6225642; Fax: 525-6225607

Address correspondence and reprints requests to:

Lourdes Massieu, Departamento de Neurociencias, Instituto de Fisiología Celular. Universidad Nacional Autónoma de México, AP 70-253, 04510-México D.F., México

Abbreviations used: AMPA, α-amino-3-hydroxy-5-methylisoxazolepropionate; ChAT, choline acetyltransferase; DHK, dihydrokainate; GABA γ-aminobutyric acid; GAD, glutamate decarboxylase; PDC, L-*trans*-pyrrolidine-2,4-dicarboxylate; MK-801, (+)-5-methyl-10,11-dihydro-5H-dibenzo-[a,d]cyclohepten-5,10-imine maleate; NBQX, 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(f)quinoxaline; NMDA, N-methyl-D-aspartate.

Abstract: It is well documented that neurons exposed to high concentrations of excitatory amino acids, such as glutamate and aspartate, degenerate and die. The clearance of these amino acids from the synaptic cleft depends mainly on their transport by high affinity sodium-dependent carriers. Using microdialysis in vivo and HPLC analysis, we have studied the effect of the administration of inhibitors of the glutamate transporter (L-*trans*-pyrrolidine-2,4-dicarboxylate, and dihydrokainate) on the extracellular concentration of endogenous amino acids in the rat striatum. In addition, we have analyzed whether the changes observed in the concentration of glutamate and aspartate were injurious to striatal cells. Neuronal damage was assessed by biochemical determination of choline acetyltransferase and glutamate decarboxylase activities, 7 days after the microdialysis procedure. In other experiments, pyrrolidine-dicarboxylate and dihydrokainate, as well as two other inhibitors of the glutamate carrier, DL-threo-β-hydroxyaspartate and L-aspartate-β-hydroxamate were microinjected into the striatum and neuronal damage was assessed, both biochemically and histologically, 5-7 days after the injection. Dihydrokainate and pyrrolidine-dicarboxylate produced a similar remarkable increase in the concentration of extracellular aspartate and glutamate. However, the former induced also notable elevations in the concentration of other amino acids. Clear neuronal damage was observed only after dihydrokainate administration, which was partially prevented by intraperitoneal injection of (+)-5-methyl-10,11-dihydro-5H-dibenzo-[a,d]cyclohepten-5,10-imine maleate

(MK-801) or by intrastriatal coinjection of 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(f)quinoxaline (NBQX). No cell damage was observed with the other three glutamate carrier inhibitors used. It is concluded that increased extracellular glutamate in vivo by disfunction of its transporter is not sufficient for inducing neuronal damage. The neurotoxic effects of dihydrokainate could be explained by direct activation of glutamate postsynaptic receptors, an effect not shared by the other inhibitors used.

Key words: Dihydrokainate - Excitotoxicity - Glutamate uptake inhibitors - Microdialysis - Striatum - L-*trans*-Pyrrolidine-2,4-dicarboxylate

Running title: Glutamate accumulation and excitotoxicity in vivo

Exposure of nerve cells to high concentrations of excitatory amino acids, such as glutamate and aspartate, leads to neuronal degeneration and death (Choi et al. 1987; Manev et al. 1989). Pioneer studies by Olney and colleagues showed that the neurotoxic properties of these amino acids are related to their ability to depolarize neurons, and suggested that their death occurs as a consequence of overexcitation (Olney, 1971; Olney et al. 1971; Rothman and Olney, 1987). Excitatory amino acids interact with at least two groups of receptors. A first group of receptors is coupled to ion channels (iontropic receptors) and is represented by the N-methyl-D-aspartate (NMDA) type and the non-NMDA type. The latter includes the kainate receptor and the α-amino-3-hydroxy-5-methylisoxazolepropionate (AMPA) receptor. A second group is that of metabotropic receptors, which are coupled to the production of the second messengers diacylglycerol and inositol triphosphate. Agonists at the three types of ionotropic receptors can elicit neuronal degeneration both in vivo an in vitro (Choi et al. 1987; Koh et al. 1990; Coyle and Schwarcz, 1976; McGeer and McGeer, 1976; Schwarcz et al. 1983; Foster et al. 1988).

The cytopathological characteristics of the damage observed after the exposure to excitatory amino acids resembles the neuropathological changes which follow brain ischemia (Brown and Brierly, 1972; Van Reempts, 1989; Simon et al, 1984) or prolonged seizure activity (Evans et al. 1984). Moreover, microdialysis studies in vivo have demonstrated that during cerebral ischemia (Benveniste et al. 1984; Globus et al. 1988; Hillered et al. 1989; Butcher et al.

1990; Baker et al. 1991; Graham et al. 1990), hypoglycemia (Sandberg et al. 1986), and cerebral trauma (Fanden et al. 1989), the extracellular concentrations of glutamate and aspartate are highly increased, reaching levels comparable to those which are neurotoxic in cell culture (Choi et al. 1987). Since several studies have shown that antagonists at both the NMDA and non-NMDA receptor subtypes protect against the neuronal damage associated to these neuropathological conditions (Gill et al, 1988; Gill et al. 1992; Bullock et al. 1990; Swan and Meldrum, 1990; Sheardown et al. 1992), it has been suggested that this type of damage occurs through the hyperactivation of glutamate receptors.

Several mechanisms can be responsible for the increase in the glutamate and aspartate extracellular concentrations during ischaemia. One factor could be an exaggerated depolarization-induced release of these amino acids from synaptic terminals, consequent to the increased external potassium concentration that has been observed during ischaemia (Astrup et al. 1977; Hansen et al. 1980; Attwell et al. 1993). Another possibility is that excitatory amino acids accumulate in the extracellular space due to the impairment of the uptake systems responsible for the clearance of glutamate and aspartate from the synaptic cleft (Fagg and Foster, 1983; Fonnum, 1984; Nicholls and Attwell, 1990), as a consequence of the energy failure accompanying ischemia (Pulsinelli and Duffy, 1983).

The purpose of the present study was to test whether the inhibition of

the aspartate/glutamate carrier in vivo might result in increased endogenous extracellular glutamate and aspartate levels, and whether such an increase might be injurious to neurons. Two inhibitors of the glutamate transport were used for this purpose, L-*trans*-pyrrolidine-2,4-dicarboxylate (PDC), which was recently proposed as a potent and selective competitive inhibitor of glutamate uptake, with no activity at the excitatory amino acid receptors (Bridges et al., 1991), and the less potent inhibitor dihydrokainate (DHK). In some experiments two other inhibitors of glutamate uptake were also used: DL-threo-β-hydroxyaspartate and L-aspartate-β-hydroxamate.

MATERIALS AND METHODS

Intrastriatal microinjection of glutamate uptake inhibitors

Male Wistar rats (220-250 g weight) were anesthetized with 3-4% halothane in a 95% O_2/5% CO_2 mixture and placed in a stereotaxic frame with the nose bar positioned at -3.3 mm. The skin was removed to expose the skull and a 1-2 mm diameter hole was drilled (coordinates: A ±0.7 mm from bregma, L ±2.8 mm from midline, and V 4.6 mm from dura, according to Paxinos and Watson, 1982). Four glutamate uptake inhibitors were tested in this experimental paradigm: PDC, DHK, DL-threo-β-hydroxyaspartate and L-aspartate-β-hydroxamate. They were dissolved in 1 M NaOH, adjusted to pH 7.0-7.5 and brought to the desired volume with 10 mM phosphate buffer pH

7.4. The final concentration of glutamate uptake inhibitors was 400 nmol/µl. In some experiments PDC was coinjected either with glutamate (1 µmol/µl) or with glycine (1 µmol/µl). For coinjection, solutions of appropriate concentrations of PDC and either glutamate or glycine were prepared and mixed. Some animals were injected with kainate (10 nmol/µl). Kainate was dissolved in 1 M NaOH, the pH adjusted to 7.0-7.5 and brought to the desired volume with 0.1 M phosphate buffer, pH 7.4. In all cases a one µl volume was injected unilaterally at a rate of 0.5 µl/min via a 27 gauge stainless steel needle connected to a Hamilton syringe, using a microinjection pump (Carnegie CMA/100). After the injection was completed the needle was left in place for two additional minutes, then removed and the skin sutured. Control animals were injected with 0.1 M phosphate buffer pH 7.4. Rats were maintained for 7 days (5 days for DHK or kainate-treated animals, since they did not survive longer) with food and water *ad libitum* and then were either decapitated or perfused for biochemical or histological analysis, respectively.

In order to test the possible protective effects of NMDA and non-NMDA receptor antagonists on the DHK or kainate-induced lesion, some animals received an i.p. injection of MK-801 (1 mg/kg), 30 min before the intrastriatal microinjection of DHK or kainate. MK-801 was dissolved in distilled water at a concentration of 1 mg/ml. Another group of animals was coinjected intrastriatally with DHK (400 nmol/µl) and NBQX (50 nmol/µl). NBQX was dissolved in 0.2 M NaOH, adjusted to pH 7.5-8.5 with HCl and brought to the

appropriate volume with 10 mM phosphate buffer pH 7.4.

Neuronal degeneration

Biochemical evaluation. Degeneration of GABAergic and cholinergic neurons in the striatum was quantitatively determined by measurement of the activity of the marker enzymes L-glutamate decarboxylase (GAD) and choline acetyltransferase (ChAT), respectively. Five to seven days after surgery, rats were decapitated and both striata were dissected and weighted. Each striatum was sonicated (Branson Sonifier 250, setting 2) for 12 sec in 25 vol (wt/vol) cold water. Aliquots were used for the determination of ChAT and GAD activities. ChAT activity was measured according to the radiometric method of Fonnum (1975) with some modifications (López-García et al. 1990). GAD activity was determined by the radiometric method of Albers and Brady (1959) slightly modified (López-García et al. 1990).

Neuronal loss was determined as the percent decrease of ChAT and GAD activities in the injected striatum relative to the contralateral uninjected striatum after subtraction of blank values (Massieu and Tapia, 1994). Statistical comparisons were made using the non-parametric Mann-Whitney U-test, unless otherwise stated.

Histological evaluation. Seven (for PDC) or five (for DHK) days after microinjection, rats were anesthetized and transcardially perfused with 250 ml 0.9% saline followed by 250 ml 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. Brains were removed, left in fixative for additional 24 h and

transferred successively to 10%, 20%, and 30% sucrose (24 h each). Coronal sections (30 µm thick) were obtained in a cryostat, collected on gelatine-coated slides and stained with cresyl violet.

Microdialysis experiments

Recovery of amino acids from standard solutions. Microdialysis cannulas 2 mm long and 0.5 mm diameter (CMA12, Bioanalytical Systems, West Lafayette, IN, USA) were used. Before implantation each probe was flushed with distilled water for 30 min at a flow rate of 5 µl/min. In order to assess the recovery of the amino acids from the dialysis membrane, some dialysis probes were immersed in 1.5 ml of an amino acid standard mixture (1 nmol/10 µl) of aspartate, glutamate, glutamine, glycine, taurine, alanine and γ-aminobutyric acid (GABA), dissolved in a Ringer-Krebs medium containing (in mM): NaCl 118, KCl 4.5, $MgSO_4$ 2.5, NaH_2PO_4 4.0, $CaCl_2$ 2.5, $NaHCO_3$ 25, and glucose 10, pH 7.4. Probes were perfused with Ringer-Krebs medium at a rate of 2 µl/min and 25 µl fractions of perfusate were collected and analyzed by HPLC as described below. The calculated recovery of amino acids was as follows (%): aspartate, 7.2 ± 1.0; glutamate, 6.2 ± 0.6; glutamine, 5.9 ± 1.6; glycine, 11.4 ± 1.0; taurine, 11.1 ± 1.9; alanine, 10.3 ± 0.9; and GABA 9.9 ± 2.3 (n=4).

Implantation of dialysis probes. Male Wistar rats (220-250 g weight) were anesthetized and placed in a stereotaxic frame as described above. Animals were implanted unilaterally in the left striatum with a microdialysis

cannula (coordinates: A +0.7 mm from bregma, L +2.8 mm from midline, and V 6.0 mm from dura, according to Paxinos and Watson, 1982) and maintained under low anesthesia (0.5% halothane) throughout the experiment. The probes were perfused continuously with the Ringer-Krebs solution described above at a flux rate of 2 µl/min using a microinjection pump (Carnegie CMA/100). After a 60 min equilibration period, 25 µl (12.5 min) consecutive fractions of perfusate were continuously collected. After the first three fractions (basal level of amino acids), DHK (50 mM), PDC (1, 25 and 100 mM) or kainate (10 mM) were perfused for the time periods indicated in results. At the end of the experiment the skin was sutured and the animals were maintained for one week for further analysis of striatal ChAT and GAD activities.

Amino acid analysis of striatal dialysates. The 25 µl collected fractions were derivatized with the same volume of *o*-phthalaldehyde (Geddes and Wood, 1984) and 3 min later 20 µl were injected into a HPLC system (Beckman). An ODS column (25 cm x 4 mm internal diameter) was used. The mobile phase was methanol/potassium acetate (0.1 M , pH 5.5) and was run at a rate of 1.5 ml/min in a linear gradient (15 min duration) from 25 to 75% methanol (Geddes and Wood, 1984). The results obtained were compared with standard samples equally processed.

Materials

DHK and PDC were purchased from Tocris Neuramin (Bristol, U.K); DL-threo-β-hydroxyaspartate, L-aspartate-β-hydroxamate and the standard

amino acids from Sigma Chemical Co. (St. Louis MO, U.S.A). 2,3-Dihydroxy-6-nitro-7-sulfamoyl-benzo(f)quinoxaline (NBQX) was generously provided by Dr. L Nordholm from Novo Nordisk A.S., Copenhagen, Denmark.

RESULTS

Behavioral observations

In all the animals tested, intrastriatal microinjection of 400 nmol DHK induced intense ipsilateral barrel rotation, which appeared between 90 and 120 min after surgery. Tonic movements of the forelimbs, chewing, salivation, and intermitent series of wet-dog shakes were also observed. Rats injected with 10 nmol kainic acid showed a similar behavior but barrel rotations were more intense and series of wet-dog shakes were more numerous. Seven out of 10 animals injected with 400 nmol PDC showed only occasional barrel rotations, chewing, tonic movements of the forelimbs and wet-dog shakes. Coinjection of glutamate or glycine (1 mmol) with PDC (400 nmol) did not potentiate the behavioral effects of PDC alone. Three of 4 rats injected with 400 nmol DL-threo-β-hydroxyaspartate and one of seven rats injected with 400 nmol L-aspartate-β-hydroxamate showed also only occasional barrel rotations and tonic movements of the forelimbs.

Neuronal degeneration

As indicated in Methods, neuronal degeneration consequent to the intrastriatal microinjection of the glutamate uptake inhibitors was assessed by

the measurement of ChAT and GAD activities 5-7 days after surgery.

Control ChAT and GAD activities measured in striatal homogenates were, respectively (nmol/mg wet weight/h), 17.5 ± 0.7 and 19.5 ± 2.4 in the buffer-injected striatum and 17.6 ± 0.55 and 19.22 ± 1.8 in the non-injected striatum (n=5). These values are in good agreement with those reported previously (Foster et al. 1988; Massieu and Tapia, 1994).

As shown in Fig. 1, intrastriatal administration of 400 nmol DHK produced a significant reduction of both ChAT (46.6% decrease) and GAD (44.5% decrease) activities relative to the contralateral non-injected striatum. In contrast, injection of 400 nmol PDC produced only a slight decrease in ChAT (23.4%) and GAD (14.9 %) activities (Table 1). Similarly, 400 nmol of DL-threo-β-hydroxyaspartate or L-aspartate-β-hydroxamate produced only small decreases (11-23%) in ChAT and GAD activities. None of these changes was statistically significant as compared to those observed in buffer-injected animals (Table 1).

To test whether PDC could potentiate the neurotoxic effect of glutamate, some animals were coinjected with 400 nmol PDC and 1 μmol glutamate or with PDC and glycine, the well established positive modulator of the NMDA receptor (Johnson and Ascher, 1987). As shown in Table 1, neither of the combined treatments resulted in significant ChAT and GAD changes. Glutamate alone at the dose used was also ineffective, as has been previously reported (McBean and Roberts, 1985).

Protective effects of NMDA and non-NMDA receptor antagonists on DHK-induced lesions. In order to elucidate if DHK-induced neuronal damage was mediated through NMDA receptors, some rats were pretreated with the NMDA receptor antagonist MK-801. As shown in Fig 1, this antagonist protected by 43.0% and 37.5% against the DHK-induced damage, as judged by ChAT and GAD activity decreases, respectively. The protective effect of MK-801 was statistically significant for both enzyme activities ($p < 0.05$).

The possible involvement of non-NMDA receptors in DHK-induced damage was tested by means of the intrastriatal injection of the non-NMDA antagonist NBQX together with DHK (400 nmol). As depicted in Fig 1, NBQX showed a slightly better protection than MK-801, 54.6% and 49.2% for ChAT and GAD activities, respectively. This protective effect of NBQX was statistically significant for both enzyme activities ($p < 0.05$).

Since DHK is a derivative of kainate, it was interesting to compare the neurotoxic effect of these two compounds. Three animals were intrastriatally injected with 10 nmol kainic acid and three were treated i.p. with MK-801 (1 mg/kg) 30 min before injection. As depicted in Fig 1, 10 nmol kainate produced a remarkable decrease (about 80%) in both ChAT and GAD activities. In contrast to the effect of DHK, pretreatment with MK-801 failed to protect against this kainate-induced damage.

For histological analysis, 8 rats were injected with DHK alone, 7 rats were pretreated with MK-801 30 min before DHK, and 8 rats were coinjected

with DHK and NBQX, at the doses mentioned above. As shown in Fig. 2, DHK produced an extensive lesion in all the animals. The characteristic histological pattern of the striatum normally observed in cresyl violet-stained sections was partially disrupted, and numerous very darkly stained pycnotic cells and glial cells were observed around the injection tract and in the surrounding tissue. Very few "normal appearing" cells with clearly observable nucleus were present (Fig 2A). Both NBQX and MK-801 protected against this damage, although not in all rats: 5 out of 8 rats coinjected with NBQX, and 5 out of 7 MK-801-pretreated animals showed the normal histological appearance of the striatum, with normal large cells and clearly defined nucleus, even in close vicinity to the injection site (Fig. 2B and 2C).

In contrast to the effects of DHK, none of the 7 rats microinjected with PDC and studied histologically showed any significant alteration. Micrographs are not shown since they are very similar to Fig. 2D.

Microdialysis experiments

In order to study the possible correlation between the accumulation of glutamate and aspartate in the extracellular space and cell damage induced by the administration of glutamate uptake inhibitors, microdialysis experiments were performed. In these experiments either PDC or DHK were administered through the dialysis probe and the extracellular levels of amino acids were monitored as described in Methods. Seven days after the experiment, neuronal damage was assessed by measuring ChAT and GAD activities in the dissected

striata. DL-threo-β-hydroxyaspartate and L-aspartate-β-hydroxamate were not studied in this experimental paradigm because these compounds produced chromatographic peaks which overlapped with glutamate.

Basal levels of amino acids in collected dialysate perfusates were in good agreement with those reported previously (Young et al. 1988; Millan et al. 1993; Hillered et al. 1989). Mean values obtained were (μM): alanine 0.9 ± 0.19; glutamate 0.70 ± 0.23; glutamine 6.6 ± 1.8; glycine 1.5 ± 0.64; and taurine 1.4 ± 0.14 (n=7). With the methodology employed the basal levels of aspartate and GABA were too low to be reliably measured.

When PDC (25 mM) was perfused through the dialysis probe, extracellular concentrations of aspartate and glutamate rised steeply, reaching values of about 4 μM and 11 μM, respectively (Fig. 3); the glutamate increase was about 12 times its basal levels. PDC induced also increases in the levels of other amino acids, but these were much more discrete: a 5-fold increase in taurine, 2-fold in glutamine and 1.4-fold in alanine. No increments were observed in glycine nor in GABA levels (GABA data not shown). The observed increases in amino acid concentrations were maintained as long as PDC was perfused, except for aspartate and glutamine, which declined slowly (Fig. 3).

Figure 4 shows the peak concentrations of the six amino acids affected by PDC, as a function of the inhibitor dose (1, 25 and 100 mM). The accumulation of aspartate, glutamate and taurine in the extracellular space followed a similar

dose-response curve. In contrast, the comparatively small rises of glutamine, glycine and alanine produced by 1 mM PDC did not increase further with higher concentrations of the drug (Fig. 4).

Administration of DHK (50 mM) through the dialysis probe resulted in increments in the extracellular concentrations of aspartate and glutamate identical to those observed during PDC perfusion (Fig 3). However, large increments in the extracellular levels of all measured amino acids were also found. The increases over basal levels were 13-fold for taurine, 6-fold for alanine and glycine, and 2-fold for glutamine. GABA levels were also increased by DHK, reaching a maximum of 13.6 ± 5.8 pmol/10 µl (data not shown). Similarly to PDC, elevated concentrations of all amino acids were maintained as long as DHK was perfused. However, it is worth to note that increments in alanine and glycine concentrations were slow and apparently did not reach a plateau up to the duration of the experiment.

ChAT and GAD activities were measured in striatal tissue from the rats perfused with PDC and DHK, 7 days after surgery. Animals perfused with either 1, 25 or 100 mM PDC during 2 h, or with 25 mM PDC during 4 h, showed no decrease at all in ChAT or GAD activities as compared to the contralateral tissue. For example, for the 4 rats perfused with 25 mM PDC during 4 h, ChAT activity values were 22.0 ± 4.4 and 19.1 ± 6.6 nmol/mg wet tissue/h in ipsilateral and contralateral striatum, respectively; corresponding values for GAD were 12.9 ± 1.7 and 9.79 ± 1.45. The differences between the ipsilateral and

contralateral tissue was not statistically significant for either GAD or ChAT activity (Student's *t* test).

In contrast to the lack of neurotoxicity of PDC, rats perfused with 50 mM DHK for 1.5 h showed a decrease of 20.0% ± 4.7 and 26.9% ± 4.9 (n = 4-6) in ChAT and GAD activities, respectively, in the perfused striatum. The differences between the ipsilateral and the contralateral tissue were statistically significant for both ChAT ($p < 0.005$) and GAD ($P < 0.05$) activities (Student's *t* test).

For comparison with DHK, the effect of kainate on the accumulation of extracellular amino acids and on ChAT and GAD activities was studied. For this purpose, 4 rats were perfused with 10 mM kainate for 90 min. No increases in any of the amino acids studied were found, except for taurine, which was elevated 13-24-fold (data not shown). One of the rats died 3 days after the surgery, and the other three showed at day 5 large decreases in both ChAT (92.2% ± 3.7) and GAD (66.8% ± 8.7) activities, relative to the contralateral tissue. Since increases in extracellular levels of glutamate after perfusion of the striatum with kainate have been reported (Young et al., 1988; Arvin et al., 1994), these experiments were repeated with lower concentrations of kainate (0.2, 1 and 5 mM). No increase in glutamate extracellular concentration was observed under any of these experimental conditions, whereas the stimulation of taurine release was always observed (data not shown).

DISCUSSION

Two experimental paradigms were followed in the present work in order to relate the concentration of extracellular glutamate with the production of neuronal damage in vivo. One was the perfusion into the striatum of inhibitors of the aspartate/glutamate carrier through microdialysis probes, coupled to the determination of the extracellular concentration of glutamate; the other was the acute striatal microinjection of the inhibitors.

Of the four glutamate uptake inhibitors used in the present study (DHK, PDC, DL-threo-β-hydroxyaspartate and L-aspartate-β-hydroxamate) only DHK reliably induced a lesion of cholinergic and GABAergic striatal neurons, both after microinjection and after microdialysis perfusion. Cholinergic and GABAergic neurons were damaged to a similar extent by administration of DHK, whereas the other three inhibitors produced decreases in ChAT and GAD activities not significantly different from those observed after vehicle injection (Fig. 1 and Table 1). McBean and Roberts (1985) reported decreases of 30% in ChAT activity and in sodium-dependent [^{3}H]GABA uptake in the rat striatum, 14 days after intrastriatal injection of 170 nmol DL-threo-β-hydroxyaspartate. One possible explanation for this discrepancy is the different post-injection times used before tissue damage was evaluated, since we allowed only 7 days. However, this is not very likely since we have previously

found in similar experimental paradigms that striatal damage mediated by excitatory amino acid receptor agonists is developed 5-7 days after the surgery (Massieu and Tapia, 1994).

The neurotoxic effect of DHK might be due to activation of both NMDA and non-NMDA receptors, since both MK-801 and NBQX partially protected striatal tissue against the damage induced DHK (Figs. 1 and 2). As discussed below, it is improbable that activation of these receptors was due to the glutamate or aspartate accumulated in the extracellular space as a consequence of DHK application, since a similar increase produced by PDC was ineffective (Fig. 3). It seems more likely that DHK directly activated these postsynaptic receptors. In fact, it has been previously reported that DHK can inhibit, although weakly (500 times less potently than kainate), the sodium-independent [^{3}H]-kainic acid binding to rat brain membranes (Johnston et al. 1979).

To our knowledge there are no data on an agonist action of DHK on NMDA receptors. However, the significant protective effect of MK-801 against DHK-induced damage suggests that such action is possible. The fact that the same concentration of MK-801 failed to protect against the neurotoxic effects of a much smaller dose of kainate (Fig. 1) favors the hypothesis that DHK behaves in vivo as a weaker and less selective agonist of glutamate receptors, as compared to kainate. The latter would be acting mainly through kainate/AMPA receptors (Massieu and Tapia, 1994), whereas the effect of the

former would be mediated by activation of both kainate/AMPA and NMDA receptors.

It is interesting that, in contrast to the effect of DHK and PDC, under our experimental microdialysis conditions no significant rise in the extracellular concentration of glutamate and aspartate was found when kainate was intrastriatally perfused. Increases in the concentration of these two amino acids induced by administration of kainate through microdialysis probes have been previously reported in the rat striatum (Young et al., 1988; Arvin et al., 1994). This discrepancy might be due to the differences in the design of the probes used and/or in the perfusion rate. In the former study the perfusion rate was 7.5 µl/min, nearly four times higher than in the present work, whereas in the latter a a long transverse dialysis probe, including both striata, was used. In any case, we must emphasize that our results with PDC, DHK and kainate were obtained using identical experimental conditions and were highly reproducible (including elevations in taurine with kainate). Furthermore, in other experiments using the same experimental paradigm, perfusion with depolarizing concentrations of K^+ have reliably shown notable increases in extracellular taurine, aspartate and glutamate (manuscript in preparation).

Taken together, the results discussed above suggest that the mechanism of neurotoxicity of DHK is different from that of kainate. Activation of postsynaptic kainate/AMPA receptors seems to be crucial for the neurotoxic

action of kainate, with much less participation of activation of NMDA receptors. It has been suggested that glutamate directly released by kainate from axon terminals of cortical neurons might be important for its neurotoxic effects in the striatum (Biziére and Coyle, 1978; McGeer and et al. 1978; McBean and Roberts, 1985; Young et al., 1988). However, our present observations showing a nil effect of kainate on the extracellular levels of glutamate and lack of protection by MK-801 against kainate-induced neurotoxicity, together with the contrasting significant protection by NBQX (Massieu and Tapia, 1994), suggests that direct activation of postsynaptic AMPA/kainate receptors by kainate might be crucial for its neurotoxic actions.

The most striking observation of the present study was the identical notable increasing effect of DHK and PDC on the levels of extracellular glutamate and aspartate, which was not correlated with the neurotoxic action of the two inhibitors: DHK was clearly neurotoxic whereas PDC showed no toxicity. The effect of PDC on the accumulation of extracellular amino acids was much more specific for these two excitatory amino acids than that of DHK. This result was not surprising, since among the drugs used here PDC is the most potent and selective inhibitor of the glutamate/aspartate transporter, while DHK is the weakest and less selective (Bridges et al. 1991; Johnston et al., 1979; Robinson et al., 1991). This suggests that the main, if not the only effect of PDC in vivo is to block glutamate and aspartate uptake, whereas DHK, as already discussed, probably exerts several actions besides inhibiting the

transporter, including a direct activation of the glutamate receptors. The difference in PDC and DHK toxicity cannot be attributed to a requirement of increased extracellular glycine, which was observed with DHK but not with PDC, since coinjection of the latter and glycine did not result in toxicity (Table 1).

The substantial accumulation of both glutamate and aspartate extracellular levels produced by PDC was long lasting (for more than 2 h) and reached concentrations similar to those reported during neuronal damage subsequent to ischemia (Globus et al. 1988; Hillered et al. 1989; Baker et al. 1991). A puzzling question is why no damage was observed under these conditions. A possible explanation might be that the extracellular glutamate and aspartate accumulated as a consequence of the blocking action of the transporter by PDC does not reach concentrations in synaptic sites sufficiently high to overactivate glutamate postsynaptic receptors. In cultured organotypic spinal cord, long-duration infusion of glutamate uptake inhibitors leads to neurotoxicity (Rothstein et al. 1993), and in cultured hippocampal cells glutamate toxicity is potentiated by short exposure to PDC (Robinson et al. 1993). It is conceivable that, differently to the *in vivo* situation, in tissue cultures the external glutamate accumulated may readily reach high concentrations close to postsynaptic glutamate receptors.

From the present results it can be concluded that substantial elevation of endogenous glutamate and aspartate in the extracellular space resulting

from blocking of their uptake system in vivo, even when sustained for several hours, appears not to be sufficient for inducing neuronal degeneration in the striatum. Additional factors that may facilitate an overactivation of the glutamate receptors by the excess glutamate, for example the energy depletion occurring during ischemia or the hyperexcitation present during seizures, seem to be necessary to induce neuronal damage.

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TABLE 1. *Lack of significant neuronal damage after the microinjection of glutamate uptake inhibitors, as assessed by changes of ChAT and GAD activities; glutamate or glycine were coinjected with PDC, as described in Methods*

	% Change in enzyme activity, relative to the contralateral striatum	
	ChAT	GAD
Vehicle (buffer)	- 4.7 ± 9.7 (5)	+ 1.8 ± 9.0 (5)
PDC	- 23.4 ± 6.9 (10)	- 14.9 ± 11.4 (10)
PDC + glutamate	- 10.9 ± 7.5 (5)	- 6.4 ± 9.6 (5)
PDC + glycine	+ 1.7 ± 3.0 (4)	+ 0.4 ± 6.8 (4)
DL-threo-β-hydroxy-aspartate	- 19.2 ± 7.9 (4)	- 23.2 ± 8.3 (4)
L-aspartate-β-hydroxamate	- 11.3 ± 11.2 (7)	- 17.2 ± 13.7 (7)
Glutamate	- 16.7 ± 8.6 (7)	+ 8.8 ± 8.5 (7)

Enzyme activities were measured 7 days after treatment. Mean values ± SEM for the number of rats shown in parentheses. None of the values was significantly different from the control injected with vehicle.

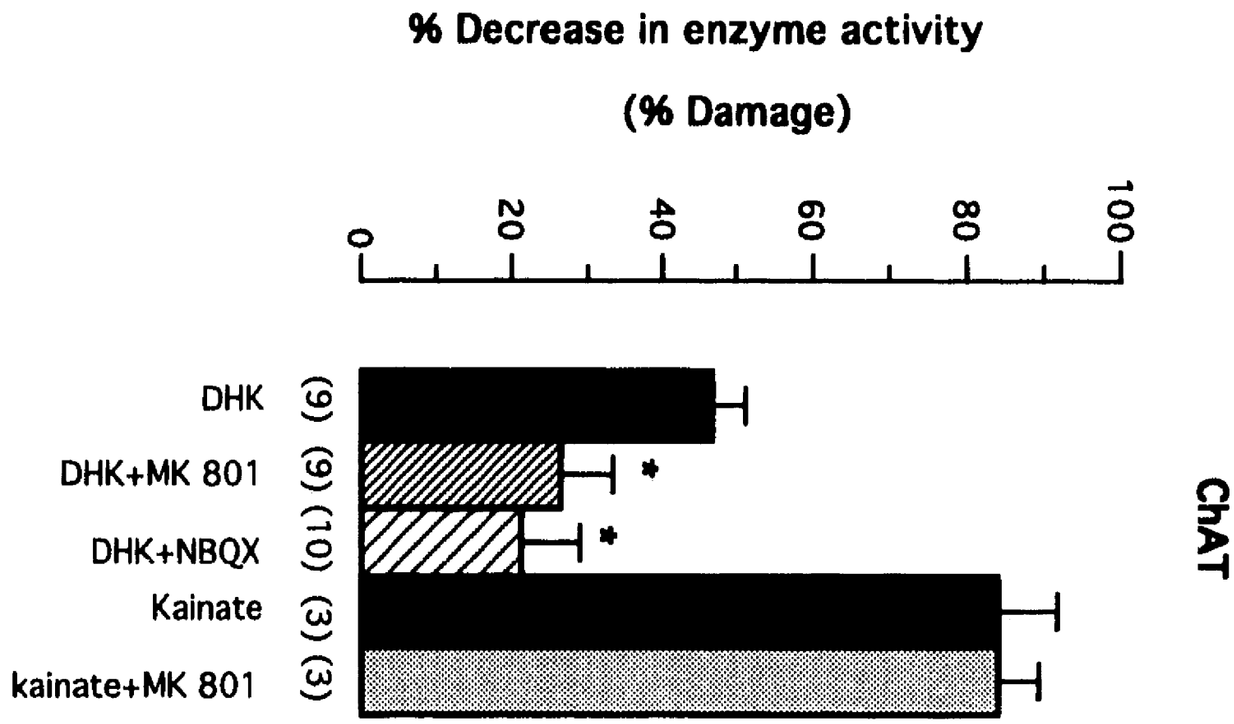
FIGURE LEGENDS

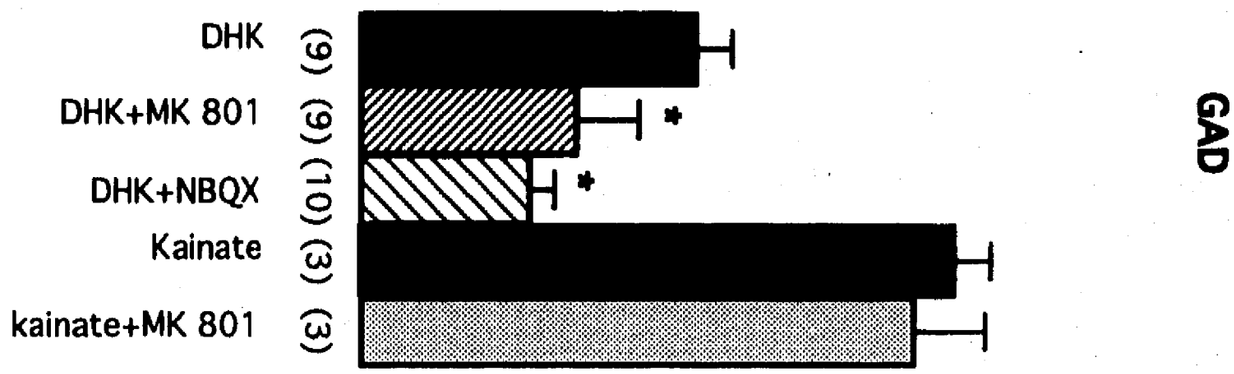
Fig 1. Neuronal damage induced by the intrastriatal microinjection of DHK (400 nmol) and kainate (10 nmol), assessed by decrease in ChAT and GAD activities, 5 days after injection. As indicated below the corresponding bar, one group of rats was pretreated i.p. with MK-801 (1 mg/kg) 30 min before administration of either DHK or kainate, and another was coinjected in the striatum with DHK (400 nmol) and NBQX (50 nmol). Damage is expressed as percent decrease of enzyme activity in the injected striatum relative to the contralateral tissue. Mean values ± SEM for the number of animals indicated in parentheses. The asterisk indicates significant differences ($P < 0.05$) as compared to DHK alone (Mann-Whitney U-test).

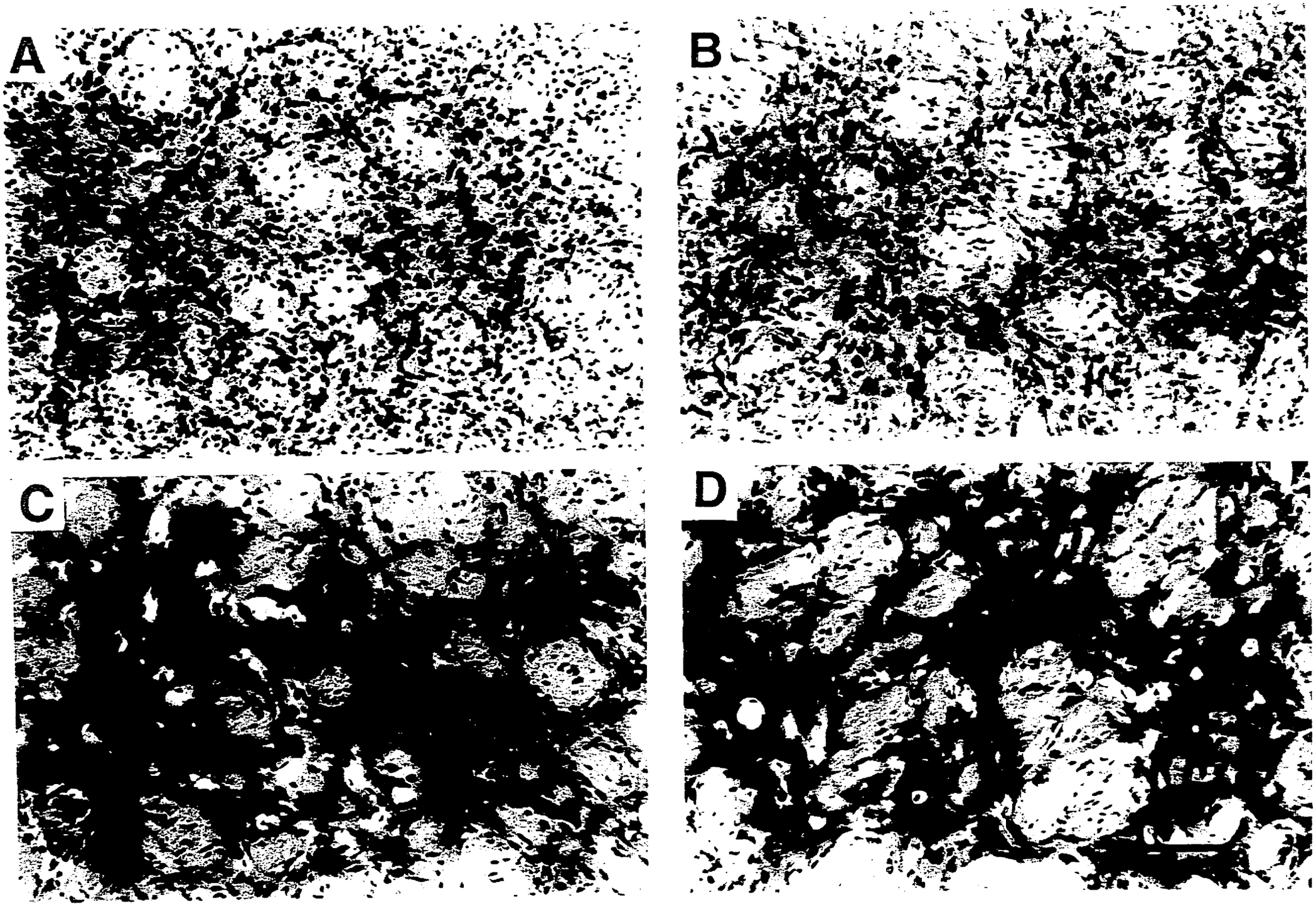
Fig. 2. Histological damage produced by DHK when injected in the rat striatum in vivo and its protection by MK-801 and NBQX. (A) Photomicrograph showing the striatum of a rat injected with DHK (400 nmol). Note the absence of normal appearing cells, which were substituted by pyknotic and glial cells. (B) Protective effect of NBQX (50 nmol) coinjected with DHK. Large cells with well defined nucleus can be seen. Five out of 8 animals were protected by NBQX. (C) Protective effect of i.p. MK-801 (1 mg/kg 30 min before DHK). Numerous normal neurons are evident. Clear protection was observed in 5 out of 7 rats. (D) Contralateral striatum of the same animal showed in (C). Scale bar = 100 μm.

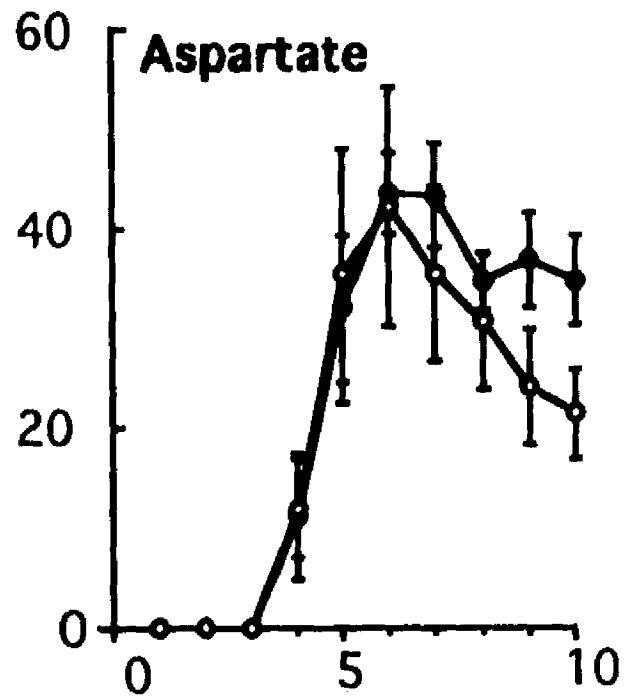
Fig 3. Effect of DHK (50 mM) and PDC (25 mM) on the extracellular concentration of endogenous amino acids in the striatum. Microdialysis probes were implanted and perfused with Ringer-Krebs solution at a rate of 2 µl/min. After a 60 min equilibration period, three 25 µl fractions were collected in order to determine basal levels of amino acids. After this period (end of fraction 3), either DHK (o) or PDC (•) was perfused through the probes during the rest of the experiment and fraction collection was continued. Mean values ± SEM for 7 rats.

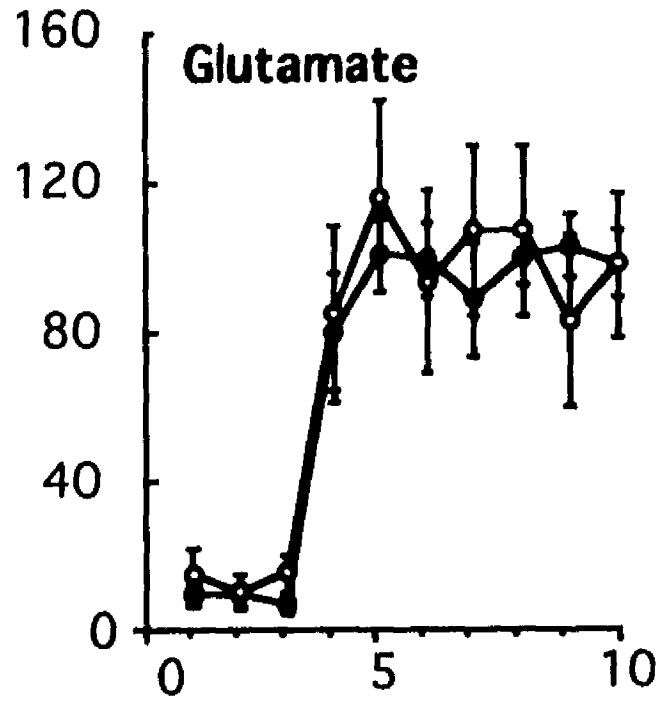
Fig. 4. Peak extracellular concentration of endogenous amino acids after perfusion of different concentrations of PDC through microdialysis probes. Experimental details as in Fig. 3. The ordinate values refer to the peak concentration obtained in a single fraction (fraction 5 or 6, see Fig. 3). Mean values ± SEM for the number of animals indicated in parentheses below the PDC concentration values.

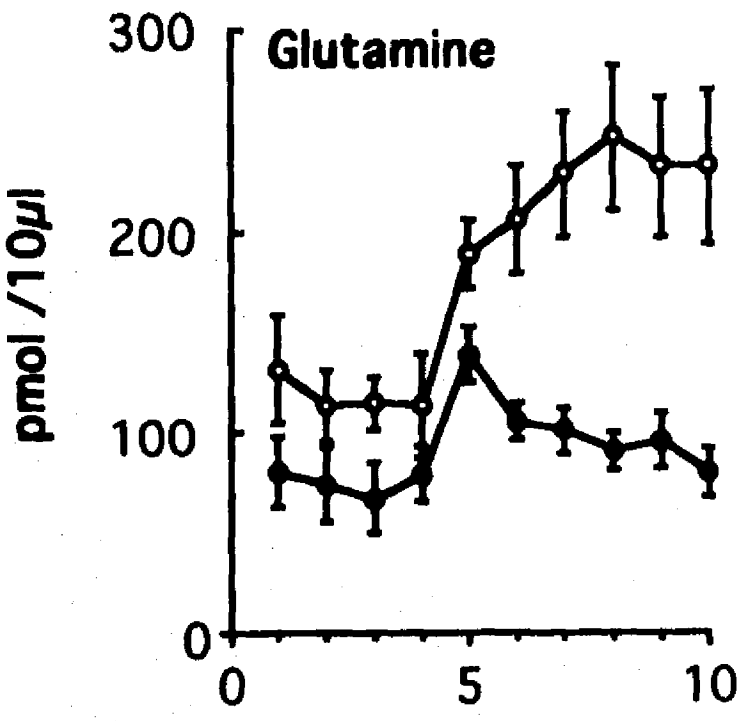

% Decrease in enzyme activity
(% Damage)
0
20
40
60
80
100
DHK (9)
DHK+MK 801 (9)
DHK+NBQX (10)
Kainate (3)
kainate+MK 801 (3)
*
*
ChAT

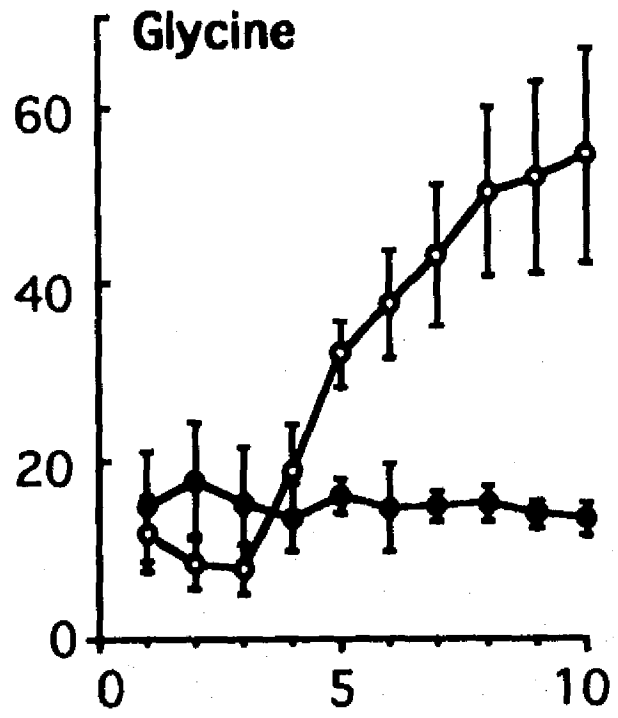

DHK (9)
DHK+MK 801 (9)
DHK+NBQX (10)
Kainate (3)
kainate+MK 801 (3)
*
*
GAD

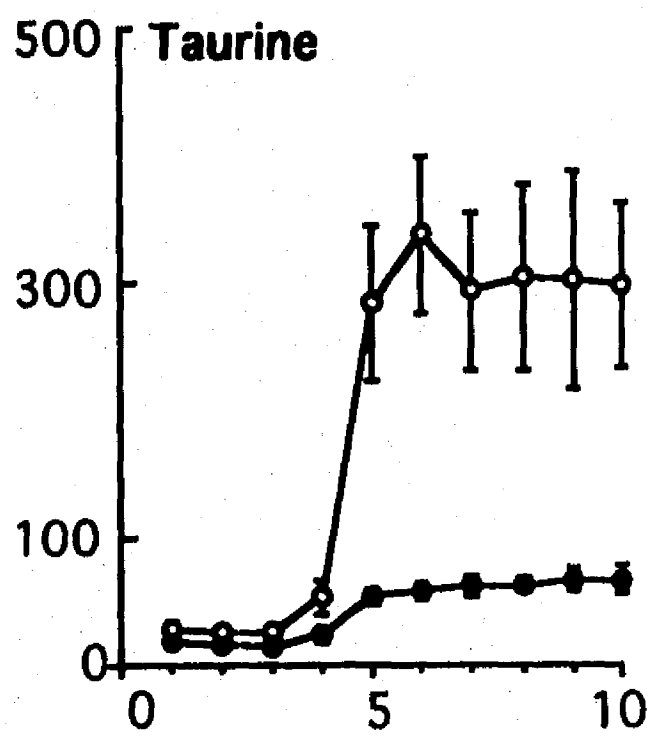

A
B
C
D

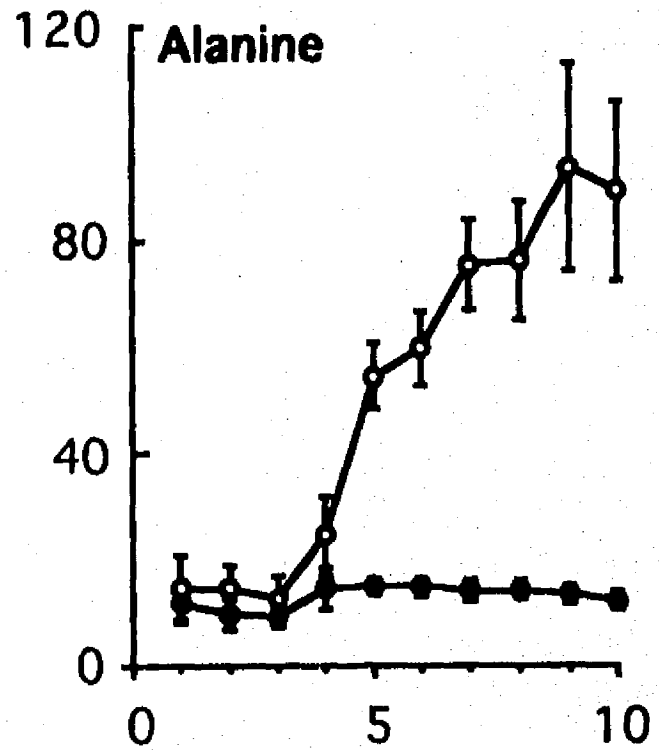




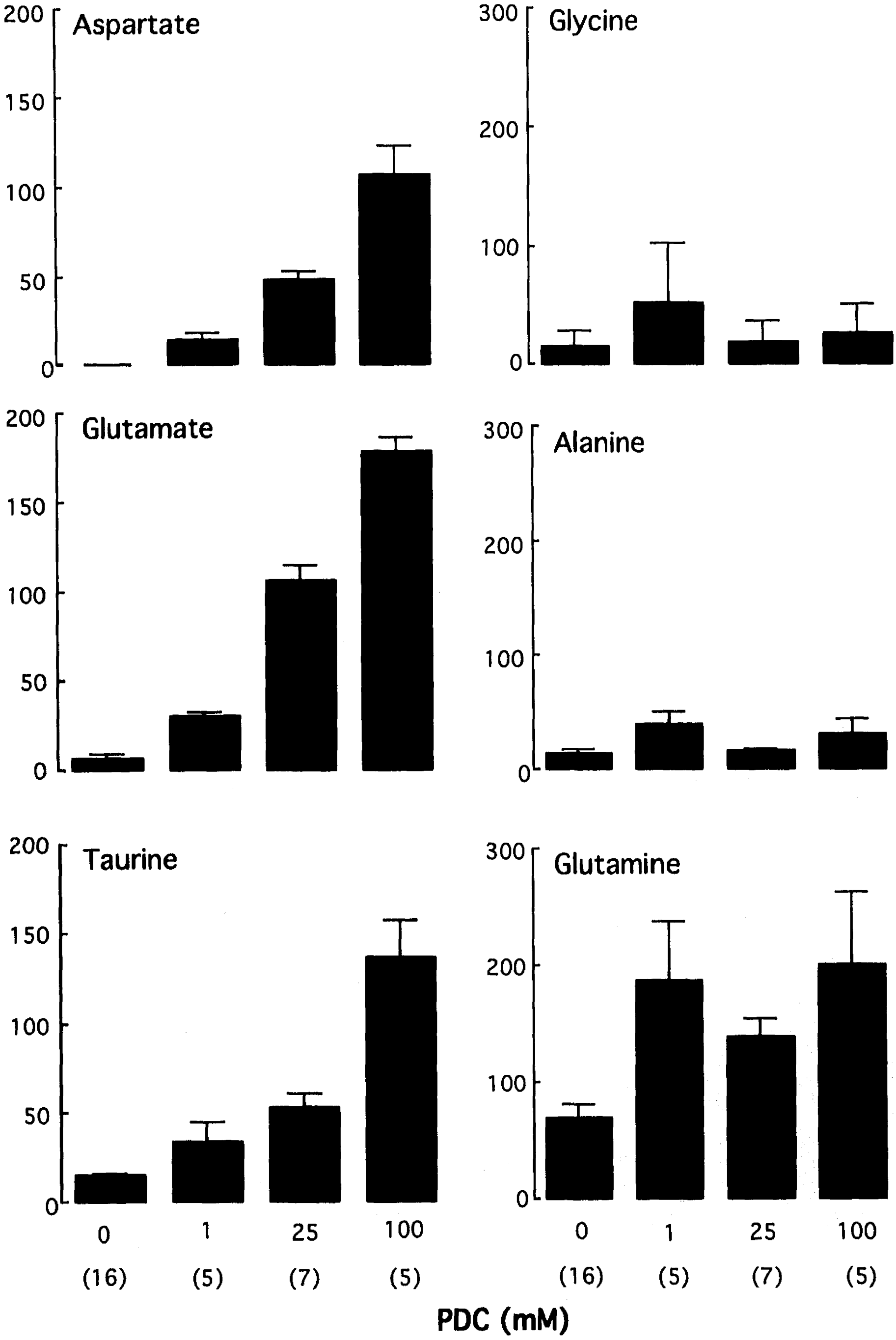








Fraction number


Aspartate
Glycine
Glutamate
Alanine
Taurine
Glutamine
pmol/10 µl
200
150
100
50
0
300
200
100
0
0
1
25
100
(16)
(5)
(7)
(5)
PDC (mM)

FALTA PAGINA

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III DISCUSION GENERAL

En cada uno de los trabajos presentados se han discutido de manera específica los resultados obtenidos, por lo que en las siguientes líneas se hará una discusión global del trabajo en su conjunto. Se discutirán lss contribuciones del trabajo al conocimiento de los posibles mecanismos que dan lugar a la neurodegeneración por excitotoxicidad, así como algunas estrategias de neuroprotección de posible relevancia clínica. Los trabajos presentados en la tesis aportan principalmente tres contribuciones: (1) Antagonistas competitivos de los receptores NMDA (CGP 37849) protegen considerablemente contra la neurodegeneración producida por excitotoxicidad *in vivo* al administrarse hasta dos horas después de iniciada la lesión. (2) El NBQX, que es un antagonista de los receptores no-NMDA, presenta potencialidad neuroprotectora de las neuronas del estriado de la rata contra la neurodegeneración producida por la administración de agonistas glutamatérgicos en esta región *in vivo.* (3) La acumulación de glutamato extracelular por inhibición de sus sistemas de recaptura no es suficiente para producir daño excitotóxico en el estriado de la rata *in vivo.*

Mecanismos de excitotoxicidad. Los resultados del primer trabajo están de acuerdo con las observaciones *in vitro* (descritas en la revisión bibliográfica que se presenta al principio de la tesis) que indican que la muerte celular por excitotoxicidad es un proceso retardado que requiere de varias horas (24 en cultivo) para completarse (Choi 1987, Rothman et al. 1987). Los datos presentados indican que es posible interrumpir el proceso excitotóxico y evitar la neurodegeneración al administrar antagonistas de los receptores NMDA después de 2 h (neuronas

GABAérgicas) o incluso 6 h (neuronas colinérgicas) de iniciada la lesión. Esto puede reflejar dos cosas: 1) que la administración de los antagonistas lo que está interrumpiendo en realidad es la propagación de la lesión (propagación de la excitación) de un foco central, constituído por las células que recibieron la mayor parte del agente excitotóxico y que están destinadas a morir, hacia las células que se encuentran en la periferia y que es posible rescatar, o 2) que el proceso de muerte neuronal es un proceso lento que requiere de la activación de varias cadenas metabólicas para su consumación. Esta última hipótesis predice que la activación de dichas cadenas que darían lugar a la muerte depende de la activación constante de los receptores glutamatérgicos, ya que los antagonistas de los receptores NMDA actúan a este nivel. Estas dos hipótesis no son mutuamente excluyentes, pues existen algunas evidencias de que es posible interrumpir el proceso excitotóxico en pasos ulteriores a la activación del receptor glutamatérgico, como es a través de la inhibición la activación de la fosfolipasa A$_2$, de la proteína cinasa C, de la xantina oxidasa y de la óxido nítrico sintasa (ver sección de mecanismos de excitotoxcicidad, p. 16).

La evaluación del daño por determinación de la actividad de enzimas neuronales, como se hizo en este estudio, no nos permite conocer los límites del área lesionada. Un estudio histológico detallado sería muy útil para conocer si la administración post-lesión de los antagonistas protege del daño a las células cercanas al sitio de inyección o sólo a aquellas que están alejadas de éste. La primera hipótesis mencionada en el párrafo anterior, predice que la propagación de la lesión dependería de la liberación de glutamato por las neuronas inicialmente

excitadas por el agonista o por escape de glutamato de neuronas que están ya parcialmente dañadas. Estas dos consideraciones se han argumentado para explicar la neurodegeneración retardada *in vitro.* De acuerdo a la hipótesis formulada por Choi (ver Choi,1988), en neuronas corticales cultivadas el establecimiento de un circuito de retroalimentación excitatora podría explicar la neurodegeneración retardada: al activarse los receptores postsinápticos por la exposición inicial de las neuronas al glutamato o a sus análogos, hay despolarización y por ende liberación de glutamato endógeno por las neuronas corticales glutamatérgicas presentes en el cultivo. El glutamato liberado, a su vez despolariza a las células adyacentes y éstas liberan glutamato estableciéndose una retroalimentación. Además, la fuga de glutamato de las células inicialmente dañadas contribuye al incremento de glutamato en el espacio extracelular, y al igual que la liberación de enzimas proteolíticas de las células dañadas, a la neurogegeneración. Si este mecanismo ocurriera *in vivo* en el estriado, sería posible predecir que la administración post-lesión de antagonistas no-NMDA (como el NBQX) también protegerían contra el daño inducido por el ácido quinolínico, ya que en el estríado están presentes también los receptores a AMPA y kainato, y el glutamato al liberarse puede actuar a través de todos sus subtipos de receptores. Sin embargo, al evocar un mecanismo similar en el estriado, tendría que considerarse que la mayor parte de la liberación de glutamato en este núcleo proviene de las terminales corticales ya que la mayoría de las interneuronas son GABAérgicas o colinérgicas, pero no glutamatérgicas (ver Wilson 1990). De aquí que la fuga de glutamato por células parcialmente dañadas probablemente no es un factor tan determinante en la neurodegeneración retardada en el estriado, como en

estructuras cerebrales que cuentan con grandes poblaciones de células glutamatérgicas intrínsecas. Por otra parte, si existe una liberación contínua de glutamato después de la aplicación inicial del agonista, ésta tendría que provenir de las terminales corticales. Estudios previos han demostrado que el efecto tóxico del kainato en el estriado depende de la presencia de la vía córtico-estriatal (Biziére y Coyle, 1978; McGeer et al. 1978) y aunque no se ha encontrado una explicación clara a este fenómeno, se ha sugerido la presencia de receptores presinápticos glutamatérgicos en las terminales corticales modulables por el kainato, que al activarse incrementan la liberación de glutamato. Es interesante mencionar que si bien, los aminoácidos excitadores tienen efectos neurotóxicos en neuronas estriatales cultivadas, éstos se potencian al co-cultivarlas con neuronas corticales (Galarraga et al. 1990).

Otra posibilidad, que explicaría la activación prolongada de los receptores glutamatérgicos *in vivo*, es que el agonista administrado exógenamente permanezca en el tejido por un tiempo lo suficientemente largo que sobrepase el periodo crítico, más allá del cual el proceso de muerte celular es irreversible. La administración de los antagonistas antes de este período podría todavía rescatar a las células de la muerte. Bakker y Foster (1991) realizaron un estudio que favorece esta última hipótesis, pues demostraron que después de administrar ácido quinolínico radiactivo éste permanece en el tejido en concentraciones los suficientemente elevadas para producir daño aún después de 5 h. Si este es el mecanismo que explica la excitotoxicidad retardada *in vivo*, se puede predecir que la protección post-lesión debe ser mayor al administrar un antagonista específico del excitotóxico aplicado (en

este caso un antagonista del receptor NMDA) que por la administración de otros antagonistas (en este caso antagonistas no-NMDA).

Los resultados presentados en la tesis demuestran que la activación de los tres subtipos de receptores glutamatérgicos (kainato>AMPA>NMDA>glutamato) produce neurodegeneración si se administran directamente en el estriado de la rata *in vivo.* Estas observaciones predicen que el glutamato al acumularse en el espacio extracelular es capaz de inducir la muerte celular por activación de cualquiera de sus receptores, si alcanza concentraciones lo suficientemente elevadas. Sin embargo, la administración directa de glutamato en el estriado no llega a producir degeneración aún cuando se apliquen concentraciones muy altas. La administración de 1 M de glutamato en el estriado de la rata *in vivo* produce un decremento de únicamente el 16% en la actividad de la ChAT y no modifica la actividad de la GAD (tercer trabajo, tabla 1), mientras que la administración de 200 mM de ácido quinolínico (primer trabajo, Fig. 3) o NMDA (segundo trabajo, Fig. 1), 50 mM de AMPA y 10 mM de kainato (segundo trabajo, Fig. 1), producen decrementos del 70% al 80 % en la actividad de ambas enzimas. Se ha argumentado que la incapacidad del glutamato para producir neurodegeneración en estas condiciones se debe a que es rápidamante capturado por las neuronas y las células gliales a través de sus sistemas de transporte. Sin embargo, en nuestras manos no fue posible obtener daño celular al administrar glutamato en presencia de L-*trans*-pirrolidin-2,4-dicarboxilato (PDC), que es un potente inhibidor de la recaptura de glutamato (tercer trabajo, tabla 1). Este resultado señala que el posible efecto tóxico del glutamato *in vivo* no está influenciado por su sistema de recaptura. Además en cultivo de células estriatales, en

donde la población de células gliales es mucho menor, el glutamato es también menos potente que otros agonistas glutamatérgicos, y se mantiene el mismo orden de potencia neurotóxica que *in vivo* : Kainato>QUIN>glutamato.

Sorprendentemente, una elevación del glutamato extracelular hasta de 12 veces sobre los niveles basales (alcanzando concentraciones de 11 µM), debida a la administración de 25 mM de PDC, no fue suficiente para producir degeneración de las células estriatales, aún manteniendo los niveles de glutamato elevados en esta magnitud por períodos hasta de 4 h. Este dato es interesante ya que se ha sugerido que la neurodegeneración subsecuente a un proceso isquémico se debe a la elevación de los niveles extracelulares de glutamato, y se ha demostrado mediante sistemas de colección y detección de aminoácidos muy similares al utilizado en este estudio (membranas de microdiális y detección por HPLC), que durante períodos isquémicos la concentración de glutamato extracelular puede elevarse desde 5 hasta 80 µM dependiendo del modelo de isquemia utilizado y las condiciones de experimentación (Benveniste et al. 1894; Globus et al. 1988; Baker et al. 1991; Fanden et al. 1989; Hillered et al. 1989; McCulloch et al. 1991).

La ausencia de daño neuronal a pesar de que en nuestros experimentos el glutamato se acumula en niveles similares a los descritos durante la isquemia, y que además esta elevación se mantuvo por períodos prolongados, puede deberse a varias causas:

(1) Es posible que el efecto tóxico del glutamato durante la isquemia sea potenciado por la depleción energética subyacente a este fenómeno. Cabe pensar, que las células al estar sometidas a un estado de estress se vuelvan vulnerables a la

toxicidad por glutamato. Además, en modelos de isquemia transitoria (isquemia global) interviene un factor muy importante que es el de la reperfusión después del período isquémico. La reoxigeneción súbita del tejido isquémico puede dar lugar a la producción de radicales libres, y esto desde luego contribuiría al daño neuronal. En este sentido, puede considerarse que el estado metabólico de las células puede ser un factor crucial para que ocurra el daño irreversible. En nuestros experimentos, la inhibición de los sistemas de recaptura del glutamato quizás no altere el estado basal de las neuronas, o no las lleve a un estado tal (posiblemente de estress metabólico), que las vuleva vulnerables a la toxicidad por glutamato como en el caso de la isquemia.

(2) Alternativamente, es posible el glutamato acumulado *in vivo*, en nuestras condiciones experimentales no llegue a ser suficiente para producir muerte neuronal. El efecto tóxico de los aminoácidos excitadores es variable en las distintas regiones cerebrales, y en las distintas preparaciones. Por ejemplo, en neuronas corticales cultivadas el glutamato es un potente neurotóxico y su efecto es dependiente de la dosis y el tiempo de exposición. Una exposición de 5 min a una concentración de 500 μM es suficiente para dañar a la mayoría de las neuronas y se calcula un ED_{50} entre 50 y 100 μM (Choi et al. 1987). Sin embargo, se ha descrito que concentraciones menores de los agonistas glutamatérgicos, requieren de períodos más prolongados de exposición para inducir el daño. Por ejemplo en rebanadas de hipocampo de ratas adultas 10 μM de NMDA induce degeneración del 50% de las neuronas con una exposición de 90 min (Siman y Card, 1988). Con exposiciones de 24 h se han calculado EC_{50s} de 20 μM para el kainato y 4 μM para el AMPA, en cultivos de

neuronas corticales (Koh et al. 1990a). E incluso, es necesario incubar a las células nerviosas durante días en presencia del ácido quinolínico para producir degeneración (Kim y Choi, 1987). Tomando en cuenta estas observaciones, es posible que una concentración de 10 µM de glutamato durante 4 h *in vivo* no sea suficiente para inducir muerte celular. Sin embargo, esto es poco probable ya que se ha demostrado que exposiciones cortas de 5 min a kainato, quinolinato y glutamato son suficientes para producir un xxxxx% de muerte neuronal en cultivo de células estriatales (Galarraga et al. 1990). En este sentido sería interesante investigar si la administración crónica de PDC durante varios días es capaz de producir neurodegeneración.

(3) Alternativamanete, como se discute en el tercer manuscrito, es posible que aunque la concentración extracelular de glutamato aumente considerablemente, quizás ésta no llege a ser lo suficientemente alta en los sitios donde se encuentran sus receptores y por ende no tenga efectos tóxicos. En este sentido, es interesante mencionar que en neuronas cultivadas de corteza cerebral nosotros hemos observado en experimentos preliminares, que el PDC ejerce efectos tóxicos dependientes de la dosis y del tiempo de exposición. Una concentración de 500 µM de PDC incubado durante 6 h es capaz de producir degeneración de aproximadamente el 50% de las células (datos no publicados). Es posible predecir que en un sistema abierto como es el cultivo, la posibilidad de que el glutamato acumulado extracelularmente alcance sus receptores es mayor que con respecto a una situación *in vivo*. Sin embargo, como se mencionó anteriormente (P. 46 primer párrafo), en los cultivos de neuronas corticales el efecto tóxico del glutamato puede

verse incrementado por la liberaciónn contínua de glutamato de las células glutamatérgicas presentes en el cultivo, o por lisis de las células dañadas (P. 46 primer párrafo), mientras que *in vivo* este elemento podría no ser tan determinante.

(4) Además de las diferencias mencionadas entre las condiciones *in vivo* y en cultivo, debe considerarse que en un sistema más íntegro como es el animal *in vivo,* existen una serie de factores intrínsecos que podrían atenuar el posible efecto tóxico del glutamato. Por ejemplo, la presencia de conexiones inhibitorias (GABAérgicas) en el animal íntegro podría contrarestar el efecto excitador del glutamato sobre las neuronas estriatales, evitando una activación prolongada de los receptores glutamatérgicos.

(5) Por otra parte, hay que considerar que los transportadores de glutamato presentes en las células estriatales (ya sea gliales o neuronales) puéden ser diferentes a los de las células corticales en el cultivo y posiblemente su farmacología sea tan bien diferente, de aquí que no se puede excluir que en el estriado existan otros sitios de recaptura no reconocidos por el PDC cuya contribución pudiera ser más determinante para contrarestar el efecto tóxico del glutamato. Este último argumento es sin embargo discutible ya que las concentraciones de glutamato se elevaron considerablemente.

(6) Por último, los presentes resultados comprueban la previamente observada falta de toxicidad del glutamato en el estriado de la rata *in vivo* , a diferencia del potente efecto tóxico de otros agonistas, especialmente el kainato. Como se mencionó anteriormente, existen datos que indican que la presencia de las terminales gluatamatérgicas córtico-estriatales es determinante para que el kainato ejerza sus

efectos neurotóxicos. Se ha sugerido que la presencia de un receptor presináptico en dichas terminales modulable por el kainato, al activarse incrementaría la liberación de glutamato, y poe ende contribuiría al efecto tóxico. Es posible, que la falta de toxicidad del glutamato *in vivo,* se deba a que presenta poca afinidad por dicho receptor, y por tanto sea incapaz de establecer este circuito. Es quizás la interacción entre el compuesto excitotóxico y las terminales corticales lo que permite que la neurona postsináptica se vuelva vulnerable a un proceso excitotóxico.

Estrategias de neuroprotección. El primer trabajo demuestra que es posible prevenir la muerte de las células est riatales a través de tratamientos post-lesión con antagonistas de los receptores NMDA. Así se define una ventana terapéutica de por lo menos dos horas, lo cual tiene importantes repercusiones clínicas ya que si el daño neuronal asociado a la isquemia y el trauma cerebral se debe a un proceso excitotóxico, se podría prevenir en cierta medida la degeneración neuronal del paciente aún cuando éste no sea atendido de manera inmediata.

Consideraciones teóricas predicen que los antagonistas de tipo competitivo son menos eficientes que los no-competitivos para prevenir el daño que se produce como consecuencia de la elevación de la concentración de glutamato extracelular (como ocurre durante la isquemia), ya que tendrían que competir con éste. Sin embargo, otra de las contribuciones del trabajo presentado es el hecho de que el CGP 37849, que es un antagonista competitivo del receptor al NMDA, no sólo no presenta ninguna desventaja en cuanto a su efecto protector del daño en relación con el antagonista no competitivo, MK-801, sino que además pudiera ser más eficiente ya

que su vida media en el cerebro es más prolongada que la del MK-801. Sin embargo, a pesar de la eficiencia de los antagonistas del receptor NMDA para proteger de la muerte celular excitotóxica, algunos estudios han demostrado que el MK-801 y drogas relacionadas a este a las dosis a las que protegen de la neurodegeneración son tóxicos *per se* para ciertas poblaciones neuronales, como la corteza del cíngulo y la restroesplenial, donde se ha visto que estos compuestos inducen la vacuolización de las células y posterior degeneración (Olney et al. 1989,1991). Además, estos compuestos, debido a que son anestésicos disociativos, producen alucinaciones.

Como alternativa terapéutica se propone a las quinoxalindionas, como el NBQX, que son antagonistas de los receptores no-NMDA. El NBQX es capaz de proteger contra el daño neuronal en modelos de isquemia global (Sheardown et al. 1990; Le pelliet et al. 1992), lo cual es relevante dado el limitado efecto protector de los antagonistas NMDA en dichos modelos (Wieloch et al. 1989; Buchnan y Pulsinelli, 1990). De aquí que se ha sugerido que el daño celular asociado a este tipo e isquemia está mediado de una manera más importante por los receptores AMPA/kainato que por los NMDA. En el presente trabajo demostramos que el NBQX es una droga con potencialidad terapéutica para proteger contra lesiones mediadas por la activación de este tipo de receptores y que es igualmente eficiente para contrarrestar el daño mediado por ambos subtipos receptores. Sin embargo los efectos secundarios asociados a esta droga han sido muy poco estudiados por lo que su consideración en la clínica es todavía incipiente. Otro antagonistas de los receptores no-NMDA que ha mostrado tener propiedades neuroprotectoras contra el daño isquémico es el GYKI 52466 cuyo mecanismo neuroprotector, por lo menos en

el estriado, aparentemente está ligado a la inhibición de la liberación de glutamato (le Pelliet et al. 1992; Arvin et al. 1992; Arvin et al. 1994). Al igual que el NBQX, los efectos secundarios de este compuesto aún no han sido estudiados. Recientemente, se ha demostrado que el kinurenato, que es un antagonista tanto de los receptores NMDA como no-NMDA, inhibe la liberación de glutamato y aspartato que se presenta durante la isquemia, tanto en el hipocampo como en el estriado (Lekieffre et al. 1992; Ghribi et al. 1993). Sin embargo, la relación entre este efecto y su acción protectora contra el daño excitotóxico y/o isquémico (Foster et al. 1984; Nozaki y Beal, 1992) no está clara.

Además de de los antagonistas de los receptores glutamatérgicos, existen otras drogas que ofrecen nuevas posibilidades de tratamientos antisquémicos, como son: inhibidores de la liberación de glutamato, como el BW1003C87 (Lusting et al. 1992; Meldrum et al. 1992; Lekieffre y Meldrum 1993; Graham et al. 1993); antagonistas del sitio de unión a la glicina del receptor al NMDA (Moroni et al. 1992; Lehmann et al. 1994); inhibidores de la proteína cinasa C (Madden et al. 1991); inhibidores de la translocación de la proteina cinasa C, como los gangliósidos (Skaper et al. 1989; Lombardi et al. 1989; Lombardi y Moroni, 1992); inhibidores de la lipoperoxidación (Monyer et al. 1990); antioxidantes (Miyamotro et al. 1989; Miyamoto y Coyle, 1990); compuestos capaces de inhibir la liberación de calcio de compartimientos intarcelulares como el dantroleno (Frandsen y Schousboe, 1992; Lei et al. 1992), y factores de crecimiento (Schumacher et al. 1991).

ABSTRACT

Exposure of neuronal cells to high concentrations of glutamate or related amino acids leads to their degeneration and death. Glutamate can produce neuronal damage through the activation of its two types of ionotropic (coupled to ionic channels) receptors: the NMDA and the non-NMDA (which include AMPA and kainate receptor subtypes) both *in vivo* and *in vitro*. The term excitotoxicity has been proposed for this type of neuronal damage since it is related to the overactivation of glutamate receptors and consequent overexcitation of neurons. Due to the discovery that NMDA antagonists are efficient neuroprotectans in ischemia models, most of the studies have focused on the role of NMDA receptors in neurodegenerative processes. Many studies have used non-competitive NMDA antagonists, such as, MK-801 due to its systemic activity. Besides theoretical considerations argue against competitive antagonists relative to non-competitive since the action of the later does not depend on the extracellular glutamate concentration (which is implicated in neuronal damage). In the first work that conforms the thesis we have done a comparative study of the neuroprotective properties of two blockers of the NMDA receptors, a non-competitive, Mk-801, and a competitive, CGP 37849. Both compounds were tested in a model of excitotoxicity *in vivo* through the administration of quinolinic acid, an endogenous agonist of the NMDA receptors, in the rat striatum. Neuronal damage was evaluated through the degeneration of GABAergic and cholinergic cells7 days after the surgery as determined by the activity of the enzymes cholin actetyltransferase (ChAT) and glutamate decarboxylase (GAD), markers of these two types of cells, respectively. Antagonists were administrated intraperitoneally as well as coinjected with quinolinic acid in the striatum, either inmediately or 1-6 h after agonist administration. Our results show that cholinergic cells can be rescued from death by the administration of the antagonists even 6 h after quinolinic acid injection, while GABAergic cells only if NMDA antagonists are administered 1-2 h after the agonist. Both antagonists were equally efficient in our experimental paradigm. When NMDA antagonists were administered directly in the striatum, cholinergic and GABAergic cells were protected even when the antagonists were injected 1-2 h post-quinolinic acid, resultiing CGP

37849 more efficient than MK-801. Our results show that both competitive and non-competitive antagonists of the NMDA receptors were equally efficient neuroprotectants in this model of excitotoxicity, and that CGP 37849 could be even more efficient due to its longer half life in brain as compared to MK-801.

The role of non-NMDA receptors in the development of neurodegenerative disorders has not been as extensively studied as NMDA receptors due to the lack of selective antagonists. In the second study of the present thesis, we have studied the neuroprotective properties of NBQX, one of the most selective antagonists of non-NMDA receptors recently sinthetized, on the excitotoxic damage induced by the administration of some glutamatergic agonists in the rat striatum *in vivo*. Neuronal damage was evaluated as described in the previous study but a quantitative histological analysis of pycnotic cells was also performed in cresyl violet stained sections. NBQX was intrastriatally coinjected with each of the glutamate agonists studied (AMPA, kainate, NMDA and quinolinic acid). NBQX protected neuronal damage induced by AMPA, kainate and NMDA but not by quinolinic acid. This antagonist was equally efficient neuroprotectant against neuronal damage induced both by AMPA and kainate. The results favor the possible therapeutic potentiality of NBQX against cell damage induced by overactivation of glutamate receptors.

The clearence of extracellular glutamate from synaptic cleft depends on the activity of its high affinity glutamate uptake system. It has been suggested that the lack of toxicity of glutamic acid *in vivo* is due to its rapid clearence from the synaptic cleft through its uptake system. Having this in mind, in the third study that conforms the thesis, we tested the effcet of the administration of two glutamate uptake inhibitors, dihydrokainate (DHK) and L-trans-pyrrolidin-2,4-dicarboxilate (PDC), on the extracellular accumulation of glutamate and cell injury. Extracellular levels of glutamate were measured from samples colected through a microdialysis membrane and by means of HPLC. High concentrations of glutamate uptake inhibitors were also acutely injected in the rat striatum and cell damage was assessed by biochemical determination of ChAT and GAD activities and by histological examination of cresyl violet stained sections. According to the results DHK (50 mM) and PDC (25 mM)

produced identical remarkable increments in the extracellular concentrations of asparate and glutamate. However, DHK, induced also notable elevations in the concentration of other amino acids. Clear neuronal damage was observed only after DHK administration, which was partially prevented by intraperitoneal injection of MK-801, or by intrastriatal coinjection of NBQX. No damage was observed when PDC or two other glutamate uptake inhibitors were tested: DL-thero-ß-hydroxyaspartate and L-aspartate-ß-hydroxamate. The results suggest that increased extracellular glutamate in rat striatum *in vivo* by disfunction of its transporter is not sufficient for inducing neuronal damage. The neurotoxic effect of DHK could be explained by direct activation of glutamate postsynaptic receptors.

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