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PAPEL DEL GLUTAMATO EN LA EPILEPSIA Y LA NEURODEGENERACION PRODUCIDAS POR LA 4-AMINOPIRIDINA EN EL HIPOCAMPO *in vivo*.

T E S I S

QUE PARA OBTENER EL GRADO DE

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P R E S E N T A :

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Dedicatoria

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INDICE

RESUMEN

A pesar de que se ha relacionado la generación de epilepsia y neurodegeneración con un incremento en la transmisión glutamatérgica, experimentos *in vivo* muestran que un aumento en los niveles extracelulares de glutamato no necesariamente induce alguna de estas condiciones patológicas. El objetivo de esta tesis es estudiar, utilizando simultáneamente las técnicas de electroencefalografía, microdiálisis cerebral y de histología, las siguientes preguntas: a) ¿Existe alguna relación entre la acumulación de glutamato extracelular, las crisis epilépticas y la neurodegeneración en el hipocampo de la rata *in vivo*? b) ¿El origen del glutamato extracelular es determinante en la producción de la epilepsia y la neurodegeneración *in vivo*? y c) ¿Cuáles estrategias de neuroprotección funcionan en estas condiciones? Los resultados muestran que el incremento en el glutamato extracelular provocado por la aplicación de tetraetilamonio o de una concentración elevada de potasio, no es suficiente para producir epilepsia o neurodegeneración en el hipocampo de la rata *in vivo*, probablemente porque este glutamato no es liberado mayoritariamente en la sinapsis. Por el contrario, la aplicación de la 4-aminopiridina (4-AP), que produce la liberación de glutamato desde terminales sinápticas, genera epilepsia y neurodegeneración que pueden deberse, al menos en parte, a la activación de receptores glutamatérgicos, ya que los antagonistas de estos receptores tienen un buen efecto neuroprotector. En el mismo sentido, la inhibición de la liberación de glutamato por la aplicación de tetrodotoxina produce una neuroprotección total. El incremento farmacológico de la transmisión GABAérgica potencia los efectos de la 4-AP, lo cual podría indicar que el GABA puede inducir un efecto excitador bajo estas condiciones, como ya se ha observado *in vitro*. Finalmente, otras estrategias de neuroprotección estudiadas en esta tesis, como los anticonvulsivantes carbamacepina y valproato, los abridores de canales de potasio diazoxida y NS1619, y el "inhibidor de la liberación de glutamato", riluzol, no tuvieron ningún efecto neuroprotector, e incluso el riluzol resultó ser muy tóxico al combinarse con la hiperexcitabilidad que produce la 4-AP.

ABSTRACT

In spite of the fact that epilepsy and neurodegeneration have been related to an increase in glutamatergic transmission, *in vivo* experiments show that an increase in the extracellular level of glutamate, does not necessarily produce these pathological conditions. The aim of the present work was to study with the use of electrencephalographic recordings, brain microdialysis and histology, the following questions: a) Is there any relation between accumulation of extracellular glutamate, epilepsy and neurodegeneration in the rat hippocampus *in vivo*? b) Is the origin of extracellular glutamate a decisive factor in the generation of epilepsy and neurodegeneration *in vivo*? c) Which neuroprotective strategies are useful in these conditions? The results show that an increase in extracellular glutamate promoted by tetraethylammonium and an elevated potassium concentration is not enough to induce epilepsy and neurodegeneration in the rat hippocampus *in vivo*, probably because this glutamate is not released at the synaptic level. On the other hand, 4-aminopyridine (4-AP) infusion, that promotes the release of glutamate from the presynaptic terminals, generates epilepsy and neurodegeneration. Both, epilepsy and neurodegeneration, which are due at least in part to activation of glutamate receptors, since glutamatergic antagonists have good neuroprotective effects. Similarly, the inhibition of glutamate release by the administration of tetrodotoxin causes a total protection. The pharmacological stimulation of GABAergic transmission enhanced the 4-AP effects, which indicates that GABA can act as an excitatory neurotransmitter under these conditions, as has been shown *in vitro*. Finally, other strategies of neuroprotection studied in this thesis, such as the anticonvulsants carbamazepine and valproate, the potassium channels openers diazoxide and NS1619, and the "glutamate release inhibitor", riluzole did not have any neuroprotective effect. In addition, riluzole was highly toxic when combined with the hiperexcitability produced by 4-AP.

ORGANIZACIÓN DE LA TESIS

Este trabajo esta dividido en cinco partes: Introducción, Antecedentes seguidos de los Objetivos; Resultados experimentales, Discusión general y Conclusiones.

En la Introducción se ubica al glutamato dentro del contexto de la transmisión sináptica y se incluye el artículo de revisión 1, titulado: "El ácido glutámico y las enfermedades neurodegenerativas" (*Ciencia* 50(4):5-13). En éste se revisan algunos padecimientos neurológicos relacionados con alteraciones en la función del glutamato.

En los Antecedentes se revisa el papel del glutamato en la generación de epilepsia y neurodegeneración, la utilidad de la 4-aminopiridina para el estudio de estos fenómenos *in vivo* y algunas estrategias de neuroprotección. Se incluye en esta sección el trabajo de revisión 2, titulado: "On the relationship between extracellular glutamate, hyperexcitation and neurodegeneration, *in vivo*" (*Neurochem Int*, 34(1)23-31) en el que se discuten los hallazgos de experimentos *in vivo* en los que se ha tratado de correlacionar un incremento en los niveles extracelulares de glutamato endógeno con la generación de epilepsia y neurodegeneración.

La sección de Resultados, que incluye la metodología utilizada, se presenta mediante dos trabajos, el primero de ellos titulado: "Relationships among seizures, extracellular amino acid changes, and neurodegeneration induced by 4-aminopyridine in rat hippocampus: A microdialysis and electroencephalographic study" (*J Neurochem* 75(5):2006-2014) y el segundo titulado: "Seizures and neurodegeneration induced by 4-aminopyridine in rat hippocampus *in vivo*: Role of glutamate- and GABA-mediated neurotransmission and of ion channels" (*Neuroscience* 101(3):547-561).

En la Discusión general se enmarcan los resultados en un contexto más amplio de lo incluido en la discusión de cada uno de los trabajos de Resultados para finalizar con las Conclusiones.

LISTA DE ABREVIATURAS

ADCI	(+)-5-aminocarbonil-10,11-dihidro-5H-dibenzo[a,d] cyclohepten-5,10-imina
AMPA	amino-3-hidroxi-5-metil-isoxasol-4-propionato
4-AP	4-aminopiridina
AP7	(±)-2-amino-7-heptanoato
CNQX	6-ciano-7-nitroquinoxalin-2,3-diona
CPP	(+)3-(2-carboxipiperazin-4il)-propil-1-fosfonato
DP	despolarización paroxística
EEG	electroencefalograma
ELT	epilepsia del lóbulo temporal
GABA	ácido γ-aminobutírico
GYKI52466	1-(4-aminofenil)-4-metil-7,8-metilenedioxi-5H-2,3-benzodiacepina
i.p.	intraperitoneal
MK-801	maleato de dizocilpina
NMDA	N-metil-D-aspartato
NS1619	1-(2hidroxi-5-trifluorometilfenil)-5-trifluoro-metil-2(3H)bencimidazolona
TEA	tetraetilamonio

I. INTRODUCCIÓN

A partir de los hallazgos de los grandes neuroanatomistas del siglo XIX como William His (1889), Rudolph Kölliker (1849) o Jan Purkinje (1838) entre muchos otros y culminando con los acuciosos estudios de Santiago Ramón y Cajal (1906) con la ayuda de la técnica de tinción argéntica desarrollada por Camilo Golgi (1873) se estableció que la neurona es la unidad estructural del sistema nervioso. Un poco más tarde se descubrió la unidad anatómica de la neurona que permite la comunicación entre las diferentes células nerviosas o entre éstas y otros tipos celulares: la sinapsis, término acuñado por Charles Sherrington en 1897. Desde entonces se ha planteado que existen dos componentes en una sinapsis, la pre y la postsinapsis. La presinapsis es la encargada de emitir la información nerviosa y la postsinapsis tiene como función responder ante dicha información.

En el sistema nervioso existen dos tipos de sinapsis, las llamadas sinapsis eléctricas y las sinapsis químicas. En las sinapsis eléctricas el impulso nervioso, que es de naturaleza eléctrica, pasa directamente de la neurona presináptica a la neurona postsináptica a través de poros proteicos llamados conexones, los cuales permiten el paso libre de la corriente y de diferentes moléculas, estableciendo así una continuidad citoplásmica entre la pre y la postsinapsis, que no existe en las sinapsis químicas.

Los hallazgos iniciales que dieron origen a la teoría química de la transmisión nerviosa fueron realizados en 1848 por Du Bois Raymond, quien postuló que la contracción muscular era causada por la secreción de una sustancia excitadora. Esta idea fue confirmada por Elliot en 1904 al descubrir que la adrenalina mimetiza la acción de los nervios simpáticos, y además sugirió que dicha sustancia podría ser liberada por las terminales nerviosas de estos nervios y actuar como neurotransmisor en la glándula suprarrenal. En 1921, Otto Lewi descubrió que la estimulación del nervio vago induce la liberación de una sustancia inhibidora del latido cardiaco, que más tarde se identificó como acetilcolina. Sin embargo, fue hasta 1929 cuando la teoría química de la transmisión nerviosa tuvo el apoyo experimental más determinante, en los estudios de Dale y sus colaboradores, quienes encontraron que al estimular los

nervios motores que inervan el músculo esquelético en mamíferos se producía la secreción de acetilcolina, lo que permitió plantear que para que la sinapsis química se lleve a cabo es necesaria la liberación de un neurotransmisor desde la presinapsis.

El neurotransmisor es liberado preferencialmente de las terminales presinápticas (Artículo de revisión 1, Fig. 1), las cuales cuentan con vesículas sinápticas que lo almacenan en altísimas concentraciones. Estas vesículas sinápticas se anclan en regiones de la membrana presináptica especializadas en la liberación del neurotransmisor, llamadas zonas activas. Los neurotransmisores almacenados en las vesículas sinápticas se liberan al espacio sináptico por medio de la exocitosis que ocurre al arribar un potencial de acción a la terminal presináptica: la despolarización que se produce abre los canales de calcio sensibles al voltaje localizados en las zonas activas y la entrada masiva de calcio por la apertura de estos canales desencadena la fusión de las vesículas y la liberación del neurotransmisor. Una vez en el espacio sináptico las moléculas del neurotransmisor difunden una distancia de entre 20-40 nm hasta unirse a sus receptores específicos localizados preferentemente en la membrana postsináptica; la unión del neurotransmisor con su receptor es el siguiente paso de la comunicación nerviosa.

Una vez que el neurotransmisor ha activado a sus receptores debe ser eliminado del espacio sináptico para terminar ese evento sináptico y permitir el siguiente. Esto se hace por medio de tres mecanismos principales: difusión, degradación enzimática y recaptura. La recaptura parece ser el mecanismo más importante en la remoción de la mayoría de los neurotransmisores, pues la difusión es un proceso que remueve una pequeña fracción del neurotransmisor y la degradación enzimática esta restringida a las sinapsis que utilizan acetilcolina y péptidos. La recaptura tiene un doble propósito, por un lado termina con la acción sináptica del neurotransmisor y por el otro permite la recuperación de estas moléculas que pueden eventualmente ser reutilizadas. Para ello es necesaria la actividad de proteínas específicas llamadas transportadores, las cuales utilizan los gradientes iónicos establecidos a través de la membrana para transportar al

neurotransmisor del espacio sináptico al interior de las neuronas y de las células gliales.

EL ÁCIDO GLUTÁMICO

Entre las muchas sustancias catalogadas como neurotransmisores podemos encontrar péptidos, aminas biogénicas, acetilcolina, trifosfato de adenosina y aminoácidos (Artículo de revisión 1, Fig. 2). Los aminoácidos utilizados como neurotransmisores son el glutamato, el aspartato, el ácido γ-aminobutírico (GABA) y la glicina. Los dos primeros son llamados aminoácidos excitadores y los últimos aminoácidos inhibidores, en función de su efecto sobre la probabilidad de disparo de la neurona postsináptica; en otras palabras, un neurotransmisor excitador aumenta la probabilidad de disparo de la neurona postsináptica y un neurotransmisor inhibidor la disminuye.

El glutamato y el GABA son los neurotransmisores más utilizados por las neuronas del sistema nervioso. Datos obtenidos a partir de diferentes líneas experimentales muestran que alrededor del 90% de las sinapsis en el cerebro utilizan uno de estos aminoácidos como neurotransmisor (Fonnum, 1984). Existen diferentes tipos de receptores para el glutamato, tanto del tipo metabotrópico, los cuales están asociados a la activación de proteínas G y a la producción de segundos mensajeros, como receptores ionotrópicos que en sí mismos son canales iónicos. Como podemos ver en la tabla 1, existen diferentes subtipos de receptores ionotrópicos a glutamato, que han sido clasificados, dependiendo de su sensibilidad farmacológica, en dos tipos: receptores tipo NMDA (aquellos activados por el N-metil-D-aspartato) y receptores no-NMDA sensibles al AMPA (α-amino-3-hydroxy-5-metilisoxasole-4-propionato) o al kainato. Todos estos receptores presentan en su estructura un poro iónico que permite el paso de cationes cuando el receptor es activado por el glutamato u otro agonista. En todos los casos los receptores ionotrópicos de glutamato tienen una alta conductancia para el Na^+ y el K^+, pero como podemos ver en la tabla 1 los receptores NMDA tienen además una alta conductancia para el Ca^{2+} (para una revisión ver Krogsgaard-Larsen, 1991)

Otra diferencia muy importante entre los receptores ionotrópicos de glutamato se encuentra en su sensibilidad al voltaje; pues a diferencia de los receptores no-NMDA, los receptores tipo NMDA son sensibles al voltaje transmembranal debido a que en condiciones de reposo su poro iónico se encuentra bloqueado por el ion Mg^{2+} y este bloqueo sólo puede ser removido por un cambio despolarizante en el potencial de membrana. Esta despolarización que remueve al Mg^{2+} del poro iónico de los receptores NMDA es provocada, muy frecuentemente, por la activación de los receptores no-NMDA (MacDonald et al., 1998).

Tabla 1. Receptores ionotrópicos de glutamato.

Tipo	Agonistas	Antagonistas	Efector
NMDA	NMDA Aspartato	MK801 D-AP5 LY233053	Incremento en la conductancia de Na^+, K^+ y Ca^{2+}
AMPA	AMPA 5-fluorowilardina	NBQX GYKI52466	Incremento en la conductancia de Na^+ y K^+
Kainato	Kainato Domoato	CNQX LY294486	Incremento en la conductancia de Na^+ y K^+

Abreviaturas: AMPA: amino-3-hidroxi-5-metil-isoxasol-4-propionato, **D-AP5:** D-amino-fosfo-valerato, **CNQX:** 6-ciano-7-nitroquinoxalin-2,3-diona, **GYKI52466:** 1-(4-aminofenil)-4-metil-7,8-metilenedioxi-5H-2,3-benzodiacepina, **LY233053**: cis-(+-)-4-[(2H-tetrazol-5-il)metil]piperidin-2-carboxilato, **LY294486:** ((3SR, 4aRS, 6SR, 8aRS)-6-((((1H-tetrazol-5-il) metil)oxi)metil)-1, 2, 3, 4, 4a, 5, 6, 7, 8, 8a-decahidroisoquinolin-3-carboxilato, **MK-801:** maleato de dizocilpina, **NMDA:** N-metil-D-aspartato.

El glutamato es el neurotransmisor excitador por excelencia, está relacionado con prácticamente todas las funciones del sistema nervioso y se encuentra presente en todas las regiones del mismo (Fonnum, 1984). Por ello, no es muy difícil pensar que una disfunción en la actividad glutamatérgica puede tener graves consecuencias para la fisiología cerebral. De hecho existen múltiples padecimientos neurológicos que pueden asociarse a alteraciones en la transmisión glutamatérgica y que se abordan en el siguiente artículo de revisión.

ARTICULO DE REVISIÓN 1

El ácido glutámico y las enfermedades neurodegenerativas

Fernando Peña
Ricardo Tapia

Las neuronas, células encargadas de recibir, procesar y transmitir la información en el sistema nervioso, tienen como característica más importante que son células excitables, es decir que, en respuesta a un estímulo de cierta magnitud, son capaces de producir potenciales de acción. Estos potenciales viajan por prolongaciones de la neurona denominadas axones, y al llegar a las terminales nerviosas estimulan la liberación de neurotransmisores, sustancias químicas cuya función consiste en excitar o inhibir otra neurona. Los sitios de comunicación entre dos neuronas reciben el nombre de sinapsis, término acuñado a principios de este siglo por Sherrington. La sinapsis se compone de una región presináptica —las terminales nerviosas—, que libera el neurotransmisor, y una región postsináptica, que responde ante la presencia del transmisor (Figura 1).

Se conocen varios tipos de moléculas —de naturaleza química muy variable— que funcionan como neurotransmisores, entre ellas, las aminas biogénicas (como la dopamina), los péptidos (como las endorfinas), los aminoácidos (como el ácido glutámico) o la acetilcolina (Figura 2). Los aminoácidos son los neurotransmisores probablemente más abundantes y más ampliamente distribuidos en las distintas regiones del sistema nervioso central. Por su acción sobre la neurona postsináptica, son agrupados en dos tipos aminoácidos inhibidores, como el ácido γ-aminobutírico (GABA) y la glicina, y aminoácidos excitadores, como los ácidos glutámico y aspártico (Figura 2).

El ácido glutámico es el neurotransmisor más abundante en el cerebro, y un compuesto fundamental en el sistema nervioso, ya que, además de su función neurotransmisora, forma parte del glutatión y de péptidos y proteínas, y participa en el metabolismo de los azúcares, de los ácidos grasos, del amonio y de otros aminoácidos como el GABA, así como en el control del volumen celular.

La acción del ácido glutámico como neurotransmisor se debe a su capacidad para activar receptores específicos en la membrana postsináptica, a través de la cual éstos permiten el paso de iones

Fernando Peña obtuvo la licenciatura en biología, en 1996, en la Facultad de Ciencias de la UNAM, donde actualmente es profesor, y es candidato al doctorado en ciencias biomédicas en el Instituto de Fisiología Celular de la UNAM. Su tema de tesis doctoral es la relación entre la epilepsia y la excitoxicidad.

Ricardo Tapia es investigador emérito en el Instituto de Fisiología Celular de la UNAM, nivel 3 en el Sistema Nacional de Investigadores, catedrático nivel I del Conacyt y director de Ciencia. Desde hace muchos años trabaja sobre los mecanismos neuroquímicos de la transmisión sináptica mediada por aminoácidos y sus alteraciones, tema del que ha publicado más de 125 trabajos internacionales y numerosos artículos de divulgación. Es también autor de los libros *Las células de la mente, Neurobiología celular* y, el recientemente publicado, *Enfermedades neurodegenerativas. Mecanismos celulares y moleculares* (con otros cuatro autores), todos ellos editados por el Fondo de Cultura Económica.

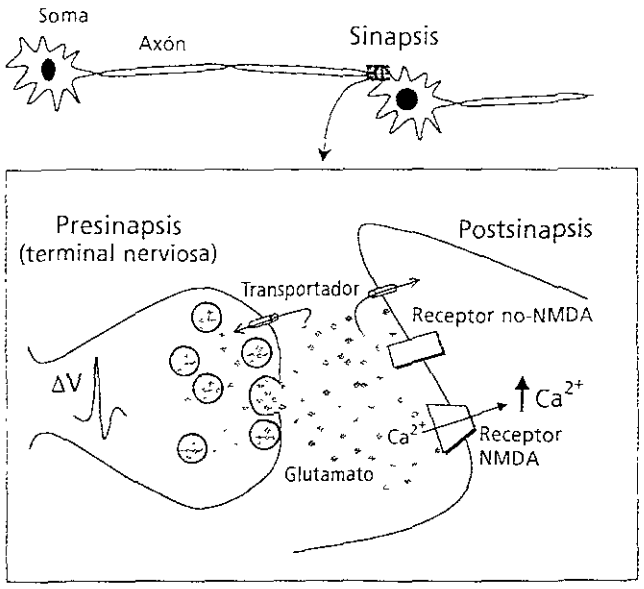


Figura 1. Neurotransmisión sináptica glutamatérgica. La parte superior muestra una neurona en comunicación con otra a través de su axón, y la parte inferior un esquema del sitio de comunicación (sinapsis), muy amplificado. El ácido glutámico (glutamato, •) es almacenado en vesículas en el interior de las terminales nerviosas y liberado por exocitosis, como consecuencia de la llegada de un potencial de acción (ΔV). El ácido glutámico liberado actúa sobre su receptor tipo NMDA y, como consecuencia, éste se abre y el Ca^{2+} penetra en la neurona postsináptica. Después, el ácido glutámico es eliminado del espacio sináptico por moléculas transportadoras

Los receptores del ácido glutámico se dividen farmacológicamente en dos grupos: los receptores sensibles a una droga llamada N-metil-D-aspartato (receptores tipo NMDA), y los receptores no sensibles a este compuesto (receptores no-NMDA). Al ser activados por el ácido glutámico, ambos tipos de receptores permiten el paso de cationes (iones cargados positivamente) a través de la membrana. Sin embargo, se diferencian en que los receptores tipo NMDA son mucho más permeables al catión calcio que los no-NMDA (Figura 1; véase también Ciencia, vol 50, número 2, junio de 1999, p 5).

La neurotransmisión mediada por el ácido glutámico (transmisión glutamatérgica) es fundamental en el funcionamiento normal del sistema nervioso, ya que este neurotransmisor participa en gran número de procesos nerviosos, tales como el desarrollo y maduración de las neuronas, la percepción, el control motor, el aprendizaje y la memoria (Figura 2), razón por la que no debe sorprender que distintas alteraciones en la transmisión glutamatérgica tengan incidencia en varios padecimientos neurológicos y psiquiátricos (Cuadro 1). En este trabajo se revisan algunos datos en relación con l forma en la que el ácido glutámico interviene e ese tipo de patologías. La información se ha obteni do por medio de estudios directos en pacientes, bien a través de modelos experimentales de las dis tintas enfermedades en animales de laboratorio particularmente en roedores.

Ácido glutámico y encefalopatías metabólicas

Las encefalopatías metabólicas son padecimientos que manifiestan diversas alteraciones de la actividad mental, de la conducta o del funcionamiento neurológico. Se pueden distinguir dos tipos de encefalopatías. En el primero, los pacientes presentan agitación, ansiedad e hiperactividad; en el segundo, se tornan tranquilos y retraídos, y en todos los casos se observa un marcado deterioro de la memoria.

Muchas encefalopatías están asociadas con la sobreproducción de ciertas sustancias químicas —que, en cantidades excesivas, pueden resultar tóxicas para el sistema nervioso (neurotoxinas)—, como el amonio o el ácido quinolínico. Algunas de estas neurotoxinas tienen relación con el ácido glutámico.

La producción de amonio aumenta cuando hay daño en el hígado, lo que al parecer es la causa de la llamada encefalopatía hepática. Algunos testimonios muestran que los aminoácidos excitadores se encuentran disminuidos en animales a los que experimentalmente se les induce incremento de amonio en los niveles sanguíneos, o en animales a

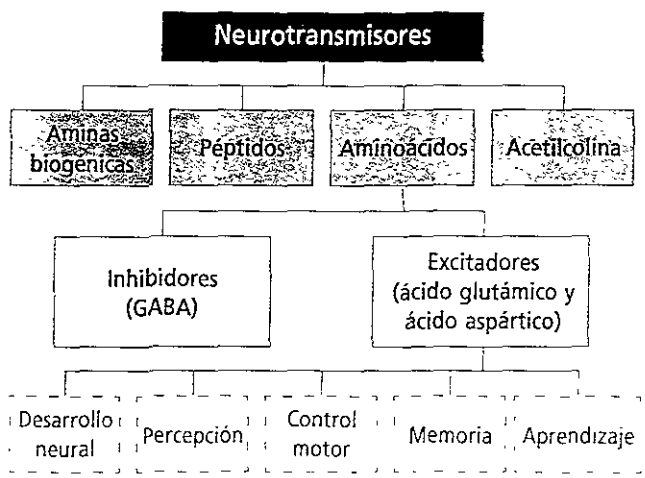


Figura 2. Algunos compuestos neurotransmisores y funciones normales del sistema nervioso en que participan de manera fundamental los aminoácidos excitadores

CUADRO 1
Enfermedades neurológicas y mentales posiblemente relacionadas con el ácido glutámico

Encefalopatías metabólicas
Alteraciones por abuso de drogas psicoactivas
Esquizofrenia
Esclerosis lateral amiotrófica
Enfermedad de Huntington
Enfermedad de Alzheimer
Isquemia cerebral
Epilepsia y daño neuronal asociado

los que se les provoca disfunción hepática. Por este motivo se ha propuesto que hay determinada disminución de neurotransmisión glutamatérgica a causa de la utilización del glutamato en el metabolismo del amonio, de lo que resulta la alteración de la conciencia y la disminución de la actividad cerebral que se observan en el primer grupo de encefalopatías antes mencionado.

En contraste, las encefalopatías que se acompañan de actividad cerebral aumentada podrían tener su origen en el incremento de la transmisión glutamatérgica, ya que otra de las neurotoxinas causantes de encefalopatía, el ácido quinolínico, puede actuar como potente agonista (molécula capaz de activar a los receptores) glutamatérgico, y de esta manera causar la hiperactividad que experimentan los pacientes.

Muchas encefalopatías están asociadas con la sobreproducción de ciertas sustancias químicas

Ácido glutámico y drogas de abuso

Existen diversas drogas psicoactivas que por sus efectos sobre el sistema nervioso inducen consumo sostenido o exagerado, por lo cual son llamadas drogas de abuso. Estas drogas producen cambios psicológicos y bioquímicos importantes, los cuales generan en el individuo dependencia física, psicológica, o ambas, es decir, adicción. La dependencia psicológica puede ser definida como el desarrollo de un deseo de la droga en razón de su efecto placentero. La dependencia física implica un cambio bioquímico-fisiológico que hace necesaria la presencia continua de la droga en el organismo para preservar la sensación de bienestar. Por ello, al interrumpir el consumo se produce el llamado síndrome de abstinencia, que consiste en la aparición de una serie de malestares físicos que pueden ser extremadamente graves e incluso constituir una amenaza para la vida. Es posible que, en algunos de estos procesos, los aminoácidos excitadores influyan de manera importante.

Una de las drogas de abuso más comúnmente utilizada en nuestra sociedad es el etanol. Esta droga actúa a muchos niveles y sobre diferentes sistemas de neurotransmisión; pero en el caso particular que nos compete, se sabe que puede actuar como inhibidor de los receptores del ácido glutámico tipo NMDA y contribuir así a sus efectos placenteros y sedantes. Después de descontinuar el consumo sostenido de alcohol, se produce el llamado síndrome de abstinencia por la droga, que incluye la aparición de crisis convulsivas que, como veremos más adelante, están muy relacionadas con la sobreactivación de receptores del ácido glutámico.

Otro caso que involucra la transmisión glutamatérgica con las drogas de abuso es el de la fenciclidina. Esta droga —conocida vulgarmente como "pelo de ángel"— es un antagonista (molécula capaz de bloquear los receptores) de los receptores tipo NMDA y, como se revisa en la siguiente sección, los efectos conductuales de este psicoestimulante son muy similares a los de la esquizofrenia. Finalmente, cabe mencionar que también se ha vinculado al ácido glutámico en los fenómenos de tolerancia y abstinencia relacionados con el consumo de opiáceos.

Ácido glutámico y esquizofrenia

La esquizofrenia es una enfermedad mental muy lacerante en términos sociales. Los pacientes esquizofrénicos, generalmente jóvenes, pierden toda capacidad asociativa y presentan deterioro de los hábitos sociales, alucinaciones auditivas o visuales

y delirios, entre los que destaca el de persecución, característico de la esquizofrenia de tipo paranoide. Otros síntomas que pueden detectarse en estos pacientes son alteraciones del pensamiento, conducta estereotipada, deterioro del aspecto y anhedonia (incapacidad de experimentar o incluso imaginar emociones o sensaciones agradables).

La neurotoxicidad producida por el ácido glutámico puede relacionarse con la destrucción neuronal aguda

Como adelantamos en la sección anterior, por los efectos de la fenciclidina se ha postulado que el ácido glutámico está asociado con la patogénesis de la esquizofrenia. Esta droga fue desarrollada por la compañía Parke-Davis en la década de los años cincuenta, y se utilizó como anestésico y como analgésico postoperatorio. Posteriormente se descubrió que era un potente antagonista de los receptores tipo NMDA y que era capaz de producir delirio, disociación, aislamiento, despersonalización, negativismo, hostilidad, apatía e incluso cierto estado cataléptico. Estos efectos no solamente son muy parecidos a los llamados síntomas primarios de la esquizofrenia, sino que además pueden ser controlados eficazmente con drogas llamadas antipsicóticas (como el haloperidol), las cuales son el tratamiento más común de la esquizofrenia.

Otros hallazgos que apuntan hacia un papel importante del ácido glutámico en la patología esquizofrénica provienen de observaciones postmortem en humanos. Estos estudios han mostrado alteraciones en los niveles de receptores del ácido glutámico en la corteza frontal y en el lóbulo temporal de pacientes esquizofrénicos. Por otro lado, se sabe que el ácido glutámico controla los niveles de otro neurotransmisor, la dopamina, en distintas regiones cerebrales, principalmente en el llamado sistema límbico. Como la hipótesis bioquímica más aceptada del mecanismo de la esquizofrenia es que ésta ocurre por incremento de la transmisión dopaminérgica, es posible que la alteración de la regulación que el ácido glutámico ejerce sobre la dopamina sea indirectamente responsable de ese incremento y, por lo tanto, del síndrome esquizofrénico.

La fenciclidina (vulgo "pelo de ángel"), como droga de abuso, produce efectos conductuales muy similares a los de la esquizofrenia

El ácido glutámico como excitotoxina

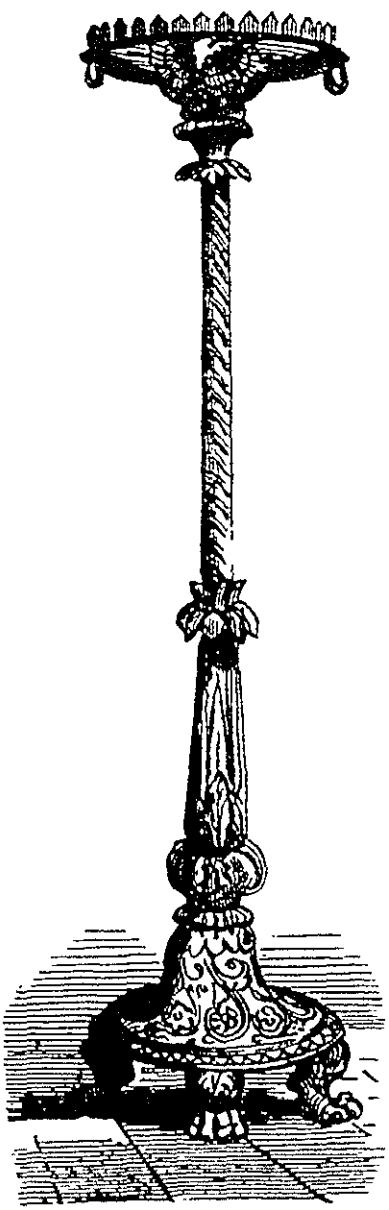
Las propiedades neurotóxicas del ácido glutámico se describieron por primera vez en 1957, cuando se demostró que la exposición de la retina a elevadas cantidades de ácido glutámico producía la muerte de las neuronas retinianas, y doce años después se encontró que el exceso de este aminoácido producía también muerte neuronal en distintas regiones del cerebro. A este fenómeno se le conoce como excitotoxicidad, pues relaciona la sobreexcitación producida por el ácido glutámico con el daño neuronal.

Hoy sabemos que la neurodegeneración por excitotoxicidad se manifiesta inicialmente, en forma aguda, por hinchamiento celular, provocado por la penetración de diferentes iones en el interior de la célula, como consecuencia de la activación de los receptores del ácido glutámico y de la excitación que esto ocasiona. Esos cambios iónicos se acompañan de la entrada de agua y, por consiguiente, del hinchamiento de las neuronas.

Más tarde, a largo plazo, el daño neuronal se desencadena por la entrada masiva de Ca^{2+} por vía de los receptores tipo NMDA (Figura 1),

ya que, en cantidades excesivas, este catión es capaz de provocar la muerte neuronal al alterar varios procesos celulares vitales. Por ejemplo, la elevación del Ca^{2+} en el citoplasma activa gran número de enzimas, entre ellas las proteasas, las lipasas y las endonucleasas, las cuales degradan, respectivamente, proteínas, lípidos y ácidos nucleicos, componentes fundamentales de cualquier célula. El Ca^{2+} en exceso también induce la producción de los llamados radicales libres, que son formas moleculares de oxígeno muy tóxicos para las membranas celulares, así como alteraciones en la función de las mitocondrias, organelos celulares encargados de la producción de energía. El resultado de todos estos procesos fisiopatológicos es la muerte celular.

La neurotoxicidad producida por el ácido glutámico puede relacionarse con la destrucción neuronal aguda que se observa en la isquemia, en el trauma mecánico o en la epilepsia, así como con la muerte de las neuronas asociada a enfermedades neurodegenerativas crónicas.



Ácido glutámico y enfermedades neurodegenerativas crónicas

En varios padecimientos neurológicos se presenta una destrucción, lenta pero progresiva, de grupos neuronales del sistema nervioso central. Esta destrucción se caracteriza por ser más o menos selectiva para ciertas neuronas, por lo que los síntomas que produce dependen de las funciones nerviosas que esas neuronas controlan. Es decir, el daño neuronal se traduce en diversas alteraciones neurológicas o conductuales, según la región del cerebro en la que la pérdida neuronal sea más extensa.

Una de las enfermedades causadas por la pérdida neuronal selectiva es la esclerosis lateral amiotrófica. Esta enfermedad se caracteriza por una parálisis muscular que empieza en la vida adulta y progresa durante años para afectar a la mayoría de los músculos. La parálisis muscular es debida principalmente a que las neuronas de la médula espinal encargadas de generar el movimiento (las motoneuronas) van muriendo de manera progresiva. Una de las hipótesis que se ha postulado para explicar esta neurodegeneración es el daño por excitotoxicidad, ya que se ha encontrado que algunos pacientes con esclerosis lateral amiotrófica tienen niveles *elevados* de ácido glutámico en el líquido cefalorraquídeo, así como deficiencias en los transportadores de ácido glutámico responsables de eliminarlo del espacio extracelular, precisamente en la médula espinal.

Otro trastorno probablemente relacionado con la neurotransmisión glutamatérgica es la enfermedad de Huntington. Este padecimiento, que tiene un componente genético muy importante, fue descrito en 1872 por el científico estadunidense que le da nombre. Se caracteriza por la aparición de movimientos coreicos y el avance progresivo de demencia. El cerebro de los pacientes está extensamente atrofiado, y presenta notable destrucción de neuronas en una estructura cerebral llamada neoestriado. En animales de experimentación

se ha visto que la administración de distintos agonistas de los receptores del ácido glutámico en esta estructura puede simular la sintomatología y la muerte neuronal que se observa en los pacientes con Huntington, aunque las características histológicas del daño no son exactamente iguales. Por otro lado, en dichos pacientes se han encontrado alteraciones en el metabolismo de aminoácidos, que resultan en un incremento en la síntesis de los ácidos kinurénico y quinolínico, los cuales pueden funcionar como agonistas glutamatérgicos.

Numerosos datos sugieren que el ácido glutámico está muy relacionado con la aparición de múltiples desórdenes neurológicos y mentales

Algunos estudios han relacionado los aminoácidos excitadores con la muerte neuronal que ocurre en las enfermedades de Alzheimer y en la de Parkinson, aunque las evidencias no son del todo convincentes. Hay datos que sugieren que las neuronas glutamatérgicas son las células más susceptibles a la formación de las llamadas "marañas neurofibrilares", que son una de las características histopatológicas más comunes de la enfermedad de Alzheimer. En algunos pacientes con Alzheimer, se ha encontrado pérdida de terminales nerviosas glutamatérgicas en la neocorteza y en el hipocampo, niveles reducidos de glutamato en algunas áreas corticales y deficiencias en los transportadores de este aminoácido en la corteza frontal. Por otro lado, como ya hemos visto, el aumento en el contenido intracelular de Ca^{2+} es un factor preponderante en el desarrollo de la excitotoxicidad. Una de las proteínas cuya aparición está muy relacionada con la enfermedad de Alzheimer es la proteína β-amiloide. Cuando esta proteína se aplica a cultivos neuronales, produce disfunción de la regulación del Ca^{2+} intracelular y aumento en la producción de radicales libres de oxígeno, lo cual desencadena la muerte de las neuronas de una manera muy parecida a la excitoxicidad por ácido glutámico. Además, hay indicios de que la proteína β-amiloide exacerba el daño neuronal producido por el ácido glutámico.

Isquemia cerebral

La isquemia cerebral, enfermedad que puede producirse por trauma cerebral, accidente vascular o paro cardiaco transitorio, tiene gran impacto a causa de la severidad de sus secuelas. En los adultos los trastornos cerebrovasculares se ubican entre las más frecuentes causas de muerte y de incapacidad permanente.

Durante la isquemia se interrumpe la llegada de sangre al cerebro en su conjunto o a una zona en particular, lo que produce muerte neuronal masiva en aquellas regiones privadas del aporte sanguíneo. A la zona cerebral adyacente a la región isquémica se le conoce como zona de penumbra, y se piensa que allí el proceso de neurodegeneración ocurre más o menos lentamente, durante las horas siguientes a la isquemia, como resultado de la excitotoxicidad producida por la liberación masiva de ácido glutámico, que a su vez es consecuencia de la destrucción neuronal en la región isquémica. Esta idea se apoya, entre otros datos, en dos observaciones experimentales: la concentración extracelular de ácido glutámico aumenta durante la isquemia, y algunos antagonistas glutamatérgicos protegen eficientemente contra el daño neuronal en la zona de penumbra.

Epilepsia

Otra condición en la que se presenta muerte neuronal es después de crisis epilépticas recurrentes. La epilepsia es un desorden neurológico que se origina por la sobreactivación de grupos neuronales que, en muchos de los casos, es capaz de propagarse a amplias zonas del cerebro y de esta forma provocar crisis convulsivas generalizadas. Una de las hipótesis más sólidas que explican el origen de la epilepsia es la de cierto desequilibrio entre la neurotransmisión inhibidora y la excitadora en alguna

región cerebral, desorden que posiblemente tiene origen en la disminución de la actividad inhibidora GABAérgica, en el aumento de la actividad del ácido glutámico o en la combinación de ambos factores.

Existen muchas evidencias que indican la participación del ácido glutámico en la patogenia de la epilepsia. Se han registrado índices elevados de aminoácidos excitadores en el líquido cefalorraquídeo de pacientes epilépticos, y algunos tipos de crisis epilépticas pueden ser controladas con antagonistas glutamatérgicos. Se ha mostrado que los niveles de ácido glutámico extracelular se encuentran elevados en los focos epilépticos y que estos niveles pueden aumentar durante una crisis, además de que en estudios en animales de experimentación se ha visto que mientras los agonistas glutamatérgicos son potentísimos convulsivantes, los antagonistas glutamatérgicos tienen notables efectos anticonvulsivantes.

En cuanto a la muerte neuronal asociada a la epilepsia, se ha encontrado que buen número de pacientes epilépticos, principalmente aquellos que padecen epilepsia del lóbulo temporal, llegan a presentar deficiencias neurológicas ocasionadas por la muerte masiva de neuronas en una estructura cerebral llamada hipocampo.

En nuestro laboratorio estamos interesados en estudiar las relaciones entre epilepsia, ácido glutámico y daño neuronal. Con este fin, hemos administrado directamente en el hipocampo de la rata un compuesto llamado 4-aminopiridina (4-AP), que es un potente convulsivante, mediante la técnica de microdiálisis, que permite aplicar la droga y estimar, al mismo tiempo, la concentración extracelular de ácido glutámico y otros aminoácidos, por medio de cromatografía líquida de alta presión (HPLC, *high presion liquid cromatography)* (véase la Figura 3). Hemos combinado esta técnica con el registro de la actividad eléctrica cerebral (electroencefalograma o EEG), y de esta manera evaluamos la aparición de actividad epiléptica y podemos correlacionarla con la liberación de ácido glutámico desde las neuronas y con la muerte neuronal, en la misma rata (Figura 3).

Algunos resultados de estos experimentos se muestran en la Figura 4. La administración de 4-AP en el hipocampo de la rata produce elevación considerable en los niveles extracelulares de ácido glutámico, al mismo tiempo que induce crisis epileptiformes y una considerable muerte neuronal. Además, hemos podido demostrar que el daño neuronal y las crisis epilépticas que produce la 4-AP se deben principalmente a la sobreactivación de los receptores del ácido glutámico tipo NMDA, ya que la aplicación de antagonistas de este receptor, como el MK801, es capaz de atenuar las crisis y proteger contra el daño neuronal (véase la Figura 4).

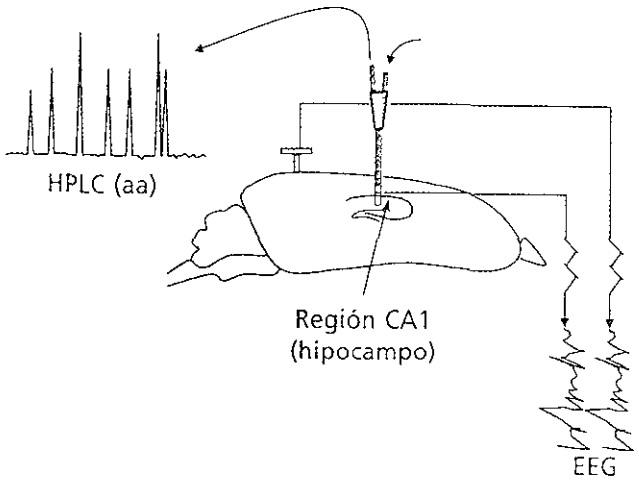


Figura 3. Diseño experimental para estudiar las relaciones entre la liberación del ácido glutámico y otros aminoácidos (aa), la aparición de epilepsia y la neurodegeneración, en el hipocampo de la rata. La cánula de microdiálisis insertada en el hipocampo permite perfundir un líquido y colectar lo que las neuronas del hipocampo liberan (como se indica por las flechas). Simultáneamente se registra la actividad eléctrica (EEG) del propio hipocampo y de la corteza cerebral. El contenido de ácido glutámico se mide por cromatografía líquida (HPLC) y cinco días después del experimento se prepara el cerebro de la rata para determinar si hubo muerte neuronal en el hipocampo. La región CA1 del hipocampo es la más afectada por la droga usada en estos experimentos, la 4-aminopiridina, como se observa en la micrografía mostrada en la Figura 4

Consideraciones terapéuticas

Como hemos visto, numerosos datos sugieren que el ácido glutámico está muy relacionado con la aparición de múltiples desórdenes neurológicos y mentales. Esas pruebas abren la posibilidad de diseñar nuevas estrategias terapéuticas para el manejo de padecimientos como las enfermedades neurodegenerativas, la isquemia y la epilepsia. Actualmente contamos con una amplia gama de antagonistas de los receptores del ácido glutámico que, teóricamente, podrían aliviar dichos males. El gran problema al que nos enfrentamos es que, como mencionamos en la primera parte de este trabajo, ese aminoácido participa de manera muy importante en prácticamente todas las funciones del sistema

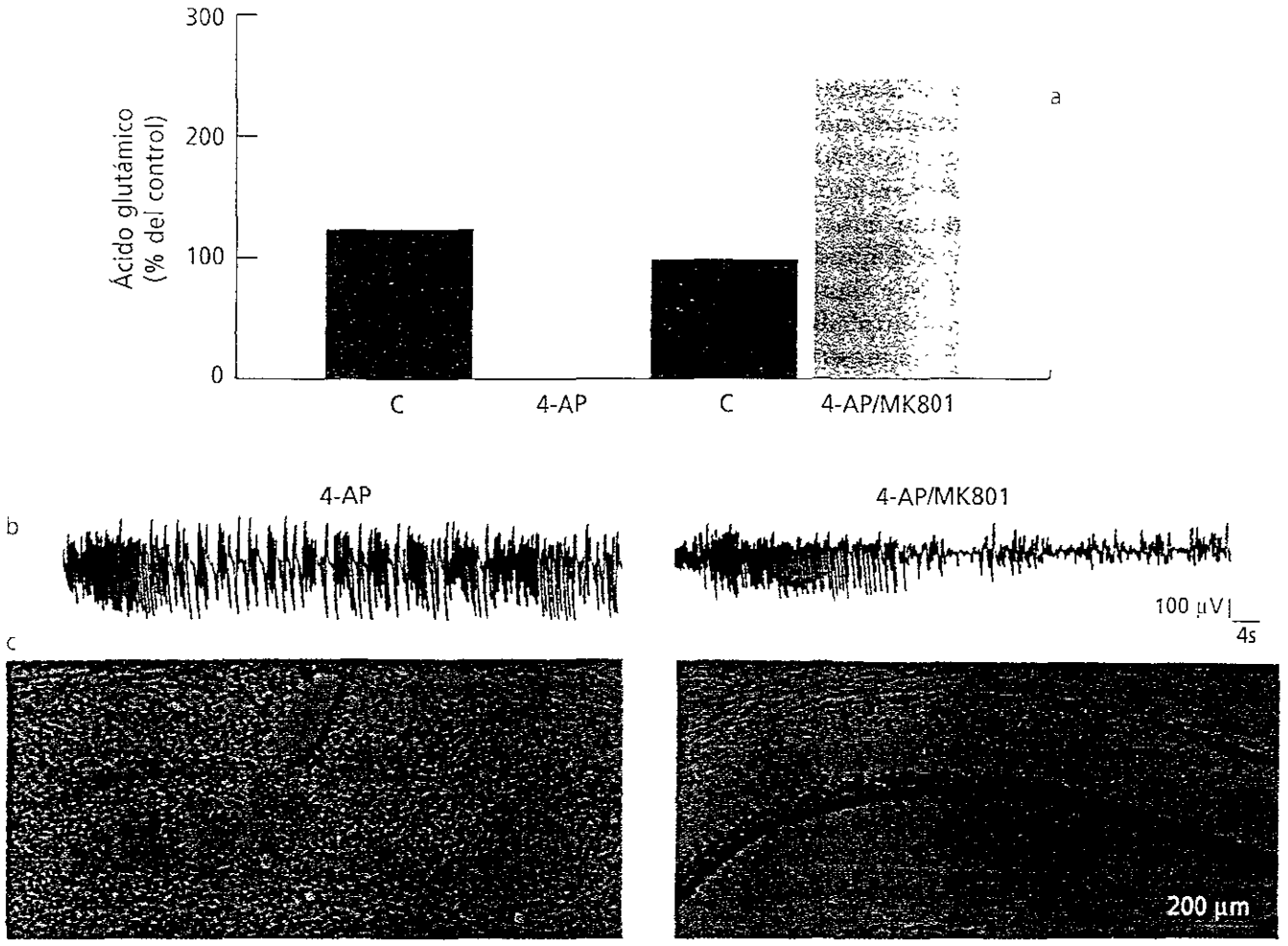


Figura 4. Efectos de la administración de 4-aminopiridina (4-AP) en el hipocampo. Resultados obtenidos siguiendo el diseño experimental mostrado en la Figura 3. La 4-AP produjo a) incremento de casi tres veces sobre el valor control (c) en la concentración de ácido glutámico extracelular (gráfica superior, parte izquierda); b) descargas de tipo epiléptico en el EEG (trazo del lado izquierdo); y c) destrucción neuronal completa en la región CA1 del hipocampo (micrografía del lado izquierdo). Los efectos b) y c) parecen deberse a la liberación de ácido glutámico y a la consecuente sobreactivación de su receptor tipo NMDA, ya que cuando se administró a la rata el compuesto MK801, que es un antagonista de ese receptor, las descargas epileptiformes en el EEG fueron mucho más cortas (compárese el EEG del lado derecho con el del izquierdo) y las neuronas de la región CA1 no se destruyeron (compárese la micrografía del lado derecho, en la que se ve la capa de neuronas normales característica de esta región, con la del lado izquierdo, en donde se observa la destrucción prácticamente completa de dicha capa de neuronas; la barra indica 200 µm). Resultados originales de los autores

nervioso, por lo cual no es sorprendente que la administración de drogas antagonistas provoque efectos colaterales indeseables a corto y mediano plazos. Entre estos efectos indeseables se encuentran el aletargamiento y diversas alteraciones en la percepción, la atención, el control motor o la memoria. Un claro ejemplo de estas acciones es la ya mencionada fenciclidina, un antagonista del receptor tipo NMDA que, como hemos visto, produce síntomas esquizoides.

El desarrollo de la biología molecular ha permitido clonar varias de las diferentes subunidades peptídicas que componen los receptores del ácido glutámico, tanto del tipo NMDA como del no-NMDA. En esta forma se ha demostrado que los receptores están constituidos por diversas combinaciones de subunidades y que se distribuyen de manera diferencial en las distintas regiones del cerebro. Sin duda, esto permitirá, en los próximos años, el desarrollo de fármacos más específicos para antagonizar la excitotoxicidad producida por el exceso de transmisión glutamatérgica, pero carentes de efectos colaterales, y así establecer procedimientos terapéuticos eficaces en los padecimientos neurodegenerativos, para los cuales aún no existe tratamiento alguno.

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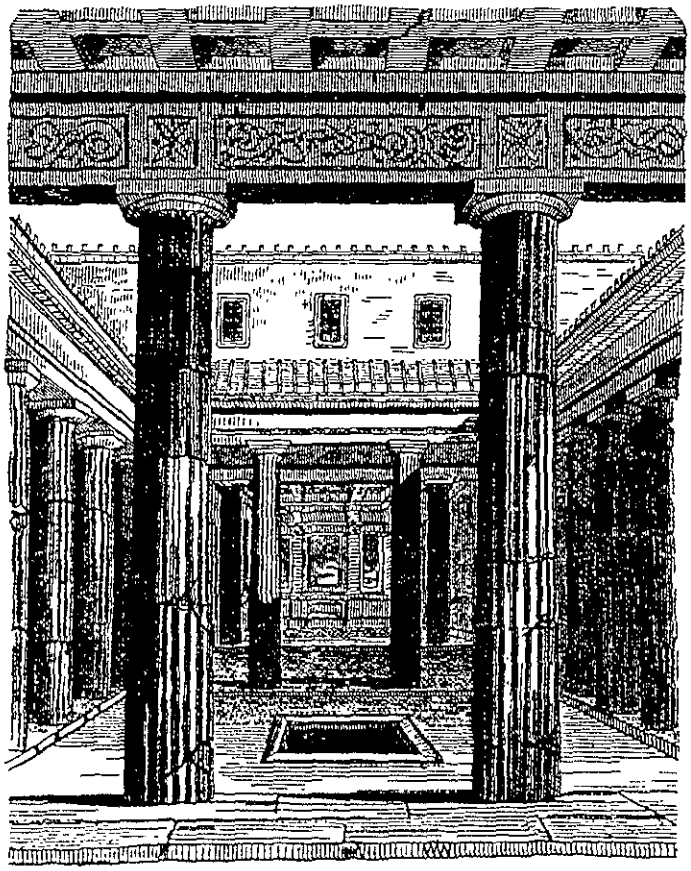

II. ANTECEDENTES

LA EPILEPSIA Y EL GLUTAMATO

La epilepsia ha sido uno de los grandes problemas de la humanidad, tanto por su alta prevalencia e incidencia, como por sus consecuencias médicas y sociales. Etimológicamente, la palabra epilepsia deriva de una preposición y de un verbo irregular griego, *epilambanein*, que significa: "ser sobrecogido bruscamente". La definición de la epilepsia ha sido muy complicada a lo largo de la historia, por lo que en 1973, la Liga Internacional contra la Epilepsia y la Organización Mundial de la Salud publicó un diccionario de epilepsia en el que se define a ésta como *una afección crónica de etiología diversa, caracterizada por crisis recurrentes, debidas a una descarga excesiva de las neuronas cerebrales (crisis epilépticas), asociadas eventualmente con diversas manifestaciones clínicas y paraclínicas.*

Existen muchas clasificaciones de la epilepsia. Pero en la actualidad se separa este padecimiento en dos grupos: epilepsia parcial y epilepsia generalizada. La epilepsia parcial se origina en un pequeño grupo de neuronas que se constituyen en un "foco epiléptico" y de esta manera la sintomatología depende de dónde se encuentra el foco epiléptico, mientras que la epilepsia generalizada involucra a toda la corteza cerebral desde su inicio, aunque no se puede descartar la presencia de un foco epiléptico. El modelo animal que se desarrollará en este trabajo se puede considerar como un modelo de epilepsia parcial, pues se induce un foco epiléptico en el hipocampo que eventualmente produce crisis convulsivas por la propagación de la actividad epiléptica a la corteza cerebral.

En el origen de un foco epiléptico pueden participar múltiple factores, tales como cambios en las propiedades intrínsecas de la membrana neuronal y/o alteraciones en la transmisión sináptica. Invariablemente cada neurona en un foco epiléptico presenta de manera sincronizada una respuesta llamada despolarización paroxística (DP). Una DP se inicia por una despolarización súbita (20-40mV) de larga duración (50-200ms), en la que se monta un tren de potenciales de acción. Esta despolarización esta mediada principalmente por la

activación de receptores a glutamato del tipo AMPA y NMDA (Jefferys y Traub, 1998)

Además del papel fundamental de los receptores glutamatérgicos en la generación de la DP, existe mucha evidencia de que una sobreactividad glutamatérgica está estrechamente relacionada con la epilepsia. La capacidad del glutamato o sus análogos estructurales para producir convulsiones cuando son inyectados en el cerebro fue puesta de manifiesto por los experimentos de Hayashi (1952); más adelante se postuló que la epilepsia podría estar asociada a un metabolismo anormal del glutamato o bien a alteraciones en su transporte, pues se encontraron niveles elevados de glutamato (hasta 3 veces) en pacientes con epilepsia generalizada (Janjua et al., 1992a) o bien en ratones genéticamente epilépticos de la cepa E1 (1.5 veces, Janjua et al., 1992b). Por medio de la técnica de la microdiálisis se han observado incrementos importantes en los niveles extracelulares de glutamato, de entre 3-16 veces, en pacientes epilépticos con crisis de origen cortical (Carlson et al., 1992; Hamberger et al., 1993) o hipocámpico (2-5 veces, During y Spencer, 1993; Wilson et al., 1996). También con el uso de la microdiálisis, se han documentado aumentos en los niveles extracelulares de glutamato en un buen número de modelos animales de epilepsia como el *kindling* (1-3 veces, Zhang et al., 1991; Kaura et al., 1995), la aplicación de ácido kaínico (3 veces, Stein-Behrens et al., 1992), pilocarpina (1.5 veces, Millan et al., 1993), o de estimulación eléctrica (1.2 veces, Walker et al., 1995).

También se ha mostrado que una expresión anormal de los receptores a glutamato o un aumento en su función juega un papel importante en varias formas de epilepsia (McNamara, 1994; Meldrum, 1994). En ratas a las que se les indujo epilepsia por el método de *kindling* se demostró alteraciones importantes en el funcionamiento del receptor tipo NMDA (Kraus et al., 1994; Lee et al., 1994), y se han encontrado incrementos en la actividad del receptor de NMDA en rebanadas corticales de pacientes epilépticos (Louvel y Pumain, 1992). En este mismo sentido se ha observado que la sobreexpresión de la subunidad GLUR6 del receptor de kainato puede producir crisis epilépticas espontáneas y neurodegeneración en el hipocampo (During et al., 1993).

Otros datos que sugieren fuertemente que el glutamato juega un papel importante en la patología de la epilepsia son aquellos obtenidos mediante el bloqueo de los receptores glutamatérgicos, lo cual resulta en una excelente estrategia anticonvulsivante. Por dar algunos ejemplos, los antagonistas competitivos de los receptores NMDA como el CPP (Löscher et al., 1988; Patel y col., 1990) o el AP7 (Czuczwar y Meldrum, 1982) o no competitivos como el MK-801 (McNamara et al., 1988; Gilbert, 1994) o el ADCI (Rogawski et al., 1991) inhiben las convulsiones producidas en una amplia variedad de modelos de epilepsia. En el caso de los receptores no-NMDA, antagonistas de los mismos como el NBQX o el GYKI52466, han probado también ser buenos anticonvulsivantes en distintos modelos (Chapman et al., 1991; Smith et al., 1991; Yamaguchi et al., 1993).

Pero no solamente el bloqueo de los receptores de glutamato ayuda a prevenir las crisis epilépticas. Chapman y colaboradores (1996) mostraron que una disminución en la expresión de los receptores NMDA producida por la aplicación de oligonucleótidos antisentido contra la subunidad NMDAR1 de los receptores de NMDA resulta en una reducción de las crisis epilépticas en ratones genéticamente epilépticos de la cepa DBA/2. Esta misma estrategia dio muy buenos resultados en ratas a las que se indujeron crisis por la aplicación de NMDA (Zapata et al., 1997).

En el caso de los transportadores de glutamato, se ha observado una disminución en la expresión de estas proteínas en un modelo animal de epilepsia (Samuelsson et al., 2000) o la aparición de crisis epilépticas letales en ratones que no expresan el transportador glial GLT-1 (Tanaka et al., 1997).

LA NEURODEGENERACIÓN Y EL GLUTAMATO

Las observaciones de Lucas y Newhouse (1957) de que la administración sistémica de glutamato en el ratón produce degeneración en la retina sugirieron que el glutamato puede actuar como una endotoxina. Posteriormente los trabajos de Olney y sus colaboradores (1971) asociaron la activación de receptores a glutamato con el daño neuronal, estableciéndose a partir de entonces el término

de excitotoxicidad para asociar la despolarización excesiva que produce el glutamato con la muerte neuronal. Posteriormente los trabajos de Rothman (1984) demostraron que el daño neuronal producido por la anoxia en cultivo de neuronas hipocampicas es provocado por la liberación de glutamato y finalmente los trabajos de Choi (1987) pusieron énfasis en el papel determinante del influjo de calcio en la excitotoxicidad.

El glutamato puede producir la excitotoxicidad cuando la acumulación de Ca^{2+} intracelular, producida fundamentalmente por la sobreactivación de los receptores NMDA, rebasa los sistemas de amortiguamiento propios de la célula (Choi, 1987). Esta actividad excitotóxica del glutamato resulta en la producción de especies reactivas de oxígeno de una manera dependiente de calcio (Dugan et al., 1995; Reynolds y Hastings, 1995; Bindokas et al., 1998) ya sea por un incremento en la respiración mitocondrial inducido por la acumulación del Ca^{2+} intracelular (Gunter et al., 1994; White y Reynolds, 1996) o por la activación de la fosfolipasa A2 y de la sintasa del óxido nítrico (Dawson et al., 1992; Coyle y Puttfarcken, 1993; Lafon-Cazal et al., 1993). La actividad de la fosfolipasa A2 genera ácido araquidónico que potencialmente puede generar radicales libres y la sintasa del óxido nítrico genera óxido nítrico (NO).

El NO puede alterar el metabolismo mitocondrial generando grandes cantidades de superóxido (Bolaños et al., 1994; Stamler, 1994; Brorson et al., 1997), que puede reaccionar con el mismo NO para formar peroxinitrito, que es un oxidante muy potente que puede modificar proteínas y producir alteraciones en el DNA (Beckman y Koppenol, 1996; Smith et al., 1997). Finalmente, otra acción generadora de radicales libres por parte del NO es la disminución drástica del NADH producida por la activación de la ADP-ribosa polimerasa, lo que disminuye el poder reductor de las células (Cosi et al., 1994).

Otro blanco posible del incremento excitotóxico en el calcio intracelular es la calpaina. La calpaina es una proteasa presente en las neuronas que es activada por altas concentraciones de calcio, similares a las que se encuentran durante el daño neuronal (Siman et al., 1989; Del Cerro et al., 1994; Wang y Yuen, 1994). Otras enzimas que pueden ser activadas por el calcio son fosfatasas de proteínas

(como la calcineurina) (Feng y Stemmer, 1999), y endonucleasas (Joseph et al., 1993). De todo lo anterior se desprende que la activación de todos estos mecanismos excitotoxicos por el Ca^{2+} resulta en la degradación de múltiples componentes fundamentales para la sobrevivencia neuronal.

EPILEPSIA Y NEURODEGENERACIÓN: LA EPILEPSIA DEL LÓBULO TEMPORAL

Una de las patologías donde más claramente se encuentra una asociación entre la epilepsia y la neurodegeneración es la epilepsia del lóbulo temporal (ELT), un tipo muy común de epilepsia intratable farmacológicamente que responde muy bien a tratamientos quirúrgicos (Thadani et al.,1995). Dado que la asociación del daño neuronal y la epilepsia con el glutamato es el tema fundamental de la presente tesis, se describirá brevemente ésta patología. En 1951, Jasper, Pertuisset y Flanigin usaron el término de ELT para agrupar una gran variedad de crisis (incluyendo a las crisis psicomotoras) que tenían como común denominador la aparición en el electroencefalograma (EEG) de actividad paroxística originada en el lóbulo temporal, que se caracteriza por espigas de alto voltaje y/u ondas lentas. Las manifestaciones ictales de este tipo de epilepsia se pueden subdividir en dos componentes: subjetivos y objetivos (Engel et al., 1997). El componente subjetivo, que generalmente inicia la crisis, recibe el nombre común de *aura*. El aura se caracteriza por sensaciones viscerales principalmente en el epigastrio, aunque también puede expresarse por el común *deja vu, jamais vu* o bien por alucinaciones olfatorias. El aura es continuada por las manifestaciones objetivas, las cuales consisten inicialmente de rigidez muscular y dilatación pupilar; en muchos casos las crisis no progresan mas allá de estas manifestaciones, pero en otros casos las crisis desembocan en la aparición de automatismos e incluso de crisis convulsivas. Desde finales del siglo XIX, más o menos en la misma época Pfleger (1880), Sommer (1880) y Bratz (1889) llamaron la atención sobre cambios histopatológicos que aparecían en el hipocampo de pacientes que presentaban las llamadas "crisis psicomotoras". Después de múltiples reportes aislados, el estudio de Margerison y Corsellis (1966) mostró claramente que la ELT esta asociada en

un alto porcentaje de los pacientes con un cambio histopatológico denominado esclerosis hipocámpica, que se caracteriza fundamentalmente por la aparición de gliosis y daño neuronal en el hipocampo. Es muy importante hacer hincapié en que el patrón de daño neuronal en esta estructura es muy consistente. Como se muestra en la figura 1, en los pacientes con ELT, se encuentra una degeneración

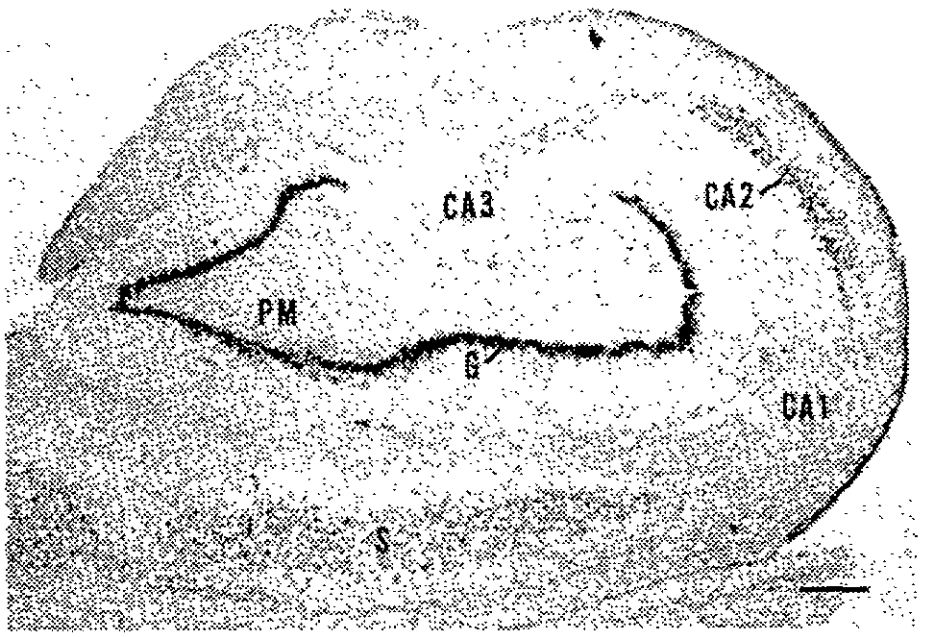

Figura 1. *Hipocampo de un paciente con epilepsia del lóbulo temporal.* Observe la completa neurodegeneración en las regiones de CA1 y CA3, así como la preservación de las neuronas de la región CA2, el giro dentado (G) y el subiculum (S). Barra = 500 µm (Tomado de Houser, 1992).

de las neuronas localizadas en las regiones CA1 y CA3 y la preservación de las "regiones resistentes" de CA2 y el giro dentado (Kim et al., 1990; Houser, 1992). Esta vulnerabilidad selectiva en el hipocampo es simulada por algunos modelos de epilepsia asociados con neurodegeneración, en muchos de los cuales se ha involucrado la activación de receptores a glutamato (Ben-Ari 1985; Stein-Behrens et al., 1994; Zhang et al., 1996; Arias et al., 1997; Grooms et al., 2000), lo que puede indicar la existencia de una estrecha correlación entre un incremento en la transmisión glutamatérgica y la inducción de epilepsia y daño neuronal, como se describe en la siguiente revisión.

ARTICULO DE REVISIÓN 2

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Review article

On the relationship between extracellular glutamate, hyperexcitation and neurodegeneration, in vivo

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1. Introduction

In recent years there has been an increasing interest in the studies on neurodegeneration, including the physiological or programmed neuronal death and the cell disruption occurring as a consequence of necrosis. This interest has been greatly stimulated by the fact that precipitate and localized neuronal destruction is a central event in the course of many acute and chronic disorders of the central nervous system (CNS). These disorders include stroke (anoxia-ischemia), hypoglycemia, cerebral trauma, epilepsy and several devastating neurodegenerative diseases, such as amyotrophic lateral sclerosis, Parkinson's disease, Alzheimer's disease and Huntington's disease

Among the cellular mechanisms possibly involved in neuronal death in the above mentioned disorders, three closely related factors seem to play important roles: (1) the generation of reactive oxygen species or free radicals, (2) the overactivation of synaptic excitatory amino acid (EAA) receptors, and (3) the increase in cytoplasmic free Ca^{2+} concentration. As shown in Fig. 1, the links between these factors are multiple and an initial event may lead, in a cascade manner, to the generation of further alterations.

One of the key events in this chain of reactions resulting in neuronal damage is an excess of the excitatory synaptic neurotransmission mediated by amino acids, mainly glutamate. Pioneer studies in the decade of the seventies showed that exposure of nervous tissue to high concentrations of glutamate and other EAA produced neuronal degeneration and death, and demonstrated that such effects are related to the ability of these neurotransmitters to depolarize the membrane and therefore to excite neurons (Olney, 1971; Olney et al., 1971; Rothman and Olney, 1987). This knowledge led to the concept of excitotoxicity, or neuronal damage due to overexcitation, whose mechanisms are presently known in some detail due to the advances in the characterization of the different types of EAA receptors and their functioning. Two groups of EAA receptors located in neuronal membranes have been identified. A first group of receptors, mainly localized postsynaptically, constitute ligand gated ion channels (ionotropic receptors) and includes the *N*-methyl-D-aspartate (NMDA) type and the non-NMDA type. The two types are activated by glutamate and aspartate, but the former is activated by NMDA whereas the latter includes a subtype recognizing kainate acid (KA) and another subtype that is activated by α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA). The second group is that of metabotropic EAA receptors, which are frequently present in the presynaptic membrane and do not form ion channels but are associated to G proteins and coupled to the production of second intracellular messengers (Hollmann and Heinemann, 1994; Miller, 1994; Michaelis, 1998).

Besides its ligand sensitivity, the NMDA receptor differs from that of non-NMDA receptors in its sensitivity to voltage changes, its requirement for glycine as a positive modulator and its ionic selectivity The NMDA receptor channel is permeable mainly to Na^+ and Ca^{2+}; in contrast, the non-NMDA receptor channels are voltage insensitive and normally permeable only to Na^+, although this permeability may vary depending on the subunit composition of the receptor (Hollmann et al., 1991; Bettler and Mulle, 1995; Pellegrini-Giampietro et al., 1997). These differences determine a complex response to glutamate of the postsynaptic neuron possessing the two types of receptors Under resting conditions, the NMDA receptor is not readily opened by the agonists, because its channel is blocked by Mg^{2+}

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However, because of the voltage sensitivity of this receptor, when the membrane is depolarized Mg^{2+} is expelled out of the channel and the receptor is able to respond to its ligand. Consequently, when the non-NMDA receptor is activated and depolarization occurs due to the entrance of Na^+ through its channel, the overactivation of the NMDA receptor is facilitated.

The hyperactivation of the NMDA receptor results in a massive entrance of Ca^{2+} (Randall and Thayer, 1992; Hartley et al., 1993), which may overcome the intracellular Ca^{2+} buffering mechanisms that, together with plasma membrane Ca^{2+}-ATPases and Na^+-Ca^{2+} exchangers, are responsible for maintaining a submicromolar intracellular concentration of the cation. The resulting accumulation of cytoplasmic Ca^{2+} eventually leads to neuronal death (Mattson, 1994; Siesjö, 1994) due to several factors, such as activation of proteases, lipases and endonucleases, membrane protein and lipid alterations, generation of toxic reactive oxygen species, mitochondrial damage, disruption of energy metabolism, and membrane depolarization. These events potentiate each other in a cascade manner to produce membrane damage and consequently cell death (Fig. 1).

2. Neurotoxicity of EAA receptor agonists

A great deal of the foregoing notions arose from a variety of experiments carried out in vivo and in neuronal cultures, by means of the administration of glutamate o of EAA receptor agonists, such as NMDA and K (Malva et al., 1998). That neurotoxic effects of the compounds are due to their interaction with EAA recep tors is usually ascertained by testing the blockade o prevention of their toxicity in the presence of antagonis specific for the different receptor subtypes. Among th compounds more frequently used are the NMDA recep tor antagonists (±)-3-(2-carboxy-piperazin-4-yl)-propy 1-phosphonic acid (CPP), (±)-2-amino-7-phosphono heptanoic acid (AP7) and (+)-5-methyl-10,11-dihydro 5H-dibenzo[a,d]cyclohepten-5,10-imine hydrogen male ate (MK-801), and the non-NMDA receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, mor effective on KA receptor), 2,3-dihydroxy-6-nitro-7-sul famoyl-benzo(f)quinoxaline (NBQX, more effective o AMPA receptor; Sheardown et al., 1990), and some 2,3 benzodiazepines (Vizi et al., 1996, 1997).

Important support for a link between the abov described mechanisms of excitotoxicity and neurona death in neuropathological disorders derives from th following findings: (a) excessive EAA-mediated neu rotransmission leads to epileptic activity (Choi, 1988 Meldrum, 1991); (b) the cytopathological characteristic of the damage observed after the exposure to EA resemble those occurring after brain ischemia (Brown and Brierly, 1972; Simon et al., 1984; Van Reempts, 1984 or prolonged seizure activity (Evans et al., 1984); (c

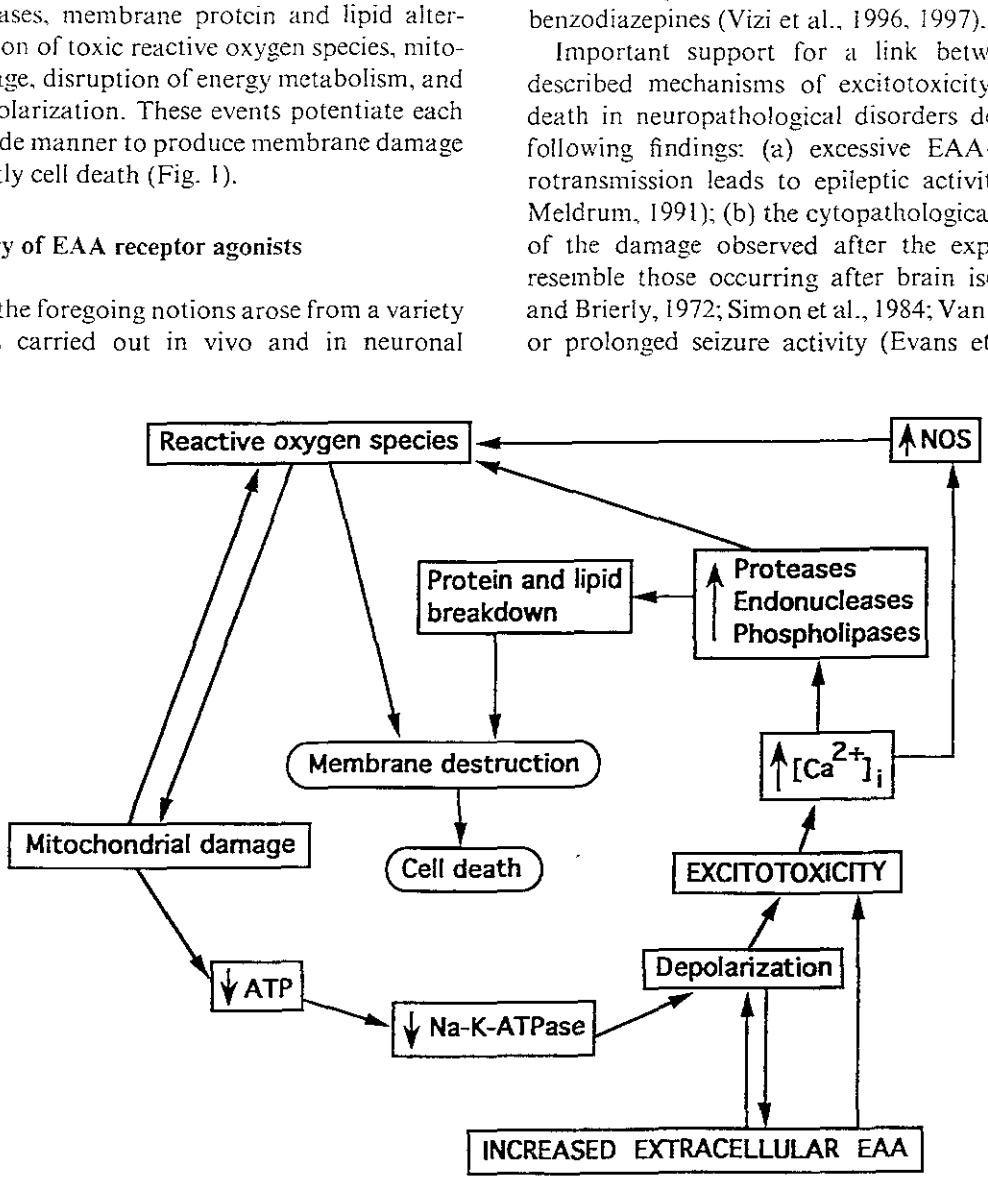

Fig. 1. Factors involved in the mechanisms of neuronal death. This scheme describes mainly death by necrosis due to membrane destruction. NOS = nitric oxide synthase, which may produce reactive oxygen species by oxidation of its product, NO.

microdialysis studies in vivo have shown that during cerebral ischemia (Benveniste et al., 1984, Globus et al., 1988; Hillered et al., 1989; Butcher et al., 1990; Graham et al., 1990; Baker et al., 1991), hypoglycemia (Sandberg et al., 1986), and cerebral trauma (Faden et al., 1989), the extracellular concentrations of glutamate and aspartate are highly increased, reaching levels comparable to those which are neurotoxic in cell cultures (Choi et al., 1987); (d) antagonists of both the NMDA and non-NMDA receptor types protect against the neuronal damage associated to these neuropathological conditions (Gill et al., 1988, 1992; Bullock et al., 1990; Sheardown et al., 1990; Swan and Meldrum, 1990).

3. Is the origin of augmented extracellular glutamate important for neurotoxicity?

Most of the studies on glutamate-induced neurotoxicity have been carried out in vitro, largely in neuronal or mixed neuronal-glial cultures (Ohno et al., 1997; Rogers and Hunter, 1997; Jensen et al., 1998), whereas in vivo the excitotoxicity has been observed in some brain regions mainly after the intracerebral microinjection of EAA receptor agonists, such as NMDA, KA, AMPA or dihydrokainate (DHK, an inhibitor of glutamate transport that seems to have a direct agonist effect on NMDA receptors) (Ben-Ari, 1985; Stein-Behrens et al., 1994, Massieu and Tapia, 1994, 1997; Massieu et al., 1995; Arias et al., 1997). These experimental conditions, however, do not necessarily reflect the situation occurring under pathological states, in which, as implied in the previous section, neuronal destruction by excitotoxicity must be due primarily to an excess of endogenous extracellular EAA capable of overactivating their postsynaptic receptors. It is clear, therefore, that in vivo EAA-induced neurodegeneration can occur only when the extracellular concentration of the amino acids increases above a critical value and/or for a certain period of time. During the normal functioning of the glutamatergic synapses this does not occur because after the release of glutamate from the presynaptic terminal its concentration in the synaptic cleft is kept low ($\sim$1 μM), due to its rapid clearance by an efficient reuptake carried out by specific high affinity transporters (Fagg and Foster, 1983; Fonnum, 1984; Nicholls and Attwell, 1990), of which five types have been cloned (Danbolt, 1994; Fairman et al., 1995; Arriza et al., 1997). It is therefore clear that an augmented glutamate release, such as that probably occurring in certain types of epilepsy, could result in hyperexcitation but would not necessarily induce neurodegeneration, because of the rapid removal of glutamate from the synaptic cleft. On the other hand, one should expect that a deficient reuptake would more easily result in extracellular glutamate levels sufficiently high and long lasting to become toxic. This has been postulated to occur during ischemia, since the transporter requires extracellular Na^+, which is co-transported with glutamate, and thus the ischemia-induced energy failure might result in diminished Na^+-K^+-ATPase activity and therefore in an impairment of the transporter. Additionally, glutamate release might occur through the reversal of the carrier-mediated Na^+-dependent uptake (Pulsinelli and Duffy, 1983; Obrenovitch and Urenjak, 1997). In fact, as already mentioned, it has been shown that extracellular aspartate and glutamate levels increase after experimental transient ischemia.

In the following sections these relationships will be discussed, focused on experiments in vivo, which should reflect more closely than those in vitro the complexities of the pathological alterations. Indeed, several important factors influencing the possibility of interaction of glutamate with its receptors, for example the volume, constraints and tortuosity of the extracellular space, particularly the synaptic cleft (Rusakov and Kullmann, 1998), may be overlooked by the in vitro experimental conditions. These and other problems make it difficult to establish a correlation in vivo between alterations in the extracellular concentration of EAA and facilitation of excitatory synaptic transmission. However, a reasonable expectation would be that if increased levels of EAA may indeed hyperactivate the receptors, when such increase occurs an enhanced neuronal excitability should be observed, behaviorally and/or electroencephalographically, accompanied by or followed by neuronal damage of the cerebral region affected.

What procedures could be appropriate to test this presumption? An obvious answer is to design experiments aimed at studying whether a stimulation of EAA release or an inhibition of its transport results in enhanced endogenous extracellular EAA and whether this is related to neuronal hyperexcitability and neurodegeneration. This can be accomplished with the use of the microdialysis technique, which has been extensively used to measure changes in the extracellular concentration of amino acids in vivo under a great variety of experimental conditions. However, the origin and consequences of an enhanced EAA concentration, as determined by this technique, have been the subject of considerable controversy (Herrera-Marshitz et al., 1996; Timmerman and Westerink, 1997). In the light of the above discussion, the heart of this controversy lies not only in ascertaining what is the cellular origin of the basal and the increased extracellular EAA (neuronal, synaptic or glial) but also in establishing that they are indeed enhancing the excitatory synaptic efficacy.

3.1 *Inhibition of glutamate uptake*

Recently, several reports have appeared studying the effect of inhibitors of EAA transporters on the concentration of extracellular amino acids in vivo. The most

used compounds are L-*trans*-pyrrolidine-2,4-dicarboxylate (PDC), DHK, and DL-*threo*-β-hydroxy-aspartate. Microdialysis experiments have shown that in the striatum and in the hippocampus the basal levels of glutamate and aspartate are notably increased by PDC (Millan et al., 1993; Bloc et al., 1995; Massieu et al., 1995; Herrera-Marschitz et al., 1996; Obrenovitch et al., 1996; Zuiderwijk et al., 1996; Massieu and Tapia, 1997; Rawls and McGinty, 1997; Lada et al., 1998), but only few investigations have addressed the correlation between the changes observed and electrical activity alterations or neurodegeneration. In one of these studies (Obrenovitch et al., 1996), it was found that PDC-induced increases of extracellular glutamate to more than 20-fold the basal level in the hippocampus did not induce electrophysiological signs of hyperexcitability. In two studies from our laboratory (Massieu et al., 1995; Massieu and Tapia, 1997), we have shown that PDC induced long lasting (over 2 h) large increases of extracellular glutamate (10–20-fold, reaching concentrations comparable to those observed during neuronal damage due to ischemia (Globus et al., 1988; Hillered et al., 1989; Baker et al., 1991; Wahl et al., 1994)), but no neurodegeneration was observed, either in the striatum or in the hippocampus. In these reports, neuronal damage was assessed by histological examination and, in the case of the striatum, by biochemical determination of choline acetyltransferase and glutamate decarboxylase activities, markers of cholinergic and GABAergic neurons, respectively. The effect of PDC was much higher on glutamate and aspartate than on other amino acids, in agreement with the described potent and selective action of this compound on the glutamate/aspartate transporter (Bridges et al., 1991; Robinson et al., 1991). The elevation of glutamate by PDC could be due to a combination of the blockade of the transporter and a carrier-mediated release of the amino acid, since it has been shown that PDC can be exchanged with glutamate (Bridges et al., 1991; Isaacson and Nicoll, 1993; Sarantis et al., 1993). The important finding was, however, that in spite of the very high levels of extracellular glutamate no neuronal damage was observed (Massieu et al., 1995; Massieu and Tapia, 1997).

In contrast to this lack of effect of PDC, under similar experimental conditions the administration of DHK or KA through the microdialysis probe produced remarkable neuronal damage in the striatum, observed 7 days after treatment. The lesion produced by DHK was partially prevented by treatment with the NMDA receptor antagonist MK-801 or with the non-NMDA receptor antagonist NBQX (Massieu et al., 1995). In addition, the microinjection of DHK in the hippocampus produced a notable neuronal destruction a few hours after treatment, which was, however, restricted to the CA1 area and prevented by MK-801 but not by NBQX (Arias et al., 1997). Altogether, these experiments with DHK suggest that the neurotoxic effect of the latter is due to a direct activation of glutamate receptors and not to the glutamate accumulated in the extracellular space.

Whatever the mechanism of the neurotoxic effect of DHK may be, the above results with PDC clearly indicate that increased extracellular glutamate in vivo by impairment of its transporter is not sufficient by itself to produce neuronal hyperexcitation or neuronal damage. One possible explanation for these unexpected findings is that the extracellular glutamate accumulated as a consequence of PDC action on the transporter does not reach sufficiently high concentrations to overactivate glutamate receptors. In view that five different glutamate carriers have been cloned and they differ in their neuronal or glial localization (Kanai and Hediger, 1992; Pines et al., 1992; Storck et al., 1992; Kanai et al., 1993; Fairman et al., 1995; Arriza et al., 1997), another possibility is that the transporter affected by PDC might not be the one closely involved in the removal of glutamate from synaptic sites. However, it has been reported that PDC inhibits glutamate transport in both synaptosomes and cultured astrocytes (Bridges et al., 1991; Robinson et al., 1991; Rauen et al., 1992; Garlin et al., 1995), and that the extracellular glutamate enhanced under the effect of PDC in vivo seems to be of neuronal origin and may possess transynaptic effects in the striatum (Rawls and McGinty, 1997).

Two other models of glutamate transport deficiencies in vivo have been described: knockout of the transporters by intracerebroventricular chronic administration in the rat of antisense oligonucleotides specific for the glial (GLAST and GLT-1) or neuronal (EAAC1) transporters (Rothstein et al., 1996), and the generation of mutant mice lacking EAAC1 (Peghini et al., 1997). In both studies, when EAAC1 was affected animals showed some motor and behavioral abnormalities, but no neurodegeneration was observed. In contrast, with the antisense oligonucleotides against GLAST and GLT-1 extracellular glutamate was increased and some signs of neurodegeneration characteristic of excitotoxicity were observed in the striatum and the hippocampus (Rothstein et al., 1996). These studies suggest that the glial glutamate transport may be more efficient in reducing the level of synaptic glutamate. In any case, it seems that in the absence of other potentiating factors the concentration of endogenous glutamate in the synapse must reach very high values in order to produce neurotoxicity (Obrenovitch et al., 1996; Obrenovitch and Urenjak, 1997), since under normal synaptic transmission it may peak at > 1 mM concentration (Clements et al., 1992; Clements, 1996).

The problems in understanding the accessibility of endogenous glutamate to the postsynaptic receptors and the role of its clearance by uptake in vivo can be illustrated by comparing the lack of neurotoxicity described in the preceding paragraphs with the results of experi-

ments in neuronal and glial cultures In these mixed cultures, exposure to PDC induced an elevation of glutamate concentration in the culture medium which, differently from the observations in vivo, resulted in neuronal damage as assessed morphologically, by the release of lactic dehydrogenase or by the mitochondrial reduction of a tetrazolium salt; this neurodegeneration was attenuated by NMDA receptor antagonists (Blitzblau et al , 1996, Velasco et al., 1996, Volterra et al., 1996). Furthermore, under these in vitro conditions the PDC-induced accumulation of extracellular glutamate resulted also in sustained neuronal depolarization (Volterra et al., 1996). Also in contrast to the microdialysis results, under these experiments in vitro DHK did not induce neurotoxicity nor produced any increase of extracellular glutamate (Velasco et al , 1996). Since DHK does not affect glutamate transport in cultured astrocytes (Garlin et al., 1995), these data support the participation of the glial transporter as the main site of removal of extracellular glutamate in the mixed cultures

The most probable explanation for the discrepancy between the lack of neurotoxicity of glutamate accumulation by uptake inhibition in vivo and its damaging effect in the mixed neuronal-glial cultures is that, whereas in the former condition the access of the extracellular glutamate to the synaptic receptor sites might be limited, in cell cultures the amino acid readily diffuses and reaches all available receptor sites.

3.2. *Excessive glutamate release' experiments with 4-aminopyridine*

The second experimental approach to increase the extracellular levels of glutamate and assess its effects on excitability and neuronal integrity is by enhancement of release Among the drugs capable of stimulating the release of neurotransmitters are certain K^+ channel blockers, such as 4-aminopyridine (4-AP) and some members of the family of dendrotoxin peptides from the venom of the black mamba snake These compounds prolong the depolarization phase of action potentials, augment the frequency of neuronal firing (Nisenbaum et al., 1994), and induce the release of neurotransmitters in several in vitro preparations, such as the neuromuscular junction (Lundh, 1978, Thesleff, 1980), brain slices (Dolezal and Tucek, 1983, Hu et al , 1991, Versteeg et al , 1995, Schechter, 1997) and synaptosomes (Tapia and Sitges, 1982; Tapia et al , 1985, Tibbs et al , 1989a,b) It is interesting that this releasing effect of both dendrotoxins and 4-AP is strictly dependent on external Ca^{2+}, which, together with other pharmacological and ionic studies in brain slices (Thesleff, 1980; Jones and Heinemann, 1987, Perrault and Avoli, 1991), indicate that the neurotransmitters released by these drugs originate from presynaptic nerve endings

Both 4-AP and dendrotoxins are also potent convulsants, that produce intense behavioral signs of hyperexcitability and EEG seizures when administered intracerebrally These effects have been observed after injection in the rat substantia nigra reticulata, hippocampus, cerebral ventricles and cerebral cortex (Gandolfo et al , 1989, Fragoso-Veloz et al , 1990, Tapia and Flores-Hernández, 1990; Bagetta et al., 1992, 1994, 1996, Fragoso-Veloz and Tapia, 1992; Morales-Villagrán et al , 1996) 4-AP produces also intense seizure activity when administered systemically in several mammalian species, including man (Schafer et al., 1973; Spyker et al., 1980, Fragoso-Veloz et al., 1990; Yamaguchi and Rogawski, 1992; Cramer et al., 1994). Furthermore, this drug induces epileptiform electrical discharges in vitro, in rat hippocampal (Perrault and Avoli, 1991, Yonekawa et al , 1995; Avoli et al , 1996) and cortical (Siniscalchi et al., 1997) slices.

An increased glutamatergic transmission in the epileptogenic action of dendrotoxins and 4-AP has been postulated on the basis that EAA receptor antagonists, of both the NMDA and the non-NMDA types, are effective anticonvulsants against seizures induced by these compounds in vivo (Gandolfo et al , 1989, Fragoso-Veloz and Tapia, 1992; Bagetta et al., 1994, 1996; Cramer et al., 1994, Morales-Villagrán et al., 1996) and, in the case of 4-AP, also in brain slices (Perrault and Avoli, 1991; Avoli et al., 1996; Siniscalchi et al., 1997). Further support for the involvement of glutamatergic transmission in the 4-AP-induced seizures stems from our recent finding that 4-AP stimulates the release of glutamate in the striatum and in the hippocampus in vivo, and that this release is correlated with intense behavioral convulsive activity (Fig. 2; Morales-Villagrán and Tapia, 1996).

The above discussed findings, particularly in the case of 4-AP, clearly suggest that, differently from the results of uptake inhibition, an increase in the extracellular glutamate resultant from an excessive release may overactivate EAA receptors and thus induce electrical and behavioral hyperexcitation and seizures. Consequently, in accord with the previous sections, it was to be expected that such an increase would produce neurodegeneration This in fact has been shown to occur with dendrotoxins, which induce notable neuronal destruction when administered in the hippocampus (Baggeta et al., 1994, 1996) Since no similar studies have been reported with 4-AP, we have carried out some experiments in the hippocampus, using microdialysis and simultaneously measuring the release of amino acids and the EEG As shown in Fig. 2, when we perfused 4-AP through a microdialysis probe in this region, we observed a correlation between the increase in extracellular glutamate collected via the same microdialysis probe and neurodegeneration of the CA1, CA3 and CA4, but not CA2, hippocampal subfields It must be emphasized that this selective neuronal damage was similar to that observed after the intrahippocampal injection of EAA receptor agonists (Ben Ari, 1985, Stein-

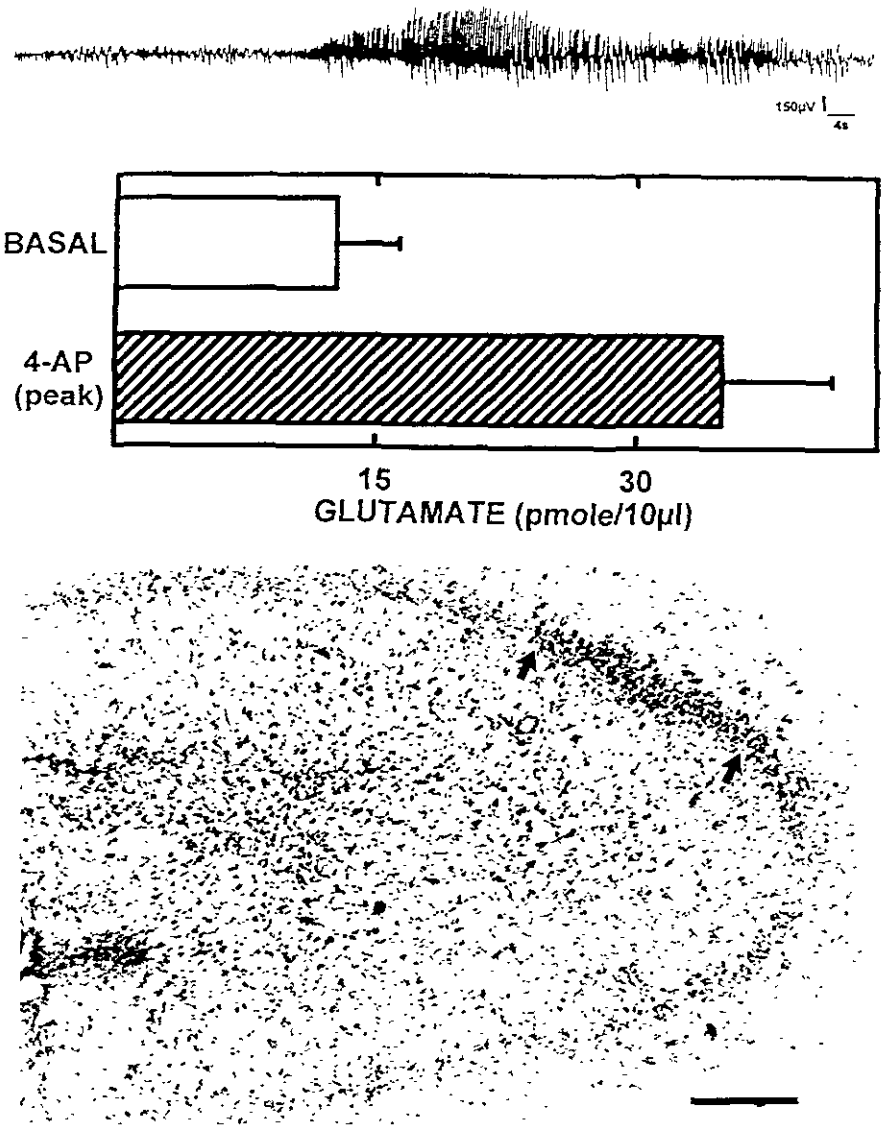

Fig. 2. Effects of microdialysis administration of 4-AP in the anesthetized rat hippocampus on electrical activity (top trace), extracellular glutamate (middle graph) and histological appearance (bottom micrograph). After obtaining basal electrical activity (using the microdialysis cannula as the electrode) and three 12.5 min microdialysis fractions (25 μl each) to determine basal extracellular glutamate values (by HPLC and fluorometric detection), 4-AP (35 mM) was perfused through the microdialysis cannulas during 12.5 min, and three subsequent fractions were collected. The electrical epileptic discharge shown lasted 18 s and occurred at 5 min after 4-AP; it is a representative example of the seizure discharges produced by this 4-AP dose, which occurred with a frequency of about one every 1.5 min and persisted until the end of the experiment. The peak increase in extracellular glutamate (values are means ± SEM for 13 rats) corresponds to the first microdialysis fraction after 4-AP, and coincides with the onset of seizure discharges. Five days after the experiment rats were fixed by transcardial perfusion and coronal brain sections were stained with cresyl violet. As can be observed in the representative example shown, a notable neuronal loss was observed in CA1, CA3 and CA4, but CA2 (between arrows) was spared. (Scale bar = 200 μm). The electrical seizures and neuronal damage were very similar in the 13 rats studied. For details on the experimental procedures (see Massieu et al., 1995; Morales-Villagrán and Tapia, 1996; Massieu and Tapia, 1997).

Behrens et al., 1994; Arias et al., 1997). These results strongly suggest that the hippocampal neurodegeneration observed after stimulation of glutamate release is related to an enhanced concentration of the amino acid at glutamatergic synapses, which could permit its interaction with the abundant EAA receptors present in the areas lesioned by 4-AP (Insel et al., 1990; Young et al., 1991).

4. Conclusion

In conclusion, the present article describes some of the problems in correlating the increases in extracellular glutamate in vivo with excitability alterations and neurodegeneration. The origin and mechanisms of glutamate accumulation in the extracellular space appear to be determinant for the access of the amino acid to its post-

synaptic receptors and consequently for the induction of excitotoxic neuronal damage. Even very high concentrations of the amino acid do not seem to be sufficient to produce neurodegeneration, unless they occur at the synaptic space. This clearly differs from the abundant data on glutamate-induced excitotoxicity in tissue culture, and casts some doubts on the generally accepted possibility that increased extracellular glutamate by itself, when originating from cell destruction or glutamate transporter deficiencies, might be responsible for neuronal death.

From the data reviewed in the present article it can be concluded that it would be an oversimplification to relate the occurrence of neurodegeneration only to an augmented concentration of endogenous extracellular glutamate or other EAA. Although there is no doubt that such increase may facilitate neuronal death through the mechanisms outlined here, other concurrent factors that accompany neurological disorders, such as mitochondrial energy deficits or chronic hyperexcitability, seem to be necessary to induce neurodegeneration.

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LA 4-AMINOPIRIDINA, LA EPILEPSIA Y EL GLUTAMATO

La 4-aminopiridina es un bloqueador de canales de potasio de amplio espectro. De hecho, aunque con diferente potencia, puede bloquear todos los canales de potasio clonados hasta el momento (Chandy y Gutman, 1995; Dolly y Parcej, 1996). A nivel fisiológico, se conoce muy bien que a diferencia del tetraetilamonio (TEA), la 4-AP bloquea preferentemente las corrientes de potasio transitorias, es decir, aquellas que se activan rápidamente y se inactivan aun en presencia del estimulo despolarizante. Estas corrientes reciben el nombre de corrientes tipo A (I_A) y tipo D (I_D) (Storm, 1993); el bloqueo de estas corrientes por la 4-AP puede prolongar la fase despolarizante del potencial de acción e inducir disparo repetitivo (Bargas et al., 1989; Nisenbaum et al., 1994; Koyano et al., 1996, Hoffman et al., 1997). Sin embargo, el principal blanco de acción de la 4-AP se encuentra en las terminales sinápticas, donde produce la liberación de neurotransmisores excitadores e inhibidores en diferentes preparaciones *in vitro*, como la placa neuromuscular (Lundh, 1978; Thesleff, 1980), rebanadas de cerebro (Buckle y Haas, 1982; Dolezal y Tucek, 1983; Hu et al., 1991; Veersteg et al., 1995; Schechter, 1997) o sinaptosomas (Tapia y Sitges 1982; Tapia et al., 1985; Tibbs et al., 1989) así como en algunas regiones cerebrales *in vivo* (Dawson and Routledge, 1995; Morales-Villagrán y Tapia 1996). La 4-AP también es un potente convulsivante que induce actividad epileptiforme *in vitro* (Gean et al., 1990; Perrault y Avoli, 1991; Avoli et al., 1996; Siniscalchi et al., 1997, Doczi et al., 1999) y convulsiones *in vivo* en la rata (Gandolfo et al., 1989; Fragoso-Veloz y Tapia, 1992, Morales-Villagrán et al., 1996; Barna et al., 2000) en el ratón (Yamaguchi y Rogawski, 1992; Cramer et al., 1994) e incluso en el humano (Spyker et al., 1980).

Múltiples líneas de evidencia muestran que el efecto convulsivante de la 4-AP está mediado por un aumento en la liberación de glutamato y la concomitante sobreactivación de sus receptores. Se ha observado que los antagonistas para los receptores NMDA y no-NMDA resultan ser muy buenos anticonvulsivantes contra la actividad epileptiforme que produce la 4-AP *in vitro* (Perrault y Avoli, 1991; Avoli et al., 1996; Siniscalchi et al., 1997; Doczi et al., 1999) e *in vivo* (Gandolfo et al., 1989; Fragoso-Veloz, 1992; Cramer et al., 1994; Morales-Villagrán et al., 1996,

Barna et al., 2000). Un estudio previo realizado en nuestro laboratorio mostró que la 4-AP libera preferencialmente glutamato cuando es administrado por microdiálisis en el estriado de la rata (Morales-Villagrán y Tapia, 1996).

Como se muestra claramente en el artículo de revisión 2, parece existir una estrecha interrelación entre un incremento en la liberación de glutamato con la inducción de epilepsia y neurodegeneración. En esta misma revisión se dejó en claro que, en el animal íntegro, un incremento en el glutamato extracelular, como el que produce el bloqueo de los transportadores de glutamato, no desencadena epilepsia y/o neurodegeneración, pues parece ser fundamental que este glutamato sea de origen neuronal y más aún de origen presináptico (Massieu et al., 1995; Obrenovitch et al., 1996; Massieu y Tapia 1997, Obrenovitch y Urenjak, 1997). En este sentido, la 4-AP puede ser una muy buena herramienta experimental, pues diferentes aproximaciones experimentales muestran que la 4-AP tiene una acción muy potente a nivel presináptico. La 4-AP es capaz de producir efectos claramente presinápticos tales como aumento en la frecuencia de potenciales espontáneos miniatura (Perrault y Avoli, 1991; Flores-Hernandez et al., 1994) disminución en la relación de pulsos pareados (Buckle y Hass, 1982; Msghina et al., 1998) despolarización medida con indicadores fluorescentes sensibles al voltaje (Barish et al., 1996) o la acumulación de calcio presináptico (Jones y Heinemann, 1987) a concentraciones mucho menores de las necesarias para producir cambios a nivel postsináptico (Buckle y Hass, 1982; Perraul y Avoli, 1991; Hoffman et al., 1997), esto debido a que los canales de potasio que son más sensibles a la 4-AP están localizados preferentemente en la presinapsis (Rudy, 1988; Southan y Robertson, 1998).

ESTRATEGIAS DE NEUROPROTECCIÓN CONTRA LA EPILEPSIA Y LA NEURODEGENERACIÓN

Con la finalidad de obtener herramientas terapéuticas contra la epilepsia y la neurodegeneración se han desarrollado múltiples estrategias de neuroprotección. Como se ha revisado en las secciones precedentes una de las hipótesis mejor fundamentadas sobre la generación de la epilepsia y la neurodegeneración

plantea que éstas se pueden originar por una sobreexcitación glutamatérgica, por lo tanto una de las estrategias de neuroprotección mas estudiadas en modelos animales es el bloqueo de los receptores glutamatérgicos (Meldrum, 1991). A pesar de que los antagonistas de los diferentes receptores ionotrópicos de glutamato tienen excelentes efectos anticonvulsivantes y neuroprotectores en modelos animales (para una revisión ver Lees, 2000), el gran problema con esta estrategia de neuroprotección es por una parte que los promisorios resultados obtenidos con animales de laboratorio no se corresponden con buenos efectos clínicos, y por otra parte, como era de esperarse, el bloqueo de la transmisión glutamatérgica afecta a prácticamente todas las funciones del sistema nervioso y por lo tanto genera una gran cantidad de efectos colaterales indeseables (Muir y Lees, 1995; Klein et al., 1999).

Dado que la inhibición sináptica es un mecanismo muy importante para regular la excitabilidad neuronal, un aumento en la inhibición podría ser un medio efectivo para contrarrestar la excitabilidad anormal que se presenta en la epilepsia y la neurodegeneración. Por ello, se ha planteado como una estrategia de neuroprotección el uso de potenciadores de la actividad GABAérgica (Lyden, 1997). El GABA es el neurotransmisor inhibidor más ampliamente utilizado en el cerebro y su efecto inhibidor se deriva de la activación de sus receptores $GABA_B$ (metabotrópico) o $GABA_A$ (ionotrópico). Los receptores $GABA_B$ reducen la liberación de distintos neurotransmisores principalmente por el bloqueo de canales de calcio a nivel presináptico (Barral et al., 2000) e inhiben postsinápticamente al generar un potencial postsináptico inhibidor por la activación de algunos canales de K^+ (Karlsson y Olpe, 1989), mientras que los receptores $GABA_A$ abren una conductancia de Cl^- que generalmente hiperpolariza e inhibe a las neuronas (Mehta y Ticku, 1989). En este sentido, se ha demostrado que la inhibición GABAérgica puede restringir la excitación provocada por el glutamato (Kanter et al., 1996) y de esta manera bloquear el influjo de Ca^{2+} promovido por la activación de los receptores NMDA y la muerte neuronal inducida por la activación de los mismos (Saji y Reis, 1987).

La acción anticonvulsivante del GABA esta ampliamente estudiada, pues algunos de los anticonvulsivantes más utilizados en la clínica como las benzodiacepinas y los barbitúricos deben probablemente su efecto antiepléptico a que incrementan las corrientes GABAérgicas (Skerritt y Macdonald, 1984; Schulz y Macdonald, 1981).

Hay mucha maneras de aumentar la transmisión GABAérgica, ya sea por inhibir su degradación, bloquear sus recaptura o simplemente activar a sus receptores (Green et al., 2000). Solo por dar algunos ejemplos, tanto los inhibidores de los transportadores de GABA (Johansen y Diemer, 1991; Inglefield et al., 1995; Phillis, 1995), los agonistas de los receptores $GABA_A$ (Lyden y Hedges, 1992; Shuaib et al., 1993; Lyden y Lonzo, 1994), las benzodiacepinas (Sternau et al., 1989; Voll y Auer, 1991; Schwartz et al., 1994) y los inhibidores de la GABA transaminasa (Ylinen et al., 1991) han mostrado efectos neuroprotectores y anticonvulsivantes en diferentes modelos *in vitro* e *in vivo*.

Otra posibilidad de neuroprotección es la inhibición de la entrada de calcio a las neuronas (Choi, 1995), pues el Ca^{2+} esta muy relacionado con el control de la excitabilidad neuronal y la generación de epilepsia (DeLorenzo, 1986; Heinemann y Hamon, 1986) y neurodegeneración (Siesjö et al., 1995). De hecho se ha mostrado que los antagonistas orgánicos de los canales de calcio tipo L (como el verapamil, la nimodipina, la flunaricina, etc.) son buenos antiepilépticos (Morocutti et al., 1986; Vezzani et al., 1988; Aicardi y Schwartzkroin, 1990; Czuczwar et al., 1992) aunque sus efectos protectores contra la muerte neuronal son muy inconsistentes (para una revisión ver Wauquier et al., 1988). Por otra parte, recientemente se ha encontrado que el bloqueo de los canales de calcio tipo N tiene muy buenos efectos neuroprotectores en modelos de isquemia global y focal (Valentino et al., 1993; Buchan et al., 1994; Zhao, 1994; Bowersox y Luther, 1998), pero aun no se han probado sus efectos en modelos de epilepsia.

Las drogas que bloquean los canales de Na^+ sensibles al voltaje son bien conocidas como anestésicos locales, antiarritmicos y anticonvulsivantes, pero más recientemente se ha explorado su papel protector contra la neurodegeneración (para una revisión ver Taylor y Meldrum, 1995). El bloqueo selectivo de los

canales de sodio con la tetrodotoxina (TTX) previene contra el daño neuronal inducido por isquemia *in vitro* (Boening et al., 1989) e *in vivo* (Yamasaki et al., 1991; Lysko et al., 1994) y previene el daño neuronal en cultivos hipocámpicos inducido por hipoglicemia y por cianuro de potasio (Tasker et al., 1992; Vornov et al., 1994) o el daño producido por el ácido 3-nitropropiónico en cultivos organotípicos (Storgaar et al., 2000). Algunos anticonvulsivantes cuyo efecto terapéutico esta asociado con la inhibición de los canales de sodio también han mostrado cierta capacidad neuroprotectora. La carbamacepina puede proteger contra el daño producido por la inyección de NMDA en ratas neonatas (McDonald y Johnston, 1990) por anoxia *in vitro* (Fern et al., 1993) e *in vivo* (Rataud et al., 1994); la fenitoina también protege contra el daño que produce la hipoxia en rebanadas de hipocampo (Taylor et al., 1995) o en el hipocampo de la rata *in vivo* (Taft et al., 1989).

Un neuroprotector que está muy relacionado con el bloqueo de los canales de Na^+ sensibles al voltaje es el riluzol (Romettino et al., 1991; Stutzmann et al., 1991; Guyot et al., 1997;O´Neill et al., 1997; Obrenovitch, 1997; Siniscalchi et al., 1999). Esta droga que ha sido catalogada como "inhibidor de la liberación de glutamato" (Louvel et al., 1997) es hasta la fecha el único fármaco que ha demostrado algún efecto terapéutico en el tratamiento de la esclerosis lateral amiotrófica (Roch-Torreilles et al., 2000), una enfermedad neurodegenerativa en la que se presenta una muerte progresiva de las motoneuronas que se ha planteado pudiera deberse a una sobreexcitación glutamatérgica (Rothstein, 1995). El riluzol también ha mostrado buenos efectos neuroprotectores contra la neurodegeneración producida por la isquemia *in vitro* e *in vivo* (Malgorius et al., 1989; Pratt et al., 1992; Wahl et al., 1993) y por la aplicación de ácido 3-nitropropiónico (Guyot et al., 1997) e incluso se le han encontrado algunos efectos anticonvulsivantes (Romettino et al., 1991; Stutzmann, 1991).

Otro grupo de drogas que se ha planteado que puede tener buenos efectos neuroprotectores son los abridores de canales de potasio (Obrenovitch, 1997) pues el abrir canales de potasio puede reducir la actividad neuronal al decrementar la excitabilidad de las neuronas y la liberación de los

neurotransmisores (Boireau, 1991; Hertaux et al., 1993). Y, efectivamente, los abridores de canales de potasio pueden proteger contra el daño neuronal producido por la isquemia (Reshef et al., 1998) o el provocado por el glutamato *in vitro* (Abele y Miller, 1990; Goodman y Mattson, 1996; Lauritzen et al., 1997) e incluso pueden funcionar como anticonvulsivantes (Alzheimer y ten Bruggencate, 1988; Gandolfo et al., 1989; Popoli et al., 1991; Katsumori et al., 1996).

OBJETIVO GENERAL

Con base en los antecedentes previos, el objetivo general de este trabajo es determinar si existe una relación entre un aumento en el glutamato extracelular producido por la 4-AP y la inducción de epilepsia y neurodegeneración en el hipocampo de la rata *in vivo*, así como probar algunas estrategias de neuroprotección relacionadas con la transmisión GABAérgica y con algunos canales iónicos.

OBJETIVOS PARTICULARES

1.- Establecer si existe correlación entre un incremento en la concentración de glutamato extracelular producido por la 4-AP con la inducción de crisis epilépticas y neurodegeneración, así como comparar estos efectos con otros inductores de la acumulación extracelular de glutamato (incremento en la concentración extracelular de K^+ y tetraetilamonio) en el hipocampo de la rata *in vivo*.

2.- Determinar si el glutamato acumulado por la acción la 4-AP es de origen presináptico y si el bloqueo de esta acumulación puede tener efectos neuroprotectores.

3.- Determinar el papel los receptores ionotrópicos de los receptores glutamato en los efectos de la 4-AP *in vivo*.

4.- Probar si un incremento de la actividad GABAérgica puede tener efectos neuroprotectores contra las alteraciones que produce la 4-AP en el hipocampo de la rata *in vivo*.

5.- Probar si moduladores de la actividad de algunos canales iónicos pueden tener efectos neuroprotectores contra las alteraciones que produce la 4-AP en el hipocampo de la rata *in vivo*.

III. RESULTADOS

TRABAJO 1

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Relationships Among Seizures, Extracellular Amino Acid Changes, and Neurodegeneration Induced by 4-Aminopyridine in Rat Hippocampus: A Microdialysis and Electroencephalographic Study

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Abstract: 4-Aminopyridine is a powerful convulsant that induces the release of neurotransmitters, including glutamate. We report the effect of intrahippocampal administration of 4-aminopyridine at six different concentrations through microdialysis probes on EEG activity and on concentrations of extracellular amino acids and correlate this effect with histological changes in the hippocampus. 4-Aminopyridine induced in a concentration-dependent manner intense and frequent epileptic discharges in both the hippocampus and the cerebral cortex. The three highest concentrations used induced also a dose-dependent enhancement of extracellular glutamate, aspartate, and GABA levels and profound hippocampal damage. Neurodegenerative changes occurred in CA1, CA3, and CA4 subfields, whereas CA2 was spared. In contrast, microdialysis administration of a depolarizing K^+ concentration and of tetraethylammonium resulted in increased amino acid levels but no epileptic activity and no or moderate neuronal damage. These results suggest that seizure activity induced by 4-aminopyridine is due to a combined action of excitatory amino acid release and direct stimulation of neuronal firing, whereas neuronal death is related to the increased glutamate release but is independent of seizure activity. In addition, it is concluded that the glutamate release-inducing effect of 4-aminopyridine results in excitotoxicity because it occurs at the level of nerve endings, thus permitting the interaction of glutamate with its postsynaptic receptors, which is probably not the case after K^+ depolarization. **Key Words:** 4-Aminopyridine—Seizures—Hippocampus—Neurodegeneration—Excitotoxicity—Microdialysis.
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An effect of K^+ channel blockers, such as 4-aminopyridine (4-AP), is to induce the release of neurotransmitters. 4-AP stimulates the release of both excitatory and inhibitory neurotransmitters in synaptosomes (Tapia and Sitges, 1982; Tapia et al., 1985), brain slices (Hu et al., 1991; Versteeg et al., 1995; Schechter, 1997), and some cerebral regions in vivo (Dawson and Routledge, 1995; Morales-Villagrán and Tapia, 1996). 4-AP is also an efficient convulsant that induces epileptiform electrical discharges in rat hippocampal (Perrault and Avoli, 1991; Yonekawa et al., 1995; Avoli et al., 1996) and cortical (Siniscalchi et al., 1997) slices and in vivo produces intense seizure activity in the rat (Gandolfo et al., 1989; Fragoso-Veloz and Tapia, 1992; Morales-Villagrán et al., 1996), mouse (Yamaguchi and Rogawski, 1992; Cramer et al., 1994), and human (Spyker et al., 1980).

Among the neurotransmitters whose release is induced by 4-AP is glutamate, and, in view of the well-established role of excitatory amino acid (EAA)-mediated synapses in convulsive and excitotoxic mechanisms (Choi, 1988; Meldrum, 1991), it has been postulated that the epileptogenic action of this drug is related to an increased glutamatergic transmission. This postulation is based on the fact that EAA receptor antagonists, of both the *N*-methyl-D-aspartate (NMDA) and the non-NMDA types, are effective anticonvulsants against the 4-AP-induced seizures, both in brain slices (Perrault and Avoli, 1992; Avoli et al., 1996; Siniscalchi et al., 1997) and in vivo (Gandolfo et al., 1989; Fragoso-Veloz and Tapia, 1992; Cramer et al., 1994; Morales-Villagrán et al., 1996). Furthermore, we have demonstrated, using microdialysis, that 4-AP preferentially stimulates the release of glutamate in the striatum and that this release is correlated with intense behavioral convulsive activity (Morales-Villagrán and Tapia, 1996).

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Abbreviations used: 4-AP, 4-aminopyridine; EAA, excitatory amino acid; NMDA, *N*-methyl-D-aspartate; TEA, tetraethylammonium

A second consequence of increased glutamatergic transmission is neuronal death, which has been linked mainly to an overactivation of NMDA and kainate receptors (Olney, 1978; Choi, 1988). However, although neuronal damage in the hippocampus has been observed after treatment with agonists of glutamate receptors (Ben-Ari, 1985; Stein-Behrens et al., 1994; Arias et al., 1997), an accumulation of endogenous extracellular glutamate, resultant from inhibition of its transporters in vivo, surprisingly did not result in neuronal damage and induced only weak signs of hyperexcitability (Massieu et al., 1995; Obrenovitch et al., 1996; Massieu and Tapia, 1997).

These findings suggest that the origin or mechanism of the augmented extracellular glutamate is an important factor for determining seizures and neuronal death. It was therefore of interest to study the relationship among seizures, enhancement of glutamate release, and neuronal damage, and this is the aim of the present work. We have administered 4-AP through microdialysis probes in the rat hippocampus and measured its effect on the extracellular concentration of glutamate and other amino acids. In addition, we have simultaneously recorded the electrical activity of the injected area and of the cerebral cortex and assessed whether the neurochemical and electroencephalographic changes correlated with cellular damage in the hippocampus. The effects of microdialysis perfusion with another K^+ channel blocker, tetraethylammonium (TEA), and with a depolarizing K^+ concentration were also tested.

MATERIALS AND METHODS

Microdialysis procedure

Adult male Wistar rats (weighing 200–250 g) were used throughout and handled according to the Rules for Research in Health Matters (Mexico), with approval of the local Animal Care Committee. Animals were anesthetized with 3–4% halothane in a 95% O_2/5% CO_2 mixture and secured in a Kopf stereotaxic frame with the nose bar positioned at −3.3 mm. Microdialysis cannulae (2 mm long and 0.5 mm in diameter; CMA/Microdialysis, Solna, Sweden; previously flushed with distilled water for 1 h at a flow rate of 40 μl/min) were implanted in the left dorsal hippocampus [A −3.6 mm, L 2.4 mm, and V 4.2 mm from bregma, according to the atlas of Paxinos and Watson (1982); with these coordinates the probe crosses the middle part of the CA1 region, and the tip occasionally reaches the dentate gyrus] Animals were maintained under low anesthesia (0.5% halothane) throughout the experiment. The probes were perfused with a Ringer–Krebs medium containing 118 m*M* NaCl, 4.5 m*M* KCl, 2.5 m*M* $MgSO_4$, 4.0 m*M* NaH_2PO_4, 2.5 m*M* $CaCl_2$, 25 m*M* $NaHCO_3$, and 10 m*M* glucose (pH 7.4) at a rate of 2 μl/min, using a microsyringe mounted in a microinjection pump (model CMA/100; Carnegie). 4-AP (Sigma, St. Louis, MO, U.S.A.) was added to the medium at different concentrations (0.7, 3.5, 7.0, 17.5, 35, and 70 m*M*), and the osmolarity was maintained by reducing the NaCl concentration proportionally When 4-AP was added at any of the concentrations tested, the final pH of the Ringer–Krebs medium was 8.2–8.5 We have previously determined that the recovery of 4-AP through the dialysis membrane is close to 11% and that the medium at this pH without 4-AP did not produce significant effects (Morales-Villagrán and Tapia, 1996). In addition, as shown in Results, the fact that no alterations in any of the parameters studied were observed with the lowest 4-AP concentration used provides a further control indicating that the microdialysis perfusion procedure was innocuous per se.

After a 1-h equilibration period, 25-μl (12.5-min) consecutive fractions of perfusate were continuously collected After the first three fractions (basal release of amino acids), 4-AP was perfused during a 12.5-min fraction, and three additional fractions with normal medium were collected. The amino acid content of the 25-μl perfusate fractions was measured by HPLC after *o*-phthaldialdehyde derivatization, as previously described (Salazar et al., 1994; Massieu et al., 1995). The values reported were not corrected for the efficiency of the dialysis membrane, which was 7–11% (Massieu et al., 1995).

For comparative purposes, in other experiments the effects of 120 m*M* TEA (Sigma) and of depolarization with 100 m*M* K^+ (added to the Ringer–Krebs medium and reducing the NaCl concentration proportionally to maintain osmolarity, as indicated above for 4-AP) were tested. TEA was perfused with the same protocol as for 4-AP, whereas KCl was perfused during two fractions, because we have previously observed that perfusion for only one fraction was not sufficient to induce significant changes in levels of extracellular amino acids (Morales-Villagrán and Tapia, 1996). Osmolarity of the medium was maintained by reducing the NaCl concentration proportionally.

EEG recording

The EEG was recorded in both the implanted hippocampus and the sensorimotor cortex, simultaneously to the microdialysis perfusate collection. For recording in the hippocampus, the microdialysis cannulae were used as electrodes, after electrically insulating them by varnishing the whole surface of the needle excluding 1 mm just above the beginning of the dialysis membrane. For cerebral cortex recording, the same animals were bilaterally implanted with epidural screws [A −6.0 mm and L ±2.4 mm from bregma (Paxinos and Watson, 1982)]. EEG recording was made using a Grass polygraph with a 3–35-Hz filter band.

Histological evaluation

At the end of the experiment the skin was sutured, anesthesia was discontinued, and rats were kept in cages with water and food ad libitum. Five days later animals were anesthetized with sodium pentobarbital and transcardially perfused with 250 ml of 0.9% NaCl, followed by 250 ml of 4% paraformaldehyde in 0.1 *M* phosphate buffer, pH 7.4. Brains were removed, left in fixative for an 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 and stained with cresyl violet for histological observations. For quantitative analysis of the neuronal loss, the morphologically undamaged neurons in a 20× microscopic field (22,600 μm^2) of each hippocampal region were counted with the help of an image analyzer system (NIH Image 1.6). Large cells (>15 μm in diameter) containing clear cytoplasm, with an appearance similar to that of the corresponding region of untreated hippocampi, were considered undamaged.

The number of animals used in each experimental series is indicated in Results (see figure legends) Either ANOVA

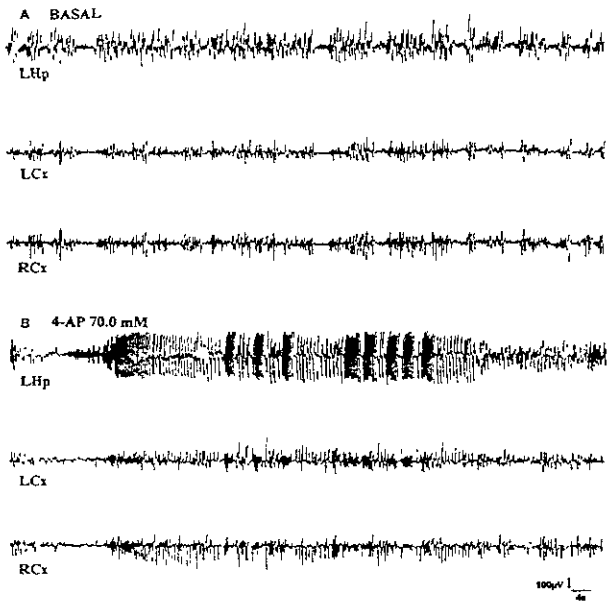

FIG. 1. Effect on EEG activity of microdialysis perfusion with 70 m*M* 4-AP during 12.5 min in the left hippocampus. All traces were obtained with the animal under halothane anesthesia. **A:** Basal activity. The trace shows slow waves and frequent spikes. **B:** At 20 min after the beginning of perfusion with 4-AP. The injected hippocampus (LHp) showed intense discharges characterized by an initial hypersynchronous activity lasting ~10 s, followed by trains of high-frequency and large-amplitude spindles during ~40 s. This activity was propagated to both the ipsilateral cortex (LCx) and the contralateral cortex (RCx), although with considerably less intensity. In all animals, as mentioned in Results, the discharges occurred approximately every 40 s and lasted for the duration of the experiment (~40 min). These recordings are representative of those observed in six rats.

followed by Tukey's test or *t* test was used for statistical analysis.

RESULTS

EEG activity

The basal EEG activity showed frequent spindles and high-amplitude slow waves, which occurred synchronously in the hippocampus and the cortex (Fig. 1A), a pattern that has been described as due to halothane anesthesia (Keifer et al., 1994; Farber et al., 1997). As shown in Fig. 1B, perfusion with 70 m*M* 4-AP in the hippocampus induced intense epileptiform discharges, characterized by an initial hypersynchronic activity lasting ~10 s, followed by trains of high-amplitude spindles, different from those produced by halothane, which lasted for ~40 s. These seizure discharges appeared with a latency of 12.2 ± 1.1 min after the beginning of 4-AP perfusion, occurred with a frequency of 0.61 ± 0.01/min, and persisted until the end of the microdialysis collection. As shown also in Fig. 1B, the discharges propagated to the ipsi- and contralateral cortex, albeit with a considerably lower intensity.

As shown in Fig. 2, with 35, 17.5, 7, and 3.5 m*M* 4-AP the characteristics of the discharges were similar to those after 70 m*M*, but the number of spindles was progressively reduced, until no EEG alterations were observed with the lowest concentration used (0.7 m*M*). The frequency of the discharges was the same at all concentrations, and a slight increase in the latency to the first discharge was observed only with 3.5 m*M* 4-AP (15.2 ± 0.36 min). As with 70 m*M* 4-AP, in all cases the hippocampal discharges were propagated to the cerebral cortex (data not shown). Immediately after the recovery from anesthesia, slight behavioral alterations were observed, mainly masticatory movements and occasional wet-dog shakes. This behavior was present in all animals displaying EEG alterations.

In contrast to 4-AP, neither the administration of 100 m*M* K^+ nor the perfusion with 120 m*M* TEA induced any behavioral or EEG alteration at any time (n = 5 for TEA and n = 6 for high K^+).

Extracellular amino acids

The basal extracellular concentrations of amino acids are similar to those previously described in the hippocampus (Massieu and Tapia, 1997). The levels of glutamate and aspartate were 12–22 and 0–2 pmol/10 μl, respectively, whereas the GABA concentration was in general below the detection limits (Fig. 3). The basal levels of the other four amino acids measured were more variable among different experiments. Taurine, glycine, and alanine concentrations were similar (20–40 pmol/10 μl), whereas glutamine was much more abundant (120–200 pmol/10 μl).

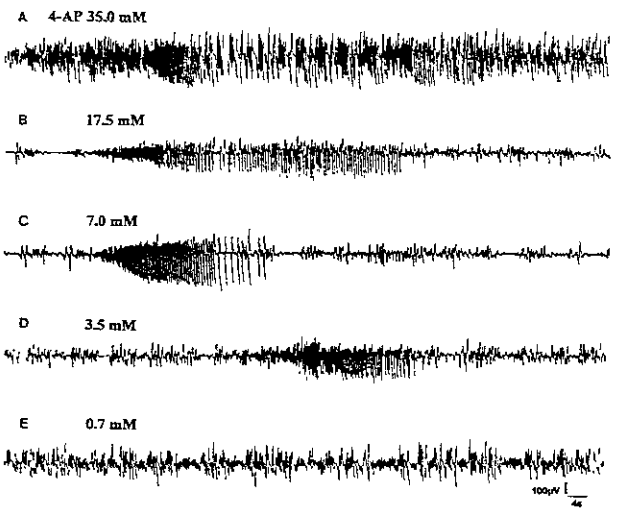

FIG. 2. Effect of different concentrations of 4-AP on EEG activity in the perfused hippocampus, as described in Fig. 1. All traces were obtained with the animal under halothane anesthesia. The traces shown correspond to 20–30 min after the beginning of 4-AP perfusion. Note the progressive reduction in the duration and intensity of the discharges with lower concentrations, until no effect was observed with 0.7 m*M* 4-AP. However, as with 70 m*M* 4-AP (Fig. 1), the discharges occurred every 40–50 s and lasted until the end of the experiment. The recordings are representative of those observed in 13 rats for 35 m*M* 4-AP and in six to eight rats for the other concentrations.

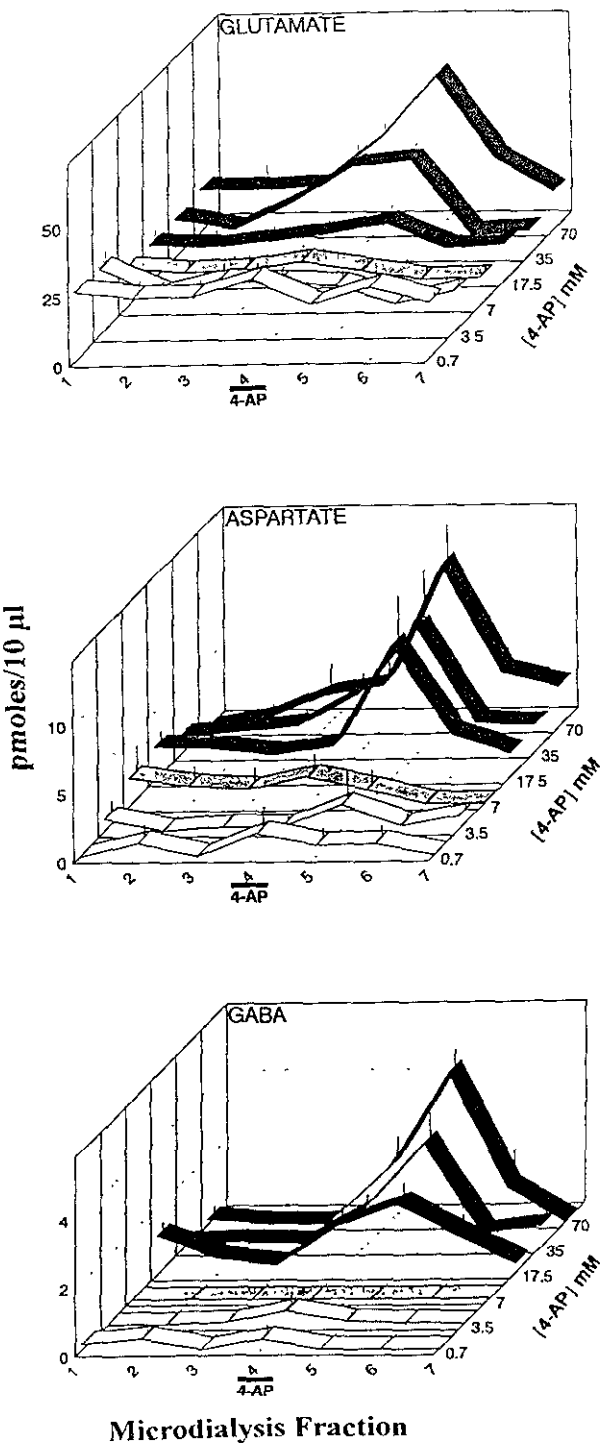

FIG. 3. Effect of microdialysis perfusion with different concentrations of 4-AP (indicated on the z-axis) on the extracellular concentration of glutamate, aspartate, and GABA (y-axis) in hippocampus. As indicated on the x-axis, 4-AP was present during the fourth fraction collected. No changes were observed with the three lowest concentrations, whereas with the three highest concentrations a dose-dependent significant increase in levels of the three amino acids was found in fractions 4 and 5, as compared with the basal values ($p < 0.05$ by ANOVA and Tukey's test). Data are mean ± SEM (bars) values, obtained in the same rats used for EEG recordings (n, as indicated in Figs. 1 and 2).

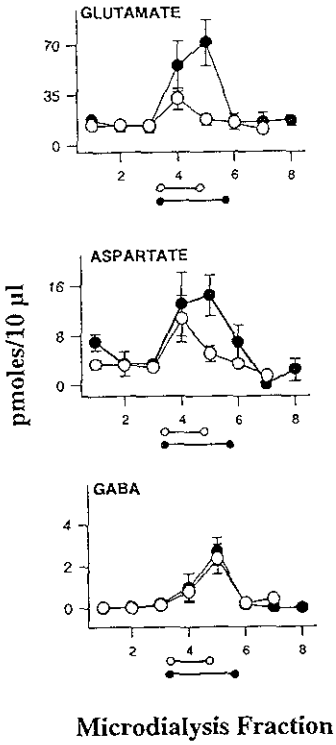

FIG. 4. Effect of microdialysis perfusion with 120 m*M* TEA during fraction 4 (○—○) and with 100 m*M* KCl during fractions 4 and 5 (●—●) on the extracellular concentration of glutamate, aspartate, and GABA in hippocampus. Data are mean ± SEM (bars) values, obtained in the same rats used for EEG recordings (n = 5 for TEA and n = 6 for high K^+). The peak increases with both treatments were significant ($p < 0.05$).

The three lowest concentrations of 4-AP tested did not significantly modify the extracellular amino acid levels, but with 17.5, 35, and 70 m*M* the drug induced in a concentration-dependent fashion a notable increase in glutamate, aspartate, and GABA (Fig. 3). These doses also induced a slight increase in glycine, taurine, and alanine and a progressive decrease in glutamine; the only significant changes were the peak increase (60%) in glycine and the peak decrease (30%) in glutamine, both with 70 m*M* 4-AP (data not shown). In all cases the peak increase occurred in the first dialysate fraction after the end of perfusion with 4-AP, coincidentally with the onset of the EEG seizures, and then decreased to reach the basal values after two fractions. The highest values for the neurotransmitter amino acids, observed with 70 m*M* 4-AP, were 57.4, 11.9, and 4.4 pmol/10 µl for glutamate, aspartate, and GABA, respectively. For glutamate the increase was ~170%, whereas for aspartate and GABA the percentages cannot be calculated because of their practically undetectable basal values (Fig. 3).

Perfusion with 100 m*M* K^+ during two fractions produced an increase in extracellular aspartate and glutamate levels similar to that observed after 70 m*M* 4-AP, although they returned to the basal values more rapidly (Fig. 4). The peak glutamate and aspartate concentrations

reached were slightly higher than those induced by 4-AP. Perfusion with high K^+ induced also an increase in GABA, equivalent to that produced by 35 m*M* 4-AP (Fig. 4), and a notable increase in taurine, which reached a peak value of 260 pmol/10 μl, threefold higher than that induced by 70 m*M* 4-AP (data not shown). A 35% reduction in glutamine was also observed after high K^+, whereas alanine and glycine levels were not significantly modified (data not shown).

As shown also in Fig. 4, 120 m*M* TEA perfusion during one fraction resulted in significant although comparatively small and transient increases in glutamate and aspartate content and in a GABA concentration increase identical to that after high K^+. Levels of the other amino acids measured were not affected by TEA.

Neuronal damage

As shown in Figs. 5 and 6, only the three highest 4-AP concentrations (17.5, 35, and 70 m*M*), those that produced large increases in levels of extracellular amino acids, induced neuronal damage in the perfused hippocampus, when assessed 5 days later. It is interesting that with these three concentrations the neurodegeneration was evident in CA1, CA3, and CA4 regions, whereas neither CA2 nor the dentate gyrus was affected, even when the microdialysis cannula was located very close to the latter structure (Figs. 5C and D and 6). The intensity of the neuronal loss was concentration-dependent: With 70 m*M* 4-AP a massive neuronal death in CA1, CA3, and CA4 was observed, whereas with 35 m*M* and particularly with 17.5 m*M* nuclei were pyknotic, and the pyramidal layer was thinner, but the cell loss was not complete (Fig. 5C–H). No damage at all was observed with 4-AP at concentrations of $\leq$7 m*M* (Figs. 5I–L and 6).

Perfusion with 120 m*M* TEA resulted in a slight thinning of the pyramidal cell layer in the same regions affected by high concentrations of 4-AP, sparing CA2, but the cell loss was similar or, in CA3, even less pronounced than that produced by 17.5 m*M* 4-AP (Figs. 6 and 7; compare Fig. 5G and H with Fig. 7A and B). No damage at all was observed after perfusion with 100 m*M* K^+ (Figs. 6 and 7C and D).

DISCUSSION

The main findings of the present work are a concentration-dependent induction of EEG seizure activity, enhanced extracellular glutamate, aspartate, and GABA levels, and neuronal damage in CA1, CA3, and CA4 hippocampal regions, after microdialysis perfusion with 4-AP. In contrast, a high concentration of TEA did not affect the electrical activity and induced only a modest increase in levels of the three amino acids and a comparatively slight hippocampal damage, whereas depolarization with a high K^+ concentration produced only an increase in levels of the amino acids but neither seizures nor damage.

The potent convulsant effect of 4-AP in vivo has been described also after systemic, intrastriatal, intracortical, or intracerebroventricular administration, and in view of the protective effects exerted by NMDA receptor antagonists under these experimental conditions, it appears that an augmented release of glutamate is related to the excitatory action of the drug (Fragoso-Veloz and Tapia, 1992; Morales-Villagrán and Tapia, 1996; Morales-Villagrán et al., 1996). The present results permit a more detailed analysis of this possible correlation, and, furthermore, they allow us to draw some conclusions regarding the relationships among seizure activity, extracellular glutamate, and neuronal death.

With the highest concentration of 4-AP used (70 m*M*) and considering the recovery of the drug through the dialysis membrane [~11% (Morales-Villagrán and Tapia, 1996)], the total amount of the drug theoretically reaching the hippocampus after 12.5 min of perfusion is 196 nmol. This dose caused the maximal effects on the three parameters analyzed, and progressively fewer changes were observed with lower doses. The lowest concentration tested (0.7 m*M*) corresponds to a theoretical total dose of 1.96 nmol and was without any effect, indicating that the observed changes were not due to the surgical procedure. The seizure activity induced by 4-AP is similar to that previously described after bolus microinjection of the drug in the CA1 hippocampal region in a dose range of 2–200 nmol (Gandolfo et al., 1989; Bagetta et al., 1992; Fragoso-Veloz and Tapia, 1992).

4-AP induced a notable release of glutamate, aspartate, and GABA. The resultant increase in extracellular glutamate content was not as dramatic as that previously observed in the striatum, and this change by itself cannot account for the convulsive activity, because it was observed only with the three highest 4-AP concentrations used, whereas the epileptiform discharges occurred also with lower concentrations. The remarkable enhancement of extracellular GABA induced by 4-AP might also be involved in the hyperexcitation, because it has been repeatedly shown in hippocampal slices that under conditions of neuronal overactivity (Staley et al., 1995; Kaila et al., 1997; Labrakakis et al., 1997), including that induced by 4-AP (Michelson and Wong, 1991; Perrault and Avoli, 1992; Lamsa and Kaila, 1997; Siniscalchi et al., 1997), the synaptic action of this neurotransmitter changes from inhibitory to excitatory. Nonetheless, as with glutamate, GABA content increases were observed only with the highest 4-AP concentrations.

The efficient reuptake of glutamate by the EAA transporters is well established. Therefore, the possibility that glutamate may increase transiently after low 4-AP doses to levels sufficient to induce epileptiform activity but below the detection limits of our assay cannot be discarded. Another likely possibility, however, would be that besides excitatory neurotransmitter release, another mechanism might also be involved in the 4-AP-induced seizure activity, as will be discussed below.

Unlike the seizure activity, the remarkable neurodegeneration produced by 4-AP seems to be related to the increased extracellular levels of glutamate. This conclusion is based mainly on two findings: that the neuronal

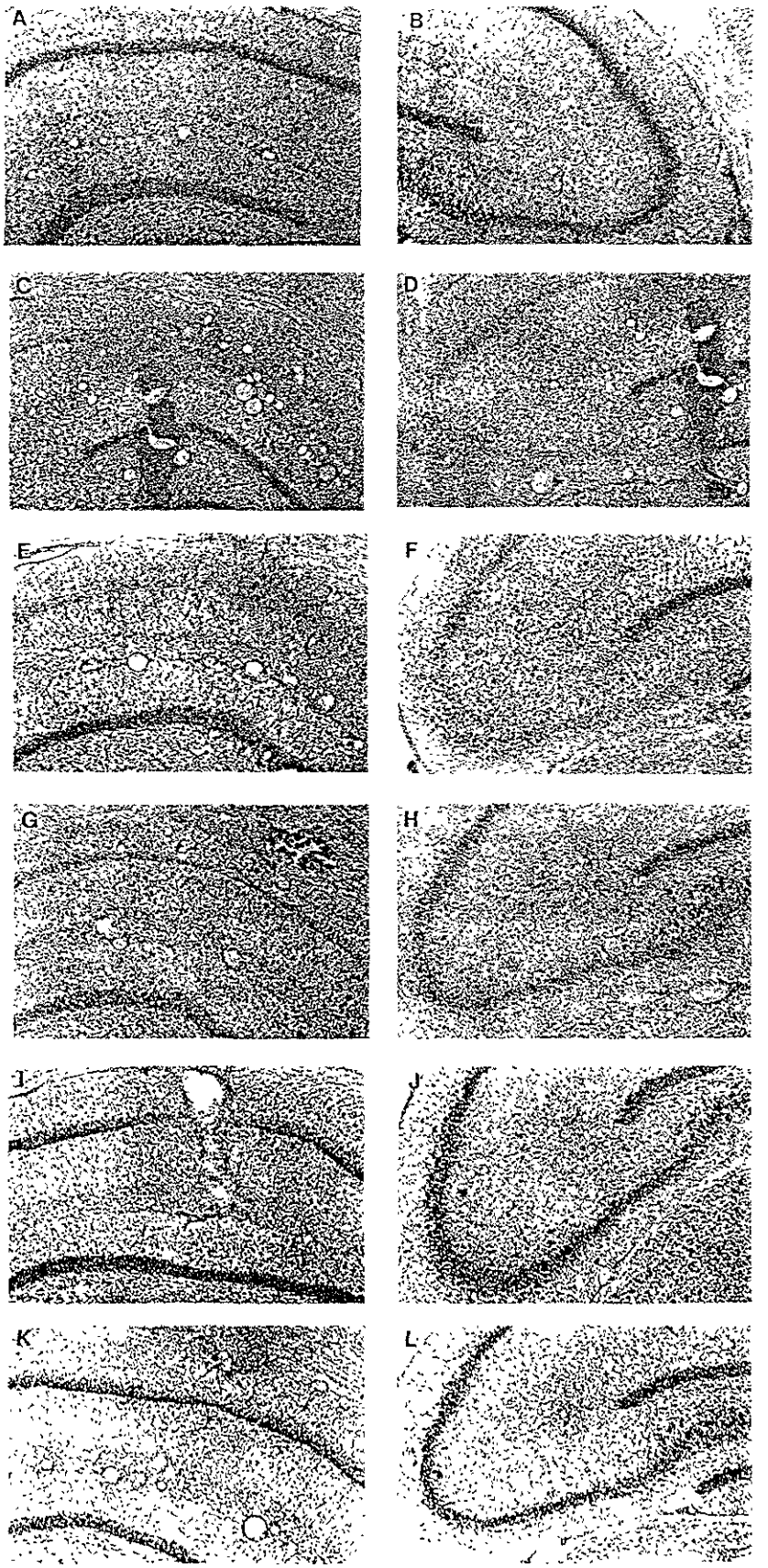

FIG. 5. Representative micrographs illustrate the effect of microdialysis perfusion with different concentrations of 4-AP on the morphological appearance of hippocampus, 5 days after the experiment: (**A** and **B**) contralateral hippocampus (control) and (**C** and **D**) 70 m*M*, (**E** and **F**) 35 m*M*, (**G** and **H**) 17.5 m*M*, (**I** and **J**) 7 m*M*, and (**K** and **L**) 3.5 m*M* 4-AP. The three lowest concentrations did not induce any apparent damage, whereas the three highest concentrations produced a dose-dependent destruction of CA1 (A, C, E, G, I, and K) and of CA3 and CA4 (B, D, F, H, J, and L), but CA2 was not affected. A very similar damage was observed in all rats in each dose group, which were the same animals used to obtain the data of Figs. 1–3. Bar = 200 μm.

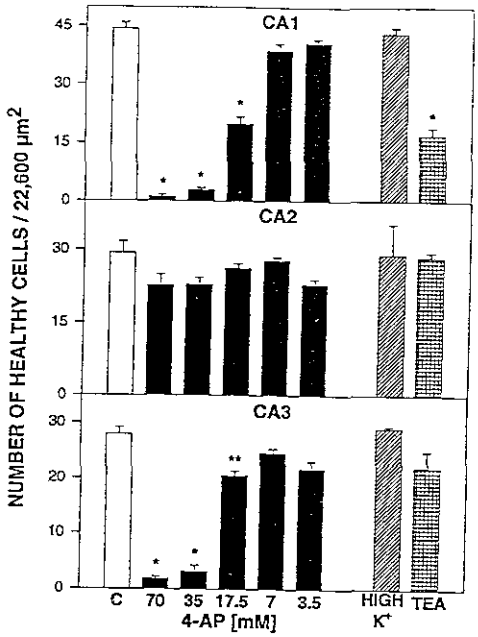

FIG. 6. Number of undamaged pyramidal neurons in one 20× microscopic field in hippocampal regions, 5 days after the microdialysis perfusion with 4-AP, high K^+, or TEA. Four rats per each 4-AP group, high-K^+, or TEA were analyzed, and for each hippocampal region two coronal histological sections 80 μm apart were counted. Data are mean ± SEM (bars) values. C, control value, obtained from five hippocampi contralateral to the tissue treated with different concentrations of 4-AP, chosen blindly. No significant differences were found between them. $^*p < 0.001$, $^{**}p < 0.01$, as compared with control by *t* test.

damage was observed only after the doses of the drug capable of inducing glutamate release and that the neuronal damage was profound in regions CA1, CA3, and CA4, which possess abundant EAA receptors (Insel et al., 1990; Young et al., 1991), whereas the CA2 region was not affected at all, even with the highest 4-AP concentration. In this respect, it is noteworthy that the resistance of CA2 to the 4-AP-induced damage is strikingly similar to that observed after intrahippocampal microinjection of agonists of different EAA receptor types (Ben-Ari, 1985; Stein-Behrens et al., 1994; Arias et al., 1997). It is interesting that some members of the family of dendrotoxins, which are also K^+ channel blockers, produce neurodegeneration in CA1, CA3, and CA4 subfields and that this damage is prevented by EAA receptor antagonists (Bagetta et al., 1994, 1996).

As mentioned in the introductory section, 4-AP is a potent blocker of several types of voltage-sensitive K^+ channels, including most of the cloned Kv types (Chandy and Gutman, 1995; Dolly and Parcej, 1996). Channels particularly affected by 4-AP are the rapidly activated–inactivated types A and D, which possess a low-voltage activation threshold (Storm, 1993). It was therefore important to compare the effects of 4-AP on the parameters studied with those of TEA and with the general depolarization induced by a high K^+ concentration. TEA is another K^+ channel blocker that acts on some of the channels affected by 4-AP, although it is considerably weaker (Chandy and Gutman, 1995; Dolly and Parcej, 1996). The results of these experiments show that, unlike with 4-AP, no seizures occurred after perfusion with 120 m*M* TEA or with high K^+. Furthermore, only a moderate neuronal damage was observed after TEA, and no damage at all occurred after high K^+. Nevertheless, both TEA and high K^+, particularly the latter, induced an increase in extracellular glutamate and aspartate levels.

To explain these results, it seems relevant to consider the physiological effects of 4-AP and TEA on neuronal firing. Both compounds considerably increase the duration of the depolarization phase of action potentials (Zhang and McBain, 1995; Chen et al., 1996; Koyano et al., 1996), but only 4-AP notably augments the frequency

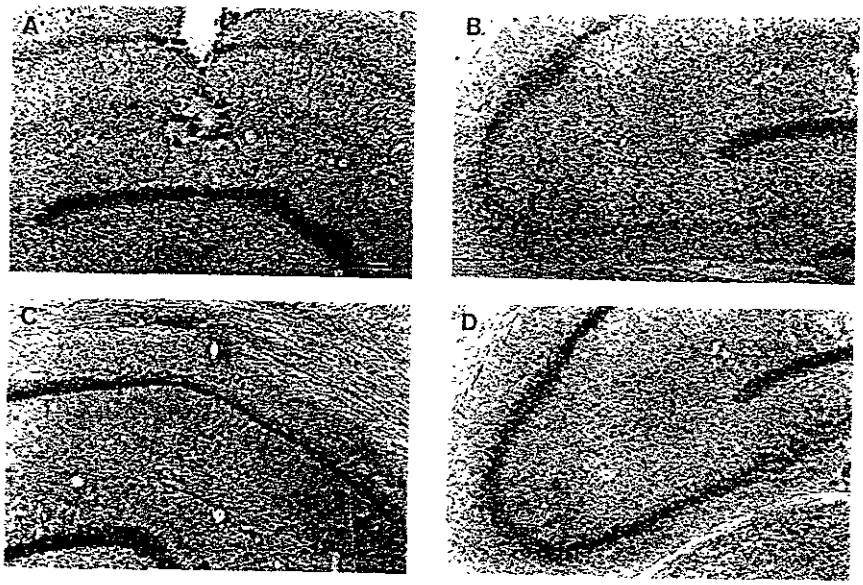

FIG. 7. Representative micrographs illustrate the effect of microdialysis perfusion with 120 m*M* TEA and with 100 m*M* KCl on the morphological appearance of the hippocampus, 5 days after the experiment. **A** and **B:** TEA-treated tissue. Note that the damage produced was comparable to that produced by 17.5 m*M* 4-AP (Figs. 5G and H and 6). **C** and **D:** High K^+-perfused tissue. No damage at all was observed (compare with the control in Figs. 5A and B and 6). Very similar results were observed in all rats in each group, which were the same animals used to obtain the biochemical and EEG data. Bar = 200 μm.

of neuronal firing (Bargas et al., 1989; Nisenbaum et al., 1994; Koyano et al., 1996). Thus, it can be concluded that the 4-AP-induced EEG discharges are the result of an increment in frequency firing, combined with an increased excitatory glutamate- and GABA-mediated synaptic transmission. Consistent with this conclusion, it has been found that dendrotoxins, like 4-AP, induce increased neuronal firing (Nisenbaum et al., 1994) and are also potent convulsants (Gandolfo et al., 1989; Bagetta et al., 1992).

On the other hand, it seems clear that a generalized depolarization, as that expected to occur after high K^+ administration, failed to induce seizure activity because it does not reproduce the increased neuronal firing probably responsible for the seizure activity, although it did induce an increased glutamate release (see below).

With regard to neurodegeneration, it is interesting that, *in contrast to 4-AP, no damage was observed with high* K^+ and that TEA induced only a moderate lesion, in spite of the fact that the three agents produced an enhancement of extracellular glutamate content. A plausible explanation for this finding is that the increased amount of the amino acid must occur at a cellular site close enough to its receptors to allow their interaction. Thus, although it is generally accepted that 4-AP acts mainly at the level of nerve endings (Thesleff, 1980; Jones and Heinemann, 1987; Perrault and Avoli, 1991), K^+ depolarization most probably acts at neuronal somas and glial cells, and therefore the augmented level of glutamate has no easy access to the glutamatergic receptors. In other words, the synaptic efficacy seems to be facilitated by 4-AP but not by high K^+ depolarization. In agreement with this interpretation, it has been demonstrated that even remarkable increases in content of extracellular glutamate, induced by inhibition of its transport in vivo, does not produce hyperexcitability or neuronal damage (Massieu et al., 1995; Obrenovitch et al., 1996). With regard to TEA, the slight damage produced by this compound, together with its relatively small effect on extracellular EAAs, suggests that although it is probably acting at the nerve endings, it behaves as a much weaker release-inducing agent than 4-AP.

In conclusion, the present report describes for the first time a correlation in vivo between neuronal death and increased extracellular concentrations of endogenous glutamate and GABA due to an enhancement of their release. This finding contrasts with the lack of endogenous glutamate neurotoxicity in vivo when its extracellular concentration is elevated by K^+ depolarization (present study) or by inhibition of its uptake (Massieu et al., 1995; Massieu and Tapia, 1997). Thus, the subcellular site of glutamate accumulation in the extracellular space seems to be determinant for the access of the amino acid to its postsynaptic receptors and consequently for the induction of excitotoxic neuronal damage. This clearly differs from the abundant data on glutamate-induced excitotoxicity in tissue culture, including those observed after uptake inhibition (Velasco et al., 1996), and casts some doubts on the generally accepted possibility that an increased level of extracellular glutamate by itself, when originating from cell destruction or glutamate transporter deficiencies, might be responsible for neuronal death. On the other hand, the present results also point out, in agreement with other studies (Obrenovitch and Urenjak, 1997), that there is a clear dissociation between hyperexcitability and neuronal damage.

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SEIZURES AND NEURODEGENERATION INDUCED BY 4-AMINOPYRIDINE IN RAT HIPPOCAMPUS *IN VIVO*: ROLE OF GLUTAMATE- AND GABA-MEDIATED NEUROTRANSMISSION AND OF ION CHANNELS

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Abstract—Infusion of the K^+ channel blocker 4-aminopyridine in the hippocampus induces the release of glutamate, as well as seizures and neurodegeneration. Since an imbalance between excitation and inhibition, as well as alterations of ion channels, may be involved in these effects of 4-aminopyridine, we have studied whether they are modified by drugs that block glutamatergic transmission or ion channels, or drugs that potentiate GABA-mediated transmission. The drugs were administered to anesthetized rats subjected to intrahippocampal infusion of 4-aminopyridine through microdialysis probes, with simultaneous collection of dialysis perfusates and recording of the electroencephalogram, and subsequent histological analysis. Ionotropic glutamate receptor antagonists clearly diminished the intensity of seizures and prevented the neuronal damage, but did not alter substantially the enhancement of extracellular glutamate induced by 4-aminopyridine. None of the drugs facilitating GABA-mediated transmission, including uptake blockers, GABA-transaminase inhibitors and agonists of the A-type receptor, was able to reduce the glutamate release, seizures or neuronal damage produced by 4-aminopyridine. In contrast, nipecotate, which notably increased extracellular levels of the amino acid, potentiated the intensity of seizures and the neurodegeneration. $GABA_A$ receptor antagonists partially reduced the extracellular accumulation of glutamate induced by 4-aminopyridine, but did not exert any protective action. Tetrodotoxin largely prevented the increase of extracellular glutamate, the electroencephalographic epileptic discharges and the neuronal death in the CA1 and CA3 hippocampal regions. Valproate and carbamazepine, also Na^+ channel blockers that possess general anticonvulsant action, failed to modify the three effects of 4-aminopyridine studied. The N-type Ca^{2+} channel blocker ω-conotoxin, the K^+ channel opener diazoxide, and the non-specific ion channel blocker riluzole diminished the enhancement of extracellular glutamate and slightly protected against the neurodegeneration. However, the two former compounds did not antagonize the 4-aminopyridine-induced epileptiform discharges, and riluzole instead markedly increased the intensity and duration of the disharges. Moreover, at the highest dose tested (8 mg/kg, i.p.), riluzole caused a 75% mortality of the rats.

We conclude that 4-aminopyridine stimulates the release of glutamate from nerve endings and that the resultant augmented extracellular glutamate is directly related to the neurodegeneration and is involved in the generation of epileptiform discharges through the concomitant overactivation of glutamate receptors. Under these conditions, a facilitated GABA-mediated transmission may paradoxically boost neuronal hyperexcitation. Riluzole, a drug used to treat amyotrophic lateral sclerosis, seems to be toxic when combined with neuronal hyperexcitation. © 2000 IBRO. Published by Elsevier Science Ltd. All rights reserved.

Key words: hippocampus, tetrodotoxin, riluzole, glutamate release, GABA transport, epilepsy.

Excitatory and inhibitory neurotransmission in the CNS is mediated mainly by glutamate and GABA, respectively. A dysfunction of any of these neurotransmitter systems may be implicated in the generation of epilepsy, since an imbalance between excitation and inhibition produced by a decrease in GABAergic and/or an increase in glutamatergic transmission has been associated with the generation of this pathological condition, both in animal models and in humans.[9,56] In addition, a considerable body of evidence has shown that an enhancement of glutamatergic transmission is involved in the excitotoxic mechanisms of neurodegeneration, mainly by overactivation of *N*-methyl-D-aspartate (NMDA) receptors.[14,55]

4-Aminopyridine (4-AP) is a K^+ channel blocker that stimulates the release of both excitatory and inhibitory neurotransmitters in different CNS preparations *in vitro*,[84,86,89] and produces intense epileptiform activity in brain slices[4,8,13,20,32,38,61,69] and *in vivo*.[17,25,59,92] An enhancement of glutamatergic transmission has been related to the convulsant action of 4-AP,[83] since excitatory amino acid receptor antagonists, of both the NMDA and the non-NMDA types, are effective anticonvulsants against 4-AP-induced seizures, both in brain slices[4,69] and *in vivo*.[17,25,59] Furthermore, we have demonstrated that the intrahippocampal perfusion of 4-AP through microdialysis probes produces intense electroencephalographic (EEG) seizures associated with neuronal damage in the CA1 and CA3 regions, effects that correlate well with an increase in the concentration of extracellular glutamate.[68]

Besides the use of glutamate receptor antagonists, one strategy to protect against seizures and neuronal damage is an enhancement of GABAergic neurotransmission, which should reduce the hyperexcitability through an increased inhibition. Following this approach, it has been found that pro-GABAergic drugs protect against ischemia- or epilepsy-induced neuronal death.[39,50,70,73,95] The first aim of the present work was therefore to evaluate whether the blockade of glutamatergic transmission or the enhancement of GABAergic

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Abbreviations: AOAA, amino-oxyacetic acid; 4-AP, 4-aminopyridine; CPP, (3-phosphonopropyl)-piperazine-2-carboxylic acid, EEG, electroencephalogram; MK-801, (+)-5-methyl-10,11-dihydro-5H-dibenzo(a,d)cyclohept-5,10-imine maleate; NBQX, 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(F)quinoxaline; NMDA, *N*-methyl-D-aspartate; NNC-711, 1-(2-{[(diphenylmethylene)amino]oxy}ethyl)-1,2,5,6-tetrahydro-3-pyridine-carboxylic acid hydrochloride; NPCA, nipecotic acid; NS1619, 1,3-dihydro-1-(2-hydroxy-5-(trifluoromethyl)phenyl)-5-(trifluoromethyl)-2H-benzamidazol-2-one; TTX, tetrodotoxin.

transmission can protect against the seizures and the neurodegeneration induced by 4-AP in the rat hippocampus *in vivo*. For this purpose, we have tested the effect of several NMDA and non-NMDA receptor antagonists, as well as pro-GABAergic drugs, such as GABA receptor agonists, GABA uptake blockers and inhibitors of GABA-transaminase, on the EEG changes, extracellular glutamate levels and neuronal damage induced by the intrahippocampal administration of 4-AP.

A role of ion channels in such 4-AP-induced, glutamate-mediated neurodegeneration and neuronal hyperexcitation is highly probable, since 4-AP itself is a K^+ channel blocker capable of depolarizing nerve endings and, in addition, its neurotransmitter-releasing action is dependent on external Ca^{2+}.[85,87] In agreement with this possibility, it has been shown that the epileptogenic action of 4-AP in hippocampal slices is prevented by some Na^+ channel blockers, such as tetrodotoxin (TTX), carbamazepine and valproate.[4,10,28,29,69] Moreover, TTX and other Na^+ channel blockers may protect against neurodegeneration after brain tissue insults thought to involve glutamate-mediated toxicity, such as ischemia, hypoxia or hypoglycemia.[63,66,71,93]

Only L-type Ca^{2+} channel antagonists have been tested against the convulsant effect of 4-AP, with contradictory results. Nifedipine and other dihydropyridines potentiated the behavioral and EEG seizures induced by the systemic and the intrahippocampal administration of 4-AP in the rat,[24] and nimodipine did not prevent the behavioral seizures and death induced by 4-AP in mice.[92] In contrast, fluspirilene and PN 200-110 protected against the effects of i.c.v. applied 4-AP.[30]

Of particular interest among the ion channel blockers is riluzole, which has been considered as a "glutamate release inhibitor" and is one of the few drugs approved for clinical use in amyotrophic lateral sclerosis, on the basis of the excitotoxic hypothesis of this disease.[47] Riluzole affects a wide variety of ion channels, including sodium,[66,79,100] calcium[66,79] and potassium[100] channels, and exerts several pre- and postsynaptic effects.[12] This drug has shown neuroprotective and anticonvulsant action in some experimental models,[36,63,66,80] but was ineffective against the hyperexcitability induced by i.c.v. 4-AP.[80]

Several K^+ channel openers are known, including diazoxide and NS1619, which activate ATP-sensitive and Ca^{2+}-sensitive channels, respectively.[11,35,63,94] These and other K^+ channel openers possess anticonvulsant and neuroprotective properties in some experimental models,[1,31,34,45,54] but they have been ineffective against 4-AP-induced hyperexcitability in hippocampal slices[54] or after i.c.v. administration.[31]

In view of the above, and since no data are available regarding the effect of ion channel blockers on the stimulatory action of 4-AP on the release of glutamate and other amino acids *in vivo*, or regarding the neurodegeneration induced by this drug, the second aim of the present work was to gain information on these questions. For this purpose, we have used several Na^+ and Ca^{2+} channel blockers, including riluzole, as well as K^+ channel openers.

EXPERIMENTAL PROCEDURES

Microdialysis procedure and measurement of extracellular amino acids

Adult male Wistar rats (200–250 g) were used throughout and handled with all precautions necessary to minimize the number of animals used and their suffering, according to the Rules for Research in Health Matters (Mexico), with approval of the local Animal Care Committee. The microdialysis procedure was carried out as described previously.[68] In brief, under halothane anesthesia, rats were implanted with microdialysis cannulae (membrane of 2 mm length and 0.5 mm diameter, CMA/12, Acton, MA, USA) in the left dorsal hippocampus (coordinates: A −3.6 mm, L 2.4 mm and V 4.2 mm from bregma[67]), and the probes were perfused with a Ringer–Krebs medium containing (in mM): 118 NaCl, 4.5 KCl, 2.5 $MgSO_4$, 4.0 NaH_2PO_4, 2.5 $CaCl_2$, 25 $NaHCO_3$ and 10 glucose (pH 7.4), at a rate of 2 μl/min. After a 1 h equilibrium period, 25 μl (12.5 min) consecutive fractions of perfusate were continuously collected. The first three fractions collected served to obtain the basal release of amino acids; 4-AP (35 mM) was then perfused during one 12.5 min fraction and three subsequent fractions with normal medium were collected.

The amino acid content of the 25 μl perfusate fractions was measured by high-performance liquid chromatography after *o*-phthaldialdehyde derivatization, as described previously.[52,75] The values reported were not corrected for the efficiency of the dialysis membrane, which as reported previously was 7–11%.[52]

Electroencephalographic recording

The EEG was recorded simultaneously and continuously during the microdialysis procedure. For this purpose, the microdialysis cannulae were used as electrodes, after electrically insulating them by varnishing the whole surface of the needle excluding 1 mm just above the beginning of the dialysis membrane. A Grass polygraph with a low-frequency filter at 3 Hz and a high-frequency filter at 100 Hz was used for EEG recording. For quantitative analysis, the latency to the first EEG discharge and the duration of the discharges during the last 10 min of perfusion, when the EEG seizures were constant, were calculated. The duration of each discharge was measured from the beginning of the hypersynchronic activity to the last high-amplitude spike in a continuous train.

Histological evaluation

Five days after the experiment, animals were anesthetized with sodium pentobarbital, transcardially perfused with 250 ml of 0.9% NaCl followed by 250 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4), and brains were removed and prepared for the histological procedure, as described previously.[68] Coronal sections (30 μm thick) were stained with Cresyl Violet, the correct location of the microdialysis probes was confirmed and the morphologically undamaged neurons (i.e. large, >15 μm neurons with clear cytoplasm, similar in appearance to those of the contralateral hippocampus) in the dorsal hippocampal regions were counted in a ×20 microscopic field (21,600 μm^2), with the help of an image analyser system (NIH Image 1.6). Two sections, 80 μm apart, were counted in each hippocampus, covering the whole CA1 and CA3 areas, which were those showing significant damage.[68]

Drugs

The following drugs were administered through the dialysis probe during the fourth 12.5 min collection fraction, mixed with 4-AP (see above), at the concentrations indicated, in order to test their possible antagonist action against 4-AP's effects: the NMDA receptor antagonist (3-phosphonopropyl)-piperazine-2-caboxylic acid (CPP; 100 μM); the non-NMDA receptor antagonist 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(F)quinoxaline (NBQX; 500 μM); the $GABA_A$ receptor agonists isoguvacine (1 mM) and muscimol (100 μM); the $GABA_B$ receptor agonist baclofen (50 μM); the $GABA_A$ receptor antagonists bicuculline methiodide (25 μM) and picrotoxin (150 μM); the $GABA_B$ receptor antagonist saclofen (750 μM); the Na^+ channel blockers TTX (100 μM) and carbamazepine (HBC complex, water-soluble preparation; 150 μM); the N-type Ca^{2+} channel blocker ω-conotoxin GVIA (100 μM); and the Ca^{2+}-dependent K^+ channel opener 1,3-dihydro-1-(2-hydroxy-5-(trifluoromethyl)phenyl)-5-(trifluoromethyl)-2H-benzimidazol-2-one (NS1619) (300 μM). In addition, the GABA uptake blockers 1-(2-{[(diphenylmethylene)amino]oxy}ethyl)-1,2,5,6-tetrahydro-3-pyridine-carboxylic acid hydrochloride (NNC-711; 40 μM) and nipecotic acid (NPCA; 5 mM) were perfused two fractions before, during and two fractions after 4-AP administration, at the concentrations indicated. Osmolarity of the medium was maintained in all cases

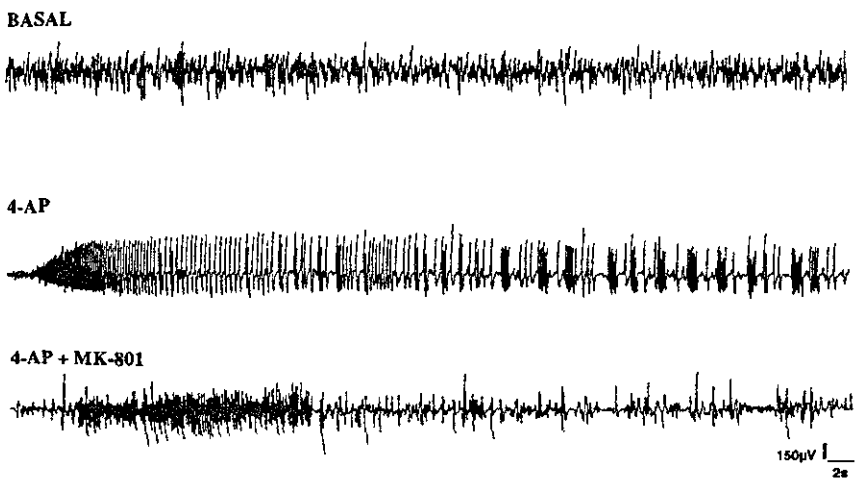

Fig. 1. EEG seizure activity induced by 4-AP and protection by MK-801. Traces are representative of the activity before 4-AP (basal) and of the changes observed once the seizure activity was stabilized. MK-801 was administered i.p. 30 min before 4-AP infusion. See Fig. 3c for quantitative evaluation.

by reducing NaCl proportionally. Furthermore, the NMDA receptor antagonist (+)-5-*methyl*-10,11-*dihydro*-5H-*dibenzo*(a,d)*cyclohept*-5,10-imine maleate (MK-801; 2 mg/kg) and the GABA-transaminase inhibitor amino-oxyacetic acid (AOAA; 50 mg/kg), magnesium valproate (300 mg/kg), riluzole (4 and 8 mg/kg) and the ATP-sensitive K^+ channel opener diazoxide (30 mg/kg) were administered i.p. 30 min before 4-AP perfusion. With the exception of AOAA, picrotoxin and TTX (Sigma, St Louis, MO, USA) and magnesium valproate (Mexican Ministry of Health Laboratories), all drugs were obtained from RBI-Sigma (Natick, MA, USA).

The doses of the drugs used were chosen on the basis of previous work showing that they were effective in other experimental models of epilepsy or neurodegeneration (see references in the introductory section and Discussion). In the case of those co-administered with 4-AP through the microdialysis probe, it should be considered that the membrane dialysis efficiency for most compounds is approximately 10%.[52,58] We verified that, when administered alone, none of the drugs used produced any significant effect on extracellular glutamate levels, EEG activity or the morphology of hippocampal neurons (groups of three or four rats were assessed for each drug).

Statistical comparisons were carried out using unpaired Student's *t*-tests. $P < 0.05$ was considered statistically significant.

RESULTS

Effect of glutamate receptor antagonists

As reported previously,[68] the intrahippocampal infusion of 35 mM 4-AP induced EEG seizures characterized by an initial hypersynchronic activity followed by trains of high-amplitude spikes (Fig. 1). The latency to the first discharge was 12.8 ± 1.07 min and the mean duration of the discharges, once established, was 59.8 ± 4.5 s (Fig. 3c).

Also as described previously,[68] the appearance of EEG discharges was accompanied by an elevation of extracellular glutamate, which in the present experiments reached a value threefold greater than the basal concentration of the amino acid (Fig. 3a), and by a notable increase in extracellular GABA levels, from practically nil values to 4.2 ± 0.34 pmol/10 μl (Fig. 4b). The changes in the other amino acids measured (aspartate, glutamine, alanine, glycine and taurine) were similar to those observed in our previous communication[68] and are not shown here. Five days after the experiment, rats treated with 4-AP showed a complete neurodegeneration of CA1 and CA3 pyramidal neurons, with little or no damage in CA2 and the dentate gyrus (Figs 2b, 3b).

The i.p. administration of MK-801 30 min before 4-AP notably reduced the intensity (Fig. 1) and the duration (79% reduction; Fig. 3c) of the EEG seizures induced by 4-AP, without affecting the latency to the first discharge, which was 14.6 ± 0.68 min. This protective effect was more evident on the high-amplitude spike phase of the discharges, while the hypersynchronic activity was less affected. MK-801 did not significantly modify the 4-AP-induced increase in extracellular glutamate concentration (Fig. 3a), but completely prevented the neurodegeneration induced by the drug, in both the CA1 and CA3 regions (Figs 2c, 3b).

Similar protective effects against seizures and neurodegeneration to those exerted by i.p. MK-801 were observed when the other competitive NMDA receptor antagonist used, CPP, or the non-NMDA receptor antagonist, NBQX, were infused together with 4-AP (Fig. 3). These drugs did not alter the latency of 4-AP-induced seizures (15.2 ± 1.52 min with CPP and 13.4 ± 0.94 min with NBQX), but reduced their intensity (not shown) and their duration (59% and 62% reductions, respectively; Fig. 3c). The protection against neurodegeneration produced by CPP and NBQX was highly significant, although less notable than that observed with MK-801 (Fig. 3b). In a different group of rats, the co-application of CPP and NBQX together did not potentiate the protective action of these drugs by themselves ($n = 4$; results not shown).

None of the glutamate receptor antagonists studied had any effect on the enhancement of extracellular glutamate (Fig. 3a) or on the changes in other amino acids induced by 4-AP.

Effect of GABAergic drugs and GABA receptor blockers

Because the pro-GABAergic drugs used included inhibitors of GABA transport, it was important to assess whether they modify the extracellular concentration of amino acids. In the groups of rats treated with these drugs alone, only NPCA and to a lesser extent NNC-711 produced an increase in GABA, which reached peak values of 18.2 and 3.16 pmol/10 μl, respectively (Fig. 4); the other amino acids measured, including glutamate, were not affected at all by these drugs (not shown). None of the pro-GABAergic drugs tested produced any alteration in the EEG or in neuronal morphology.

As shown in Fig. 4a and b, when NPCA or NNC-711 were

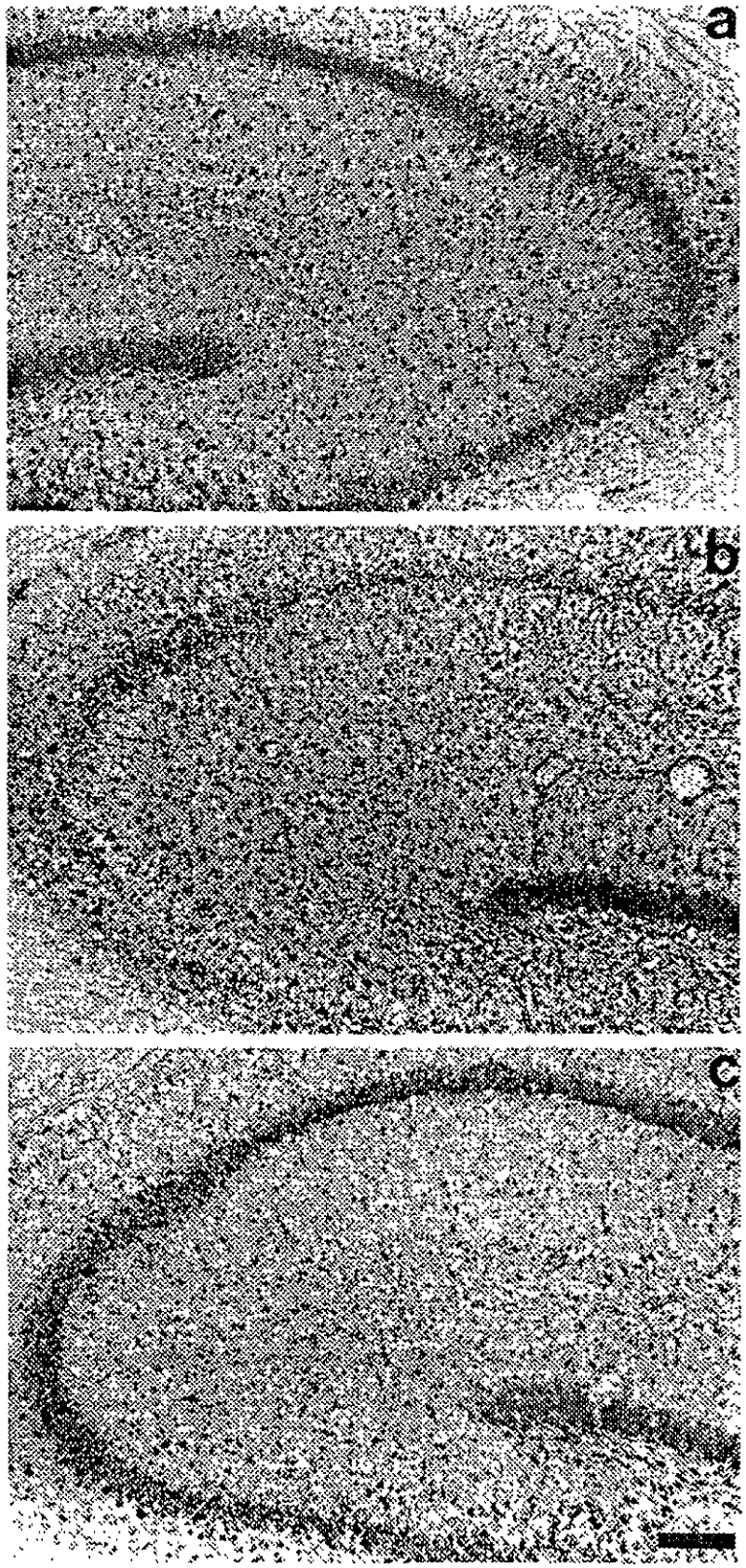

Fig. 2. Hippocampal neurodegeneration induced by 4-AP five days after treatment and protection by MK-801. (a) Non-infused hippocampus. (b) 4-AP-infused hippocampus Note the nearly complete destruction of CA1 and CA3, and the preservation of CA2 and of the visible fragment of the dentate gyrus. (c) 4-AP-infused hippocampus in a rat treated with MK-801 30 min before. For quantitative data see Fig. 3b Scale bar = 160 μm.

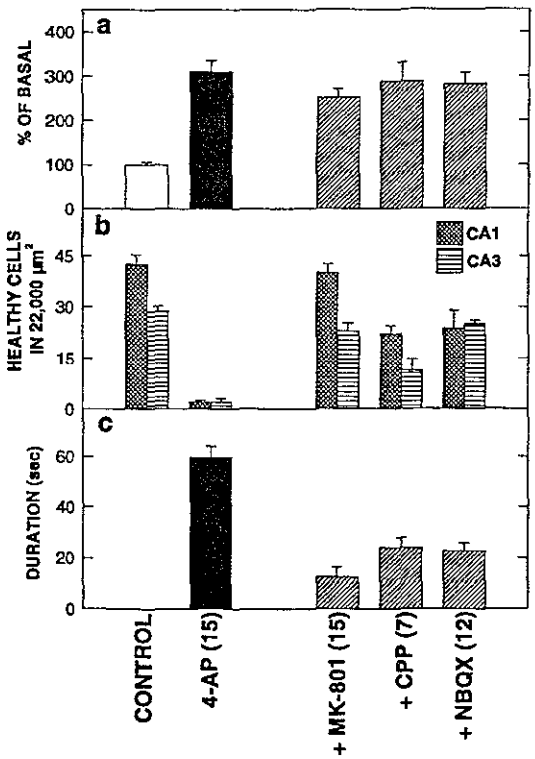

Fig. 3. Extracellular glutamate increase (a), neuronal damage in the CA1 and CA3 hippocampal regions (b), and duration of EEG discharges (c) induced by 4-AP and protection by glutamate receptor antagonists. In panel a, the control 100% value is the basal glutamate concentration (absolute values were 17.2 ± 2.2 pmol/10 μl); the other four bars represent the percentage peak values observed in the microdialysis fraction following 4-AP infusion, with 4-AP alone and in rats treated with the glutamate receptor antagonists indicated (MK-801, CPP, NBQX). The neurodegeneration data in panel b were obtained five days after 4-AP infusion; control values were obtained in non-infused hippocampi. Data are mean values ± S.E.M. for the number of rats shown in parentheses. Only rats in which the three kinds of data were obtained were included in the calculations. In panels b and c, all values with the glutamate receptor antagonists were significantly different from those with 4-AP alone (at least $P < 0.05$).

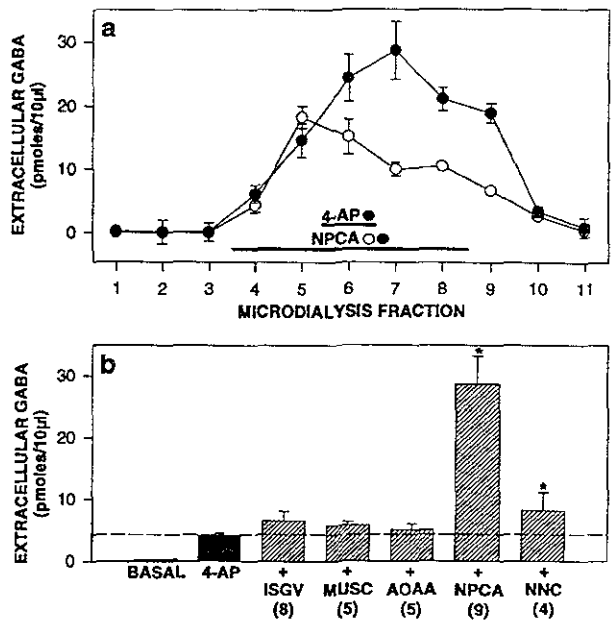

Fig. 4. (a) Changes in extracellular GABA levels induced by NPCA alone and combined with 4-AP; the horizontal bars indicate the time of infusion of the drugs through the microdialysis probe. Data are mean values ± S.E.M. for four rats for NPCA alone and nine rats for 4-AP plus NPCA. (b) Basal and peak extracellular GABA levels induced by 4-AP alone and combined with the pro-GABAergic drugs indicated (ISGV, isoguvacine; MUSC, muscimol; NNC, NNC-711). Data are mean values ± S.E.M. for the number of rats shown in parentheses. $^*P < 0.05$ compared to 4-AP alone.

administered with 4-AP the increase of extracellular GABA levels was potentiated, so that the peak levels attained were approximately the sum of the action of the drugs alone. In contrast, with the other pro-GABAergic drugs tested, muscimol, isoguvacine and AOAA, no significant modification of GABA besides that induced by 4-AP was observed (Fig. 4b).

Neither NNC-711 nor AOAA, muscimol or isoguvacine significantly modified the latency, intensity and duration of the 4-AP-induced EEG seizures. In contrast, surprisingly, NPCA increased by 40% the mean duration of the EEG discharges (Figs 5, 7c) without affecting the latency (12.4 ± 1.57 min). This potentiation of the convulsant effect of 4-AP by NPCA could be due to a depolarizing action of the excess extracellular GABA, since it has been shown in hippocampal slices[4,44,57,69] that, during epileptiform activity induced by 4-AP, GABA depolarizes neurons through the activation of $GABA_A$ receptors. To test this possibility, we applied the $GABA_A$ receptor antagonist bicuculline, at a subconvulsant dose that did not alter the EEG, on the NPCA-induced potentiation of seizures. As shown in Fig. 7c, this treatment did not reduce the potentiation. Similarly, neither bicuculline (Fig. 5) nor picrotoxin, another $GABA_A$ receptor blocker, affected *per se* the 4-AP-induced EEG discharges (Fig. 7c), indicating that the enhancement of extracellular GABA produced by 4-AP is not a determining factor in the induction of seizures. In agreement with this interpretation, it was observed that both bicuculline and picrotoxin significantly reduced the latency to seizures (from 12.8 to 8.77 ± 0.42 and 7.25 ± 0.41 min, respectively, $P < 0.05$).

None of the pro-GABAergic drugs tested had any protective effect on the neurodegeneration induced by 4-AP (Figs 6, 7b). On the contrary, and similarly to its effect on the EEG discharges, NPCA seemed to potentiate such damaging action, as indicated not only by the decrease in the number of healthy neurons in CA1 and CA3 (Fig. 7b), but also by producing a significant decrement of approximately 44% in the number of healthy cells in the CA2 region, with respect to 4-AP alone ($P < 0.05$; see Fig. 6b).

In agreement with the lack of protective effect of the pro-GABAergic drugs used on the seizures and neurodegeneration induced by 4-AP, these compounds did not prevent the extracellular accumulation of glutamate (Fig. 7a) or the changes induced by 4-AP in other amino acids. In contrast, the two $GABA_A$ receptor antagonists, bicuculline and picrotoxin, reduced the accumulation of extracellular glutamate. In addition, bicuculline also diminished the accumulation of extracellular aspartate and GABA and the reduction of glutamine induced by 4-AP[58] (not shown). We cannot offer an explanation for this finding, but one possibility is that the already mentioned depolarizing action resulting from $GABA_A$ receptor activation may be blocked by the antagonists, thus decreasing the neuronal excitation and consequently the release of glutamate.

We also assessed the possible role of $GABA_B$ receptors, since some reports suggest that these receptors are involved in

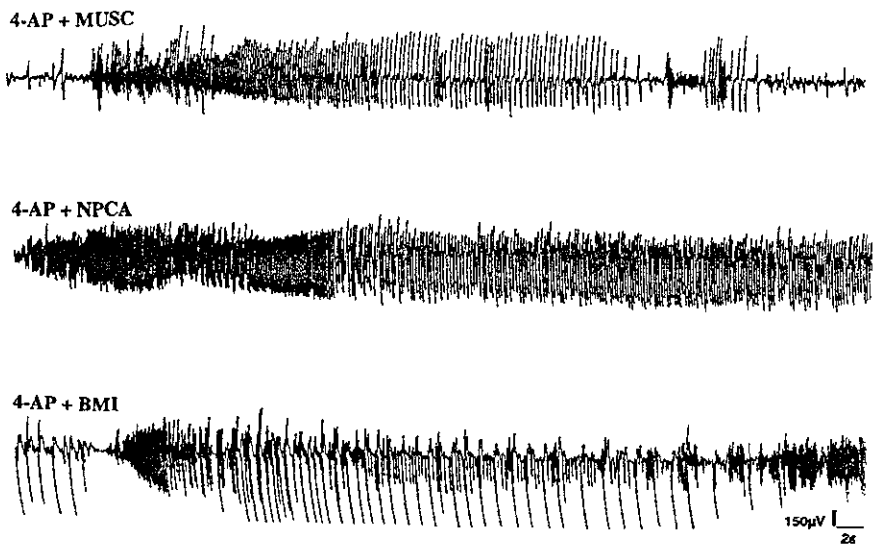

Fig. 5. EEG seizure activity induced by 4-AP in rats treated with muscimol (MUSC), NPCA and bicuculline (BMI). Note the lack of effect of muscimol and bicuculline, and the potentiation of seizures by NPCA (compare with Fig. 1). See Fig. 7c for quantitative evaluation.

the generation of epileptiform activity by 4-AP *in vitro*.[8,61] At the doses used, neither the $GABA_B$ receptor agonist baclofen nor the $GABA_B$ receptor antagonist saclofen significantly modified the effects of 4-AP. With baclofen, the extracellular accumulation of glutamate was 295.7 ± 63.1% of basal, the latency to seizures was 9.88 ± 0.57 min and the duration of discharges was 51.2 ± 2.17 min. With saclofen, these values were 310.1 ± 81.6%, 12.9 ± 1.49 and 48.9 ± 3.82 min, respectively (results of eight rats for baclofen and seven rats for saclofen). Concerning neurodegeneration, the hippocampal CA1 and CA3 subfields of the rats treated with 4-AP plus baclofen or saclofen showed very similar damage to that observed with 4-AP alone (results not shown).

Effect of Na^+ *channel blockers and related drugs*

When TTX was co-administered with 4-AP, the intensity of the 4-AP-induced EEG epileptic discharges was drastically reduced (Fig. 8; compare with the traces of the unprotected animals treated with carbamazepine or ω-conotoxin, and with those after 4-AP alone; Fig. 1). The latency to the first EEG discharge was increased from 12.8 ± 1.07 to 21.3 ± 2.11 min ($P < 0.05$), and their duration was diminished by 90%, compared to 4-AP alone (Fig. 10c). As shown in Figs 9a and 10b, TTX also almost completely protected the hippocampal neurons against the neurodegeneration induced by 4-AP, in both the CA1 and CA3 regions. These protective effects of TTX can be correlated with a significant decrease of about 65% in the 4-AP-induced enhancement of extracellular glutamate (Fig. 10a).

In contrast with the neuroprotective effects of TTX, as shown in Figs 8–10, neither carbamazepine (co-infused with 4-AP) nor valproate (injected i.p. 30 min before 4-AP infusion) significantly modified any of the effects of 4-AP on the three parameters studied.

Effect of ω-conotoxin GVIA

The co-administration of the N-type Ca^{2+} channel blocker ω-conotoxin GVIA did not affect the EEG seizures induced by 4-AP (Figs 8, 10c). The latency to the first discharge was 11.3 ± 1.73 min. This conotoxin diminished by 74% the enhancement of extracellular glutamate induced by 4-AP (Fig. 10a), and significantly protected against the neurodegeneration produced by 4-AP in the CA1 region. This protection, however, was weaker than that exerted by TTX (Figs 9c, 10b).

Effect of riluzole

We initially tested the effects of riluzole i.p. at a dose of 8 mg/kg. In a group of four rats, at this dose riluzole alone did not affect any of the parameters studied. However, when injected 30 min before 4-AP it was extremely toxic, since 24 of the 32 rats studied died during the night following the experiment. Moreover, in all 32 rats we observed a marked potentiation of the convulsant action of 4-AP: there was a notable increment in the intensity of the EEG seizures compared to 4-AP alone (Fig. 11) and the mean duration of the discharges nearly doubled (Fig. 13c), although the latency to the first discharge was not affected (13.4 ± 1.21 min). This occurred in spite of the fact that the enhancement of extracellular glutamate induced by 4-AP was abolished by riluzole (Fig. 13a), suggesting that at this dose the toxic effect of riluzole does not involve glutamatergic transmission. In fact, in the eight surviving rats that could be studied histologically after five days, the neuronal damage was significantly reduced in both the CA1 and CA3 regions, particularly in the latter (Figs 12a, 13b). It is important to mention that riluzole also abolished the increase in extracellular GABA and aspartate induced by 4-AP. With 4-AP alone, the GABA concentration increased from negligible values to 4.2 ± 0.34 pmol/10 μl, and aspartate from 1.56 ± 0.16 to 4.99 ± 0.77 pmol/10 μl, whereas in riluzole-treated animals GABA increased to only 0.8 ± 0.48 and aspartate to 2.86 ± 1.55 pmol/10 μl.

In view of the above results, another group of rats was treated i.p. with riluzole at half the previous dose (4 mg/kg), 30 min before 4-AP. With this dose, all animals survived until the fifth day, with no apparent behavioral alterations. However, as shown in Fig. 4, riluzole did not suppress but

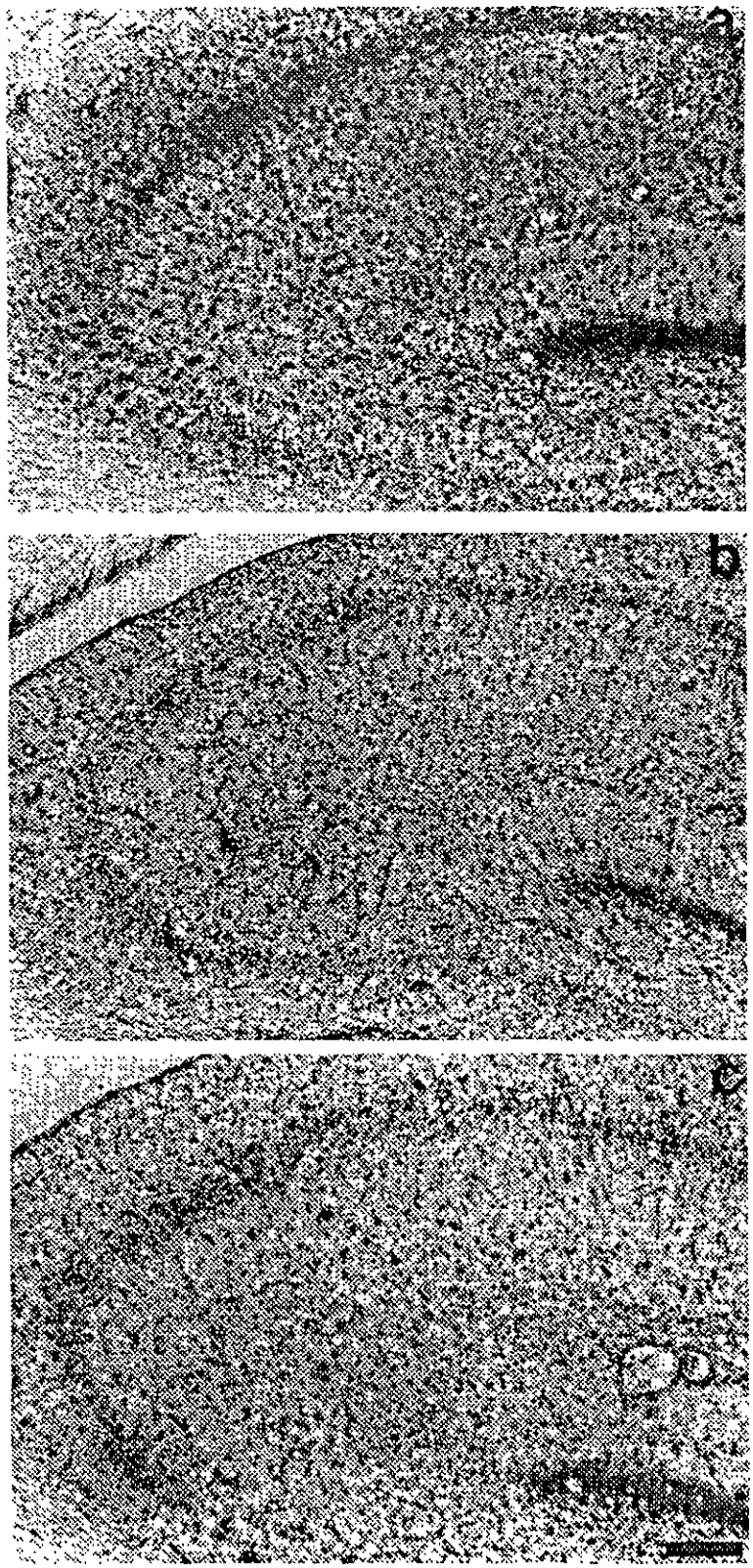

Fig 6. Lack of protective effect of pro-GABAergic drugs against the hippocampal neurodegeneration induced by 4-AP: (a) muscimol, (b) NPCA, (c) bicuculline Note that with NPCA the CA2 region seems to be more damaged than with 4-AP alone (compare with Fig. 2) and with the other drugs. For quantitative data see Fig. 7b Scale bar = 160 μm.

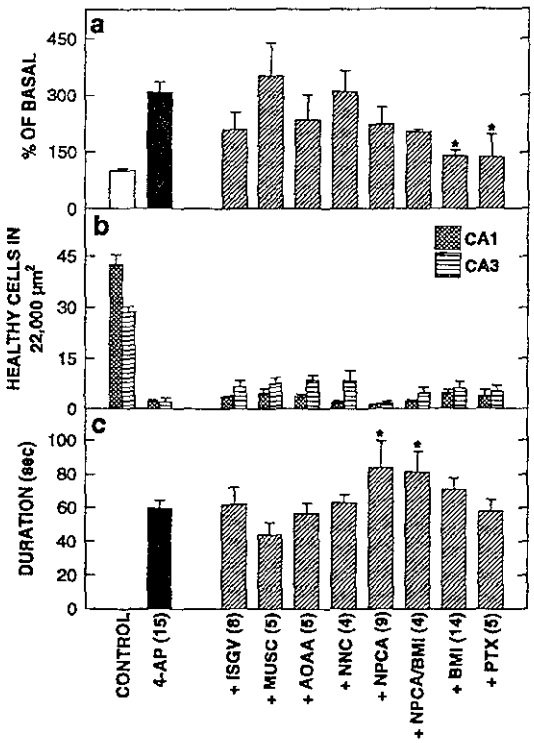

Fig. 7. Extracellular glutamate increase (a), neuronal damage in the CA1 and CA3 hippocampal regions (b), and duration of EEG discharges (c), induced by 4-AP, and effects of pro-GABAergic and anti-GABAergic drugs. Details as in Fig. 3. PTX, picrotoxin. Data are mean values ± S.E.M. for the number of rats indicated in parentheses. $^*P < 0.05$ compared to 4-AP alone.

altered the pattern of the EEG discharges induced by 4-AP. No initial hypersynchronic activity occurred and the frequency of the high-amplitude spikes decreased. Nevertheless, once established, the trains of these spikes were continuous during the rest of the experiment, so that the duration of the discharges could not be calculated. The latency to the first discharge increased to 20.8 ± 1.73 min. At this low dose, riluzole also significantly blocked the 4-AP-induced increase in extracellular glutamate, albeit to a lesser extent than after 8 mg/kg (Fig. 13a), and protected against the hippocampal damage similarly to the latter (Fig. 13b). The increase in extracellular GABA and aspartate induced by 4-AP was also reduced in the animals treated with 4 mg/kg riluzole (64% decrease for GABA and 33% for aspartate, compared to 4-AP alone).

Effect of K^+ channel openers

The Ca^{2+}-dependent K^+ channel opener NS1619 did not significantly modify any of the effects of 4-AP studied (Figs 12, 13), whereas the ATP-sensitive K^+ channel opener diazoxide did not suppress the seizures but inhibited by 55% the extracellular glutamate increase and slightly protected against neuronal death in both the CA1 and CA3 regions (Figs 12, 13).

DISCUSSION

Effect of glutamate receptor antagonists and GABAergic drugs

The results of the present work strongly support the previously proposed close relationship between an increased level of synaptic extracellular glutamate, seizures and neurodegeneration, in the hippocampus *in vivo*,[68,83] after 4-AP treatment. Since such a postulation implies that an overactivation of glutamate receptors is responsible for the epileptogenic and neurotoxic effects of 4-AP, it would be expected that the blockade of glutamate receptors should protect against such effects of 4-AP. This expectation was fulfilled, since we observed that both MK-801 and CPP, two antagonists of NMDA receptors, as well as NBQX, an antagonist of non-NMDA receptors, clearly inhibited the 4-AP-induced convulsions. Such anticonvulsant action is in agreement with previous results obtained with a bolus injection of 4-AP in the hippocampus[25] or in the lateral cerebral ventricle,[59] as well as with the protection by the non-NMDA receptor antagonist GIKY-52466 against seizures induced by the local application of 4-AP on the somatosensory cortex.[6]

Besides their anticonvulsant effect, the three glutamate

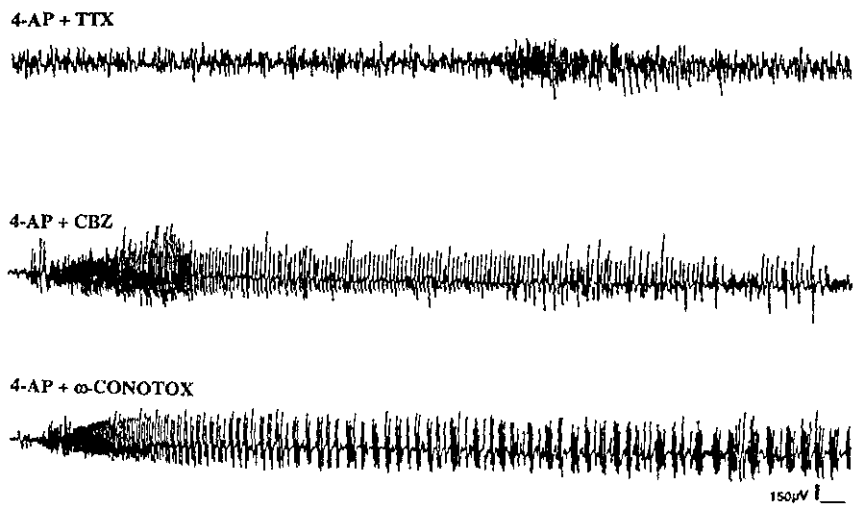

Fig. 8. Protection by TTX against the EEG seizure activity induced by 4-AP, and lack of protection by carbamazepine (CBZ) and ω-conotoxin. Traces are representative of the changes observed once the seizure activity was stabilized. Drugs were co-infused with 4-AP through the microdialysis probe. See Fig. 10c for quantitative evaluation.

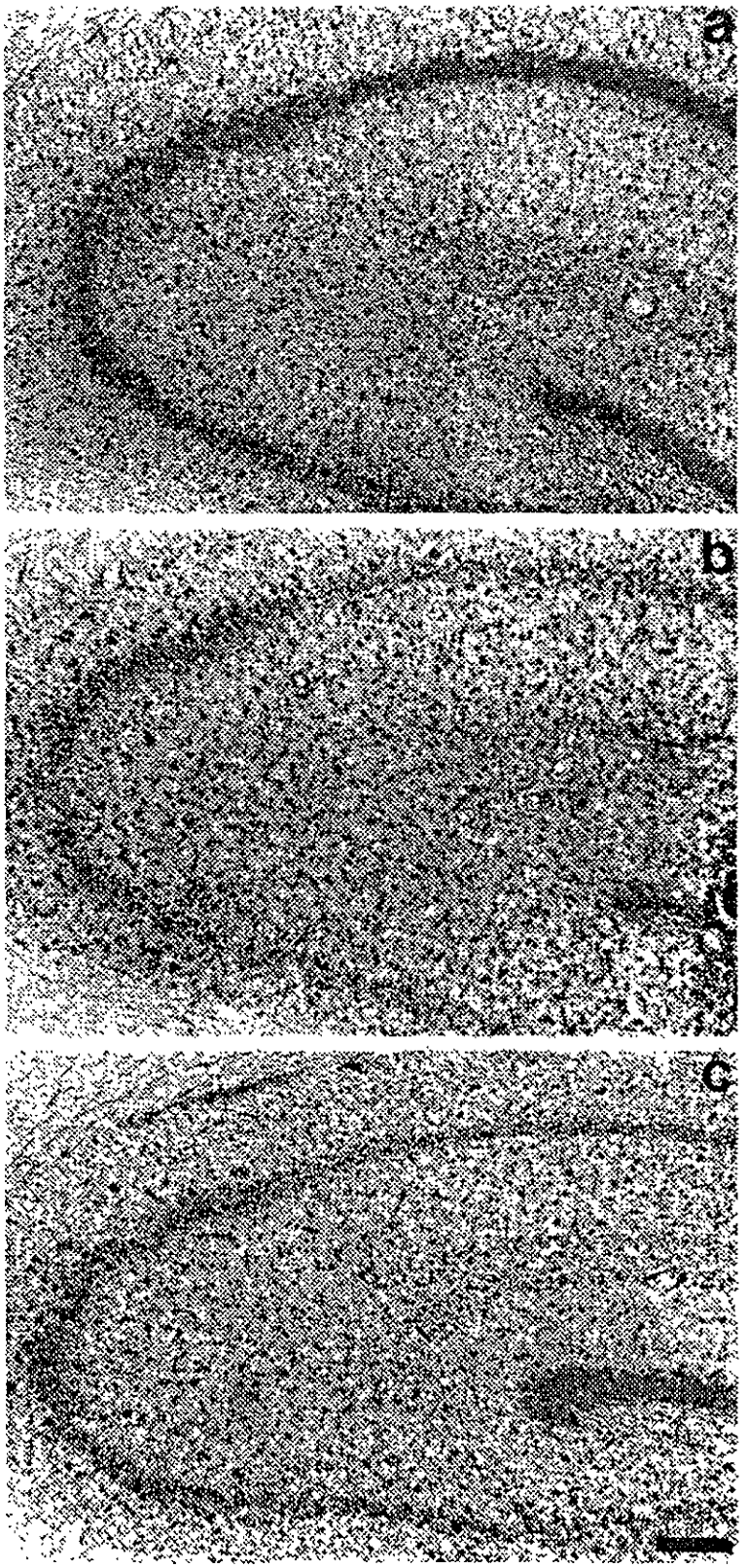

Fig. 9. Effects of TTX (a), carbamazepine (b) and ω-conotoxin (c) against the hippocampal neurodegeneration induced by 4-AP, five days after treatment. Note the nearly complete protection of CA1 and CA3 in the TTX-treated hippocampus, the partial protection by ω-conotoxin and the lack of effect of carbamazepine (compare with Fig. 2). For quantitative data see Fig 10b. Scale bar = 160 μm

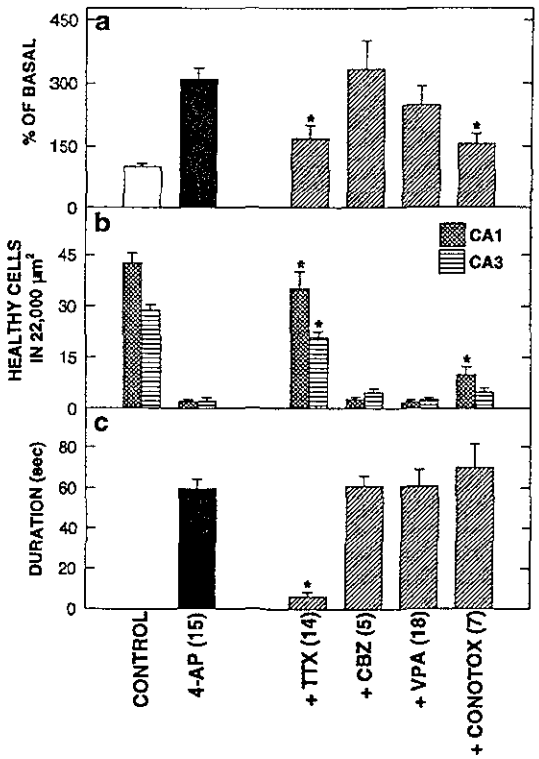

Fig. 10. Extracellular glutamate increase (a), neuronal damage in the CA1 and CA3 hippocampal regions (b), and duration of EEG discharges (c) induced by 4-AP, and effect of TTX, carbamazepine (CBZ), valproate (VPA) and ω-conotoxin (CONOTOX). Details as in Fig. 3. $^{*}P < 0.01$ for TTX and $P < 0.05$ for ω-conotoxin, compared to 4-AP alone.

receptor antagonists used, particularly MK-801, notably prevented neuronal death in the CA1 and CA3 regions, without affecting the increase in extracellular glutamate (Fig. 3). Interestingly, these protective effects were obtained in spite of the fact that MK-801 was administered systemically (i.p.), while CPP and NBQX were co-infused with 4-AP through the dialysis probe. This correlation between the antiepileptic and neuroprotective effects can be clearly seen in Fig. 3. It must be emphasized that, in these groups, as well as in all other experimental groups, the three parameters, glutamate levels, EEG and neuronal damage, were studied simultaneously in the same animal, which permits one to establish their relationships with a high degree of certainty.

A relevant aspect of the relationship between glutamate and the overactivation of its receptors that results in excitotoxicity *in vivo* is the origin of the augmented extracellular concentration of the neurotransmitter. In fact, several kinds of evidence indicate that increased extracellular glutamate resulting from inhibition of its transport or by high-K^+ depolarization does not induce epileptiform activity or neurodegeneration.[52,53,58,65,68,83] In contrast, it has been repeatedly shown in several preparations that the enhancement of extracellular glutamate induced by 4-AP is due to a stimulation of its release from presynaptic nerve endings.[69,84,86,89] It therefore seems that, at this synaptic level, the excess glutamate released by 4-AP is able to reach the receptors and overactivate them. In support of this interpretation is the observation that the damaged hippocampal regions are mainly CA1 and CA3, with more limited or no damage in CA2 and the dentate gyrus. This pattern of damage after excitotoxic insults has been amply described,[3,68,83,98] and seems to be due to differences in the distribution of glutamate receptors[40,97] and/or to the comparatively higher concentration of calcium-binding proteins, such as calbindin, in the neurons of the resistant regions.[5,76]

Strong support for the above interpretation stems from the finding that the blockade of voltage-sensitive Na^+ channels by the highly specific blocker TTX prevents all the effects of 4-AP studied: the extracellular accumulation of glutamate, the EEG discharges and the hippocampal neurodegeneration (Fig. 10). In fact, such an inhibitory effect of TTX is clearly

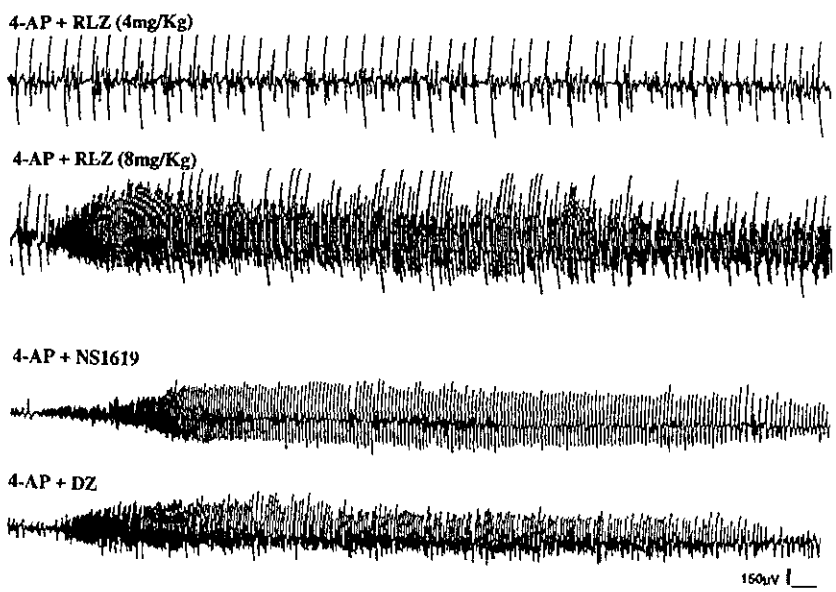

Fig. 11. EEG seizure activity induced by 4-AP in rats treated with two different doses of riluzole (RLZ), or with the K^+ channel openers NS1619 and diazoxide (DZ). Note the lack of effect of NS1619 and diazoxide, and the potentiation of seizures by riluzole (compare with Fig. 1). See Fig. 13c for quantitative evaluation (the traces after 4 mg/kg riluzole were continuous during practically the entire recording period, and therefore the duration of the discharges is not shown in Fig. 13c).

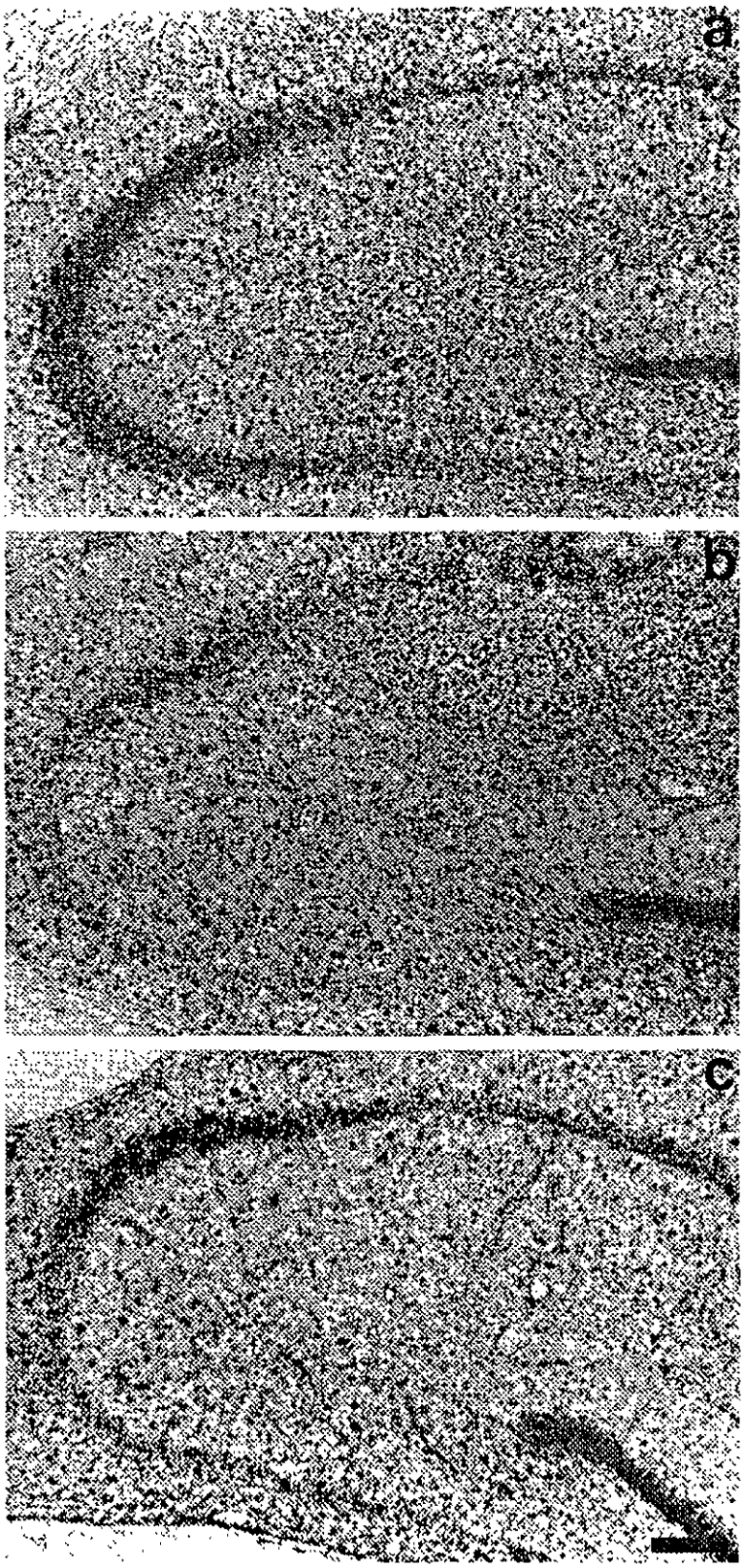

Fig. 12. Partial protective effect of riluzole (8 mg/kg; a) and of diazoxide (c) against the hippocampal neurodegeneration induced by 4-AP. NS1619 (b) did not show any effect. Compare with Fig. 1 and with the complete protection exerted by TTX (Fig. 9a). For quantitative data see Fig. 13b. Scale bar = 160 μm

indicative of a neuronal origin of the 4-AP-induced neurotransmitter release,[37,43,88] which is confirmed by the observed inhibition of glutamate release by ω-conotoxin, since the Ca^{2+} dependence of the effect of 4-AP is well established[85,87] and N-type Ca^{2+} channels are mostly responsible for the Ca^{2+}-dependent transmitter release.[48,82] In agreement with

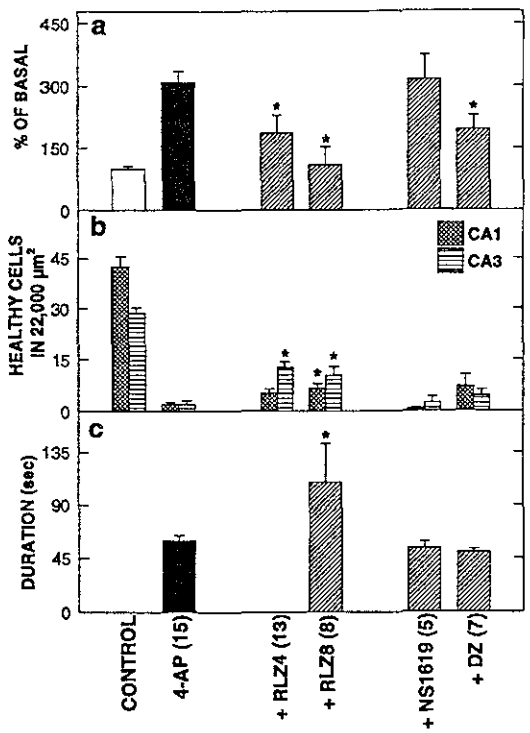

Fig. 13. Extracellular glutamate increase (a), neuronal damage in the CA1 and CA3 hippocampal regions (b), and duration of EEG discharges (c) induced by 4-AP, and effects of riluzole (RZL, 4 and 8 refer to the i.p. dose, mg/kg), NS1619 and diazoxide (DZ). Details as in Fig. 3. The traces after 4 mg/kg riluzole were continuous during practically the entire recording period, and therefore the duration of the discharges is not shown. Data are mean values ± S.E.M. for the number of rats indicated in parentheses. *$P < 0.05$ compared to 4-AP alone.

these observations, it has been reported that TTX prevents both the release of neurotransmitter amino acids[23,69] and the epileptiform activity[62,69] induced by 4-AP in hippocampal or striatal slices. TTX also showed protective properties against hypoglycemia–hypoxia-induced damage in neuronal cultures[71] and against ischemia in the hippocampus *in vivo*.[93]

None of the pro-GABAergic drugs tested here was able to prevent the increase in extracellular glutamate, the EEG seizures or the neurodegeneration induced by 4-AP. These results agree with the lack of effect of diazepam, vigabatrin and tiagabine against convulsions induced by the i.p. administration of 4-AP in mice,[92] whereas in other animal models of epilepsy the anticonvulsant action of GABAergic compounds is well established.[15,33,60,77,81] In hippocampal slices, activation of $GABA_A$ receptors with muscimol[13] or with some anticonvulsant lactams[38] prevents the epileptiform electrical activity induced by 4-AP. However, in this *in vitro* preparation there is controversy regarding the role of GABAergic transmission in the epileptogenic action of 4-AP, since it has been shown repeatedly that the blockade of GABAergic transmission results, paradoxically, in some protection.[4,8,61,69] The probable explanation for these findings is that, during neuronal hyperexcitation induced by 4-AP[4,44,57,69] or by high-frequency electrical stimulation of CA1 afferents,[41,78] the synaptic response to GABA is depolarization rather than hyperpolarization. In this respect, it seems relevant that 4-AP *per se* also induces an increase in the levels of extracellular GABA in the hippocampus (Fig. 4; also see Ref 68).

The foregoing discussion provides an explanation for our finding that the infusion of NPCA, which largely increased the concentration of extracellular GABA, enhanced the intensity and duration of EEG seizures, as well as the neuronal damage, produced by 4-AP. In support of this interpretation is the potentiation of the depolarizing action of GABA in hippocampal slices by GABA uptake blockers such as NPCA[2] or tiagabine.[41] Also in agreement with this postulation is the paradoxical proconvulsant action *in vivo* of some GABA uptake blockers, such as NPCA, NNC-711, tiagabine and 2,4-diaminobutyric acid, in other epilepsy models.[15,33,60,77] An excitatory action of GABA is also suggested by our present finding that the $GABA_A$ receptor blockers picrotoxin and bicuculline reduced the extracellular accumulation of glutamate and that the latter prevented the changes in other extracellular amino acids induced by 4-AP. Nevertheless, the experiments designed to test this hypothesis, namely the co-administration of 4-AP, bicuculline and NPCA, failed to confirm it, since bicuculline did not prevent the potentiation by NPCA of the 4-AP-induced EEG seizures.

In accordance with the lack of anticonvulsant action of pro-GABAergic drugs, these compounds did not protect against the neuronal death induced by 4-AP in the CA1 and CA3 regions of the hippocampus, and some potentiation was even observed in the rats treated with 4-AP and NPCA. A similar toxic effect of GABA or $GABA_A$ receptor agonists has been reported in cortical cultures, where they induce an acceleration of glutamate receptor-mediated excitotoxicity,[21] as well as in hippocampal cultures under depolarizing conditions.[49] *In vivo*, although an enhancement of GABAergic transmission can protect neurons against neurodegeneration induced by ischemia[50,73] and electrically induced epilepsy,[95] the hippocampal damage produced by kainate was not prevented by vigabatrin.[70]

Effect of ion channel blockers

In contrast to the above-mentioned results with TTX, the Na^+ channel blockers carbamazepine[26,42,51,72] and valproate[22,27,46,72] were ineffective in preventing the extracellular accumulation of glutamate, the EEG discharges and the neurodegeneration produced by 4-AP. A possible explanation for this difference is that the mechanism of blockade of the channel is different to that of TTX,[42,72,99] and therefore *in vivo* these drugs are much less efficient. In agreement with this postulation, valproate is ineffective and carbamazepine is only weakly effective in inhibiting the release of amino acid neurotransmitters, both in brain slices and *in vivo*.[7,18,19,46,74,90] Regarding the antiepileptic action, valproate was ineffective against epileptiform activity induced by 4-AP in hippocampal slices and carbamazepine only partially reduced it,[96] although in other studies *in vitro* these drugs showed some protection against 4-AP-induced epileptiform activity.[10,28,29,91] *In vivo*, both compounds at doses similar or equivalent to those used here are good anticonvulsants against a variety of seizure models,[16,46] but their mechanisms of action seem to be multiple and are not completely understood.[22,26,27,51]

In agreement with the well-known inhibitory effects of the N-type Ca^{2+} channel blocker ω-conotoxin GVIA on neurotransmitter release,[48,82] this toxin considerably reduced the 4-AP-induced enhancement of extracellular glutamate. However, differently from TTX, this action was not accompanied by a protective effect on the EEG discharges, although a

significant protection against neurodegeneration was observed, particularly in the CA1 region. In order to reconcile these results with those with TTX, we propose that, as we have suggested previously,[68] the convulsant effect of 4-AP involves an increased excitatory glutamate-mediated transmission combined with an increment in neuronal firing frequency. TTX should be expected to inhibit both types of action, whereas the action of ω-conotoxin is restricted to the presynaptic region. This interpretation may also account for the results obtained with riluzole and with diazoxide, which, similarly to ω-conotoxin, inhibited glutamate release but were unable to suppress the EEG seizures and neuronal death. Interestingly, however, all three drugs partially protected against neurodegeneration, suggesting that this effect of 4-AP is closely related to the increased extracellular concentration of glutamate, as suggested previously.[68]

Some reports have shown protective effects of riluzole against ischemia- or nitropropionic acid-induced neurodegeneration.[36,63,66] However, riluzole failed to protect against the convulsant action of i.c.v. 4-AP.[80] In this respect, our finding that riluzole at the highest dose tested (8 mg/kg) potentiated the convulsant action of 4-AP and produced a high mortality of the rats is worth noting, insofar as riluzole is practically the only known drug with some beneficial action in the treatment of amyotrophic lateral sclerosis.[47] Moreover, half the dose of riluzole, although not inducing mortality, also seemed to potentiate the 4-AP-induced EEG discharges, which became continuous. Although these doses are higher than those used clinically (about 1–3 mg/kg orally on a daily basis), their effect was observed after a single administration. Hence, the present results indicate that the clinical use of riluzole should be considered with caution. In addition, as shown in the present work and has been stressed previously,[64] riluzole reduces the extracellular accumulation not only of glutamate, but also of GABA and aspartate, and the possible adverse consequences of this effect cannot be ignored. Finally, riluzole acts on a great variety of ion channels, including Na^+, Ca^{2+} and K^+ channels,[66,79,100] which may add other undesirable side-effects.

CONCLUSIONS

The findings of the present work indicate that an overactivation of glutamate receptors, mainly of the NMDA type, is involved in the generation of epileptiform activity and in the neurodegeneration produced by the infusion of 4-AP in rat hippocampus *in vivo*, and that ionic channels play a relevant role. In addition, our data clearly show that the glutamate-mediated effects of 4-AP are a consequence of an increased release of the transmitter from excitatory nerve endings. Such increased release is directly related to the neurodegeneration of hippocampal regions CA1 and CA3. Since increased extracellular glutamate levels by inhibition of its transport or by K^+ depolarization do not result in excitotoxicity,[52,53,65,68] we conclude that the site of origin of augmented extracellular glutamate is determinant for the overactivation of glutamate receptors. We also conclude that, under conditions of hippocampal hyperexcitation, an enhancement of GABAergic transmission may result in a potentiation of excitotoxicity, possibly via a depolarizing action mediated by $GABA_A$ receptor activation. Finally, although riluzole did reduce the release of glutamate and this partially protected against neurodegeneration, this compound may be toxic when combined with neuronal hyperexcitation, and thus its clinical use should be considered with caution.

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IV. DISCUSIÓN GENERAL

Mediante la combinación de técnicas neuroquímicas, electrofisiológicas e histológicas, en este trabajo se estudió la correlación que existe entre un incremento en la concentración extracelular de glutamato con la inducción de epilepsia y la neurodegeneración. Con los resultados obtenidos podemos afirmar que la acumulación de glutamato extracelular debida a un incremento en su liberación desde terminales nerviosas participa en la producción de crisis epilépticas y neurodegeneración en el hipocampo de la rata *in vivo*, pero que este incremento en el glutamato extracelular no es el único factor que las desencadena.

EL ORIGEN DEL GLUTAMATO EXTRACELULAR COMO DETERMINANTE EN LA INDUCCIÓN DE CRISIS EPILÉPTICAS Y NEURODEGENERACIÓN IN VIVO.

Como se muestra en el trabajo número 1 de resultados, y como ya se había mostrado previamente en otros paradigmas experimentales (Massieu et al., 1995; Morales-Villagrán y Tapia, 1996; Massieu y Tapia, 1997; Obrenovicth et al., 1996), no es suficiente provocar la acumulación extracelular de glutamato para producir neurodegeneración *in vivo*. En una serie de estudios previos se ha demostrado que el incrementar los niveles extracelulares de glutamato *in vivo* (de 15 a 20 veces por encima de sus niveles basales) por inhibición de su recaptura no es suficiente para la inducción de epilepsia (Obrenovitch et al., 1996) o neurodegeneración (Massieu et al., 1996; Massieu y Tapia, 1997). Coincidentemente, en el presente trabajo se muestra que al aplicar por *microdiálisis una concentración alta de* K^+ (50 mM) o bien tetraetilamonio (TEA) 120 mM, los cuales producen un importante aumento en la concentración de glutamato extracelular (450% y 200% del basal respectivamente, Trabajo 1, Fig. 4a), no se observó la inducción de epilepsia ni neurodegeneración considerables. Como veremos más adelante, a diferencia de la 4-AP, el glutamato liberado por el TEA y el alto K^+ parece que no proviene en su mayoría de las terminales nerviosas y por lo tanto es ineficaz para la inducción de epilepsia y daño neuronal, pues no es liberado en el espacio sináptico y la probabilidad de alcanzar los receptores glutamatérgicos es baja (Obrenovitch y Urenjak, 1997; Tapia et al., 1999). Existe

evidencia de que además de las terminales sinápticas, los neurotransmisores pueden ser liberados, en situaciones particulares, de las células gliales (Patterson et al., 1995; Herrera-Marschitz et al., 1996; Obrenovitch y Urenjak, 1997; Timmerman y Westernink, 1997; Lada et al., 1998) y de los somas neuronales (Castel et al., 1996; Rice et al., 1997; Zaidi y Matthews, 1997; Bunin y Wightman, 1998; Jaffe et al., 1998; Hoffman y Gerhardt, 1999, Zaidi y Matthews, 1999)

El TEA bloquea canales de potasio distintos de los que bloquea la 4-AP (Rudy, 1988, Storm, 1993; Chandy y Gutman, 1995; Dolly y Parcej, 1996) y además afecta a una menor proporción de los canales de potasio localizados en la presinapsis (Jones y Heinemann, 1987; Southan y Robertson, 1998), por lo cual parece tener efectos presinápticos de mucho menor magnitud que la 4-AP (Hu et al., 1991; Boireau et al., 1991; Schechter, 1997). También existen diferencias funcionales a nivel postsináptico, pues aunque la 4-AP y el TEA aumentan la duración de la fase despolarizante del potencial de acción (Kocsis et al., 1987; Zhang y McBain, 1995; Koyano et al., 1996), solamente la 4-AP es capaz de inducir disparo repetitivo en las neuronas (Kocsis et al., 1987; Bargas et al., 1989; Nisenbaum et al., 1994; Koyano et al., 1996; Hoffman et al., 1998), lo cual también puede explicar que el TEA no genere crisis epilépticas.

Un incremento en la concentración extracelular de potasio, al igual que el TEA, resulta ser menos eficiente que la 4-AP para liberar glutamato de terminales sinápticas (Müller et al., 1999), ya que despolariza todos los componentes celulares y por consiguiente puede liberar neurotransmisores tanto de los somas neuronales (Hoffman y Gerhardt, 1999) como de las células gliales (Patterson et al., 1995). Nuevamente, es muy probable que este glutamato liberado fuera del espacio sináptico no pueda tener acceso a receptores postsinápticos y por lo tanto no puede inducir epilepsia y/o daño neuronal (Obrenovitch y Urenjak, 1997; Tapia et al., 1999).

A diferencia del alto K^+ y del TEA, múltiples experimentos *in vitro* han mostrado que la 4-AP libera neurotransmisores actuando casi exclusivamente en terminales sinápticas (Thesleff, 1980; Buckle y Haas, 1982; Jankowska et al., 1982; Tapia y Sitges, 1982; Tibbs et al., 1989; Perrault y Avoli, 1991; Flores-

Hernandez et al., 1994, Barish et al., 1996). En la presente tesis se muestra que el glutamato liberado por la 4-AP *in vivo* sí contribuye a la generación de las crisis epilépticas y al daño neuronal. En el trabajo 1 de Resultados podemos ver que aquellas concentraciones de 4-AP capaces de producir incrementos cuantificables en el glutamato extracelular pueden inducir epilepsia y neurodegeneración de una intensidad bastante considerable, pero aquellas concentraciones de 4-AP que no produjeron cambios en el glutamato extracelular, cuantificables por la microdiálisis, produjeron crisis epilépticas de muy poca intensidad y no indujeron neurodegeneración alguna.

Además de lo anterior, en el trabajo número 2 se muestra claramente que la gran mayoría del glutamato liberado por la 4-AP es de origen presináptico, pues esta liberación se inhibió por la aplicación de TTX (Trabajo 2, Fig. 10b), la cual es una toxina específica que bloquea los canales de sodio sensibles a voltaje. Reforzando la afirmación de que el glutamato liberado por la 4-AP es de origen presináptico, se encontró también que la ω-conotoxina GVIA, que bloquea los canales de calcio tipo N, también reduce la liberación de glutamato (Trabajo 2, Fig. 10b). La apertura tanto de los canales de Na^+ como de los canales de calcio tipo N es un evento esencial para inducir la liberación de los neurotransmisores en las terminales nerviosas (Luebke et al., 1993; Takahashi y Momiyama, 1993; Meir et al., 1999), por lo cual se ha planteado que aquella liberación bloqueada por antagonistas de estos canales es de origen neuronal y proviene principalmente de terminales nerviosas (Herrera-Marschitz et al., 1996; Timmerman y Westernink, 1997; Lada et al., 1998).

EL PAPEL DEL GLUTAMATO LIBERADO POR LA 4-AMINOPIRIDINA EN LA INDUCCIÓN DE CRISIS EPILÉPTICAS

Existen múltiples evidencias que muestran que el glutamato participa en la generación de crisis epilépticas (para revisiones ver Bradford, 1995; Meldrum, 1995) En este trabajo de tesis se muestra que el glutamato liberado por la 4-AP juega un papel muy importante en la aparición de crisis epilépticas en el hipocampo *in vivo*. Como se puede ver en los trabajos 1 y 2, las crisis epilépticas

inducidas por la 4-AP se caracterizan por una fase inicial de actividad hipersincrónica que se continúa con trenes de espigas de alto voltaje. El primer resultado que muestra el papel del glutamato en la generación de estas crisis es el obtenido al aplicar antagonistas para los diferentes receptores ionotrópicos de glutamato. Estas drogas reducen de manera significativa la duración de las crisis epilépticas que induce la 4-AP a expensas de la disminución de los trenes de espigas de alto voltaje (Trabajo 2, Figs. 1 y 3c), lo cual coincide con resultados previos en los que se muestra que antagonistas para los receptores a glutamato, tanto del tipo NMDA como no-NMDA, previenen contra las crisis epilépticas inducidas por la 4-AP *in vivo* (Gandolfo et al., 1989; Fragoso-Veloz, 1992; Cramer et al., 1994; Morales-Villagrán et al., 1996, Barna et al., 2000) e *in vitro* (Perrault y Avoli, 1991; Avoli et al., 1996; Siniscalchi et al., 1997; Doczi et al., 1999). El segundo resultado que evidencia el papel del glutamato liberado por la 4-AP en la generación de las crisis epilépticas es el obtenido por la aplicación de la TTX. Este bloqueador de los canales de sodio sensibles a voltaje reduce la liberación de glutamato y protege completamente contra las crisis epilépticas. Es tan potente el efecto de la TTX que se bloquean los dos componentes característicos de las crisis inducidas por la 4-AP, la actividad hipersincrónica y los trenes de espigas de alto voltaje (Trabajo 2, Fig. 8). Esto coincide con trabajos *in vitro* que muestran que la TTX bloquea completamente la actividad epileptiforme inducida por la 4-AP en rebanadas de cerebro de rata (Müller y Misgeld, 1991; Perrault y Avoli, 1991).

A pesar de que lo anterior muestra que el glutamato juega un papel importante en la generación de las crisis epilépticas inducidas por la 4-AP, los resultados obtenidos en esta tesis indican que no es el único factor que interviene en la generación de las crisis. En efecto, en el trabajo 1 se observa que a bajas concentraciones (7 y 3.5 mM) la 4-AP no induce un aumento cuantificable en la liberación de glutamato y aun así es capaz de producir crisis epilépticas, aunque no podemos descartar que con nuestro sistema experimental no sea posible cuantificar algunos incrementos transitorios en el glutamato extracelular. Por otra parte, podemos ver en el trabajo número 2 que el bloqueo de los receptores glutamatérgicos reduce las crisis epilépticas que produce la 4-AP a expensas de

disminuir la ocurrencia de las espigas de alto voltaje, pero no elimina completamente estas crisis, pues regularmente la actividad hipersincrónica no se ve afectada (Trabajo 2, Fig. 1). En apoyo de esta conclusión de que además de un incremento en la liberación de glutamato existen otros factores que intervienen en la generación de epilepsia por la 4-AP, tenemos los resultados obtenidos con la aplicación de ω-conotoxina, riluzol, diazoxida e incluso con los antagonistas de receptores $GABA_A$, picrotoxina y bicuculina, donde se observó que a pesar de que estas drogas bloquearon, con distinta intensidad, la liberación de glutamato inducida por la 4-AP, no fueron capaces de prevenir contra las crisis epilépticas (Trabajo 2, Figs. 7, 10 y 13).

¿Cuáles pueden ser esos otros factores, además del aumento en la liberación de glutamato, que parecen estar contribuyendo a la generación de crisis epilépticas por la 4-AP? Una posibilidad que se abordará mas adelante es que otros neurotransmisores además del glutamato, particularmente el GABA, puedan participar en la epileptogénesis inducida por la 4-AP, ya que esta droga provoca no solo la liberación de glutamato sino de prácticamente todos los neurotranmisores (Lundh, 1978; Thesleff, 1980; Buckle y Haas, 1982; Tapia y Sitges 1982; Dolezal y Tucek, 1983; Tapia et al., 1985; Tibbs et al., 1989; Hu et al., 1991; Dawson y Routledge, 1995; Veersteg et al., 1995; Morales-Villagrán y Tapia 1996; Schechter, 1997). Otra posibilidad, que no excluye las anteriores, es que en el efecto epileptogénico de la 4-AP, además de su acción presináptica ya descrita, participen factores postsinápticos. En este sentido se encuentran trabajos que muestran que la 4-AP, a nivel postsináptico, aumenta la duración de la fase despolarizante del potencial de acción (Kocsis et al., 1987; Zhang y McBain, 1995; Chen et al., 1996; Koyano et al., 1996) y es capaz de inducir disparo repetitivo en las neuronas (Kocsis et al., 1987; Bargas et al., 1989; Nisenbaum et al., 1994; Koyano et al., 1996; Hoffman et al., 1998), lo cual comúnmente se asocia con la generación de hiperexcitabilidad y epilepsia (Jefferys y Traub, 1998).

De lo anterior se desprende que la generación de las crisis epilépticas por la 4-AP se puede deber a la suma de un efecto presináptico (aumento en la liberación de glutamato) y un efecto postsináptico (incremento en el disparo

neuronal). Los antagonistas glutamatérgicos protegen parcialmente contra las crisis porque sólo bloquean la acción del glutamato sobre sus receptores, pero no interfieren con el incremento en el disparo neuronal, mientras que la TTX bloquea completamente las crisis epilépticas porque es capaz de revertir tanto la liberación de glutamato (Flores-Hernández et al., 1994; Tibbs et al., 1991, Perraul y Avoli 1991; trabajo 2), como el incremento en la frecuencia de disparo neuronal que produce la 4-AP (Hoffman et al., 1998).

EL PAPEL DEL GLUTAMATO LIBERADO POR LA 4-AP EN LA INDUCCIÓN DE NEURODEGENERACIÓN

El papel del glutamato en la generación de daño neuronal está muy bien establecido (ver Olney, 1978; Choi, 1988). Los primeros datos que apuntan a que el glutamato es determinante en la neurodegeneración producida por la 4-AP, vienen del trabajo número 1. En primer lugar se muestra que solamente aquellas concentraciones de 4-AP capaces de incrementar el glutamato extracelular pueden, además de inducir crisis epilépticas, producir una intensa neurodegeneración en el hipocampo (Trabajo 1, Figs. 5C-H y 6); con concentraciones bajas de 4-AP, que no aumentan el glutamato extracelular cuantificable por la microdiálisis (Trabajo 1, Fig. 3), se generan crisis epilépticas de intensidad moderada pero nunca se produce ningún daño neuronal. En ese mismo trabajo se muestra que el patrón de daño producido por la 4-AP consiste en una neurodegeneración masiva en las neuronas piramidales de CA1 y CA3, con la preservación de las regiones CA2 y el giro dentado (Trabajo. 2, Fig. 5 y 6). Este patrón de daño neuronal en el hipocampo parece ser un indicativo de sobreactivación glutamatérgica, pues es muy similar al que se observa después de aplicar agonistas glutamatérgicos en el hipocampo (Ben-Ari 1985; Stein-Behrens et al., 1994; Zhang et al., 1996; Arias et al., 1997; Grooms et al., 2000) o bien en el hipocampo de pacientes con epilepsia del lóbulo temporal (Fig. 1 de los Antecedentes), donde se ha hipotetizado que la excitotoxicidad juega un papel muy importante (Kim et al., 1990; Houser, 1992). En este mismo sentido, se ha planteado que la diferencia entre las regiones sensibles (CA1 y CA3) y resistentes

(CA2 y el giro dentado) al daño producido por el glutamato en el hipocampo, se debe a una expresión diferencial de receptores a glutamato (Insel et al., 1990; Young et al., 1991) o de proteínas atrapadoras de Ca^{2+} (Baimbridge y Miller, 1982; Muñoz, 1990; Sloviter et al., 1991), los cuales son muy importantes para el proceso excitotóxico (ver Antecedentes).

Ya se ha mostrado ampliamente que los antagonistas glutamatérgicos protegen contra la excitotoxicidad en diferentes paradigmas experimentales (Massieu y Tapia, 1994; Arias et al., 1997; Massieu et al., 2000). Confirmando el papel del glutamato en la neurodegeneración producida por la 4-AP, en la presente tesis se muestra que los antagonistas a los receptores de este neurotransmisor, principalmente el MK-801, además de reducir las crisis epilépticas, previenen contra la neurodegeneración que produce la 4-AP (Trabajo. 2, Figs. 2 y 3b). El efecto neuroprotector de los antagonistas glutamatérgicos se debe a que bloquean el efecto del glutamato sobre sus receptores postsinápticos, pues no afectan la liberación de glutamato que produce la 4-AP (*Trabajo. 2, Fig.* 3a). En cambio, como ya se mencionó, la TTX sí reduce la liberación de glutamato y de esta manera protege contra la neurodegeneración que produce la 4-AP, al igual que en otros modelos de daño neuronal tanto *in vitro* (Weber y Taylor, 1994; Strijbos et al., 1996; Probert et al., 1997) como *in vivo* (Yamasaki et al., 1991; Lysko et al., 1994).

De manera similar a la TTX, la ω-conotoxina GVIA, el riluzol, la diazoxida y los antagonistas del receptor $GABA_A$ inhibieron la liberación de glutamato que produce la 4-AP(Trabajo 2, Figs. 7,10 y 13), pero tuvieron un efecto neuroprotector muy reducido, en el caso de la ω-conotoxina GVIA, el riluzol y la diazoxida, o nulo en el caso de los de los antagonistas del receptor $GABA_A$. Esto marca una diferencia muy clara con el efecto neuroprotector de la TTX, que podemos explicar si observamos que todas estas drogas, a diferencia de la TTX, no previnieron contra las crisis epilépticas y en algunos casos las potenciaron. Todo esto sugiere que no basta con reducir la liberación de glutamato para proteger contra la neurodegeneración que produce la 4-AP, pues si las crisis epilépticas persisten es difícil lograr la neuroprotección. Apoyando esta idea, existe evidencia que indica

que las crisis epilépticas contribuyen por sí mismas a la generación del daño neuronal (Inoue et al., 1992; Fernandes et al., 1999; Dube et al., 2000), probablemente porque mantienen a las células en un estado funcional límite, que las hace más vulnerables (Fernandes et al., 1999; Folbergrova et al., 1999). Puede ser que esta condición de vulnerabilidad favorezca la inducción del daño neuronal por el glutamato liberado por la 4-AP. Incluso se ha mostrado que cuando se produce una deficiencia en los niveles energéticos (como puede ser la inhibición de la glicólisis) los niveles basales de glutamato son suficientes para producir neurodegeneración (Zeevalk et al., 1990; Zeevalk et al., 1992).

Esta misma asociación entre labilidad celular y daño neuronal producido por el glutamato, la podemos observar en otro modelo de neurodegeneración donde se ha encontrado que el glutamato acumulado por la inhibición de su recaptura no produce daño neuronal (Massieu et al., 1995), pero si previamente se induce una disfunción en el metabolismo energético con la aplicación de ácido 3-nitropropiónico o de ácido iodoacético, entonces este glutamato acumulado por la inhibición de la recaptura sí puede inducir daño neuronal (Sánchez-Carbente y Massieu, 1999; Massieu et al., 2000). Resumiendo, podemos afirmar que en la neurodegeneración producida por la 4-AP participa la activación de receptores a glutamato (ver protección por antagonistas glutamatérgicos) y que las crisis epilépticas contribuyen a esta neurodegeneración de manera muy importante, muy probablemente por inducir un estado de vulnerabilidad neuronal bajo el cual el glutamato puede ejercer sus efectos excitotóxicos con mayor facilidad.

PAPEL DE LA TRANSMISION GABAERGICA EN LA EPILEPSIA Y LA NEURODEGENERACIÓN PRODUCIDAS POR LA 4-AP IN VIVO.

Una de las hipótesis más interesantes para explicar la inducción de crisis epilépticas y neurodegeneración, es que éstas pueden deberse a una disfunción producida por un desequilibrio entre la transmisión glutamatérgica y la GABAérgica. Este desbalance se puede originar por un incremento en la transmisión glutamatérgica (excitadora), una disminución en la actividad GABAérgica (inhibidora) o ambas cosas (para revisiones ver Bradford, 1995;

Meldrum, 1995). En este sentido, se ha propuesto que el potenciar la actividad GABAérgica resulta en protección contra las crisis epilépticas y la neurodegeneración (para una revisión ver Lyden, 1997; Green et al., 2000).

En este trabajo se probaron diferentes estrategias para potenciar la transmisión GABAérgica: la inhibición de la recaptura de GABA (NC-711 y ácido nipecótico), inhibición de su degradación (ácido aminooxiacético) o agonistas tanto para su receptor ionotrópico (isoguvacina) como para su receptor metabotrópico (baclofén). Como se puede ver claramente en el trabajo 2, la mayoría de estas drogas no tuvieron ningún efecto significativo sobre el aumento en el glutamato extracelular, la epilepsia o la neurodegeneración que induce la 4-AP *in vivo* (Trabajo 2, Fig. 7). Un resultado que llama mucho la atención es que el ácido nipecótico, un inhibidor del transportador de GABA, que produce un incremento masivo en la concentración extracelular de GABA que llega hasta 3 µM (Trabajo 2, Fig. 4), potenció las crisis epilépticas que produce la 4-AP (Trabajo 2, Fig. 5), lo que pudiera indicar que el GABA, en estas condiciones, favorece la inducción de crisis epilépticas. Esto concuerda con los hallazgos *in vitro* donde se demuestra que bajo condiciones de hiperexcitabilidad inducida por estimulación de alta frecuencia (Staley et al., 1995; Jackson et al., 1999) o bien por la inducción de crisis epileptiformes con 4-AP (Michelson y Wong, 1991; Perrault y Avoli, 1991; Avoli et al., 1996; Lamsa y Kaila, 1997) el GABA contribuye a la generación de las crisis epileptiformes, porque en éstas condiciones la activación de los receptores $GABA_A$ además de producir su potencial inhibidor característico, desencadena un potencial despolarizante de larga duración que parece estar mediado por la salida de iones HCO_3^- por el receptor mismo y por la acumulación de K^+ extracelular (Staley et al., 1995; Lamsa y Kaila, 1997). Se ha observado que este efecto despolarizante del GABA *in vitro* se ve incrementado cuando se aplica ácido nipecótico (Alger y Nicoll, 1982) u otro inhibidor de la recaptura de GABA llamado tiagabina (Jackson et al., 1999).

En virtud de que un posible efecto despolarizante del GABA pudiera estar involucrado en la inducción de las crisis epilépticas producidas por la 4-AP o en su potenciación por el ácido nipecótico, se probó el efecto de un antagonista de los

receptores $GABA_A$, la bicuculina, sobre la potenciación de las crisis epilépticas por ácido nipecótico o bien sobre las crisis que produce la 4-AP por sí sola. Como se puede ver en la figura 10 del segundo trabajo, la bicuculina no tuvo ningún efecto protector contra estas crisis. Esto no coincide con los resultados obtenidos *in vitro*, donde se muestra que el antagonismo de los receptores $GABA_A$ bloquea la aparición de potenciales despolarizantes en las crisis epileptiformes inducidas por la 4-AP en rebanadas de cerebro (Perrault y Avoli, 1991; Avoli et al., 1996; Siniscalchi et al., 1997). El único dato obtenido en este trabajo que pudiera indicar un papel excitador del GABA durante las crisis producidas por la 4-AP, es que los antagonistas de los receptores $GABA_A$ (bicuculina y picrotoxina) inhibieron la liberación de glutamato producida por la 4-AP (ver trabajo 2, fig. 3), lo cual pudiera indicar que la bicuculina y la picrotoxina bloquean el efecto despolarizante del GABA y así reducen la liberación de glutamato, aunque no tenemos ninguna evidencia directa de que esto sea así.

PRECISIONES SOBRE LA OTRAS ESTRATEGIAS DE NEUROPROTECCIÓN ESTUDIADAS.

Ya se discutió previamente el papel neuroprotector que tienen los antagonistas de receptores a glutamato y el bloqueo de los canales de sodio sensibles a voltaje con la TTX, sobre la epilepsia y la neurodegeneración que induce la 4-AP, además del posible efecto potenciador que tiene el incremento de la transmisión GABAérgica sobre estos procesos. Sólo resta discutir por qué otras estrategias de neuroprotección no dieron buenos resultados en este modelo.

En el trabajo 2 se probaron estrategias de protección más cercanas a la clínica o bien de uso más reciente. Partiendo de los efectos neuroprotectores de la TTX sobre las crisis epilépticas y la neurodegeneración producidas por la 4-AP, se probó el efecto de la carabamacepina y el valproato, dos anticonvulsivantes ampliamente utilizados en la clínica para el tratamiento de diferentes tipos de epilepsia y cuyo efecto protector parece deberse a su capacidad de bloquear, al igual que la TTX, los canales de sodio sensibles a voltaje (Fromm, 1992a,b; Fariello et al., 1995; McDonald, 1995; Ragsdale y Avoli, 1998; Löscher, 1999),

además de que *in vitro* han demostrado buenas cualidades antiepilépticas contra las crisis que produce la 4-AP (Fueta y Avoli, 1992; Watts y Jefferys, 1993; Fueta et al., 1995; Brückner y Heinemann, 2000). En los resultados obtenidos en este trabajo se muestra que estos anticonvulsivantes no se comportaron como la TTX, pues no previnieron ni la acumulación de glutamato extracelular, ni las crisis epilépticas, ni la neurodegeneración que produce la 4-AP *in vivo* (Trabajo 2, Fig. 10). De aquí se puede concluir que su efecto anticonvulsivante no necesariamente ésta relacionado con los canales de sodio sensibles a voltaje, pudiendo tener mayor importancia sus efectos sobre sistemas GABAérgicos (Fromm, 1992 a,b; Löscher, 1999), o bien que el tipo de bloqueo que presentan estas drogas sobre los canales de sodio sensibles a voltaje no les permite actuar como neuroprotectores en este modelo (Zona y Avoli, 1990; Kuo et al., 1997; Ragsdale y Avoli, 1998).

Otra droga que prometía ser un buen neuroprotector es el riluzol. El riluzol ha mostrado tener *muy buenos efectos* neurprotectores y algunos anticonvulsivantes en distintos paradigmas experimentales, y se ha planteado que su efecto neuroprotector estriba en que es un "inhibidor de la liberación de glutamato" por funcionar como un bloqueador de los canales de sodio (Romettino et al., 1991; Stutzmann et al., 1991; Guyot et al., 1997;O´Neill et al., 1997; Obrenovitch, 1997; Siniscalchi et al., 1999). Sin embargo, el riluzol tiene muy diversos efectos, entre el ellos bloqueo de canales de calcio (Huang et al., 1997, O´Neill et al., 1997, Stefani et al., 1997) y de potasio (Zona te al., 1998), el aumento (Azbill et al., 2000) o la inhibición (Samuel et al., 1992; Mantz et al., 1994) de la recaptura de glutamato y de otros neurotransmisores, e incluso la inhibición de la proteína cinasa C (Noh et al., 2000). Por otra parte el riluzol no solo inhibe la liberación de glutamato, sino como se muestra en este trabajo y en otros (Martin et al., 1993; Keita et al., 1997, Obrenovitch, 1998; Jehle et al., 2000), este compuesto bloquea la liberación de otros neurotransmisores, incluyendo el GABA, mostrando así que el riluzol es un compuesto poco específico y que puede actuar a muy distintos niveles.

Cuando se probó inicialmente la dosis más comúnmente utilizada de riluzol (8 mg/kg i.p.), se encontró que tal como se había reportado previamente, el riluzol inhibe la liberación de glutamato provocada por la 4-AP (Trabajo 2, Fig. 13), pero se encontró también que a esta concentración el riluzol resultó ser muy tóxico, pues en primer lugar produjo una potenciación importante en las crisis epilépticas que induce la 4-AP (Trabajo 2, Fig. 11) y en segundo lugar produjo una alta mortalidad en los animales (75%), los cuales murieron en el transcurso de las 24 horas siguientes al experimento. En el caso del 25% de animales que sobrevivieron los cinco días que transcurrieron antes del análisis histológico, se observó una ligera neuroprotección (Trabajo 2, Figs. 12 y 13). Después probamos una dosis menor de riluzol (4 mg/kg) que también redujo la liberación de glutamato producida por la 4-AP, pero no revirtió las crisis epilépticas, aunque si cambió el patrón de las crisis, y produjo un ligera neuroprotección (Trabajo 2, Fig. 13). Estos resultados apoyan la conclusión señalada arriba de que es necesario, además de reducir la liberación de glutamato, prevenir contra las crisis epilépticas para lograr una completa neuroprotección en el modelo de la 4-AP. Cabe mencionar que estos efectos del riluzol deben de ser tomados en cuenta, pues es el único fármaco que ha mostrado tener algún efecto protector contra la esclerosis lateral amiotrófica (Louvel et al., 1997; Roch-Torreilles et al., 2000) y bajo condiciones de hiperexcitabilidad (como las crisis epilépticas) puede resultar muy tóxico.

Finalmente, se utilizó a los abridores de canales de potasio como estrategia de neuroprotección, por dos razones fundamentales: la primera es que ya se ha mencionado que el efecto primario de la 4-AP es bloquear canales de potasio, por lo que esto podría contrarrestares con abridores de canales para este mismo ion. La segunda, es que estas drogas ya habían mostrado efectos neuroprotectores en paradigmas que involucran la activación de receptores a glutamato (Gandolfo et al., 1989; Abelle y Miller, 1990; Mattia et al., 1994; Goodman y Mattson, 1996; Lauritzen et al., 1997). Se probó el NS1619, que es un abridor de canales de potasio dependientes de calcio (Gribkoff et al., 1996; Cai et al., 1998) y la diazoxida, que abre canales de calcio dependientes de ATP (Obrenovitch, 1997; Ye et al., 1997). En términos generales ninguna de estas drogas mostró un efecto

protector contundente, pero en el caso de la diazoxida, se inhibió la liberación de glutamato pero al igual que la ω-conotoxina, los antagonistas $GABA_A$ y el riluzol, como no redujo las crisis epilépticas, no protegió contra la neurodegeneración que produce la 4-AP (Trabajo 2, Fig. 13).

V. CONCLUSIONES

1.- La acumulación de glutamato extracelular producida por el TEA o por el alto K^+ en el hipocampo de la rata in vivo, no es suficiente para la inducción de crisis epilépticas y neurodegeneración.

2.- La 4-AP puede incrementar los niveles extracelulares de glutamato, muy probablemente por inducir su liberación desde terminales sinápticas, y en estas condiciones puede producir crisis epilépticas y neurodegeneración.

3.-Bajas concentraciones de 4-AP sólo producen crisis epilépticas de corta duración, sin producir ni acumulación de glutamato extracelular, ni neurodegeneración.

4.- El bloqueo de los receptores glutamatérgicos previene la neurodegeneración que produce la 4-AP y reduce la duración de las crisis epilépticas.

5.- La tetrodotoxina reduce la acumulación de glutamato extracelular y previene conmpletamente contra las crisis epilépticas y el daño neuronal producidos por la 4-AP.

6.- La reducción en la liberación de glutamato con ω-conotoxina, los antagonistas $GABA_A$, el riluzol y la diazoxida no es suficiente para prevenir la neurodegeneración, pues en estas condiciones persisten las crisis epilépticas.

7.-El incremento en la actividad GABAérgica puede tener efectos potenciadores sobre las crisis epilépticas y la neurodegeneración que produce la 4-AP.

8.- En la generación de las crisis epilépticas por la 4-AP pueden estar participando un incremento en la liberación de glutamato desde terminales sinápticas, un incremento en la frecuencia de disparo a nivel postsináptico y un efecto despolarizante del GABA.

9.- La neurodegeneración producida por la 4-AP puede deberse a una sobreactivación de los receptores glutamatérgicos y al estrés celular que subyace a las crisis epilépticas.

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