11281

17

UNIVERSIDAD NACIONAL AUTONOMA DE MEXICO

INSTITUTO DE FISIOLOGIA CELULAR

DOCTORADO EN CIENCIAS BIOMEDICAS

Regulación de los Transportadores Membranales de Glicina en la Glía de Müller de la Retina

T E S I S

que para obtener el grado de

Doctora en Ciencias

p r e s e n t a

Biól. Ana Eva Gadea Pérez

Directora de Tesis:
Dra. María López Colomé

Comité Tutoral:
Dra. María Sitges Berrondo
Dr. Miguel Pérez de la Mora

Ciudad Universitaria **2002**

TESIS CON
FALLA DE ORIGEN

UNAM – Dirección General de Bibliotecas
Tesis Digitales
Restricciones de uso

DERECHOS RESERVADOS ©
PROHIBIDA SU REPRODUCCIÓN TOTAL O PARCIAL

Todo el material contenido en esta tesis esta protegido por la Ley Federal del Derecho de Autor (LFDA) de los Estados Unidos Mexicanos (México).

El uso de imágenes, fragmentos de videos, y demás material que sea objeto de protección de los derechos de autor, será exclusivamente para fines educativos e informativos y deberá citar la fuente donde la obtuvo mencionando el autor o autores. Cualquier uso distinto como el lucro, reproducción, edición o modificación, será perseguido y sancionado por el respectivo titular de los Derechos de Autor.

Este trabajo se llevó a cabo en el Departamento de Neurociencias del Instituto de Fisiología Celular de la UNAM, bajo la asesoría de la Dra. Ana María López Colomé.

La investigación fue parcialmente financiada por el Consejo Nacional de Ciencia y Tecnología (Proyecto 31812-N) y por la Dirección General de Asuntos del Personal Académico, UNAM (Proyecto IN216501). Además se contó con el apoyo de una beca de estudios de Doctorado (febrero 1997 a enero 2002) y dos donativos (PAEP 1998-201332 y PAEP 1999-102322), por parte de la Dirección General de Estudios de Posgrado, UNAM.

El Comité Tutoral estuvo conformado por los Doctores Miguel Pérez de la Mora, María Sitges Berrondo y Ana María López Colomé.

Los Doctores Marcelino Cereijido Matioli, Mauricio Díaz Muñoz, Arturo Hernández Cruz, Ana María López Colomé, Julio Morán Andrade, Herminia Pasantes Morales e Ivone Rosenstein Azoulay, revisaron críticamente este documento y conformaron el Jurado de Examen de Grado.

TESIS CON
FALLA DE ORIGEN

Agradecimientos

A Ana María, por brindarme la oportunidad de participar en este proyecto. Por sus enseñanzas, su amistad e invaluable apoyo incondicional.

A los miembros del jurado Dr. Marcelino Cereijido Matioli, Dr. Mauricio Díaz Muñoz, Dr. Arturo Hernández Cruz, Dra. Ana María López Colomé, Dr. Julio Morán Andrade, Dra. Herminia Pasantes Morales y Dra. Ivone Rosenstein Azoulay, por sus comentarios y correcciones.

A los Doctores Miguel Pérez de la Mora y María Sitges Berrondo, miembros de mi Comité Tutoral, por participar en mi formación durante el Doctorado.

A Edithe por su valiosa ayuda en el laboratorio.

TESIS CON
FALLA DE ORIGEN

CONTENIDO

RESUMEN 1

ABSTRACT 4

ABREVIATURAS 6

INTRODUCCIÓN 8

CÉLULAS GLIALES 8

Tipos de Células Gliales 9

Funciones Generales 11

RETINA 15

Morfología 15

Neurotransmisión en la Retina 18

CARACTERÍSTICAS MORFOLÓGICAS Y FUNCIONALES DE LA GLÍA DE MÜLLER 19

TRANSPORTADORES DE NEUROTRANSMISORES 28

Función en el Sistema Nervioso 28

Mecanismos Moleculares del Transporte 29

Importancia Clínica 31

TRANSPORTADORES DE GLICINA 33

Localización Celular e Histológica 36

Estequiometría y Propiedades de Canal 38

Regulación de los Transportadores de Glicina 39

Transportadores de Glicina y Neuropatologías 40

OBJETIVOS 42

MATERIALES Y MÉTODOS 44

CULTIVO PRIMARIO DE LA GLÍA DE MÜLLER DE RETINA DE POLLO 44

CULTIVO PRIMARIO DE NEURONAS DE LA RETINA DE POLLO 45

ENSAYOS DE TRANSPORTE DE GLICINA 45

TESIS CON
FALLA DE ORIGEN

MEDICIÓN DEL CALCIO INTRACELULAR 46

RESULTADOS **49**

CARACTERIZACIÓN BIOQUÍMICA Y FARMACOLÓGICA DEL TRANSPORTE DE GLICINA 49

Curso Temporal y Cinética del Transporte de Glicina..49

Dependencia Iónica y Especificidad del Transporte de Glicina...49

REGULACIÓN DEL TRANSPORTE DE GLICINA POR MENSAJEROS INTRACELULARES 56

Regulación del Transporte de Glicina por Calmodulina..62

Papel del Calcio en la Regulación del Transporte de Glicina 64

Regulación del Transporte de Glicina por el Citoesqueleto..69

Regulación del Transporte de Glicina por la CaMKII 72

DISCUSIÓN **77**

CARACTERIZACIÓN DEL TRANSPORTE DE GLICINA...77

REGULACIÓN DEL TRANSPORTE DE GLICINA EN LA GLÍA DE MÜLLER 79

Regulación del Transporte de Glicina por Calmodulina...80

Papel del Calcio en la Regulación del Transporte 81

Activación de la Calpaína por la entrada de Calcio a través de Ionóforos 83

La CaMKII Regula el Transporte de Glicina de Alta Afinidad en la Glía de Müller....85

Regulación del Transporte de Glicina por el Citoesqueleto: Actina y Fodrina 88

CONCLUSIONES **90**

BIBLIOGRAFÍA...**92**

APÉNDICE I **112**

ARTÍCULOS PUBLICADOS **113**

TESIS CON
FALLA DE ORIGEN

RESUMEN

Las células gliales constituyen más de la mitad del volumen total del cerebro y superan en número a las neuronas, a pesar de lo cual han atraído poco la atención de los neurofisiólogos desde su descripción por Virchow en 1846. Sin embargo, en los últimos años se ha progresado considerablemente en la comprensión de la fisiología glial y las interacciones glía-neurona, lo cual ha llevado a un replanteamiento de los mecanismos que subyacen el funcionamiento del sistema nervioso. Un excelente modelo para el estudio de la fisiología glial es la glía de Müller de la retina. Uno de los aspectos más interesantes sobre la fisiología glial es su capacidad de modular las respuestas neuronales al controlar los niveles extracelulares de neurotransmisores a través de sistemas de transporte de alta afinidad. Dado que la captura de glicina en la glía de Müller podría participar en la modulación de la transmisión excitadora en la vía vertical de la retina a través de los receptores de NMDA (N-metil-D-aspartato), así como en la inhibición lateral a través de los receptores de glicina inhibidores, el objetivo de este trabajo fue el de demostrar la presencia de transportadores de glicina de la glía de Müller y estudiar las vías de mensajeros intracelulares involucradas en la regulación de los transportadores. Nuestros resultados demuestran que la glía de la retina en cultivo expresa tanto transportadores de glicina de alta afinidad del tipo GLYT1 (i.e., alta especificidad, dependencia de Na^+ y Cl^- y sensibilidad a la sarcosina) como transportadores de baja afinidad con características del sistema de transporte A, ya que se inhibe por MeAIB (ácido metilaminoisobutírico) y AIB (ácido aminoisobutírico) y depende únicamente de la presencia de Na^+ en el medio externo.

Una de las aportaciones más importantes de nuestro trabajo es que el sistema de transporte de glicina en la glía de Müller de la retina está regulado por enzimas dependientes de Ca^{2+}/CaM, entre ellas la CaMKII, a diferencia de otros sistemas de transporte para aminoácidos neurotransmisores como el glutamato y el GABA, regulados por cAMP o PKC. La CaM requiere de Ca^{2+} para activarse, por lo

que estudiamos el papel de este catión en la regulación del transporte. El ATP, el carbacol y la cafeína, agentes que inducen la elevación moderada de la $[Ca^{2+}]i$ a través de su liberación de pozas intracelulares, y que pueden disparar la propagación de ondas de Ca^{2+} en la glía de Müller. Estos compuestos incrementan el transporte de glicina a través de la activación de la CaM y la CaMKII, ya que la inhibición de la CaMKII con KN62 inactiva por completo a los transportadores de alta afinidad. Por otro lado, la entrada masiva de Ca^{2+} inducida por ionóforos activa un proceso mediado por proteasas, que inhibe el transporte. Nuestros resultados demuestran que la calpaína, cuyos principales sustratos son elementos del citoesqueleto, es una de las enzimas involucradas. Aunque el incremento en la $[Ca^{2+}]i$ inducido por los ionóforos podría reflejar condiciones patológicas, proporcionó información valiosa sobre la relación entre los transportadores de glicina y el citoesqueleto. Demostramos que la despolimerización de la actina por citocalasinas, así como la proteólisis de la fodrina por la calpaína o la toxina Pet, inhiben el transporte de glicina. Proponemos entonces que la fodrina es una de las proteínas responsables de estabilizar los transportadores en la membrana y establecer el vínculo con el citoesqueleto de actina. Estos resultados demuestran por primera vez, que la accesibilidad de los transportadores en la membrana podría controlarse a través del citoesqueleto. La regulación de los transportadores de glicina por la CaMKII parece involucrar un mecanismo indirecto, esto es, la activación de un sistema de señalamiento que module subsecuentemente a los transportadores de glicina o bien, la fosforilación de proteínas del citoesqueleto implicadas en el tráfico y/o estabilización de los transportadores en la membrana, por ejemplo la fodrina.

La localización anatómica de las células de Müller envolviendo a las sinapsis, así como la presencia de un sistema específico y de alta afinidad de transporte de glicina, apoyan la idea de que la glía juega un papel importante en la modulación de la neurotransmisión. La regulación de este sistema puede tener importantes implicaciones fisiológicas, pues la concentración de glicina en el espacio sináptico influencia tanto la actividad de los receptores de NMDA,

involucrados en la transmisión excitadora por glutamato en las vías verticales de la retina, como los receptores inhibidores de glicina que participan en la inhibición lateral en la capa plexiforme interna.

ABSTRACT

In the retina, Müller cells are the most abundant glial cell type, its processes ensheathing excitatory synapses at the plexiform layers. In addition to their structural and nutritional functions, the precise anatomical localization of Müller cells has suggested a role in the modulation of neurotransmission. Glycine (Gly) is considered an obligatory coagonist at NMDA receptors. Müller glia from the retina harbor functional NMDA receptors, as well as high affinity Glu and GABA transporters. We here studied the characteristics and regulation of Gly transport in primary cultures of Müller glia, since this process could contribute to the modulation of NMDA receptor activity at glutamatergic synapses in the retina. We have characterized two Gly transport systems showing high (K_m = 27 μM) and low affinity (K_m = 1.7 mM); the high affinity transporter was identified as GLYT1, inhibited by sarcosine. We demonstrate that neither glutamate stimulation nor the activation or inhibition of protein kinases A or C modify transport. In order to assess a function for Ca^{2+} and calmodulin (CaM)-dependent processes in the regulation of Gly transport, we explored the participation of Ca^{2+} concentration, CaM and Ca^{2+}/CaM-dependent enzymes on Gly transporter activity. ATP and carbachol, known to induce Ca^{2+} waves in Müller cells, as well as caffeine-induced Ca^{2+} release from intracellular stores stimulated transport, whereas Ca^{2+} chelation by BAPTA-AM markedly reduced transport. CaM inhibitors W-7, ophiobolin A, R-24571 and trifluoperazine, induced a specific dose-dependent inhibition of transport. The inhibition of CaMKII by the autocamtide-2 related inhibitory peptide or by KN62 caused a decrease in transport which, in the case of KN62, was due to the abolition of the high affinity component, ascribed to GLYT1. Our results further suggest that Gly transport is under cytoskeletal control, since activation of calpain by major increases in $[Ca^{2+}]i$ induced by ionophores, as well as actin destabilization clearly inhibit uptake. We here demonstrate for the first time the participation of CaM, CaMKII and the actin cytoskeleton in the regulation of Gly transport in glia. Ca^{2+} waves are induced in Müller cells by distinct neuroactive compounds released

by neurons and glia, hence the regulation of [Gly] by this system may be of physiological relevance in the control of retinal excitability.

ABREVIATURAS

AC	Adenilato ciclasa
AIB	Ácido 2-aminoisobutírico
AIP	Péptido inhibidor de la CaMKII
AMPA	Ácido α-amino-3-hidroxi-5-metilisoxazol-4-propiónico
ATP	Adenosín trifosfato
BAPTA-AM	Acetoximetil éster del ácido 1,2-bis(2-aminofenoxi)etano-N,N,N',N'-tetracético
8-Br-cAMP	8-bromo adenosín monofosfato cíclico
8-Br-cGMP	8-bromo guanosín monofosfato cíclico
CaM	Calmodulina
CaMKII	Cinasa dependiente de Ca^{2+}/Calmodulina II
cAMP	Adenosín monofosfato cíclico
CaN	Calcineurona o proteína fosfatasa 2B
cGMP	Guanosín monofosfato cíclico
DIV	días *in vitro*
DMSO	Dimetil sulfóxido
DOG	1,2-Dioctanoil-rac-glicerol
EGTA	Ácido etilenglicol bis(β-aminoetil éter)-N-N-N'-N'-tetra acético
GABA	Ácido γ-aminobutírico
GFAP	Proteína fibrilar acídica de la glía
GLAST	Transportador de glutamato/aspartato
Glu	Glutamato
Gly	Glicina
GLYT1	Transportador de glicina 1
H-7	Dihidrocloruro de 1-(5-Isoquinolinasulfonil)-2-metilpiperazina
HA-1004	Hidrocloruro de N-(2-Guanidinoetil)-5-isoquinolinasulfonamida
IP_3	Inositol 1,4,5-trifosfato
KA	Kainato
KN62	1-(N,O-bis-[5-isoquinolina-sulfonil]-N-metil-L-tirosil)-4-fenil piperazina
KRB	Krebs-Ringer Bicarbonato
L-AP4	Ácido L(+)-2-amino-4-fosfonobutírico
mRNA	Ácido ribonucleico (RNA) mensajero
MDL-12330A	Hidrocloruro de N-(cis-2-fenilciclopentil)azaciclotridecano-2-imina
MeAIB	Ácido metilamino isobutírico
ML-9	1-(5-Chloronaftaleno-1-sulfonil)-1H-hexahidro-1,4-diazepina
MLCK	Cinasa de las cadenas ligeras de miosina
NMDA	N-metil-D-aspartato
NOS	Sintasa del óxido nítrico
PKC	Proteína cinasa C

PLC	Fosfolipasa C
PMA	Forbol 12-myristato 13-acetato
PMSF	Fluoruro de fenilmetilsulfonilo
R24571	Calmidazolium
RE	Retículo endoplásmico
SNC	Sistema nervioso central
SNP	Sistema nervioso periférico
SQ-22536	9-(Tetrahidro-2-furanil)-9H-purin-6-amina
t-ACPD	ácido trans-aminociclopentano-1,3-dicarboxílico
TFP	Trifluoperazina
W7	N-(6-aminohexil)-5-cloro-1-naftalen sulfonamida

INTRODUCCIÓN

CÉLULAS GLIALES

Desde principios del siglo XX se sabía que el sistema nervioso consistía de células que eran eléctricamente excitables: las neuronas. Se pensaba que las neuronas formaban la única base para la integración de la información en el cerebro comunicándose a través de las sinapsis. Esto dio lugar a la doctrina neuronal, sustentada en los estudios histológicos de Santiago Ramón y Cajal (1909; 1911), en la que se resalta la individualidad de las neuronas y su excitabilidad. Desde entonces prevalece la convicción de que las neuronas son las unidades funcionales del cerebro, cuyo poder integrativo lleva al comportamiento y finalmente a la conciencia.

Durante la primera mitad del siglo XX, la investigación sobre los mecanismos que subyacen la excitabilidad neuronal avanzó rápidamente, culminando con el modelo explícito de la generación de potenciales de acción (Hodgkin y Huxley, 1952). Este modelo reforzó la doctrina neuronal y sostenía que la información, contenida únicamente en potenciales de acción de neuronas individuales, se transmitía e integraba con la de otras neuronas a través de las sinapsis.

No obstante, el sistema nervioso también incluye a las células gliales, que a pesar de que constituyen más de la mitad del volumen total del cerebro y superan en número a las neuronas, han atraído poco la atención de los neurofisiólogos. Los estudios morfológicos más detallados de las células gliales se hicieron varias décadas después de haber sido descritas por Rudolf Virchow en 1846, quien las llamó *neuro glia* (del griego *glia*, "pegamento"). La característica distintiva de estas células era la carencia de axón, por lo que se consideraron como elementos pasivos del sistema nervioso. Entre las funciones que se les asignaba estaban la de nutrir y la de aislar a las neuronas.

No fue sino hasta mediados del siglo XX que resurgió el interés por la biología celular y la fisiología de las células gliales. Desde entonces, los avances

han sido espectaculares. Con el desarrollo de nuevas técnicas de biología molecular y electrofisiología se revelaron propiedades funcionales de las células gliales que, como se verá, sugerían su participación activa en los procesos de transmisión del sistema nervioso. Recientemente, la idea de que la glía juega un papel importante en la actividad nerviosa ha comenzado a ganar aceptación.

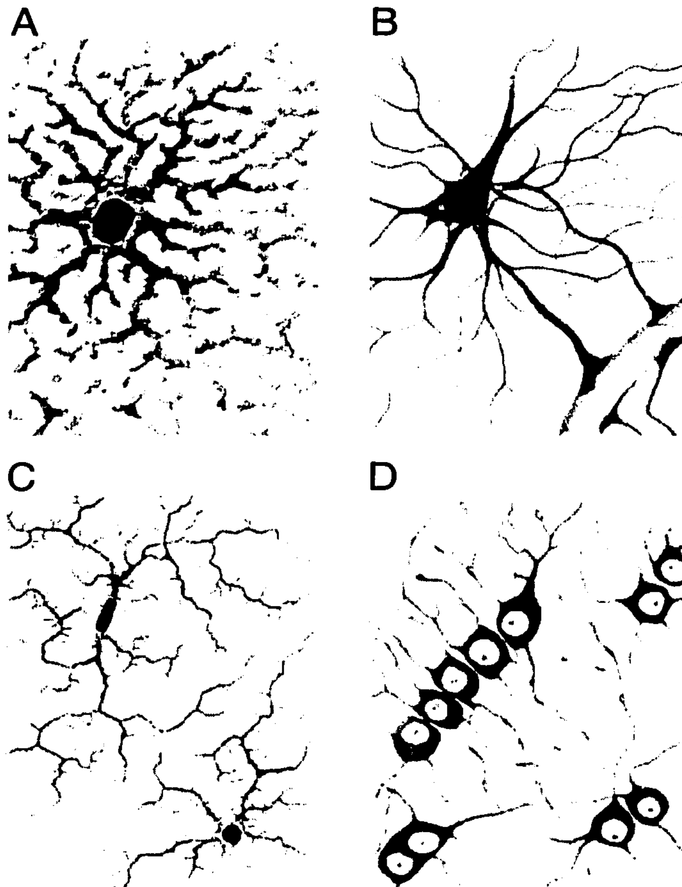
Tipos de células gliales

La mayoría de las neuronas en el sistema nervioso se encuentran rodeadas por células gliales. Se calcula que las células gliales son entre 10 y 50 veces más numerosas que las neuronas en el sistema nervioso de vertebrados (Nicholls *et al.*, 1992).

La neuroglía del sistema nervioso de vertebrados se ha dividió en dos grandes clases: la microglía y la macroglía (Fig. 1). La microglía está formada por células fagocíticas que se movilizan después de una lesión o infección y son de origen mesodérmico. La macroglía son células de origen ectodérmico y se divide en tres clases funcionales: la glía ependimal, los astrocitos y la glía mielinizante. Su número y grado de diferenciación se incrementa durante el desarrollo y conforme se asciende en la escala evolutiva.

La glía ependimal ó glía radial, se caracteriza por presentar una forma alargada durante las primeras etapas del desarrollo del cerebro formando filamentos alargados sobre los cuales las neuronas en desarrollo migran hacia su destino final (Parnavelas y Nadarajah, 2001). La glía radial incluye a las células ependimales, a las células gliales de Bergmann en el cerebelo y a las células de Müller en la retina (Bartlett et al., 1981).

La glía mielinizante incluye a los oligodendrocitos y a las células de Schwann, que son células pequeñas con pocos procesos y que se encargan de formar la vaina de mielina alrededor de los axones de las neuronas. Esta vaina de mielina aísla eléctricamente a los axones, de manera que se incrementa la velocidad de conducción de las señales eléctricas. Los oligodendrocitos se encuentran en el sistema nervioso central y pueden envolver a varios axones,



TESIS CON
FALLA DE ORIGEN

Figura 1. Tipos de células gliales en el SNC. A) astrocitos protoplásmicos de la materia gris. B) astrocitos fibrosos de la materia blanca. C) microglía. D) oligodendrocitos. Tomado de Del Río Hortega (1920).

mientras que las células de Schwann son exclusivas del sistema nervioso periférico y envuelven a un solo axón (Witt y Brady, 2000).

Los astrocitos son el más abundante de los tres grupos que comprende la macroglía; sus cuerpos celulares son irregulares y presentan largos procesos. Una de las primeras funciones que se les atribuyó es la de intervenir en la formación de la barrera hematoencefálica, pues al hacer contacto con los vasos sanguíneos provocan la formación de uniones estrechas entre las células endoteliales que filtran el paso de sustancias de la sangre al cerebro (Goldstein, 1988). Algunos astrocitos

presentan pies terminales que hacen contacto con neuronas y vasos sanguíneos, lo que ha hecho pensar que también tienen la función de nutrir a las neuronas. Por otro lado, parece ser que, al igual que la microglía, eliminan los restos neuronales y ayudan a cicatrizar el tejido nervioso dañado después de una lesión (Fawcett y Ascher, 1999).

Funciones Generales

Hallazgos recientes indican que las células gliales, aunque no tienen la habilidad de generar potenciales de acción, realizan muchas más funciones de las que se pensaba, y que modulan la actividad neuronal. Por medio de registros intracelulares se observó que los potenciales de reposo de las células gliales son mucho más negativos que los de las neuronas a las que rodean (Barres, 1991). En los vertebrados, los potenciales de membrana más altos registrados en neuronas oscilan entre -70 y -75 mV, mientras que los valores para las células gliales se acercan a los -90 mV. Este potencial de reposo de las células gliales está determinado por el potencial de equilibrio del K^+, ya que presentan una alta permeabilidad a estos iones, al mismo tiempo que presentan una baja permeabilidad a los iones Na^+ y $Ca2^+$ (Sontheimer, 1994). Como consecuencia de la actividad neuronal prolongada, la concentración de K^+ en el espacio periaxonal aumenta de dos a cinco veces; un incremento de esta magnitud influye sobre el potencial de membrana y las propiedades metabólicas de las células gliales, así como sobre las respuestas neuronales alterando, por ejemplo, la cantidad de neurotransmisor liberado por las neuronas presinápticas, modulando las interacciones neuronales recíprocas o afectando el umbral de activación de las células postsinápticas (Sykova, 1983). Las concentraciones de K^+ iniciales (pre-estímulo) deben de ser restablecidas para que un segundo impulso nervioso pueda llevarse a cabo. Las células gliales regulan la concentración de K^+ del medio extracelular gracias a su alta conductancia para este ión, capturándolo en las

regiones con una alta concentración y liberándolo en regiones distales donde su concentración es menor (Philippi et al., 1996).

Se ha demostrado que las células gliales tienen sistemas de recaptura de neurotransmisores y, ya que por lo general las sinapsis se encuentran encapsuladas por los procesos de las células gliales, se ha propuesto que juegan un papel importante en la terminación de la transmisión (Barres, 1991a). La recaptura de neurotransmisores también proporciona aislamiento a las sinapsis, ya que evita que el neurotransmisor difunda a sinapsis cercanas. Entre los sistemas de transporte que se han localizado en las células gliales está el de Glu, que se cotransporta con dos iones Na^+, al mismo tiempo que se transportan en sentido contrario un ión K^+ y un grupo OH^- o bien un HCO_3^-. Por lo tanto, la captura de Glu provoca la alcalinización del medio extracelular, de manera que las células gliales pueden regular los cambios extracelulares de pH inducidos por la actividad neural (Bouvier *et al.*, 1992), además de mantener bajos los niveles de Glu en el medio extracelular. De acuerdo con la hipótesis de la excitotoxicidad, concentraciones elevadas de Glu inducen la degeneración de poblaciones neuronales en el sistema nervioso central, como ocurre por ejemplo en la isquemia y en la hipoxia (Siliprandi *et al.*, 1992).

La glía presenta una enzima característica, la glutamino sintetasa (GS), que cataliza la aminación del glutamato en glutamina, precursor de varias vías biosintéticas, como de la síntesis de los neurotransmisores Glu y GABA (Hertz et al., 1999). Otra enzima característica de las células gliales es la anhidrasa carbónica, que cataliza la hidratación del dióxido de carbono y por lo tanto está involucrada en las funciones respiratorias vitales. Esta enzima también participa en la regulación del movimiento iónico y el balance de fluidos, por lo que juega un papel importante en la homeostasis y, como influencia los equilibrios de Na^+ y K^+, afecta indirectamente la actividad neuronal (Deitmer, 2001)

Uno de los descubrimientos más sorprendentes en el estudio de las células gliales fue el de la presencia de receptores funcionales para distintos neurotransmisores, neuromoduladores y hormonas. Se ha demostrado tanto *in vitro*

como *in situ* que las células gliales responden a numerosos neurotransmisores con cambios en el potencial de membrana. Entre los receptores reportados están los de Glu, GABA, Gly, acetilcolina y monoaminas, cuyas características son similares a aquellos de las neuronas (Verkhratsky y Steinhauser, 2000). La glía en cultivo también expresa receptores para neurotransmisores que activan sistemas de señales intracelulares como son: el incremento en las concentraciones intracelulares de Ca^{2+}, diacilglicerol, fosfatos de inositol, AMPc y GMPc los cuales, a su vez, pueden modular la actividad de canales iónicos y enzimas (Barres, 1991b). La presencia de receptores en la glía sugirió la comunicación entre neuronas y glía a través de neurotransmisores, y más recientemente se ha demostrado la activación de las células gliales perisinápticas durante la neurotransmisión. Tanto en el SNC como en el SNP, se ha demostrado que la estimulación de la glía por neurotransmisores como el Glu, GABA, noradrenalina, acetilcolina y dopamina, induce una elevación de la concentración intracelular de Ca^{2+} ($[Ca^{2+}]i$; Grosche et al., 1999; Kang et al., 1998; Dzubay et al., 1999; Kulik et al., 1999; Rochon et al., 2001). Dicha elevación de la $[Ca^{2+}]i$ genera las llamadas ondas de Ca^{2+} (incremento transitorio de la $[Ca^{2+}]i$) que se propagan intra e intercelularmente, a través de la liberación de Ca^{2+} de las pozas intracelulares (Finkbeiner, 1993). Este tipo de respuesta podría representar una forma de excitabilidad de las células gliales que les permite integrar señales extracelulares, comunicarse entre sí a larga distancia, e intercambiar información con las neuronas adyacentes (Verkhratsky y Kettenmann, 1996).

Las células gliales sintetizan y, en ocasiones, liberan neurotransmisores. Tal es el caso de las células de Schwann del axón gigante de calamar, que normalmente sintetizan y liberan acetilcolina (Heumann *et al.*, 1981). Las células gliales no contienen vesículas sinápticas, por lo que se ha sugerido que la liberación de neurotransmisores se lleva a cabo a través de la actividad inversa de los transportadores de neurotransmisores (Amara y Kuhar, 1993). Sin embargo, se ha demostrado que una de las consecuencias de la elevación en la $[Ca^{2+}]i$ en la glía es la liberación de neurotransmisores. En diversos sistemas se ha reportado que en la glía, el incremento en la $[Ca^{2+}]i$ induce la liberación de glutamato dependiente

de Ca^{2+} e independiente de los transportadores de Glu (Araque et al., 2000; Parpura et al., 1994; Bezzi et al., 1998; Pasti et al., 1997; 2001; Parpura y Haydon, 2000). Más aún, se ha propuesto que esta liberación dependiente de Ca^{2+} se da a través de un proceso semejante a la exocitosis neuronal: es sensible a bloqueadores como la toxina tetánica, la toxina botulínica y la bafilomicina A1 (Araque et al., 2000; Bezzi et al., 1998; Pasti et al., 2001) y la cinética de las corrientes provocadas en células transfectadas para expresar receptores de Glu, se correlaciona con eventos de liberación cuántica (Pasti et al., 2001). Aunque no se han observado vesículas "sinápticas" en las células gliales, se ha identificado en las mismas, a la sinaptobrevina y a la sintaxina 1A, proteínas que forman parte de la maquinaria exocitótica (Maienschein et al., 1999; Calegari et al., 1999).

Ya que las células gliales presentan un alto grado de plasticidad en respuesta a las señales neuronales, cambios a largo plazo en las propiedades de la membrana de las células gliales podrían mediar cambios a largo plazo en la función sináptica.

RETINA

En la retina de los vertebrados se ha identificado dos tipos de células gliales: las células de Müller y los astrocitos, estas últimas presentes sólo en retinas vascularizadas (Rasmussen, 1974). En la retina de las aves, las células de Müller son el único tipo de gliocito presente y combinan funciones que en otras partes del sistema nervioso están compartimentalizadas en diversos tipos de células gliales, por lo que resultan un excelente modelo experimental para el estudio de las células gliales. Antes de abordar el tema de la glía de Müller, revisaremos los aspectos generales de la retina.

Morfología

La retina, que comparte el origen embrionario con el SNC, es una capa de tejido nervioso de aproximadamente 300 μM de espesor localizada en la parte posterior del ojo, que lleva a cabo la recepción de los estímulos visuales y los primeros pasos del procesamiento de información de la vía visual (Shepherd, 1974). La organización laminar y el número limitado de tipos celulares que presenta facilitan la identificación y localización de distintas funciones bioquímicas y fisiológicas. En este sentido, la retina de los vertebrados ha sido considerada un buen modelo para estudiar al sistema nervioso central (SNC) (Farber y Adler, 1986). La retina de las aves es un modelo que ofrece grandes ventajas: contiene un sólo tipo de células gliales, las células de Müller; a diferencia de la retina de los mamíferos, la de las aves es avascular y carece de células endoteliales y elementos sanguíneos; las retinas de embriones de pollo de edad temprana pueden continuar su desarrollo *in vitro* bajo condiciones de cultivo relativamente simples. Por último, el tejido de la retina de las aves, en etapas definidas del desarrollo, puede ser fácilmente disociado en una suspensión de células para luego desarrollar cultivos adherentes en (Moscona, 1983).

La retina de los vertebrados está constituida por tres tipos celulares: neuronas, células del epitelio pigmentado y células gliales (Wheater *et al.*, 1987).

Existen 5 tipos de neuronas: los fotorreceptores (conos y bastones), las células bipolares, las células horizontales, las células amacrinas y las células ganglionares, y dos tipos de células gliales: las células de Müller y los astrocitos (Masland, 2001).

Histológicamente, la retina de los vertebrados es un tejido estratificado (Fig. 2). La capa más externa es el epitelio pigmentado, cuyas células se organizan en una monocapa que descansa sobre la membrana de Bruch y que separa a la retina neural de los capilares sanguíneos coroideos. La segunda capa incluye los segmentos externo e interno de los conos y bastones. A ésta le sigue la capa nuclear externa, formada por los cuerpos celulares de los fotorreceptores. Entre estas dos capas, se localiza la membrana limitante externa formada por la unión de los pies terminales de las células de Müller. Después se encuentra la capa plexiforme externa, en la que establecen sinapsis los fotorreceptores con las células bipolares y las células horizontales, seguida de la capa nuclear interna donde se encuentran los cuerpos neuronales de las células horizontales, bipolares y amacrinas. La siguiente capa es la plexiforme interna donde se establecen sinapsis entre las células bipolares, amacrinas y ganglionares, y por último, la capa de células ganglionares cuyas fibras eferentes convergen en un punto para formar el nervio óptico. Funcionalmente la retina neural de los vertebrados se divide en retina externa (fotorreceptores y capa plexiforme externa) y retina interna (capa nuclear externa y siguientes).

El segmento externo de los conos y bastones (por su forma) contiene una serie de discos membranosos formados en un 90% por las moléculas de pigmento encargadas de absorber la luz. En el caso de los bastones este pigmento es la rodopsina, que absorbe la luz a una longitud de onda de 500 nm y están especializados para la recepción de luz tenue. Los conos contienen los pigmentos específicos para la visión en color y están especializados para la recepción de luz brillante (Ebrey y Koulatos, 2001).

Las células bipolares, en general, establecen conexiones directas entre uno o más fotorreceptores y una o más células ganglionares, transmitiendo en sentido vertical la información visual. Las células horizontales presentan varios procesos

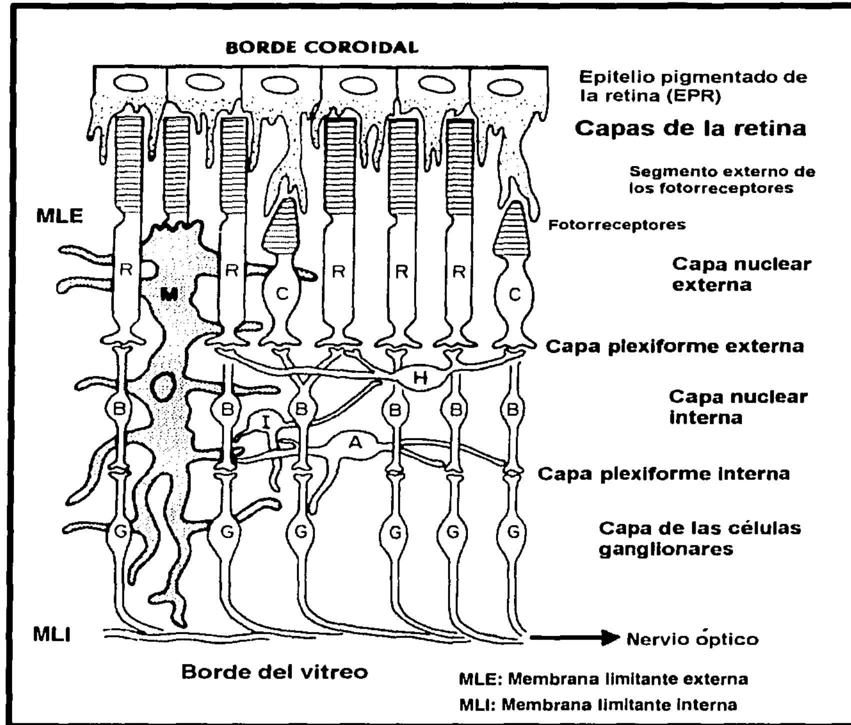


Fig 2. Retina de los vertebrados

Diagrama:

diferentes capas y relaciones sinápticas.

Tipos celulares.

R: bastones
C: conos
H: horizontales
B: bipolares
A: amacrinas
I: interplexiformes
G: ganglionares
M: Müller

cortos, y uno más delgado y largo que los demás considerado como el axón. Estos procesos conectan lateralmente, en la capa plexiforme externa, a conos y bastones contiguos o lejanos. Las células amacrinas poseen uno o dos árboles dendríticos cuyas ramificaciones se conectan con células bipolares y células ganglionares en la capa plexiforme interna y, en ocasiones, con fotorreceptores en la capa plexiforme externa (Wheater *et al*, 1987).

Las células de la glía de Müller atraviesan todo el espesor de la retina, desde la membrana limitante interna hasta los fotorreceptores (Farber y Adler, 1986). Su núcleo se encuentra en la capa nuclear interna y a través de largos procesos citoplásmicos rodean a las neuronas, llenando así los espacios intercelulares (Wheater *et al*, 1987).

TESIS CON
FALLA DE ORIGEN

Neurotransmisión en la retina

Estudios farmacológicos y registros electrofisiológicos han proporcionado pruebas de que los aminoácidos excitadores son importantes neurotransmisores en la retina de los vertebrados. Estos estudios sugieren que los fotorreceptores, las células bipolares y algunas células amacrinas utilizan glutamato y/o aspartato como neurotransmisor, por lo que la vía vertical de la retina que va de los fotorreceptores a las células bipolares y de éstas a las células ganglionares es excitadora (Copenhagen, 1991).

La elevada concentración de la aspartato aminotransferasa, enzima que sintetiza aspartato y glutamato, presente en fotorreceptores, células bipolares y algunas amacrinas, así como la presencia de sistemas de transporte de alta afinidad para estos aminoácidos, constituyen evidencia adicional que apoya la idea de que los aminoácidos excitadores son los transmisores en la vía vertical de la retina (Iuvone, 1986).

Los dos principales neurotransmisores inhibidores son el GABA (ácido γ-aminobutírico) y la glicina (Gly). Las células horizontales y las amacrinas llevan a cabo el procesamiento lateral de la información en las capas plexiforme externa e interna, respectivamente. En todos los vertebrados, el GABA y la Gly se localizan en algunas células amacrinas, y en vertebrados inferiores, el GABA se localiza también en algunas células horizontales (Daw *et al.*, 1989).

La Gly está presente en la retina de vertebrados a concentraciones muy similares a las encontradas en el SNC (Pasantes-Morales *et al.*, 1972). Las concentraciones más altas de Gly dentro de la retina se han encontrado en la capa plexiforme interna, en la capa de células ganglionares y en las células amacrinas (Iuvone, 1986). En la retina se ha demostrado la liberación de Gly, tanto acumulada como endógena, estimulada por despolarización y dependiente de Ca^{2+} (López-Colomé *et al.*, 1978).

Entre los compuestos identificados como neurotransmisores en la retina de los vertebrados se encuentran también la dopamina y la acetilcolina, localizadas principalmente en subpoblaciones de células amacrinas (Masland, 1988).

CARACTERÍSTICAS MORFOLÓGICAS Y FUNCIONALES DE LA GLÍA DE MÜLLER

Las células de Müller de la retina de los vertebrados son células orientadas radialmente que se extienden a todo lo largo de la retina, desde la capa de fibras del nervio óptico hasta el nivel de los segmentos internos de los fotorreceptores (Fig. 3). El pie terminal de la célula de Müller es una expansión cónica que termina en la membrana limitante interna de la retina, formada por fibras de colágena y glicoproteínas a las cuales se adhieren las células de Müller. El tronco principal pasa verticalmente a través de la capa de fibras ópticas, de la capa de células ganglionares y de la capa plexiforme interna hasta llegar a la capa nuclear interna, donde se localiza el núcleo de la célula de Müller, por lo que a este nivel se observa un ensanchamiento del tronco principal. Los procesos laterales que derivan del tronco principal forman una matriz que rodea al pericarion de las células ganglionares. Distal al núcleo, al nivel de la membrana limitante externa, la célula de Müller forma una serie de microvellosidades que penetran entre los cuerpos de los fotorreceptores al espacio subretinal. A estas estructuras se les ha llamado canastas de Schultze y contribuyen a la formación de la membrana limitante externa. Las células de Müller emiten numerosas ramificaciones horizontales en ambas capas plexiformes, que por lo general están íntimamente asociadas a dendritas neuronales y sinapsis. En la capa nuclear interna, las células de Müller envuelven parcialmente a las neuronas adyacentes, mientras que en la capa nuclear externa y en la capa de células ganglionares las envuelven totalmente (Robinson y Dreher, 1990).

A pesar de su relación anatómica con las neuronas, las células de Müller se consideraron durante mucho tiempo como células pasivas relacionadas con funciones de mantenimiento en la retina, sin una participación importante en el proceso visual. En las última dos décadas, esta visión ha cambiado radicalmente, al demostrarse que las células de Müller no sólo tienen una relación metabólica con las neuronas sino que podrían intervenir directamente en el procesamiento de la información en la retina.

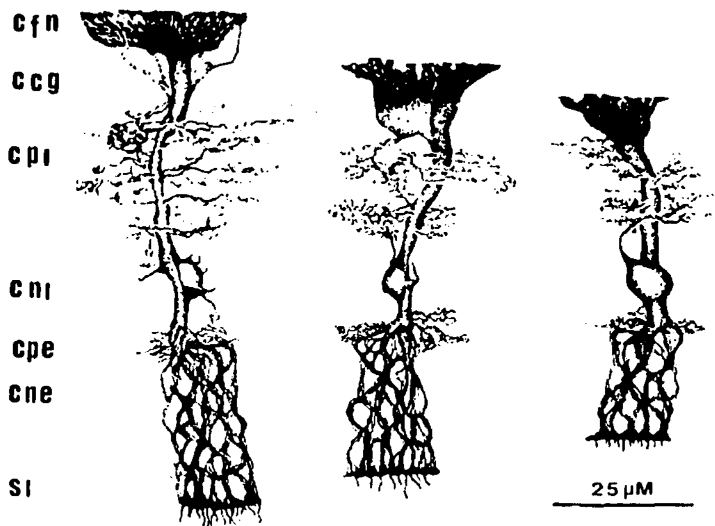


Figura 3. Dibujos en la cámara lúcida de células de Müller y su ubicación con respecto a las diferentes capas de la retina. CFN, capa de fibras nerviosas; CCG, capa de células ganglionares; CPE, capa plexiforme externa; CNI, capa nuclear interna; CNE capa nuclear externa; SI, segmento interno de los fotorrecepotres (Robinson y Dreher, 1990).

Con base en estudios citoquímicos y estructurales Magalhaes y Coimbra (1972) identificaron tres regiones en las células de Müller: (1) la porción interna de la célula, que se extiende desde la membrana limitante interna hasta el límite entre las capas plexiforme interna y nuclear interna, rica en microfilamentos, retículo endoplásmico liso y gránulos de glucógeno; (2) la porción media de la célula, situada en la capa nuclear interna, que contiene al núcleo, así como retículo endoplásmico rugoso abundante y el aparato de Golgi; y (3) la porción externa de la célula que se extiende hasta la capa de fotorreceptores y se caracteriza por presentar numerosos microtúbulos y mitocondrias. Por estas características se concluyó que la porción interna de la célula es responsable de la síntesis,

TESIS CON
FALLA DE ORIGEN

almacenamiento y liberación de glucógeno, la porción media, de la síntesis y transporte de proteínas estructurales y de secreción, y la porción externa está especializada en la absorción y en el transporte intracelular activo.

Las células de Müller juegan un papel importante en el desarrollo de la retina. Aparecen tempranamente, y su relación anatómica con las neuronas en desarrollo, sugirió su participación en la estratificación de la retina (Moscona, 1983), misma que se demostró posteriormente mediante estudios en cultivos rotatorios. En este sistema, las células disociadas de la retina embrionaria, pueden reagregarse y establecer una organización tridimensional histotípica. En estas estructuras llamadas retinoesferoides, se mantiene la estratificación de todas las capas de la retina pero sin la orientación apropiada. Sin embargo, en presencia de células de Müller o de medio condicionado por las mismas, los retinoesferoides adquieren la orientación laminar correcta de la retina in vivo (Willbold et al., 2000).

No se aprecian diferencias morfológicas significativas entre células de Müller de retinas vascularizadas y avasculares (Rasmussen, 1974). El potencial de membrana también varía muy poco de una especie a otra y es de alrededor de -80 mV. Este potencial de membrana está dado por la concentración externa de K^+, ya que las células de Müller son altamente permeables a este ion.

Una de las funciones mejor caracterizadas de la glía de Müller es la regulación de la homeostasis del K^+ ($[K^+]e$) en la retina. La actividad neuronal ocasiona un incremento en la $[K^+]e$ en ambas capas sinápticas (Karowsky y Proenza, 1977), que debe amortiguarse con el fin de limitar fluctuaciones inapropiadas en la excitabilidad neuronal. Las células de Müller remueven el exceso de K^+ del espacio extracelular por procesos tanto pasivos (captura de K^+ y Cl-) como activos (ATPasa Na^+/K^+; Reichenbach et al., 1992) comunes a otras células gliales. El K^+ también se remueve del espacio extracelular por un mecanismo de amortiguamiento espacial a través de canales rectificadores entrantes de K^+, el canal predominante en la glía de Müller (Newman, 1984). A diferencia de otros canales sensibles al voltaje, estos canales están abiertos al potencial de reposo de la membrana. La dependencia de voltaje y de K^+ de estos canales permite que

en regiones donde se eleva la $[K^+]e$, la conductancia del canal se incremente (Newman, 1993). Existe evidencia de que la regulación de la $[K^+]e$ por la glía de Müller está bajo control neuronal, ya que los neurotransmisores y otros factores pueden modular la conductancia de los canales de K^+ en la glía de Müller (Puro y Stuenkel, 1995; Schwartz, 1993; Biedermann et al., 1995).

En los anfibios, una gran proporción de los canales rectificadores entrantes de K^+ en estas células se localiza en el pie terminal y en la superficie de la retina, por lo tanto, la corriente de amortiguamiento espacial sale de la glía de Müller preferentemente por el pie terminal. El resultado de esta forma especializada de homeostasis denominada "sifón de K^+" (Newman et al., 1987), es que el exceso de K^+ liberado por la actividad neuronal se transfiere al humor vítreo. El patrón de las corrientes de amortiguamiento espacial es más complejo en los mamíferos, en los que el exceso de K^+ se dirige tanto al humor vítreo como al fluído que rodea a los fotorreceptores (espacio subretinal; Frishman et al., 1992). En especies con retina vascularizada, la conductancia es mayor en la porción de la célula que corresponde a la capa nuclear interna, donde los capilares rodean a los procesos de las células de Müller, por lo que se propone que en esta zona se libera el K^+ hacia los vasos sanguíneos (Newman, 1987).

A través de un proceso semejante al descrito, llamado "sifón de CO_2", las células de Müller amortiguan la concentración de CO_2 (Newman; 1994). Las células de Müller poseen la enzima anhidrasa carbónica, así como un sistema de cotransporte de Na^+/HCO_3^- en la membrana del pie terminal (Newman, 1996) que, en conjunto, amortiguan los cambios en el pH extracelular generados por el CO_2 producido durante de la actividad neuronal. Esto es muy importante ya que 1) en la retina de la salamandra, cambios de pH tan pequeños como 0.05 U pH producen una reducción del 24% en la transmisión sináptica entre los fotorreceptores y las neuronas postsinápticas (Barnes et al., 1993) y 2) cambios en el pH extracelular de hasta 0.1 U pH se han detectado en la retina como consecuencia de la actividad neuronal (Oakeley y Wen, 1989).

Las células de Müller están involucradas en el reciclaje de los fotopigmentos, ya que al igual que las células del epitelio pigmentado, expresan la proteína que une al retinal (CRALBP; Bunt-Milam y Sari, 1983). La glía de Müller une al trans-retinal (vitamina A), lo convierte en 11-cis-retinol, y lo libera al espacio extracelular para su recaptura por los conos (Edwards et al., 1992).

Otra molécula importante que se acumula en la glía de Müller es el tripéptido glutatión (glutamil cistenil glicina). La concentración de glutatión reducido (GSH) excede a la forma oxidada (GSSG) por un factor de entre 7 y 9 en la retina de los mamíferos (Kern et al., 1994), y juega un papel crucial de protección contra radicales libres y especies reactivas de oxígeno dado que el GSH puede ser oxidado de manera reversible a GSSG. En la retina, el GSH se concentra en la glía de Müller (Huster et al., 1998; Pow y Crook, 1995), y su síntesis depende de la disponibilidad de glutamato y cisteína (Reichelt et al., 1997).

La glía de Müller proporciona apoyo metabólico a las neuronas retinianas. Las reservas de glucógeno de la retina están restringidas a la glía de Müller, la cual expresa la enzima fosforilasa del glucógeno (Pfeiffer et al., 1994). En cultivos de células de Müller se demostró que el alto K^+ inducía la glucogenólisis (Reichenbach et al., 1993). La actividad neuronal también estimula la glucogenólisis y se ha demostrado la transferencia directa de lactato de la glía de Müller a las neuronas (principalmente a los fotorreceptores; Poitry-Yamate et al., 1995).

La retina, como el cerebro, debe de estar protegida contra cambios en la composición y los compuestos neurotóxicos contenidos en la sangre. En el cerebro, los astrocitos envuelven a los capilares y están involucrados en el mantenimiento de las propiedades de las células endoteliales que constituyen la barrera hematoencefálica (Risau y Wolburg, 1990). En las retinas vascularizadas de los mamíferos, los astrocitos están circunscritos a la capa de fibras nerviosas donde envuelven los vasos sanguíneos más internos (Schnitzer, 1987), mientras que los vasos en las capas externas de la retina están envueltos por los procesos de la glía de Müller (Nagelhus et al., 1998). Se ha demostrado que la glía de Müller participa en el establecimiento de la barrera hematorretiniana (Tout et al., 1993) y se ha

propuesto que puede regular el flujo sanguíneo de los vasos retinianos en respuesta a cambios en la actividad neuronal.

Los neurotransmisores liberados por las neuronas constituyen un mecanismo de señalamiento a través del cual la actividad neuronal puede modular el comportamiento de las células de Müller. Estas células, por otra parte, expresan una gran variedad de receptores, incluyendo receptores para aminoácidos neurotransmisores, catecolaminas, péptidos neuroactivos, hormonas y factores de crecimiento (Newman, 1996). Generalmente, estos receptores exhiben afinidad de unión y propiedades farmacológicas similares a las descritas en neuronas. En la mayoría de los casos, la unión del ligando resulta en la despolarización de la célula, ya sea por la apertura directa de canales iónicos, como en el caso del receptor $GABA_A$, o por la activación de sistemas de segundos mensajeros. Por ejemplo, el glutamato, la dopamina y la trombina, a través de sistemas de segundos mensajeros, reducen la conductancia al K^+ despolarizando a la glía de Müller (Schwarz, 1993; Biedermann et al., 1995; Puro y Stuenkel, 1995).

Otro ejemplo importante de la activación de sistemas de segundos mensajeros en la glía de Müller como consecuencia de la actividad neuronal, es la elevación de la $[Ca^{2+}]i$ inducida tanto por la elevación del K^+ como por la activación de receptores ionotrópicos (Wakakura y Yamamoto, 1994; Keirstead y Miller, 1995). En células de Müller disociadas de la retina de salamandra, la elevación de la $[K^+]e$ resulta en la entrada de Ca^{2+}, probablemente a través de canales de Ca^{2+} sensibles al voltaje (Keirstead y Miller, 1995), mientras que en las células de conejo en cultivo, el glutamato provoca la entrada de Ca^{2+} a través de receptores no-NMDA (Wakakura y Yamamoto, 1994). La estimulación mecánica de las células de Müller de la salamandra, en ausencia de Ca^{2+} extracelular, induce la liberación de Ca^{2+} de las pozas intracelulares; el incremento en la $[Ca^{2+}]i$ consecuente viaja desde el extremo apical de la célula a manera de onda hacia el pie terminal (Keirstead y Miller, 1995). Las ondas intracelulares de Ca^{2+} pueden estimularse por K^+, glutamato y ATP, así como por cafeína y ryanodina (Keirstead y Miller, 1995; 1997; Newman y Zahs, 1997; Wakakura y Yamamoto, 1994). Aunque

un blanco lógico de la $[Ca^{2+}]i$ podría ser la sintasa del óxido nítrico (NOS) y las células de Müller de la salamandra y del pez expresan esta enzima (Liepe et al., 1994), la liberación de ON de estas células no se ha demostrado. Esta información abre la posibilidad de que las ondas de Ca^{2+} en las células de Müller, a través de la liberación de neurotransmisores, proporcionaran una segunda vía, independiente de la red neuronal, para la retransmisión de señales desde la retina externa hasta la retina interna, como se ha demostrado para el glutamato en astrocitos (Parpura et al., 1994).

Las células gliales juegan un papel importante en la remoción de los neurotransmisores del espacio extracelular después de su liberación de la terminal sináptica. Esta recaptura (ver Transportadores de Neurotransmisores) es esencial para la terminación de la transmisión sináptica así como para evitar la difusión de transmisores fuera del espacio sináptico. Las células de Müller poseen sistemas de recaptura de alta afinidad para varios neurotransmisores, por lo que podrían regular su concentración extracelular en la retina. La aposición cercana de los procesos de las células de Müller con las sinapsis en ambas capas plexiformes, apoyan esta posibilidad.

El sistema de transporte para el glutamato en la glía de Müller se ha estudiado extensamente (Brew y Attwell, 1987; Schwartz y Tachibana, 1990). El L-Glu, L-Asp y el D-Asp comparten un sistema de alta afinidad, dependiente de Na^{+}, caracterizado inicialmente en cultivos primarios de glía de Müller (Somohano y López-Colomé, 1991). Más recientemente se demostró la expresión del transportador GLAST (del inglés glutamate/aspartate transporter) en estas células (Derouiche y Rauen, 1995) y se comprobó que la remoción de Glu en la retina se lleva a acabo principalmente por las células de Müller (Rauen et al., 1998). En ratones transgénicos en los que se elimina el gene que codifica para GLAST, el daño isquémico de la retina se exacerba considerablemente (Harada et al., 1998). El transportador GLAST es electrogénico, por lo que la captura de glutamato puede variar o incluso invertirse, dependiendo del voltaje (Szatkowsky et al., 1990). El

glutamato se cotransporta con iones OH^-, por lo que la captura de glutamato depende del pH y conlleva la alcalinización extracelular (Bouvier et al., 1992).

Las células de Müller también poseen sistemas de transporte de alta afinidad para el GABA (Sarthy, 1982). Se ha demostrado la expresión del transportador GAT3 en estas células. El transporte es electrogénico y se ha propuesto una estequiometría de $2Na^+/Cl^-$ por molécula de GABA transportada. El GABA se libera principalmente de las células horizontales y ciertos tipos de amacrinas. En algunas especies de mamíferos como el conejo, las células horizontales están desprovistas de transportadores de GABA por lo que las células de Müller son las encargadas de remover el GABA del espacio extracelular (Brecha y Weigmann, 1994).

La recaptura de glutamato y GABA en la glía de Müller es un paso inicial importante en el proceso de reciclaje de neurotransmisores. Las células de Müller poseen sistemas enzimáticos para la conversión o degradación de estos neurotransmisores como la glutamina sintetasa, la GABA transaminasa (GABA-T) y la deshidrogenasa del semialdehído succínico (SSA) (Witkovsky et al., 1985). La glutamino sintetasa, enzima que transamida al glutamato en glutamina, se localiza exclusivamente en la glía de Müller en la retina. La glutamina sintetizada en la glía de Müller sirve como precursor para la síntesis de glutamato en las neuronas. La inhibición de esta enzima ocasiona la pérdida completa de la función neuronal, lo que demuestra el papel crucial que juega la glía de Müller en la neurotransmisión de la retina (Rauen et al., 1998).

La glía de Müller puede modular significativamente la actividad neuronal mediante el control de la concentración de sustancias neuroactivas en el espacio extracelular. La acumulación de K^+ en el espacio extracelular, así como variaciones en el pH inducidas por cambios en la actividad glial, podrían modificar la actividad neuronal y la transmisión sináptica. Adicionalmente, las células de Müller pueden controlar la actividad neuronal de manera más directa, por la inversión del transporte de glutamato inducido por despolarización (Szatkowski et al., 1990). La liberación de Glu por este proceso puede contribuir al daño excitotóxico en las neuronas en condiciones patológicas. Al igual que en el caso del Glu, la

despolarización de las células de Müller puede inducir la liberación de GABA (Sarthy, 1983). Es importante mencionar que la liberación de glutamato y GABA de las células de Müller se ha demostrado únicamente en células que han sido precargadas con los transmisores. Queda por demostrarse que la liberación de transmisores se lleve a cabo en condiciones in vivo.

TRANSPORTADORES DE NEUROTRANSMISORES

Función en el Sistema Nervioso

Una de las principales funciones que desempeñan los sistemas transportadores de neurotransmisores es la terminación de la transmisión sináptica. La neurotransmisión química se lleva a cabo en cuatro pasos: (1) la síntesis del neurotransmisor, (2) el almacenamiento y liberación de este neurotransmisor en la terminal sináptica, (3) la interacción del neurotransmisor con sus receptores en la membrana postsináptica y, (4) la eliminación del neurotransmisor del espacio sináptico, crítica para el proceso, ya que la persistencia del transmisor en el espacio sináptico impediría la transmisión de una nueva señal. Existen tres mecanismos para terminar la acción del transmisor liberado: la *difusión* del neurotransmisor del espacio sináptico a través del fluido extraneuronal; la *degradación enzimática* del neurotransmisor, mecanismo utilizado por el sistema colinérgico, y la *recaptura* del neurotransmisor por medio de proteínas acarreadoras específicas, presentes tanto en la membrana de la neurona presináptica como en la membrana de las células gliales que se encuentran rodeando a la sinapsis (Kanner, 1994).

Los transportadores de neurotransmisores dependientes de sodio son el principal medio por el cual se elimina al neurotransmisor del espacio sináptico (Nelson y Lill, 1994). La concentración intracelular de iones Na^+ es menor que la extracelular tanto en neuronas como en glía, por lo que el co-transporte de un neurotransmisor con iones Na^+, a favor del gradiente electroquímico del ión, proporciona la energía necesaria para transportar al neurotransmisor hacia el interior de la célula, en contra de su gradiente de concentración (Kanner, 1994).

La inactivación rápida del neurotransmisor mediante su recaptura, evita la difusión del neurotransmisor y minimiza el riesgo de que el neurotransmisor actúe sobre sinapsis adyacentes (Uhl y Johnson, 1994).

La recaptura de diversos neurotransmisores en la terminal nerviosa y su posterior compartimentalización vesicular, permite el reciclaje del mismo con el

consecuente ahorro energético (Jursky *et al.*, 1994). Estudios recientes han demostrado que los transportadores de neurotransmisores, en ciertas condiciones tanto fisiológicas como patológicas, pueden funcionar en sentido inverso, llevando a cabo la liberación no vesicular, independiente de Ca^{2+}, del neurotransmisor (Attwell *et al*, 1993).

Ya que los transportadores localizados en la membrana plasmática de las células neuronales y gliales regulan las concentraciones locales de neurotransmisor, es evidente que juegan un papel importante en la modulación de la actividad de los receptores, además de participar en funciones como destoxificación, protección de sustancias reactivas y nutrición (Jursky *et al.*, 1994).

Mecanismos Moleculares del Transporte

La energía necesaria para el ciclo de la neurotransmisión la proporcionan dos tipos de ATPasa. a) La ATPasa de H^+ vacuolar de las vesículas sinápticas, que proporciona la fuerza impulsora para la acumulación de los neurotransmisores en las mismas (Nelson, 1993). b) La ATPasa de Na^+/K^+ de la membrana plasmática de las células neuronales y gliales, cuya actividad genera gradientes electroquímicos de sodio y potasio dirigidos hacia el interior y el exterior de la célula, respectivamente. La actividad de los transportadores de la membrana plasmática se impulsa por el gradiente de sodio, por el de potasio, o por ambos. Aunque la fuerza motriz principal es el gradiente de sodio generado por la ATPasa Na^+/K^+, existen variantes. Los transportadores de Glu son electrogénicos, pues co-transportan dos Na^+ con cada molécula de Glu, al mismo tiempo que contra-transportan un K^+ y un OH^- hacia el exterior de la célula. Los transportadores de Gly y GABA también son electrogénicos, co-transportan dos Na^+ y un Cl^- por cada molécula de transmisor, hacia el interior de la célula (la concentración extracelular de Cl^- también es mayor que la intracelular) (Fig. 4). Por medio del uso diferencial de estas fuerzas, el transportador puede ser controlado y actuar de manera específica en cada espacio sináptico o célula glial (Nelson y Lill, 1994).

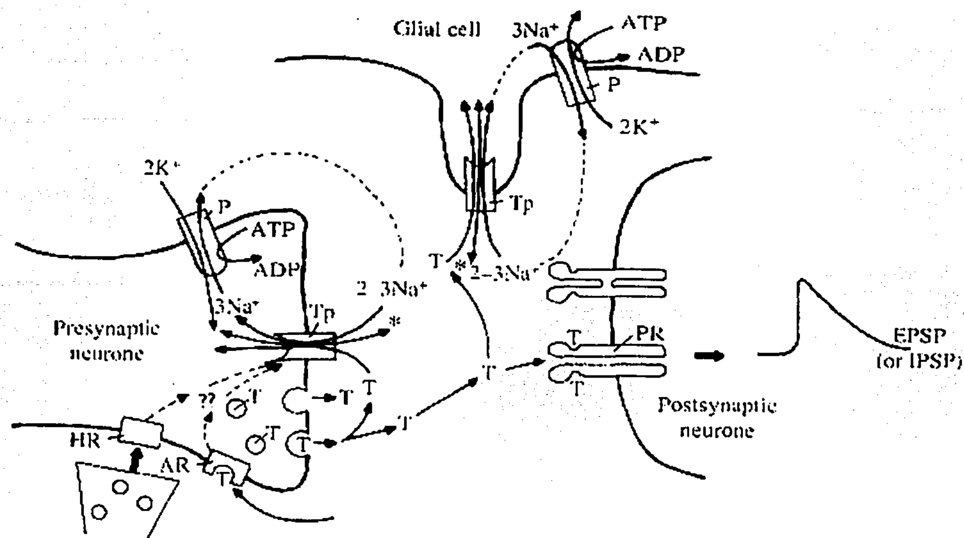


Figura 4. Mecanismos de acción de los transportadores de neurotransmisores. El neurotransmisor (T) que se encuentra almacenado en las vesículas sinápticas, se libera al espacio sináptico al fusionarse éstas con la membrana plasmática presináptica. Después de su difusión a través del espacio sináptico, el transmisor se une a receptores postsinápticos (PR) específicos que pueden ser ionotrópicos o metabotrópicos. Posteriormente, el transmisor debe de ser eliminado del espacio sináptico para que pueda tener lugar un segundo impulso. Los transportadores de neurotransmisores (Tp) recapturan al transmisor hacia la terminal sináptica o hacia las células gliales vecinas junto con Na^+ y otros iones (*). En el caso de los transportadores de Gly y otros, * es cloro, que se mueve en la misma dirección que el sodio y el neurotransmisor. En el caso de los transportadores de Glu, * representa al potasio, que se mueve en dirección opuesta al sodio y al Glu. La fuerza motriz para este proceso es el gradiente electroquímico de los iones sodio, que se mantiene por la ATPasa Na^+/K^+. La regulación fisiológica de los transportadores tal vez se de a través de receptores para el mismo neurotransmisor (autorreceptores, AR) o para otros (heterorreceptores, HR) (Kanner, 1994).

La actividad de los transportadores específicos coincide con la localización de los transmisores liberados, lo que sugiere que los transportadores se expresan de manera específica para cada sistema de neurotransmisión (Uhl y Hartig, 1992).

TESIS CON
FALLA DE ORIGEN

Importancia Clínica

Los transportadores de neurotransmisores tienen una gran importancia médica, debido a que regulan la actividad de los neurotransmisores eliminándolos del espacio sináptico. Ciertos inhibidores específicos de estos transportadores se utilizan como fármacos para tratar diversos padecimientos neurológicos, ya que incrementan la concentración del neurotransmisor en la sinapsis y/o prolongan su acción. Ejemplos notables son el uso de inhibidores del transporte de GABA en el tratamiento de la epilepsia, o de los inhibidores del transportador de serotonina para reducir los procesos depresivos (Kanner, 1994; Barondes, 1994).

Los transportadores son, asimismo, blancos moleculares de ciertos psicoestimulantes como la cocaína, que se une al transportador de dopamina inhibiendo su recaptura (Kuhar *et al.*, 1991); y de algunos antidepresivos, como el Prozac, que inhibe la recaptura de serotonina (Barondes, 1994).

Concentraciones elevadas de aminoácidos excitadores inducen muerte neuronal por excitotoxicidad, fenómeno involucrado en numerosas patologías convulsivas y neurodegenerativas (Matute et al., 1999; Shaw, 1999; Greene, 1999; Chapman, 2000). La entrada de Ca^{2+} a través de los receptores de NMDA es un evento crítico para la muerte neuronal inducida por glutamato (Choi, 1987). Una de las principales funciones de los transportadores de Glu en el sistema nervioso es la de evitar que la concentración de Glu extracelular se eleve a niveles neurotóxicos. En cultivos organotípicos de médula espinal se demostró, por primera vez, que los transportadores de glutamato protegen a las neuronas de la toxicidad inducida por glutamato (Rothstein et al., 1993). Más aún, se encontró que si se eliminan los transportadores de glutamato gliales, se induce la degeneración neuronal por excitoxicidad, mientras que si se eliminan los transportadores neuronales el daño no se presenta (Rothstein et al., 1996; Tanaka et al., 1997).

Los transportadores de Gly podrían relacionarse con la excitotoxicidad producida por el Glu a través de los receptores de NMDA, ya que la Gly actúa como coagonista potenciando el efecto del Glu sobre estos receptores (Smith *et al.*, 1992). En este sentido, se ha comprobado que la Gly potencia la muerte neuronal

excitotóxica producida por la activación crónica del receptor de NMDA en neuronas corticales y estriatales (McNamara y Dingledine, 1990; Morons et al., 1992). La capacidad del 7-clorokinurenato (antagonista del sitio de la glicina en el receptor de NMDA) de reducir la neurotoxicidad del Glu en cultivos celulares, pone en evidencia el potencial de la Gly en estados patológicos. En ciertas etapas de las enfermedades neurodegenerativas se presenta una pérdida de receptores de NMDA, por lo que la transmisión es subóptima. Se ha propuesto que el elevar los niveles de Gly endógena disminuyendo su transporte, podría resultar benéfico por incrementar la transmisión mediada por los receptores de NMDA (Fletcher *et al.*, 1990). En la esclerosis amiotrófica lateral (ALS), caracterizada por la pérdida de neuronas motoras en la médula espinal y tallo cerebral, y de células piramidales en la corteza motora, se presenta una disminución tanto en el transporte de Glu como en el transporte de Gly, registrándose altos niveles de estos aminoácidos en el fluido cerebroespinal. La sensibilidad de las neuronas motoras a altas concentraciones de Glu y Gly podría explicar la vulnerabilidad selectiva de esta población celular en ALS (Virgo y Belleroche, 1995).

TRANSPORTADORES DE GLICINA

Los transportadores de neurotransmisores se han clasificado en tres familias: (1) transportadores dependientes de sodio y cloro que operan en la membrana plasmática de células gliales y neuronales; (2) transportadores dependientes de sodio/potasio que funcionan en la membrana plasmática, específicamente transportadores de Glu; y (3) transportadores vesiculares que llevan a cabo la captura al interior de vesículas sinápticas y gránulos (Nelson y Lill, 1994).

Los transportadores de Gly pertenecen a la familia de transportadores que dependen de Na^+ y Cl^-, junto con los de GABA, prolina, betaína, taurina y aminas biogénicas. Todos los miembros de esta familia presentan una estructura y topología similares. En general, están constituidos por 600 aminoácidos arreglados en 12 segmentos transmembranales; tanto el extremo carboxilo-terminal como el extremo amino-terminal se encuentran del lado citoplásmico de la membrana. Estas regiones contienen sitios de fosforilación que pueden estar involucrados en la regulación del transporte. También presentan una gran asa extracelular entre los segmentos transmembranales III y IV donde se localizan de 2 a 4 sitios de glicosilación (Amara y Kuhar, 1993).

La comparación de la secuencia de aminoácidos de varios miembros de esta familia reveló que ciertos segmentos de estas proteínas presentan un mayor grado de homología que otros. Las regiones más conservadas son: el segmento transmembranal I junto con el asa extracelular que lo conecta con el segmento transmembranal II, y el segmento transmembranal V junto con la pequeña asa intracelular que lo conecta con el segmento IV y el asa extracelular más grande que lo conecta con el segmento VI (Fig. 5). Se ha propuesto que estos dominios están involucrados en la estabilización de la estructura terciaria que es esencial para el funcionamiento de estos transportadores, así como en la translocación de iones sodio. La parte de la proteína compuesta por los ocho primeros segmentos transmembranales está más conservada que las que componen el resto de los segmentos; se piensa que este dominio está involucrado en la translocación de los

diferentes sustratos. Las regiones menos conservadas son los extremos amino- y carboxilo-terminal, por lo que se propone que estas áreas están involucradas en la regulación del transporte (Kanner, 1994).

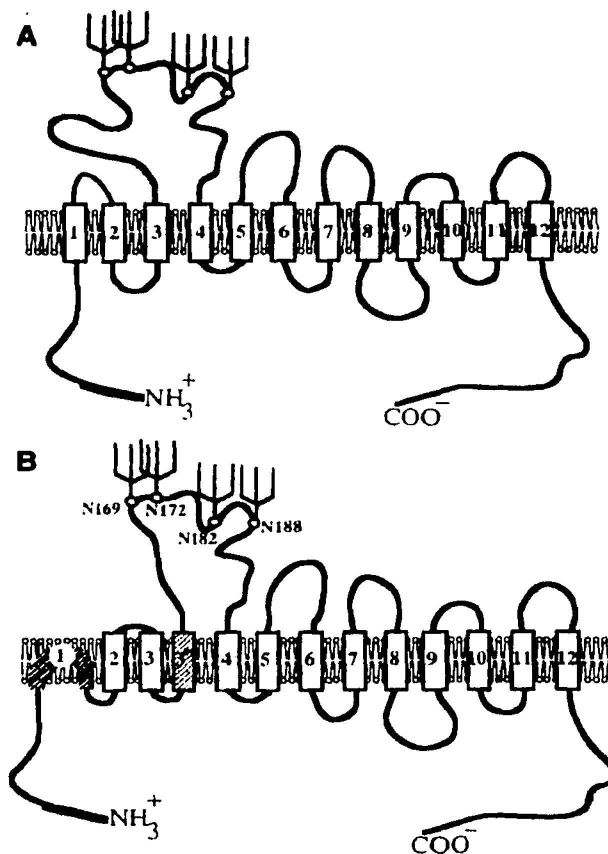


Figura 5. Topología membranal de los transportadores de Glicina. A) GLYT1. B) GLYT2. Se muestran los 12 segmentos transmembranales. Los extremos amino y carboxilo terminales son intracelulares. El asa extracelular entre los segmentos 3 y 4 presenta sitios consenso de glicosilación (Zafra et al., 1997).

TESIS CON
FALLA DE ORIGEN

La glicina (Gly) es un aminoácido no esencial que, además de tener diversas funciones metabólicas, es un neurotransmisor inhibidor clásico que actúa en la médula espinal, tallo cerebral y retina (Daly, 1990). Ejerce su efecto inhibidor a través de un canal de cloro controlado por ligando, y este efecto se antagoniza competitivamente por la estricnina (alcaloide convulsivo; Betz et al., 1993). Además de su papel inhibidor en el sistema nervioso, la Gly también modula la neurotransmisión excitadora actuando como coagonista del glutamato en los receptores del tipo NMDA. El sitio de unión de la Gly en estos receptores es insensible a la estricnina, mientras que la D-serina y la D-alanina reproducen su efecto (Fletcher et al., 1990).

Desde la década de los 60's se demostró que varios aminoácidos, incluyendo la glicina, eran incorporados por el tejido nervioso. Aprison y Werman (1965) fueron los primeros en proponer la existencia de un mecanismo de captura para la Gly que la eliminara de las sinapsis inhibidoras. Años después, la clonación de los transportadores para el GABA (Guastella, et al., 1990) y la norepinefrina (Pacholczyk et al., 1991) permitió el aislamiento de cDNAs homólogos que codifican para otros transportadores dependientes de Na^+ y Cl^-, entre ellos los de glicina. Se identificó a genes que codifican para los transportadores de glicina denominados GLYT1 y GLYT2. Por un proceso de empalme alternativo y/o uso diferencial del promotor, la transcripción del gene GLYT1 produce tres isoformas de RNAm: GLYT1a, GLYT1b y GLYT1c, que difieren en el extremo amino-terminal (Kim et al., 1994). La transcripción de las variantes GLYT1a y GLYT1b está mediada por el uso diferencial del promotor y no por el empalme alternativo de exones transcritos a partir de un promotor común (Borowsky y Hoffman, 1998). GLYT1a y GLYT1b se clonaron en rata y en ratón (Guastella et al., 1992; Liu et al., 1992; Smith et al., 1992; Borowsky et al., 1993), mientras que la isoforma GLYT1c se clonó en el humano (Kim et al., 1994). Recientemente se informó la expresión en la retina de dos isoformas; GLYT1e y GLYT1f, que difieren respectivamente de GLYT1a y GLYT1b en el extremo carboxilo terminal, como resultado del empalme

alternativo de exones (Hanley et al., 2000). Estas nuevas isoformas interactúan con la subunidad ρ1 del receptor de GABA a través del extremo carboxilo terminal.

El gene GLYT2 codifica para dos isoformas denominadas GLYT2a y GLYT2b generadas por empalme alternativo de la región 5' (Ponce et al., 1998). Ambas isoformas son muy similares en cuanto a sus características cinéticas y su distribución en el sistema nervioso, sin embargo, cuando se expresan en células COS transfectadas, GLYT2a captura glicina, mientras que GLYT2b sólo parece intercambiarla o liberarla.

Existe una distribución anatómica diferencial de GLYT1 y GLYT2 en el SNC; ambos transportadores son altamente específicos para la glicina. A pesar de que GLYT1 y GLYT2 presentan diferencias estructurales y de localización, la única diferencia farmacológica que se conoce es la sensibilidad de las isoformas de GLYT1 a la inhibición por sarcosina (n-metilglicina; Liu et al., 1993). Recientemente se reportó que la amoxapina (antidepresivo tricíclico) inhibe selectivamente a GLYT2a, mientras que su efecto sobre la actividad de GLYT1 es insignificante (Nuñez et al., 2000).

Localización Celular e Histológica

Además del cerebro, la expresión de las isoformas de GLYT1 se ha demostrado en otros tejidos. En la rata, tanto el RNAm para GLYT1b (Smith et al., 1992) se identificó exclusivamente en el hígado, mientras que en el ratón, se identificó GLYT1a en el hígado, pulmón, bazo, estómago y útero, y GLYT1b sólo se detectó en el cerebro (Borowsky et al.,1993). La expresión de GLYT1c sólo se ha demostrado en el cerebro (Kim et al., 1994); la de GLYT2 se restringe al cerebro y médula espinal en la rata (Liu et al., 1993), única especie examinada.

La distribución de los transportadores GLYT1 y GLYT2 en regiones específicas del sistema nervioso se estableció mediante el uso de técnicas de hibridación *in situ*, "northern blot" e inmunoensayos. Utilizando sondas que hibridan con el RNAm de todas las isoformas de GLYT1, se observó que la mayor expresión de este transportador se presenta principalmente en las células gliales

(Adams et al., 1995) del cerebelo, bulbo olfatorio, tallo cerebral y médula espinal (Guastella et al., 1992; Liu et al., 1992; Smith et al., 1992; Jursky et al., 1994). Sin embargo, su expresión en neuronas del SNC también está documentada (Borowsky et al., 1993). Asimismo se encontró inmunoreactividad para GLYT1 en las células amacrinas de rata, macaco, gato, conejo y pollo (Zafra et al., 1995, Pow and Hendrickson, 1999). Es importante mencionar que en ninguno de estos estudios fue posible localizar a GLYT1 en las células gliales de la retina. La distribución de las isoformas GLYT1e y GLYT1f no se ha estudiado.

La expresión de GLYT2 está restringida a las neuronas, localizándose principalmente en los axones (Zafra et al., 1995; Spike et al., 1997). Los niveles más altos se detectaron en las hastas dorsal y ventral de la médula espinal, en el sistema auditivo y en los núcleos de los nervios craneales (Luque et al., 1995).

La localización de los GLYTs en el cerebro sugiere funciones específicas relacionadas con la neurotransmisión. Por su localización específica en terminales glicinérgicas (Zafra et al., 1995), se considera a GLYT2 como un marcador confiable de neuronas glicinérgicas (Poyatos et al., 1997). A este respecto, se ha demostrado en el cerebro y en la médula espinal, la localización de GLYT2 con los receptores inhibidores de glicina (Luque et al., 1995; Jursky y Nelson, 1995), lo que sugiere que este transportador juega un papel importante en la terminación de la transmisión glicinérgica. Como ya se mencionó, recientemente se demostró que el gene GLYT2 da lugar a dos isoformas, GLYT2a y GLYT2b, las cuales se expresan en las mismas regiones anatómicas (Ponce et al., 1998). Debido a su baja concentración, el RNAm de GLYT2b se pudo identificar únicamente por RT-PCR y no por "northern blot" o por ensayos de protección de RNAsas, por lo que estudios previos sobre la localización de GLYT2 con "Northern blot" e hibridación *in situ*, probablemente corresponden a la distribución del RNAm de GLYT2a.

Con base en los estudios de Smith y col. que demuestran la colocalización de GLYT1 con los receptores de NMDA (Smith et al., 1992), se ha propuesto su participación en la neurotransmisión glutamatérgica, idea que se refuerza por la localización de GLYT1 en áreas no relacionadas con vías glicinérgicas (Borowsky et

al., 1992; Liu et al., 1992, 1993; Luque et al., 1995; Zafra et al., 1995). No obstante, la expresión de GLYT1 también se ha demostrado en células gliales asociadas a sinapsis presumiblemente glicinérgicas (Zafra et al., 1995). Es posible que isoformas particulares de GLYT1 estén asociadas a los receptores de NMDA mientras que otras estén relacionadas con los receptores inhibidores de glicina.

Estequiometría y Propiedades de Canal

Se considera que los transportadores acoplados a Na^+/Cl^-, en general, transportan $2Na^+/Cl^-$ con cada molécula de sustrato (Nelson y Lill, 1994). Los primeros estudios del sistema de transporte de glicina de alta afinidad en vesículas de membrana plasmática sináptica demostraron el cotransporte de 2 iones Na^+ y un Cl^- junto con el sustrato en cada ciclo de transporte (Aragón et al., 1987). Un estudio posterior expresando los transportadores de glicina en células HEK sugirió que la isoforma GLYT1b requiere de 2 iones Na^+ para el transporte de glicina, mientras que GLYT2 requiere de 3 iones Na^+ (López-Corcuera et al., 1998). Recientemente Roux y Supplisson (2000) corroboraron estos resultados, y demostraron que los transportadores neuronales y gliales de glicina difieren en su capacidad para funcionar en sentido inverso: GLYT2 tiene una restricción cinética para el transporte inverso, lo que limita su capacidad de liberar glicina. Esta asimetría en los flujos de glicina puede ser esencial para mantener alta la concentración de neurotransmisor en el interior de las neuronas presinápticas durante los períodos de actividad. En contraste, GLYT1 no exhibe tal limitación: en ovocitos expresando GLYT1b, la acumulación de glicina genera una corriente saliente neta cuando se remueve la glicina del medio extracelular. Esta liberación no vesicular e independiente de calcio podría ser particularmente importante en las sinapsis glutamatérgicas, donde la liberación vesicular de glicina no se ha podido demostrar (Chaudhry et al., 1998).

A diferencia de los transportadores de GABA y glutamato que presentan propiedades de canales iónicos activados por ligando (revisado en Gadea y López-Colomé, 2001a,b), en los transportadores de glicina el flujo iónico y el flujo del

sustrato están estrechamente acoplados. Esto es particularmente cierto para GLYT1b, el cual no exhibe fuga de corriente y el número de cargas que se translocan con cada molécula de glicina es totalmente independiente del voltaje (Roux y Supplisson, 2000).

Attwell y col. (1993) propusieron que en las células gliales, la despolarización y el aumento en la [Na^+]i por activación de los receptores de AMPA, es suficiente para revertir el transporte de GLYT1b, incrementando la [Gly]o y potenciando así a los receptores de NMDA. En este sentido, la liberación de glicina por parte de la glía se ha demostrado en distintos sistemas, lo que ha llevado a la idea de que, la liberación de glicina independiente de calcio de las células gliales en la vecindad de las sinapsis glutamatérgicas, contribuye a la neurotransmisión excitadora (Holopainen y Kontro, 1989; Galli et al., 1993; Saransaari y Oja, 1994; Sakata et al., 1997).

Regulación de los Transportadores de Glicina

Existe muy poca información disponible sobre los aspectos reguladores de los sistemas de transporte de glicina. Se ha demostrado que el ácido araquidónico liberado por la fosfolipasa A_2, inhibe varios sistemas de transporte dependientes de Na^+, incluyendo los de glicina: el transporte de glicina de alta afinidad en la línea celular de glioma C6 se inhibe por ácido araquidónico debido a la perturbación del dominio lipídico que rodea a los transportadores (Zafra et al., 1990).

Sato y col. (1995) demostraron que la activación de la PKC por ésteres de forbol disminuye la actividad de GLYT1b expresado en células HEK-239 debido a una reducción en la Vmax del transporte. La eliminación de todos los sitios consenso de fosforilación por la PKC no abolió el efecto del tratamiento con ésteres de forbol, lo que sugiere que la modulación del transporte por la PKC podría involucrar un mecanismo indirecto. A este respecto, Geerlings y col. (2000) demostraron recientemente una interacción física y funcional de ambos transportadores, GLYT1 y GLYT2, con la sintaxina 1A (proteína SNARE). La cotransfección de la sintaxina 1A y GLYT1 o GLYT2 en células COS disminuye el

número de proteínas transportadoras en la membrana plasmática, pero no su expresión total. Estudios de inmunoprecipitación corroboraron una interacción física entre los GLYTs y la sintaxina 1A; es posible entonces, que la PKC regule la interacción entre los GLYTs y la sintaxina 1A, como en el caso de los transportadores de GABA (Horton y Quick, 2001).

Las neuronas pueden regular la expresión glial de GLYT1: Las células gliales de la médula espinal de rata no expresan GLYT1 en cultivos puros, pero sí lo hacen en cultivos mixtos de neuronas y glia. Si se elimina a las neuronas por medio de tratamientos citotóxicos, se reduce la expresión en la glía de GLYT1 (Zafra te al., 1997). Estos hallazgos sugieren que es importante la interacción entre neuronas y glía para la regulación de la expresión de GLYT1.

Transportadores de Glicina y Neuropatologías

La glicina podría participar en procesos de excitotoxicidad al potenciar el efecto del glutamato en los receptores de NMDA, así como en la patogénesis de enfermedades relacionadas con los receptores inhibidores de glicina (Lloyd et al., 1983; Simpson et al., 1995). En cuanto a la transmisión excitadora, se ha demostrado que los transportadores de glicina juegan un papel importante en mantener la concentración local de glicina por debajo de los niveles de saturación para los receptores de NMDA, de manera que un incremento en la concentración extracelular de glicina potencie la actividad de estos receptores (Supplisson y Bergman, 1997; Bergeron et al., 1998). Dicho incremento puede producirse por la difusión de la glicina proveniente de sinapsis vecinas, dependiente de calcio, o por la inversión del transporte, mecanismo independiente de calcio, como en condiciones isquémicas en las que ocurre la reducción del gradiente electroquímico del Na^+ y del Cl^- (Baker et al., 1991). Esta liberación independiente de calcio proviene muy probablemente de los transportadores de glicina gliales cuya estequiometría, como ya se mencionó, es de $2Na^+/Cl^-/Gly$, a diferencia de los neuronales: $3Na^+/Cl^-/Gly$ (Roux y Supplisson, 2000).

En humanos con esclerosis amiotrófica lateral (ALS), existe la pérdida de GLYT1 en la médula espinal (Virgo y Belleroche, 1995); probablemente el déficit en el RNAm de GLYT1 afecta la inactivación sináptica de la glicina y potencia la acción del glutamato sobre los receptores de NMDA en las motoneuronas, contribuyendo de esta manera a la condición neurotóxica.

Los bloqueadores de canal de alta afinidad de los receptores de NMDA, como la fenilciclidina, reproducen los síntomas de la esquizofrenia en humanos, por lo que se ha sugerido que la hipofunción del sistema glutamatérgico está involucrada en esta enfermedad (Tsai et al., 1998). La disminución en la liberación de glutamato estimulada por NMDA que se ha demostrado en preparaciones de sinaptosomas provenientes de pacientes esquizofrénicos, es otro indicio de hipofunción glutamatérgica en esta enfermedad (Danysz y Parsons, 1998; Olney et al., 1999). El uso clínico de agonistas del receptor de NMDA en el tratamiento de este padecimiento se ha descartado debido a su potencial neurotóxico y convulsivo. En este sentido, los transportadores de glicina podrían representar un blanco adecuado para el diseño de drogas antiesquizofrénicas. Por lo tanto, el estudio farmacológico de los transportadores de glicina podrá proporcionar herramientas útiles para el diseño de fármacos específicos que afecten su recaptura.

Dado que la glicina tiene diversas funciones en el SNC, es evidente que la regulación de su concentración extracelular por los transportadores puede tener fuertes implicaciones para los procesos de neurotransmisión en el sistema nervioso. La manera en la que la regulación de la concentración extracelular de glicina por parte de la glía afecta condiciones fisiológicas y patológicas sigue siendo tema de investigación.

OBJETIVOS

Las células gliales constituyen más de la mitad del volumen total del cerebro y superan en número a las neuronas, a pesar de lo cual han atraído poco la atención de los neurofisiólogos desde su descripción por Virchow en 1846. La propuesta de Virchow de que las células gliales no son más que un "pegamento nervioso" siguió siendo, hasta hace pocos años, el punto de vista de muchos neurofisiólogos. Sin embargo, en los últimos años se ha progresado considerablemente en la comprensión de la fisiología glial y las interacciones glía-neurona lo cual ha llevado a un replanteamiento de los mecanismos que subyacen el funcionamiento del sistema nervioso. Un excelente modelo para el estudio de la fisiología glial es la glía de Müller de la retina.

Uno de los aspectos más interesantes sobre la fisiología glial es su capacidad de modular las respuestas neuronales mediante el control de los niveles extracelulares de neurotransmisores a través de sistemas de transporte de alta afinidad.

La captura de glicina en la glía de Müller podría participar en la modulación de la transmisión excitadora en la vía vertical de la retina a través de los receptores de NMDA, así como en la inhibición lateral a través de los receptores de glicina inhibidores, el primer objetivo de este trabajo fue:

- **Demostrar la presencia de transportadores de glicina de la glía de Müller**

Se dispone de muy poca información sobre los mecanismos reguladores de los transportadores de glicina. Las células gliales responden a la actividad neuronal a través de cambios en las concentraciones de mensajeros intracelulares. Estos cambios modifican las funciones de la glía. Una de las funciones modificadas

podría ser su capacidad de capturar neurotransmisores, por lo tanto, nuestro segundo objetivo consistió en:

- **Estudiar las vías de señalamiento intracelular involucradas en la regulación de los transportadores de glicina en la glía de Müller de la retina.**

MATERIALES Y MÉTODOS

CULTIVO PRIMARIO DE LA GLÍA DE MÜLLER DE RETINA DE POLLO

El método de cultivo para la glía de Müller se basa en el desarrollo diferencial de neuronas y glía. Las células gliales son las primeras en aparecer durante el desarrollo de la retina, pero su diferenciación es posterior al de las neuronas. Las neuronas en cultivo comienzan a morir a los 7 días *in vitro* (DIV), mientras que las células gliales siguen dividiéndose y creciendo, de manera que a los 13 DIV, tiempo al que la glía deja de dividirse, el cultivo está libre de neuronas.

Las células de Müller se cultivaron según la técnica descrita por Adler y colaboradores (1982) y modificada por López-Colomé y Romo-de-Vivar (1991). Las retinas se obtienen de embriones de pollo de 7 días. Se extraen separándolas del epitelio pigmentado y se lavan dos veces en solución de Hank libre de Ca^{2+} y Mg^{2+} (NaCl 0.8 g, KCl 0.04 g, KH_2PO_4 0.006 g, Na_2HPO_4 0.0125 g, rojo de fenol 0.002 g y glucosa 0.1 g en 100 ml de H_2O). Después de lavadas las retinas se incuban 10 minutos en tripsina al 0.25% en solución de Hank a 37°C. La reacción enzimática se detiene con L-MEM (1.338 g de medio Eagle, modificación de Dubelcco (ver apéndice), 0.05 g de glucosa, 0.022 g de $NaHCO_3$ en 100 ml, al cual se le agregó 0.025% de PNS (penicilina, neomicina, estreptomicina) y 0.025% de gentamicina y 1% de albúmina bovina (BSA). Las células se disocian mecánicamente utilizando una pipeta Pasteur, se filtran a través de una red de nylon de 50 μM y se resuspenden en H-MEM (L-MEM más 0.22 gr de $NaHCO_3$) sin suero fetal bovino. Se cuantifica el número de células con un contador Coulter modelo ZB1 (Coulter Electronics, Inc.) y la suspensión celular se diluye a un millón de células/ml. Las células se siembran en placas de 12 y de 24 pozos (de 24 y 18 mm de diámetro respectivamente), en las placas de 12 pozos se siembran 500,000 células por pozo y en las de 24 pozos se siembran 250,000 células por pozo, colocando el mismo volumen de medio H-MEM con 20% de suero fetal bovino. Se incuban a 37°C y 5% de CO_2 durante 12 ó 13 DIV (días *in vitro*), tiempo al cual el cultivo llega a

confluencia. El medio de cultivo (H-MEM 10% de suero fetal bovino) se cambia cada tercer día.

Las células de los cultivos se identificaron como glía de Müller ya que los cultivos resultaron inmunonegativos para el anticuerpo policlonal contra la NSE (enolasa específica de neuronas) e inmunopositivos para el anticuerpo policlonal contra la GFAP (proteína fibrilar acídica de la glía) que también es un marcador de astrocitos. Ya que la retina de las aves es avascularizada y no presenta astrocitos (Rasmussen, 1974), la marca corresponde a la glía de Müller (López-Colomé y Romo-de-Vivar, 1991).

CULTIVO PRIMARIO DE NEURONAS DE RETINA DE POLLO

Los cultivos de neuronas de la retina se preparan de retinas obtenidas de embriones de pollo de 7 días. Las retinas se incuban durante 35 minutos a 37°C en tripsina al 0.5% en solución de Hank a 37ºC libre de Ca^{2+} y Mg^{2+}. Las células se disocian mecánicamente utilizando una pipeta Pasteur. La suspensión celular se filtra a través de una red de nylon de 50 µM y se resuspende en H-MEM (L-MEM más 0.22 gr de $NaHCO_3$) sin suero fetal bovino. Se cuantifica el número de células con un contador Coulter modelo ZB1 (Coulter Electronics, Inc.). Las células se siembran a baja densidad (0.6×10^6 células por pozo) en placas de 12 pozos previamente tratadas con poli-DL-ornitina. Las neuronas crecen en OPTI-MEM-I (medio con suero reducido, modificación del medio MEM de Eagle) que contiene 3% de suero fetal de bovino y se mantienen a 37°C en una atmósfera húmeda de 5% CO2 : 95% aire. La pureza de los cultivos se determinó como se describe para la glía (Somohano y López-Colomé, 1991). Más del 90% de las células resultaron NSE^+ y $GFAP^-$. Los ensayos de transporte se llevaron a cabo en cultivos de neuronas de 5 DIV.

ENSAYOS DE TRANSPORTE DE GLICINA

Los ensayos de transporte se realizaron con cultivos confluentes de células de Müller de 12 ó 13 DIV. Al inicio de todos los ensayos, el medio de cultivo se

eliminó por aspiración con una pipeta Pasteur y los cultivos se lavaron 3 veces con 1 ml (placas de 12 pozos) o con 0.5 ml (placas de 24 pozos) de Ringer Krebs Bicarbonato (RKB) que contenía NaCl 118 mM, KH_2PO_4 1.2 mM, KCl 4.7 mM, $CaCl_2$ 2.5 mM, $NaHCO_3$ 25 mM, glucosa 5.6 mM y $MgSO_4$ 1.4 mM, a 37ºC. En seguida se agregó 1 ml ó 0.5 ml de KRB que contenía [^{3}H]-Gly/Gly 1:25000 (41.1 Ci/mmol) en el cual se llevaron a cabo los distintos ensayos.

Al final de cada ensayo, los cultivos se lavaron 3 veces con las mismas cantidades de KRB, pero esta vez a 4ºC. Posteriormente se agregó a cada pozo 1 ml de HCl 1N, se desprendió la monocapa celular con un gendarme y se aspiraron los pozos colocando el contenido en viales, los cuales se taparon y se calentaron a 100ºC para hidrolizar el tejido. Una vez que el tejido se disoció, se le agregó a cada vial 1ml de NaOH 1N para neutralizar al HCl y posteriormente 5 ml de Tritosol (Fricke, 1975). La radiactividad se cuantificó en un contador de centelleo líquido (Beckman). Todos los ensayos se realizaron a 37ºC.

Todos los experimentos se hicieron por triplicado y se repitieron al menos tres veces. De cada experimento se determinó la cantidad de proteína de 1 pozo, por el método de Lowry *et al.* (1951).

Los resultados se analizaron con la ayuda del programa Graph Pad INPLOT (versión 3.1, San Diego, California, EUA). En la mayoría de los casos se aplicó la prueba "t de Student" para comparar cada condición con el control.

MEDICIÓN DEL Ca^{2+} INTRACELULAR

El método es el descrito anteriormente por Hernández-Cruz y colaboradores (1997). Las células de Müller, sembradas en cubreobjetos, se cargaron con fura 2-AM (Molecular Probes, Eugene, OR, EUA), concentración final 2 μM, sin agentes dispersores. Las células se cargaron durante 30-45 minutos a 37°C y después se lavaron con superfusión continua durante 5 minutos antes del experimento. El cubreobjetos se colocó en una cámara de registro (Mod. RC-25; Warner Instruments, Hamden, CT) en un microscopio invertido (Nikon Diaphot TMD; Nikon Corp., Tokyo, Japón).

Los niveles de Ca^{2+} se determinaron registrando pares de imágenes con un objetivo para UV (Nikon UV-F 100X, 1.3 N.A.) y una cámara intensificada CCD (c2400-87, Hamamatsu, Bridgewater, NJ, EUA). Se utilizó iluminación alternada de dos lásers de nitrógeno (Laser Science, Inc.), sintonizados a 340 nm y 380 nm de excitación (BioLase Imaging, Newton, MA, EUA). La tasa de muestreo (proporción 340/380) fue de 2.5 Hz. Se tomaron imágenes del fondo a 340 y 380 nm de iluminación para restar el ruido. La adquisición de imágenes y el procesamiento se llevó a cabo con el programa BioLase's FL-2. Las determinaciones de la concentración intracelular de Ca^{2+} ($[Ca^{2+}]i$) se calcularon con la fórmula:

$$[Ca^{2+}] = K_D(F_f/F_b)(R-R_{min})/(R_{max}/R)$$

donde la constante de disociación (K_D) del fura-2 por el Ca^{2+} es 300 nM, F_f/F_b es la razón de los valores de fluorescencia para el indicador-libre-de-Ca^{2+}/indicador-Ca^{2+}-unido excitando a 380 nm, R es la razón de fluorescencia a 340/380 nm para la $[Ca^{2+}]$ desconocida, y R_{min}, R_{max} son las razones para la fluorescencia del fura-2 a 340/380 nm de fura-2-libre-de-Ca^{2+} y fura-2-Ca^{2+}-unido, respectivamente. Los valores de F_f/F_b, R_{min}, y R_{max} para el Ca^{2+} se determinaron empíricamente utilizando soluciones de calibración que contenían 50 μM fura-2 (sal de pentapotasio; Molecular Probes), y concentraciones de Ca^{2+} en el intervalo de 0 a 40 μM. La $[Ca^{2+}]_i$ basal se definió promediando en el tiempo la $[Ca^{2+}]_i$ medida en ausencia de estímulo durante un período de 3 min. La amplitud máxima del incremento intracelular de Ca^{2+} se determinó como la diferencia entre el pico absoluto de la $[Ca^{2+}]_i$ y $[Ca^{2+}]_i$ basal (pre-estímulo). El procesamiento de los datos se llevó a cabo con el programa Origin 3.78 (Microcal software, Northampton, MA).

Soluciones

Las células se superfundieron continuamente con KRB (~1 ml/min). Las soluciones con el tratamiento se aplicaron por presión (10 psi) a través de pipetas de vidrio independientes (diámetro de la punta ~ 2 μm), colocadas a 100 μm de las células examinadas. La aplicación de las soluciones probadas se controló por

medio de un dispositivo Picospritzer (General Valve, Fairfield, NJ). Las soluciones utilizadas fueron: 1) cafeína 5 mM disuelta en KRB y 2) ionomicina 10 mM disuelta en KRB. Los experimentos se llevaron a cabo a 22-23°C.

RESULTADOS

CARACTERIZACIÓN BIOQUÍMICA Y FARMACOLÓGICA DEL TRANSPORTE DE GLICINA

Curso Temporal y Cinética del Transporte de Glicina

El transporte de glicina (1mM, concentración final) en la glía de Müller es saturable; no se observó mayor acumulación después de 30 minutos (figura 1). Las curvas de saturación para el transporte se obtuvieron midiendo la tasa de incorporación de glicina a distintas concentraciones (de 10 μM a 2 mM, [^{3}H]-Gly/Gly 1:5000). El análisis cinético de los datos por el método de Eadie-Hofstee, reveló la presencia de dos sistemas de transporte (figura 2), uno de alta afinidad y otro de baja afinidad por la glicina. En dicha situación las actividades de ambos sistemas se sobreponen, esto es, cuando se mide la tasa de incorporación de glicina a bajas concentraciones, predomina el sistema de transporte de alta afinidad, sin embargo el sistema de baja afinidad también contribuye al transporte. Lo mismo ocurre a altas concentraciones de glicina: el sistema de baja afinidad predomina, pero el sistema de alta afinidad también está activo. Para evitar errores al calcular las constantes cinéticas de ambos sistemas, se calcularon las constantes modificadas por el método de Neal (1972). Los valores de K_m y V_{max} para los sistemas de alta y baja afinidad son $K_m = 26.86 \pm 7.4$ μM, $V_{max} = 2.65 \pm 0.87$ nmol/mg proteina/10 min, y $K_m = 1.66 \pm 0.38$ mM, $V_{max} = 29.91 \pm 5.26$ nmol/mg proteina/10min, respectivamente.

Dependencia Iónica y Especificidad del Transporte de Glicina

Los transportadores de glicina de alta afinidad descritos en el sistema nervioso pertenecen a la familia de transportadores dependientes de Na^+/Cl^- (Nelson y Lill, 1994), mientras que los transportadores de baja afinidad dependen exclusivamente de la presencia de Na^+ en el medio extracelular para llevar a cabo el transporte (Christensen, 1984). La dependencia iónica del transporte de glicina

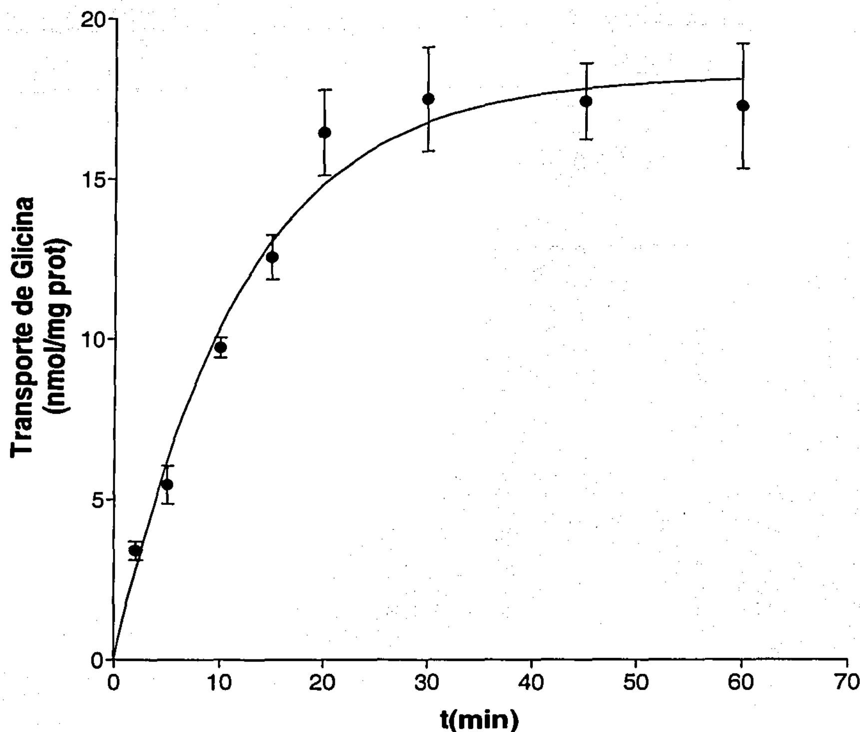


Figura 1. Curso temporal del transporte de glicina. Las células de Müller se incubaron en medio KRB en presencia de glicina 1 mM ([^{3}H]-Gly/Gly 1:25000) durante los tiempos indicados. Cada punto es el promedio de tres experimentos por triplicado. Los resultados se expresan como la media ± E.S.

en la glía de Müller se estudió utilizando concentraciones isosmóticas de cloruro de colina y de litio (medio sin Na^+), así como de gluconato de sodio (medio sin Cl^-) para sustituir al NaCl. La figura 3 muestra que ambos sistemas de transporte dependen de Na^+ para llevar a cabo la captura, mientras que la ausencia de Cl^- abolió específicamente al componente de alta afinidad (figura 4).

TESIS CON
FALLA DE ORIGEN

Km_1=27 μM Km_2=1.7 mM
$Vmax_1$=3 nmol/mg prot/10 min $Vmax_2$=30 nmol/mg prot/10 min

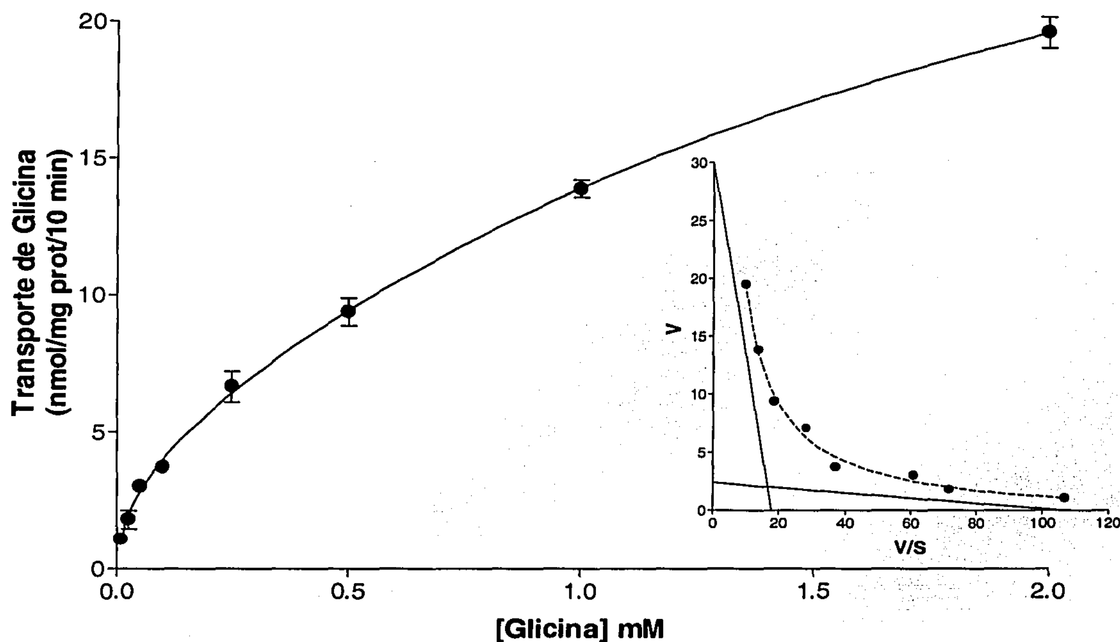


Figura 2. Cinética del transporte de glicina. Los cultivos se incubaron en medio KRB durante 10 minutos en presencia de glicina a distintas concentraciones ([^{3}H]-Gly/Gly 1:5000). Cada punto es el promedio de por lo menos dos experimentos por duplicado ± E.S. En la gráfica interior se muestra el análisis de Eadie-Hofstee.

TESIS CON
FALLA DE ORIGEN

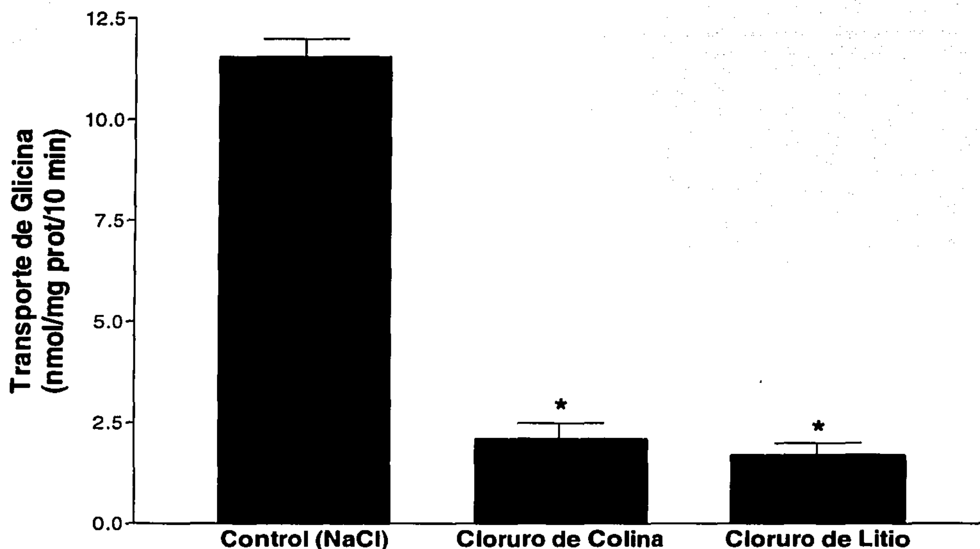


Figura 3. Dependencia iónica del transporte: Na^+. Los cultivos se incubaron durante 10 minutos con glicina 1 mM ([^{3}H]-Gly/Gly 1:25000) en medio KRB que contenía NaCl 118 mM, ó sin Na^+; el Na^+ se reemplazó por cloruro de litio o cloruro de colina 118 mM. Los resultados se muestran como la media de tres experimentos por triplicado ± E.S. *Significativamente distinto del control ($p<0.001$, prueba "t" de Student.

TESIS CON
FALLA DE ORIGEN

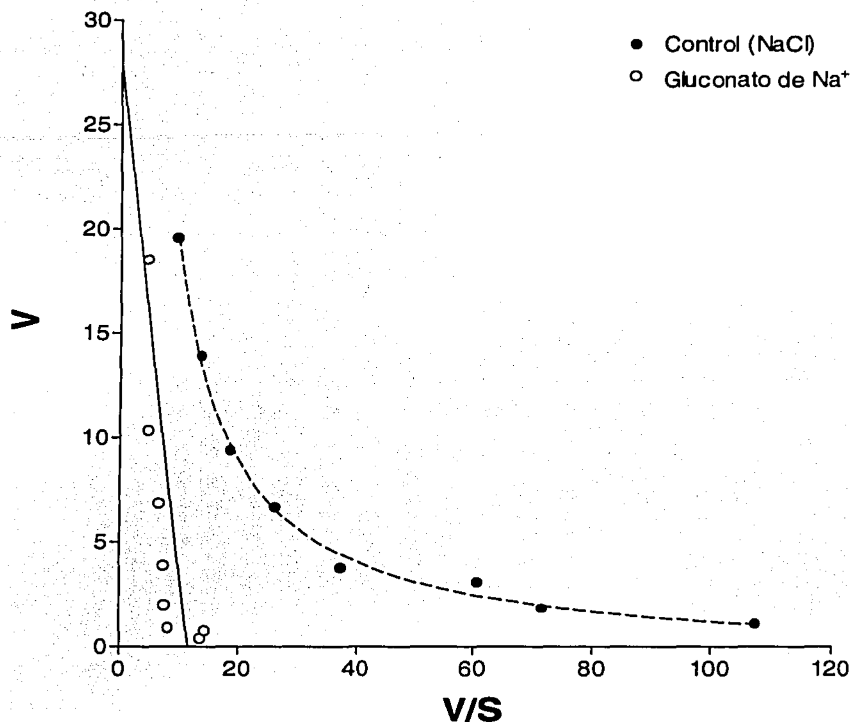


Figura 4. Dependencia iónica del transporte: Cl^-. Los cultivos se incubaron durante 10 minutos en presencia de distintas concentraciones de glicina (0.01-2 mM) ([^{3}H]-Gly/Gly 1:25000) en los siguentes medios: KRB control que contenía NaCl 118 mM (•) ó KRB en el que el NaCl se reemplazó por gluconato de sodio118 mM (O). La gráfica que se muestra es el análisis de los datos por el método de Eadie-Hofstee y los valores son la media de tres experimentos por triplicado.

TESIS CON
FALLA DE ORIGEN

Tabla 1. Especificidad farmacológica del transporte de glicina en la glía de Müller[1]

Compuesto probado	Alta afinidad (I)		Baja afinidad (II)	
	(nmol/mg proteína/10 min))			
Control	2.12 ± 0.24	(100)	12.11 ± 0.37	(100)
D-serina	2.14 ± 0.16	(101)	12.17 ± 0.74	(100.4)
GABA	2.31 ± 0.32	(109)	11.87 ± 0.49	(98)
Taurina	2.13 ± 0.15	(100)	12.19 ± 0.59	(100.6)
Sarcosina	0.51 ± 0.05***	(24)	7.52 ± 0.38**	(62)
MeAIB	2.08 ± 0.09	(98)	5.81 ± 0.48***	(48)
AIB	2.22 ± 0.10	(104)	6.19 ± 0.53***	(51)

[1]Los experimentos de captura se realizaron en presencia de distintos aminoácidos y derivados (5 mM); sarcosina 100 μM en I y 1 mM en II; glicina 25 μM (I) ó 1 mM (II). MeAIB, ácido metilaminoisobutírico; AIB, ácido aminoisobutírico. Los valores representan la media ± ES de tres experimentos por triplicado. Los valores en paréntesis representan el porcentaje del transporte con respecto al control. Para comparar los valores obtenidos con el control, se aplicó una prueba de t de Student: *Significativamente distinto del control ($P<0.05$, $**P<0.02$, $***P<0.001$).

Para determinar la especificidad farmacológica de ambos sistemas, se probó el efecto de distintos aminoácidos y derivados sobre el transporte. La glicina es sustrato de diversos sistemas de transporte de aminoácidos, por lo tanto, se examinó la relación del sistema de transporte en las células de Müller con sistemas previamente identificados. Los resultados de la Tabla 1 muestran que la sarcosina, sustrato de los transportadores GLYT1, inhibió el transporte de alta afinidad 76% (100 μM) y el de baja afinidad 38% (1 mM). El MeAIB y el AIB, sustratos del sistema A, sistema de transporte de aminoácidos de baja afinidad, inhibieron el transporte de glicina (50%). El transporte de alta afinidad lleva a cabo el 25% del transporte total a una concentración de glicina 1 mM (condiciones para el transporte de baja afinidad; Neal, 1972). Ya que la sarcosina (1 mM) inhibió el

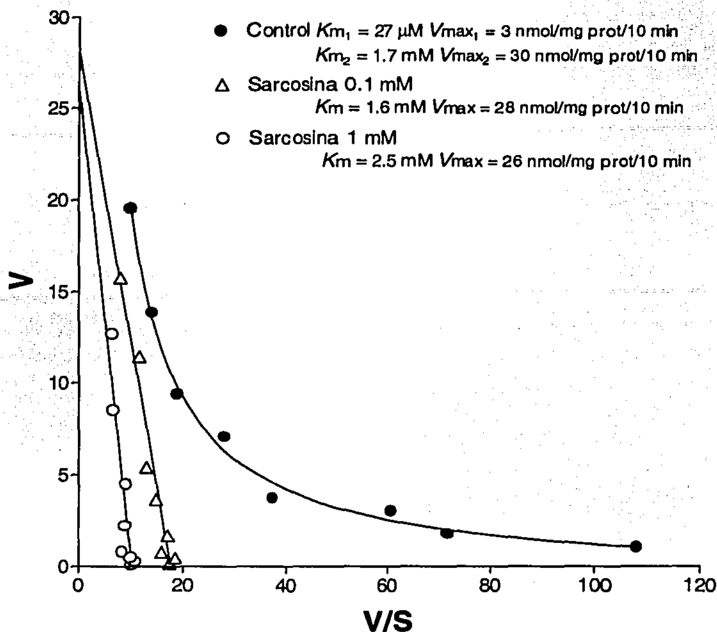


Figura 5. Efecto de la sarcosina sobre la cinética del transporte. Los cultivo incubaron durante 10 minutos con distintas concentraciones de glicina (0.01-2 ([^{3}H]-Gly/Gly 1:25000), y en ausencia (control, ●) ó en presencia de sarcosina (0.1 Δ ó 1 mM, ○). Los datos se analizaron por el método de Eadie-Hofstee y los va son la media de tres experimentos por triplicado.

transporte de baja afinidad en un 38% (Tabla 1), se estudió el efecto de distintas concentraciones de sarcosina sobre la cinética del transporte. Como se muestra en la figura 5, la sarcosina, a una concentración de 0.1 mM, abolió por completo el transporte de alta afinidad mientras que el de baja afinidad no se afectó. A concentraciones más altas (1 mM), la sarcosina inhibió ligeramente el transporte de baja afinidad incrementando el valor de la K_m a 2.55 mM.

Estos resultados demuestran que la captura de glicina en la glía de Müller se lleva a cabo por dos sistemas de transporte, uno de alta afinidad con características de los transportadores GLYT1 presentes en las células gliales e involucrados en los procesos de neurotransmisión del SNC, y uno de baja afinidad, con características del sistema A descrito en diversos tejidos.

TESIS CON
FALLA DE ORIGEN

REGULACIÓN DEL TRANSPORTE DE GLICINA POR MENSAJEROS INTRACELULARES

Cuando iniciamos el estudio de la regulación del transporte de glicina en la glía de Müller, se disponía de muy poca información sobre los aspectos reguladores de los sistemas de transporte de neurotransmisores. Se sabía que el ácido araquidónico, liberado por la fosfolipasa A_2, inhibe varios sistemas de transporte dependientes de Na^+, incluyendo los de glicina (Zafra et al., 1990) y los de glutamato (Barbour, 1989). Asimismo se había estudiado la modulación de los transportadores de glutamato (Casado et al., 1993) y glicina (Sato, et al., 1995) por la PKC, así como la regulación por AMPc de los transportadores de GABA (Gomeza et al., 1991).

Con el fin de determinar la vía de segundos mensajeros involucrada en la regulación del transporte de glicina en la glía de Müller, se estudió el efecto de diversos fármacos, tanto inhibidores como activadores de distintas vías, sobre la captura de glicina. Se probaron los activadores de la PKC, PMA y DOG, así como los inhibidores estaurosporina, H7 y queleritrina (Tabla 2). Ninguna de estas drogas tuvo efecto sobre el transporte de glicina.

Asimismo, se estudió la participación del AMP cíclico en este proceso. La figura 6 muestra el efecto de agentes que incrementan directamente los niveles intracelulares de AMPc, la forskolina y la toxina colérica, así como del 8-Br-cAMP. Ninguna de estas drogas tuvo efecto sobre el transporte de glicina. Se probaron entonces los inhibidores de la adenilato ciclasa (AC) SQ-22536 y MDL-12330A. Sólo este último mostró un efecto inhibidor sobre el transporte (40% de inhibición con respecto al control). La figura 7 muestra la captura de glicina en presencia de concentraciones crecientes de MDL-12330A y el efecto inhibidor parece ser dependiente de la dosis con una IC_{50} de 50 μM.

Tabla 2. Efecto de drogas relacionadas a vías intracelulares de señalamiento sobre el transporte de glicina en la glía de Müller de la retina[1]

Compuesto	Concentración	t (min) incubación	Captura de Glicina (% c/r al control)
Forskolina	7.5 μM	30 y 60 min	99 ± 4
8-Br-cAMP	1 mM	30 min	98 ± 4
8-Br-cGMP	1 mM	30 y 60 min	100 ± 5
SQ-22536	100 μM	30 y 60 min	99± 6
HA-1004	100 μM	60 min	98 ± 5
Toxina Colérica	1μg/ml	30 y 60 min	100 ± 3
Toxina Pertusis	1μg/ml	30 y 60 min	101 ± 6
PMA	0.5 μM	30 y 60 min	100 ± 8
DOG	100 μg/ml	30 y 60 min	101 ± 4
Queleritrina	1 μM	30 y 60 min	100 ± 4
Estaurosporina	100 nM	45 y 60 min	97 ± 5
H-7	50 μM	30 min	100 ± 6
Polimixina B	400 μM	30 min	99 ± 6
Neomicina	110 μM	30 min	99 ± 8
Mepacrina	50 μM	20 min	98 ± 8
Acido Okadaico	100 nM	30 y 60 min	98 ± 7
Deltametrina	20 μM	30 y 60 min	98 ± 7
Ciclosporina A	50 nM-20 μM	30 y 60 min	99 ± 6
Tautomicina	300 nM-1μM	30 y 60 min	97 ±8
Caliculina A	1 μM	60 min	100 ± 3
ML-9	5 μM-200 μM	60 min	100 ± 4
Wortmanina	1 μM	30 y 60 min	101 ± 7
Genisteina	50 μM	60 min	100 ± 5

[1]Las células de Müller se preincubaron con los compuestos indicados (ver apéndice para efectos farmacológicos de los compuestos). Los ensayos de captura se llevaron a cabo como se describe en Materiales y Métodos, en presencia de glicina 1mM. HA-1004, hidrocloruro de N-(2-Guanidinoetil)-5-isoquinolinasulfonamida; SQ-22536, 9-(Tetrahidro-2-furanil)-9H-purin-6-amina; PMA, Forbol 12-myristato 13-acetato; DOG, 1,2-Dioctanoil-rac-glicerol; H-7, dihidrocloruro de 1-(5-Isoquinolinasulfonil)-2-metilpiperazina; ML-9, 1-(5-Chloronaftaleno-1-sulfonil)-1H-hexahidro-1,4-diazepina. Los valores se expresan como el porcentaje del transporte de glicina con respecto al control y son la media de al menos tres experimentos independientes por triplicado ± E.S. No se observaron diferencias significativas con respecto al control.

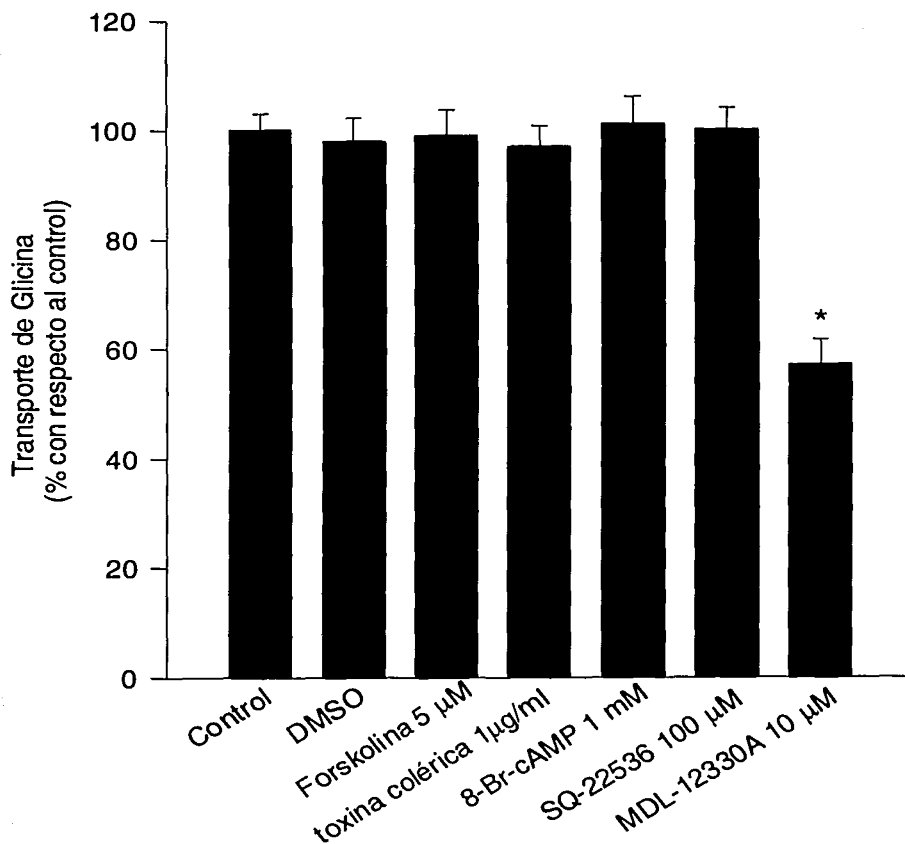


Figura 6. Participación del AMPc en la regulación del transporte de glicina. Las células de Müller se preincubaron durante 20 minutos en presencia de los compuestos indicados en KRB. Ya que el MDL-12330A se disolvió en DMSO, se añadió un control con 10 µl de DMSO en 0.5 ml de KRB. El ensayo de transporte se llevó acabo durante 10 minutos en medio KRB que contenía glicina 1 mM ([^{3}H]-Gly/Gly 1:25000). Los valores se expresan como la media ± E.S. de tres experimentos por triplicado. * Significativamente distinto del control (p<0.001, prueba "t" de Student).

TESIS CON
FALLA DE ORIGEN

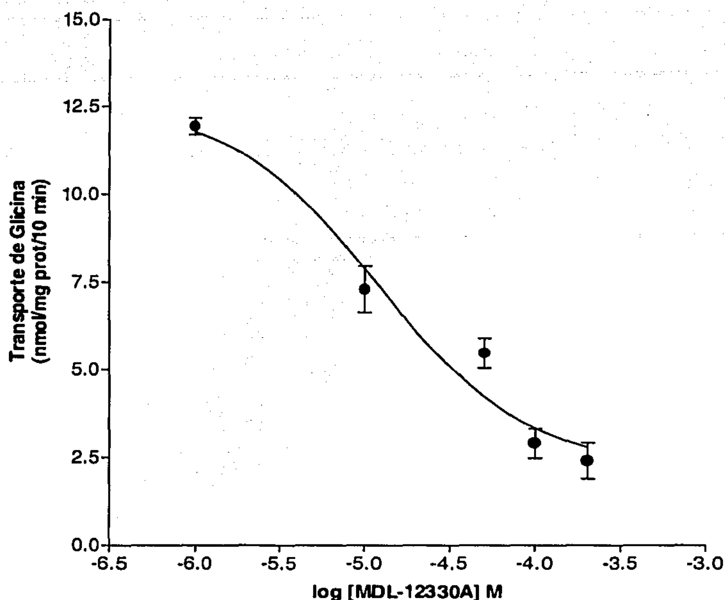


Figura 7. Curva dosis-respuesta del transporte de glicina en presencia de distintas concnetraciones de MDL-12330A. Los cultivos se preincubaron durante 20 minutos en presencia de MDL-12330A 1, 10, 50, 100 y 200 μM. El ensayo de transporte se llevó a cabo durante 10 minutos en medio KRB que contenía glicina 1 mM ([^{3}H]-Gly/Gly 1:25000). Los valores se expresan como la media ± E.S. de tres experimentos por triplicado.

Con el fin de determinar la especificidad de este efecto, se midió la captura de otros aminoácidos en presencia de forskolina, SQ-22536 y MDL-12330A. Tanto la captura de leucina como la captura de D-aspartato se inhibieron con MDL-12330A 10 μM (60% y 80%, respectivamente), mientras que la forskolina y el SQ-22536 no tuvieron efecto (figura 8).

TESIS CON
FALLA DE ORIGEN

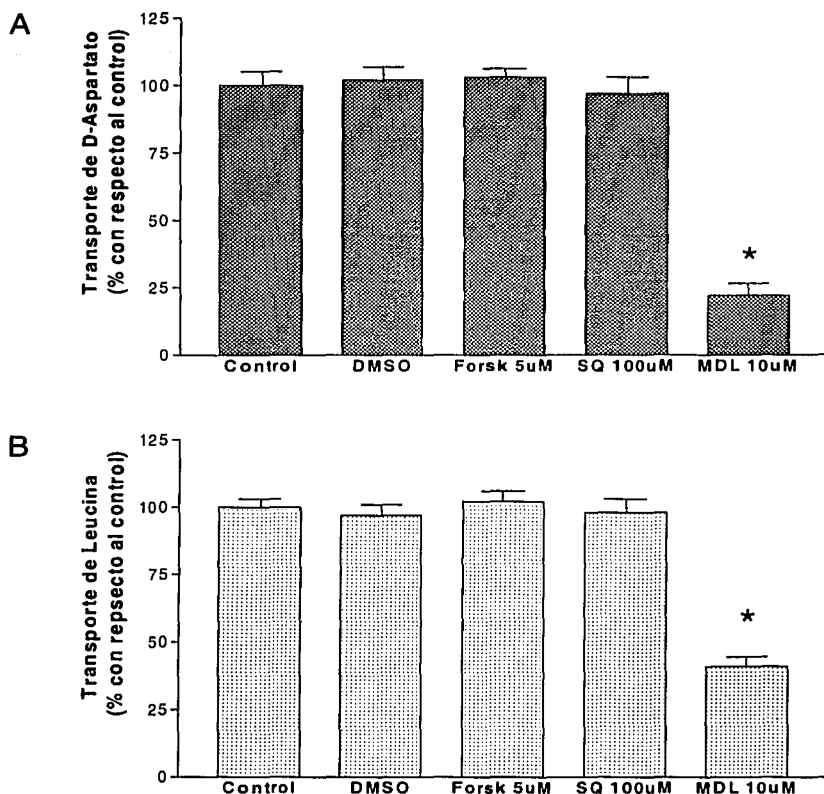
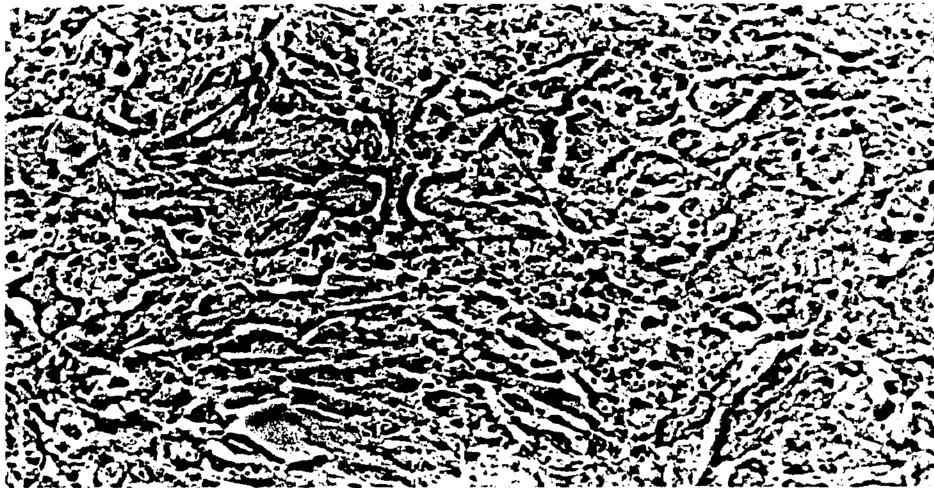


Figura 8. Efecto del MDL-12330A sobre el transporte de Leucina y D-Aspartato. Las células de Müller se preincubaron durante 20 minutos en presencia de los compuestos indicados en KRB. Ya que el MDL-12330A se disolvió en DMSO, se añadió un control con 10 µl de DMSO en 0.5 ml of KRB. Los ensayos de transporte se llevaron acabo durante 10 minutos en KRB que contenía [A] D-Aspartato ([^{3}H]-D-Asp/D-Asp 1:50) 250 nM ó [B] Leucina ([^{3}H]-Leu/Leu 1:25000) 1 mM. Los valores se expresan como la media ± E.S. de tres experimentos por triplicado.
*Significativamente distinto del control ($p<0.001$, prueba "t" de Student).

TESIS CON
FALLA DE ORIGEN

A



B

Figura 9. Efecto del MDL-12330A sobre la viabilidad celular. [A] cultivo control de células de Müller. [B] cultivo de glía de Müller después del tratamiento con MDL-12330A 50 μM, 40 minutos. El porcentaje de viabilidad celular se determinó por medio de la técnica de exclusión de azul tripano.

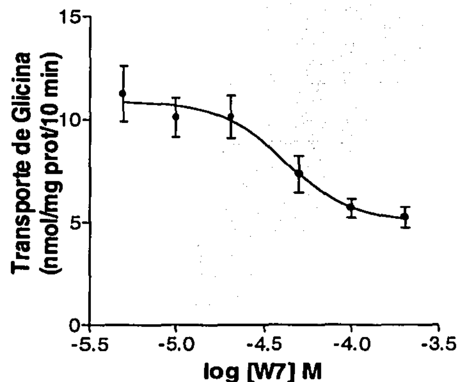
TESIS CON
FALLA DE ORIGEN

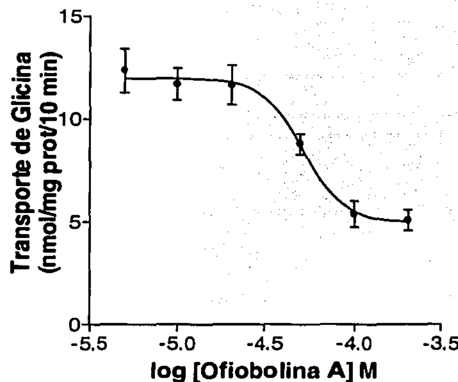
Ya que el efecto del MDL-12330A resultó inespecífico para el transporte de aminoácidos, se cuantificó la viabilidad celular después del tratamiento con este compuesto (50 μM) por medio de la técnica de exclusión de azul tripano. A los 20 minutos, el efecto inhibidor del MDL-12330A no se acompaña de un cambio en la viabilidad celular; sin embargo, a los 40 minutos de incubación, la inhibición que ejerce la droga está asociada a una disminución considerable en la viabilidad celular (70% con respecto al control), lo cual demuestra claramente un efecto tóxico de este compuesto (figura 9), independiente de la inhibición de la AC ya que el tratamiento con SQ-22536 (100 μM) durante 20 ó 40 minutos no afectó la viabilidad celular (estos datos no se muestran).

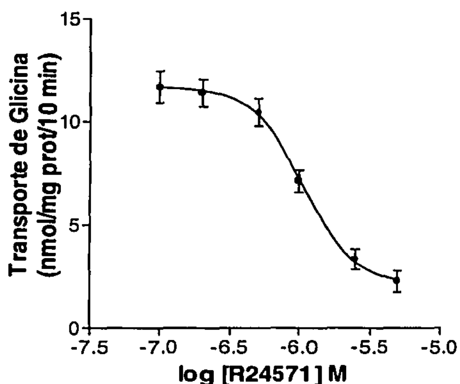
Regulación del Transporte de Glicina por Calmodulina

Se estudió entonces la posible participación de la calmodulina (CaM) y enzimas dependientes de Ca^{2+}/CaM en la regulación del transporte de glicina. La trifluoperazina (TFP; fenotiazina antipsicótica; Weiss et al., 1980), el W7 (N-(6-aminohexil)-5-cloro-1-naftalen sulfonamida; Kanamori et al., 1981), el R24571 (o calmidazolium, derivado del antimicótico miconazol; Gietzen et al., 1981) y la ofiobolina A (fitotoxina fungal; Leung et al., 1984) inhiben reacciones enzimáticas y procesos biológicos estimulados por Ca^{2+}/calmodulina. Todas estas drogas anticalmodulínicas inhibieron el transporte de glicina. La figura 10 muestra las curvas dosis-respuesta para el W-7 (inhibición máxima 40%), la ofiobolina A (inhibición máxima 45%), la TFP (inhibición máxima 80%) y el R-24571 (inhibición máxima 82%). Las IC50s calculadas en cada caso fueron 40 ± 2.5 μM, 50 ± 1.2 μM, 2.5 ± 0.5 μM y 1 ± 0.2 μM, respectivamente.

Se ha visto que las drogas anticalmodulínicas pueden tener efectos inespecíficos e interactuar con otras proteínas de manera independiente de la calmodulina, como es el caso de los receptores de dopamina D2 (Wilson et al., 1998) y los receptores de NMDA (Lidsky et al., 1997). Con el fin de determinar si el efecto inhibidor observado sobre el transporte de glicina es específico, se estudió el efecto de estos compuestos sobre el sistema de transporte dependiente de Na^+ para







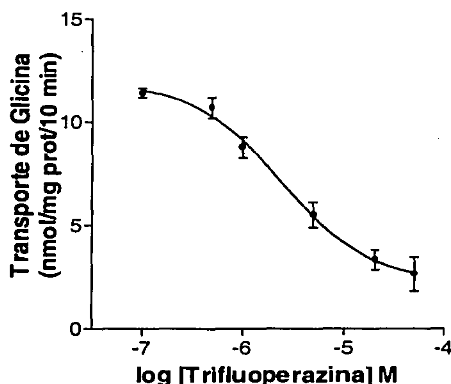


Figura 10. Papel de la CaM en la regulación del transporte. Las células de Müller se incubaron con concentraciones crecientes de W7 (inhibición máxima 40%, IC50 40 ± 2.5 μM), ofiobolina A (inhibición máxima 45%, IC50 50 ± 1.2 μM), R24571 (inhibición máxima 82%, IC50 1 ± 0.2 μM) y Trifluoperazina (inhibición máxima 80%, IC50 2.5 ± 0.5 μM) durante 20 minutos en medio KRB. El ensayo de transporte se llevó a cabo durante 10 minutos en medio KRB que contenía glicina 1 mM ([^{3}H]-Gly/Gly 1:25000). Los valores se expresan como la media ± E.S. de tres experimentos por triplicado. Las IC50s se calcularon en cada caso con el programa INPLOT (versión 3.1) de Graph PAD.

el GABA y sobre el sistema de transporte de leucina, el cual no depende de Na^+. No se encontró modificación alguna de estos compuestos sobre los sistemas de transporte de GABA o leucina (Tabla 3).

TESIS CON
FALLA DE ORIGEN

Tabla 3. Efecto de los inhibidores de la calmodulina y la CaMKII sobre el transporte de leucina y GABA en las células de Müller y sobre el transporte de glicina en neuronas de la retina[1]

Control	R24571	TFP	W7	KN62
Transporte de Leucina (% c/r al control)	100 ± 8.9	71.6 ± 14.1	81.3 ± 10.0	83.5 ± 8.7
Transporte de GABA (% c/r al control)	100 ± 5.4	87.0 ± 12.8	97.2 ± 7.4	99.1 ± 4.8
Transporte de Gly en neuronas (% c/r al control)	100 ± 17	95.9 ± 22	86.1 ± 16	96.8 ± 15

[1]Las células de Müller se trataron con R-24571 5 μM, trifluoperazina (TFP) 25 μM, W7 10 μM, ó KN62 20 μM durante 20 min. El ensayo de transporte de se llevó a cabo durante 10 minutos in KRB que contenía 1 mM Leucina, o 1 mM GABA. Los cultivos de neuronas se trataron con estos mismos compuestos durante 20 min, el ensayo de transporte de glicina se llevó a cabo en presencia de glicina 1mM durante 10 min. Los valores se expresan como porcentaje con respecto al control ± ES de tres experimentos independientes por triplicado. *Significativamente distinto del control ($p < 0.001$, Student "t" test).

La recaptura de glicina se lleva acabo tanto por las células gliales, como por las neuronas, por esta razón se evaluó la participación de la calmodulina en la regulación del transporte de glicina en cultivos enriquecidos de neuronas de la retina. Como se muestra en la Tabla 3, el tratamiento con los inhibidores de la calmodulina no tuvo efecto alguno sobre el transporte de glicina neuronal.

Papel del Ca^{2+} en la Regulación del Transporte de Glicina

Ya que la calmodulina depende de Ca^{2+} para activarse, se estudió el papel del calcio en la regulación del transporte. Para este propósito, se probó el efecto del dantroleno, bloqueador de la liberación de calcio de las pozas intracelulares, del EGTA, quelante de calcio extracelular, del BAPTA-AM, quelante de calcio

intracelular y de la tapsigargina, inhibidor de las ATPasas de Ca^{2+} del retículo endoplásmico. Como se muestra en la figura 11, el BAPTA-AM inhibió el transporte de glicina 40%.

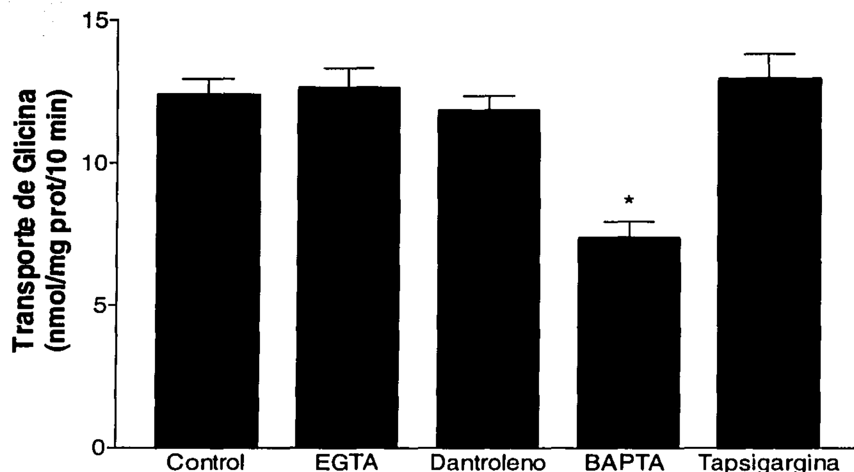


Figura 11. Papel del calcio en la regulación del transporte de glicina. Las células de Müller se incubaron en medio KRB, Ca^{2+} nominal cero, y EGTA 1mM o BAPTA-AM 10 μM más EGTA 0.5 mM durante 30 min. El dantroleno (30 mM) y la tapsigargina (2 mM) se probaron en KRB normal durante 30 min. El ensayo de transporte se llevó a cabo durante 10 minutos en estos mismos medios en presencia de glicina 1 mM ([^{3}H]-Gly/Gly 1:25000). Los valores se expresan como la media ± E.S. de tres experimentos por triplicado.

Con el fin de producir un incremento en la concentración intracelular de Ca^{2+} y, de esta manera, estimular a la calmodulina, se incubó a las células en presencia de los ionóforos A23187 y ionomicina (figura 12). El efecto de los ionóforos sobre el transporte fue contrario al esperado, pues el influjo de Ca^{2+} inhibió de manera considerable el transporte de glicina (60% y 78%, respectivamente).

TESIS CON
FALLA DE ORIGEN

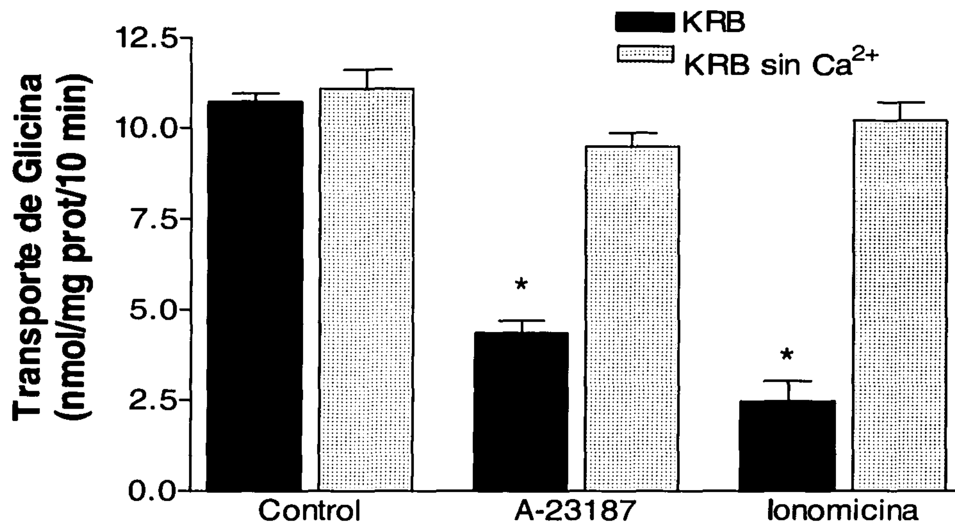


Figura 12. Efecto de ionóforos de calcio A23187 y ionomicina sobre el transporte de glicina. Los cultivos se incubaron con ionomicina ó A23187 10 μM durante 15 min en KRB normal (2.5 mM $CaCl_2$; barras negras) ó en KRB sin Ca^{2+} (Ca^{2+} nominal cero; barras punteadas). Este medio se eliminó y el ensayo de transporte se llevó a cabo durante 10 minutos en medio KRB que contenía glicina 1 mM ([^{3}H]-Gly/Gly 1:25000). Los valores se expresan como la media ± E.S. de tres experimentos por triplicado. * Significativamente distinto del control ($p<0.001$, prueba "t" de Student).

En la glía de Müller se ha demostrado que el alto K^+, el glutamato, el carbacol y el ATP así como la cafeína elevan las concentraciones intracelulares de Ca^{2+} (Keirstead y Miller, 1995; 1997; Newman y Zahs, 1997; Wakakura y Yamamoto; 1994). Para explorar el efecto de un incremento moderado en la concentración de Ca^{2+} intracelular sobre el transporte de glicina, se incubó a las células en presencia de cafeína 5 mM, carbacol 2 mM ó ATP 1mM. Como se muestra en la Tabla 4, la cafeína, el carbacol y el ATP incrementaron el transporte de glicina 50%, 65% y 30% respectivamente. La estimulación del transporte de glicina inducida por cafeína se abatió incubando previamente con tapsigargina 2

TESIS CON
FALLA DE ORIGEN

Tabla 4. Efecto de agentes que inducen ondas de Ca^{2+} en la glía sobre el transporte de glicina en la glía de Müller[1]

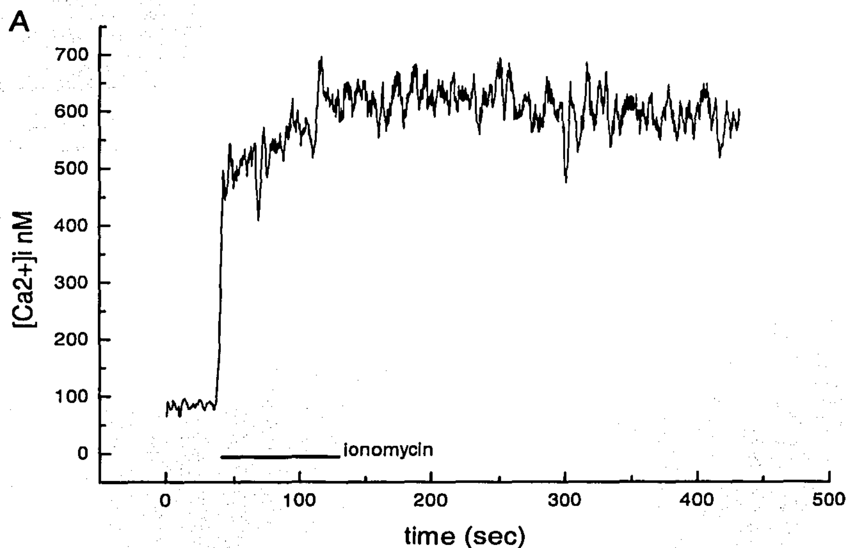
	Transporte de Glicina (% c/r al control)
Control	100 ± 8
Cafeína	150 ± 14*
Tapsi + Cafeína	101 ± 10**
ATP	165 ± 28*
Carbacol	130 ± 7*
Glu	106 ± 9
NMDA	93 ± 10
AMPA	102 ± 9
KA	104 ± 13
L-AP4	98 ± 8
t-ACPD	95 ± 14

[1]Las células de Müller se preincubaron con cafeína 5 mM, ATP 1 mM o carbacol 2 mM durante 15 min en KRB sin Ca^{2+} (Ca^{2+} nominal cero) más EGTA 0.5 mM. Cuando se probaron juntas, la cafeína 5 mM se añadió 15 min después de la preincubación con la tapsigargina 2μM en KRB sin Ca^{2+} más EGTA 0.5 mM. En otro grupo de experimentos, Las células de Müller se preincubaron durante 1hr con 1mM de glutamato (Glu), N-metil-D-aspartato (NMDA), ácido alfa-amino-3-hidroxi-5-metilisoxazol-4-propiónico (AMPA), kainato (KA), ácido L(+)-2-amino-4-fosfonobutírico (L-AP4), o ácido trans-aminociclopentano-1,3-dicarboxílico (t-ACPD) durante 1 hr en KRB. Los ensayos de captura se llevaron a cabo como se describe en Materiales y Métodos, en presencia de glicina 1mM. Los valores se expresan como el porcentaje del transporte de glicina con respecto al control y son la media de al menos tres experimentos independientes por triplicado ± E.S. *Significativamente distinto del control ($p < 0.005$, prueba "t" de Student). **Significativamente distinto de la cafeína ($p < 0.005$, prueba "t" de Student).

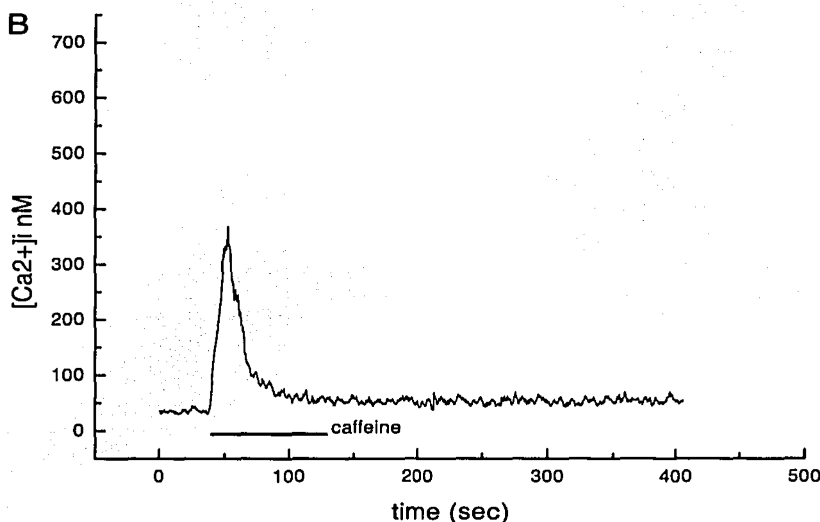
μM. También se examinó la posible regulación del transporte de glicina a través de la activación de receptores a glutamato. Ni el glutamato, ni los agonistas NMDA, KA, AMPA, L-AP4 y t-ACPD modificaron el transporte de glicina (Tabla 4).

El efecto opuesto de la cafeína y la ionomicina sobre el transporte de glicina podría deberse a diferencias en la magnitud del incremento en la concentración intracelular de Ca^{2+} ($[Ca^{2+}]i$) que inducen estos agentes. Con el fin de comparar el incremento en la $[Ca^{2+}]i$ inducido por la ionomicina con el de la cafeína, se

TESIS CON
FALLA DE ORIGEN



Ionomicina
703 ± 55



Cafeína
315 ± 23 nM

Figura 13. Incremento en la $[Ca^{2+}]_i$ inducido por ionomicina y cafeína en la glía de Müller. Las mediciones de Ca^{2+} intracelular se llevaron a cabo como se describe en Materiales y Métodos. Se muestran las respuestas de 2 células representativas. A) incremento en el Ca^{2+} intracelular inducido por ionomicina 10 μM en una célula de Müller, B) incremento en el Ca^{2+} intracelular inducido por cafeína 5 mM en una célula de Müller. Los recuadros muestran los valores promedio de amplitud máxima inducida por ionomicina y por cafeína. Los datos representan la respuesta promedio ± E.S. de 60 células tratadas con ionomicina y de 62 células (de 155) que respondieron a cafeína.

TESIS CON
FALLA DE ORIGEN

llevaron a cabo mediciones fluorométricas utilizando como indicador de Ca^{2+} al fura-2/AM. Como se muestra en la figura 13, la ionomicina produjo un incremento pronunciado y sostenido en la concentración intracelular de Ca^{2+} en todas las células examinadas ($n = 60$). La cafeína resultó menos consistente en elevar el Ca^{2+} intracelular produciendo un incremento más pequeño y transitorio en 45% de las células examinadas ($n = 155$). La amplitud máxima del incremento intracelular de Ca^{2+} producido por la ionomicina fue 703 ± 55 nM, mientras que para la cafeína fue 315 ± 23 nM (figura 13).

El Ca^{2+} es un mensajero intracelular que participa en diversos procesos celulares tanto fisiológicos como patológicos, de manera que la entrada masiva de Ca^{2+} a través de un ionóforo podría, además de activar a la calmodulina, producir otras respuestas celulares relacionadas a condiciones patológicas. En este sentido, se ha demostrado que el Ca^{2+} regula el citoesqueleto de las células gliales activando su proteólisis por medio de proteasas dependientes de Ca^{2+} (Finkbeiner, 1993). También en células gliales, se ha demostrado que la elevación en la $[Ca^{2+}]_i$ asociada a patologías específicas del cerebro activa a la calpaína (proteasa de cisteínas dependiente de Ca^{2+}; Du et al., 1999; Shields y Banik, 1999). Por esta razón estudiamos la participación de proteasas en el efecto producido por los ionóforos. Como se muestra en la figura 14, la preincubación de las células de Müller con los inhibidores permeables de la calpaína III y V, disminuye 30% y 37%, respectivamente, la inhibición del transporte inducida por ionomicina, mientras que el inhibidor de proteasas de serina, fenilmetilsulfonilfluoruro, no tiene efecto sobre el transporte.

Regulación del Transporte de Glicina por el Citoesqueleto

En las células gliales se han descrito varios procesos mediados por calpaína como la proteólisis de los componentes del citoesqueleto (Finkbeiner, 1993). La actina, así como la fodrina (espectrina) y la anquirina, proteínas del citoesqueleto que se unen a la actina, son los principales sustratos de la calpaína (Villa et al., 1998). La anquirina y la fodrina poseen sitios de unión de alta afinidad para

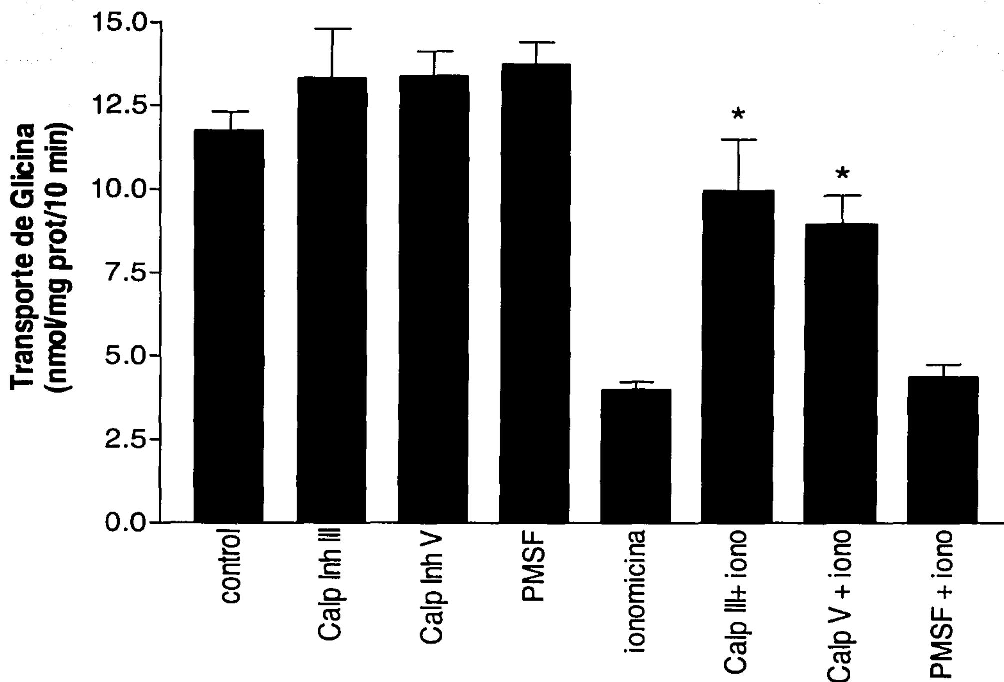


Figura 14. El efecto inhibidor de la ionomicina se revierte por los inhibidores de la calpaína. Los cultivos se preincubaron durante 90 min con los inhibidores de la calpaína III y V 50 µM ó con el PMSF 0.5 mM. Posteriormente se añadió la ionomicina al medio (10 µM, 15 min). El ensayo de transporte se llevó a cabo durante 10 minutos en este mismo medio en presencia de glicina 1 mM ([^{3}H]-Gly/Gly 1:25000). Los valores se expresan como la media ± E.S. de tres experimentos por triplicado. * Significativamente distinto de la ionomicina ($p<0.001$, prueba "t" de Student).

TESIS CON
FALLA DE ORIGEN

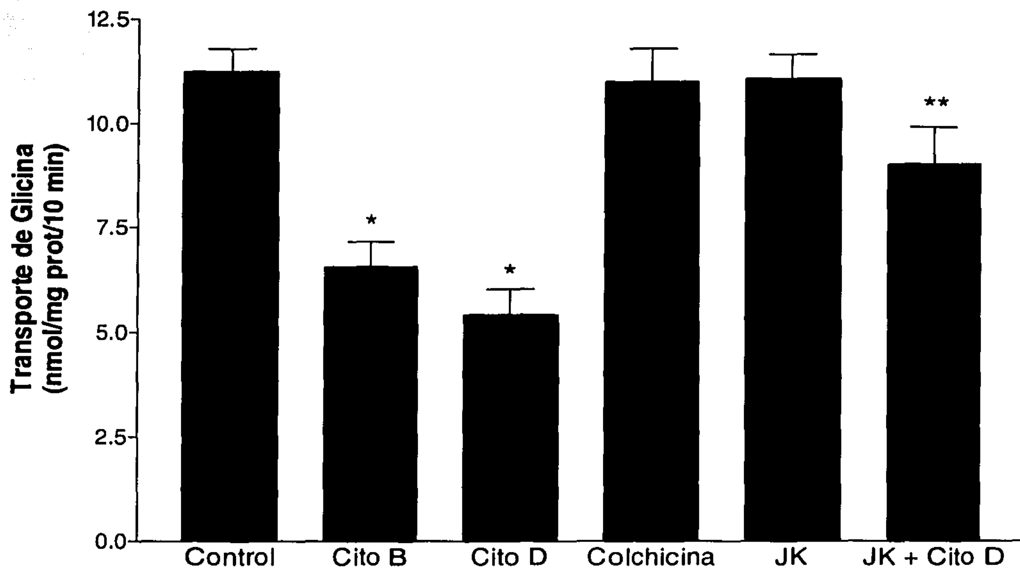


Figura 15. El citoesqueleto de actina regula el transporte de glicina en la glía de Müller. Los cultivos se incubaron 2 hrs con citocalasina B 10 μM, citocalasina D 10 μM, colchicina 25 μM o 1 hr con jasplakinolida (JK) 1 μM. Cuando se probaron juntos, la jasplakinolida se añadió 30 minutos antes de la citocalasina D. Los valores se representan como la media ± E.S. de tres experimentos por triplicado. * Significativamente distinto del control ($p<0.001$, prueba "t" de Student). **Significativamente distinto de la citocalasina D ($p<0.001$, prueba "t" de Student).

proteínas integrales de membrana y regulan diversas funciones de las proteínas membranales (Beck y Nelson, 1996; Mills y Mandel, 1994), entre ellas el transporte a través de la membrana (Handlogten et al., 1996; Nelson y Hammerton, 1989; Zharikov y Block, 2000). El tratamiento de las células de Müller con citocalasina B o D (10 μM), alcaloides que promueven la despolimerización de la actina al inhibir el crecimiento de filamentos nuevos (Cooper, 1987), tuvo un efecto inhibidor sobre el transporte de glicina (41% y 50%, respectivamente; figura 15). El pretratamiento de los cultivos con la jasplaquinolida, péptido permeable que estabiliza a la actina (1 μM; Bubb et al., 1994; figura 15), disminuyó de manera significativa el efecto de

TESIS CON
FALLA DE ORIGEN

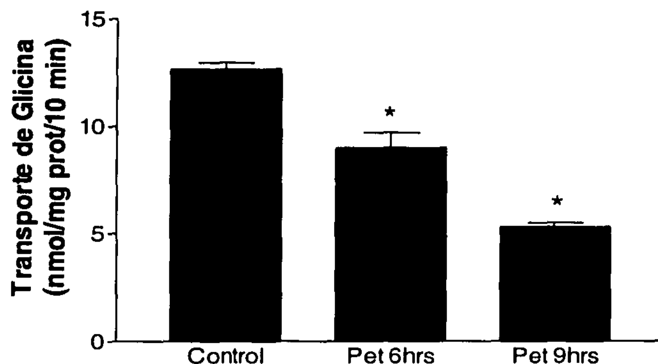


Figura 16. La degradación de la fodrina inhibe el transporte de glicina. Las células de Müller se preincubaron con la toxina Pet (600 mg/ml) a los distintos tiempos en medio el medio de cultivo D-MEM. Este medio se eliminó y el ensayo de transporte se llevó a cabo durante 10 minutos en medio KRB que contenía glicina 1 mM ([^{3}H]-Gly/Gly 1:25000). Los valores se expresan como la media ± E.S. de tres experimentos por triplicado. *Significativamente distinto del control ($p<0.001$, prueba "t" de Student).

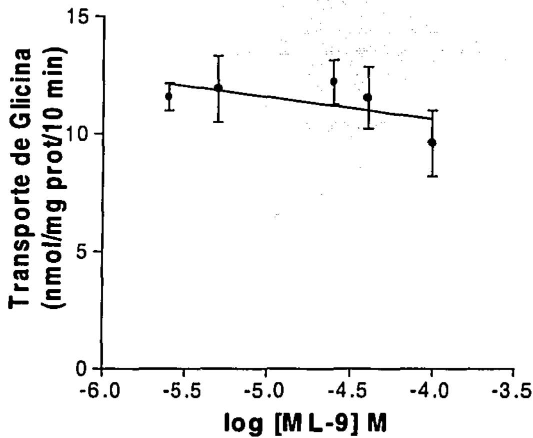
la citocalasina D. La colchicina (25 μM), agente que despolimeriza los microtúbulos, no modificó el transporte de glicina.

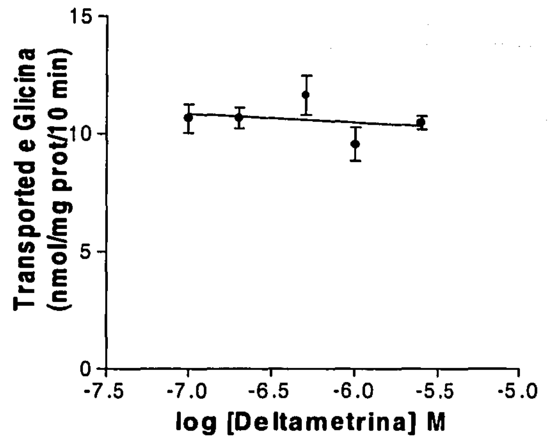
Como ya se mencionó, uno de los principales sustratos de la calpaína es la fodrina. Recientemente se encontró que una toxina aislada de Escherichia coli, denominada Pet, degrada específicamente a la fodrina (Villaseca et al., 2000). Con el fin de determinar si el efecto inhibidor de la calpaína sobre el transporte de glicina podría involucrar a la fodrina, se trató a las células de Müller con la toxina Pet. Como se muestra en la figura 16, este tratamiento inhibió el transporte de glicina 60%.

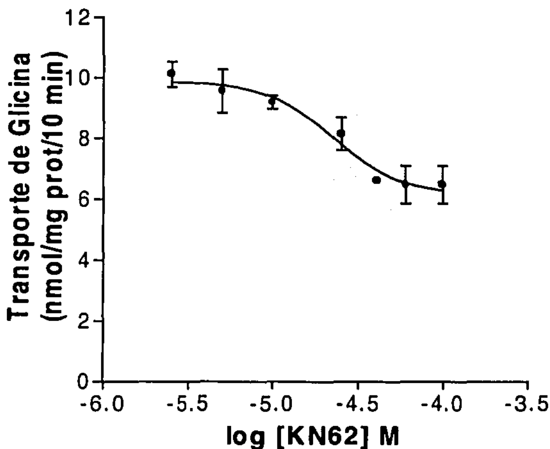
Regulación del Transporte de Glicina por la CaMKII

La calmodulina es una proteína intermediaria que acopla las señales de Ca^{2+} a respuestas bioquímicas intracelulares por medio de la activación de una gran variedad de enzimas. Entre las cinasa y fosfatasas reguladas por Ca^{2+}/calmodulina, la CaMKII (cinasa dependiente de Ca^{2+}/calmodulina II), la MLCK (cinasa de las

TESIS CON
FALLA DE ORIGEN







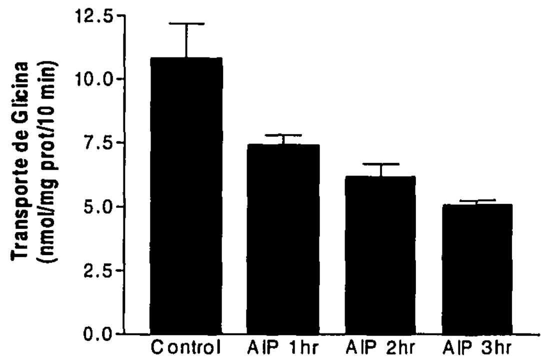
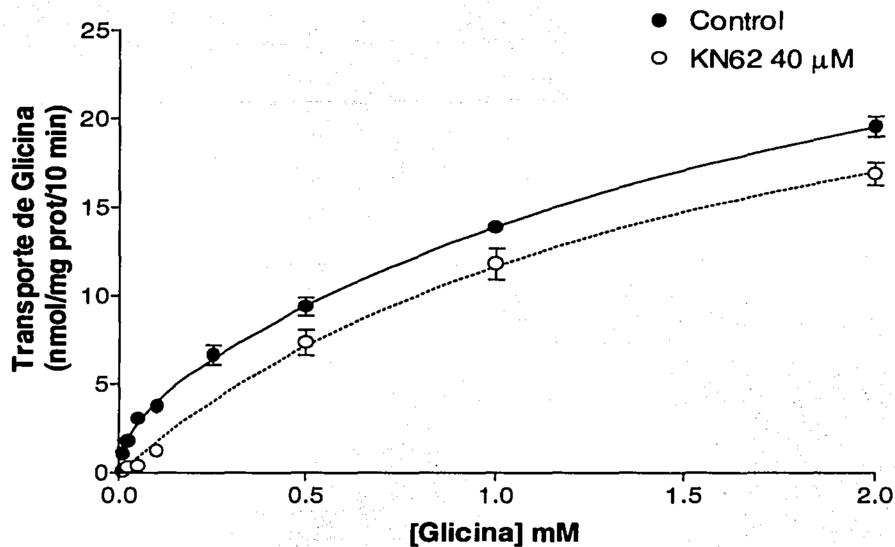


Figura 17. Regulación del transporte de glicina por la CaMKII. A) Las células de Müller se preincubaron con el inhibidor de la MLCK, ML-9 a distintas concentraciones (5, 10, 25, 50 y 100 µM) durante 20 minutos en medio KRB. B) Los cultivos de glía se trataron con el inhibidor de la CaN, deltametrina a distintas concentraciones (0.1, 0.2, 0.5, 1 y 25 µM) durante 20 min en medio KRB. C) La curva dosis respuesta con el inhibidor de la CaMKII se llevó a cabo con distintas concentraciones de KN62 (5, 10, 20, 40, 60, 100 y 200 µM; inhibición máxima 40%, IC50 20 ± 1.3 µM) preincubando 20 min en KRB. D) Los cultivos se incubaron con el péptido inhibidor de la CaMKII AIP (50 µM) a los distintos tiempos. Los ensayos de transporte se llevaron a cabo durante 10 minutos en medio KRB que contenía glicina 1 mM ([^{3}H]-Gly/Gly 1:25000). Los valores se expresan como la media ± E.S. de tres experimentos por triplicado.

TESIS CON
FALLA DE ORIGEN

cadenas ligeras de miosina) y la CaN (calcineurina ó proteína fosfatasa 2B), se expresan en las células gliales y se ha visto que interactúan con el citoesqueleto (Takeuchi et al., 2000; Vallano et al., 2000; Cotrina et al., 1998; Edelman et al., 1992; Matsuda et al., 1998; Vinade et al., 1997). El tratamiento de las células de Müller con los inhibidores de la CaN, deltametrina (Tabla 2) y ciclosporina A (figura 17a), no modificó el transporte de glicina, como tampoco lo hizo el tratamiento con el inhibidor de la MLCK, ML-9 (figura 17b). Sin embargo, el tratamiento con KN-62, inhibidor de la CaMKII, tuvo un efecto inhibidor sobre el transporte de glicina. Como se muestra el la curva dosis respuesta para el KN-62 (figura 17c), la inhibición máxima del transporte fue de 40% y la IC50 calculada 20 ± 1.3 μM. Se ha reportado que el KN62 inhibe también a la CaMKIV (cinasa dependiente de Ca^{2+}/calmodulina IV), por esta razón se probó el efecto del AIP, péptido miristoilado que corresponde al dominio de autofosforilación de la CaMKII, y que por lo tanto inhibe a esta enzima de manera altamente específica (Ishida et al., 1995). Como se muestra en la figura 17d, el AIP tuvo un efecto inhibidor sobre el transporte similar al del KN62.

Ya que el transporte de glicina en la glía de Müller se lleva a cabo por dos sistemas, uno de alta afinidad que corresponde a GLYT1, y otro de baja afinidad, se estudió el efecto del KN-62 sobre la cinética del transporte. Como se muestra en la figura 19, el tratamiento con KN62 inactiva por completo al transporte de alta afinidad llevado acabo por GLYT1, mientras que el transporte de baja afinidad no se ve afectado. Hasta la fecha no se han reportado secuencias consenso de fosforilación para la CaMKII en los transportadores de glicina (Liu et al., 1993). Se esperaría que la fosforilación directa de los transportadores produjera un cambio en la afinidad sin afectar la velocidad máxima, reflejo del número de transportadores en la membrana, no obstante la inhibición de la CaMKII elimina al transporte de alta afinidad. Estos resultados sugieren que la regulación del transporte por la



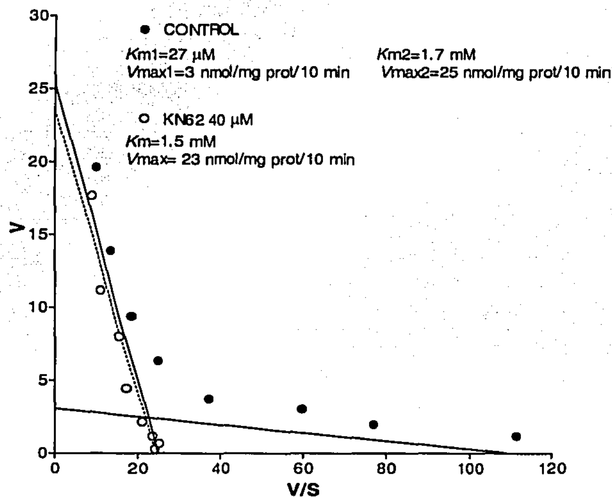


Figura 18. La inhibición de la CaMKII abate la actividad de GLYT1. El efecto del KN62 sobre la cinética del transporte se estudió incubando los cultivos de glía de Müller durante 10 minutos con distintas concentraciones de glicina (0.01-2 mM) ([^{3}H]-Gly/Gly 1:25000), y en ausencia (control, •) o en presencia de KN62 40 μM, 20 min (o). Los datos se analizaron por el método de Eadie-Hofstee (control = líneas sólidas, KN62 = línea punteda) y los parámetros cinéticos se calcularon con la ayuda del programa Graph Pad (versión 3.1). Los valores son la media de tres experimentos por triplicado.

TESIS CON
FALLA DE ORIGEN

CaMKII involucra un mecanismo indirecto, esto es, la activación de un sistema de señalamiento que subsecuentemente modula el transporte de glicina, o la fosforilación de proteínas del citoesqueleto involucradas en el tráfico o en la localización en la membrana de los transportadores de glicina.

DISCUSIÓN

CARACTERIZACIÓN DEL TRANSPORTE DE GLICINA

Las células de Müller de la retina forman parte de la glía radial y atraviesan todo el espesor de la retina. En la retina de las aves, las células de Müller son el único tipo de gliocito presente, por lo que llevan a cabo numerosas funciones que, en otras regiones del sistema nervioso, desempeñan diversos tipos de células gliales. Estudios previos demostraron que las células de Müller, además de expresar canales sensibles al voltaje y receptores de neurotransmisores, a través de los cuales reconocen una gran variedad de señales neuronales, expresan sistemas de transporte de alta afinidad para la recaptura de sustancias neuroactivas como el Glu, el GABA y el K^+.

El primer objetivo de este trabajo fue el de demostrar que la glía de Müller, además de la función ya descrita de regular las concentraciones extracelulares de Glu y GABA, podría regular las concentraciones de glicina a través de transportadores de alta afinidad específicos para este aminoácido, como los descritos en células gliales del sistema nervioso central.

Hemos demostrado por primera vez la presencia de sistemas de transporte de glicina en la Glía de Müller de la retina, a pesar de estudios previos que sugerían que, en la retina, el único tipo celular que presenta transportadores de glicina son las células amacrinas (Zafra et al., 1995).

Encontramos que la captura de glicina en la glía de Müller se lleva a cabo por dos sistemas de transporte, uno de alta afinidad cuyas características corresponden a las de los transportadores GLYT1: dependencia de Na^+ y Cl^-, alta especificidad por la glicina y sensibilidad a la sarcosina; y uno de baja afinidad con características del sistema de transporte A, ya que se inhibe por MeAIB y AIB y depende únicamente de la presencia de Na^+ en el medio externo.

La localización de los sistemas de transporte de aminoácidos en la retina, entre ellos los de glicina, se estudió inicialmente con técnicas de autorradiografía.

Estos estudios sugerían que sólo las células amacrinas y algunas células bipolares acumulaban glicina (Bruun y Ehinger, 1972; Jager y Wassle, 1987; Pourcho y Goebel, 1987). Estos resultados pueden explicarse a la luz de un estudio reciente sobre el transporte de Glu en la glía de Müller. A pesar de que estas células son las principales responsables de la captura de Glu en la retina, la localización autorradiográfica de [^{3}H]Glu en estas células fue posible sólo después de inhibir irreversiblemente a la enzima glutamino sintetasa (Rauen et al., 1998), lo que sugiere que los niveles del aminoácido necesarios para su detección por esta técnica deben rebasar los niveles fisiológicos.

Los estudios sobre la localización celular de los transportadores de glicina utilizando técnicas de inmunocitoquímica, reportaron la presencia de GLYT1 en el soma de las células amacrinas y en la capa plexiforme interna y sostenían que en la retina, a diferencia del sistema nervioso central, GLYT1 no es un transportador glial (Zafra et al., 1995; Pow y Hendrickson, 1999). Sin embargo, en la capa plexiforme interna, las ramificaciones de la glía de Müller son muy abundantes, están en íntima asociación con las sinapsis y forman tres estratos bien definidos (Rasmussen, 1974; Robinson y Dreher, 1990). Ya que el patrón de inmunorreactividad para GLYT1 en la capa plexiforme interna también presenta tres estratos bien definidos (Pow y Hendrickson, 1999), es muy posible que los transportadores de glicina también se localicen en los procesos de la glía de Müller. Experimentos de doble marcaje utilizando simultáneamente marcadores gliales y anticuerpos contra GLYT1 ayudarán a resolver este punto.

En el SNC, estudios de hibridación in situ demostraron que la distribución de GLYT1 se relaciona con las áreas donde predomina la transmisión excitadora y en las que los receptores de NMDA son abundantes (Smith et al., 1992; Zafra et al., 1995). El receptor de NMDA requiere para su activación de la interacción tanto de Glu como de Gly. Recientemente se demostró que los transportadores gliales de glicina GLYT1 juegan un papel importante en mantener la concentración local de glicina por debajo de los niveles de saturación para los receptores de NMDA, de manera que el incremento en la concentración extracelular de glicina es capaz de

potenciar la actividad de estos receptores (Supplisson y Bergman, 1997; Bergeron et al., 1998). En la retina, la localización de los receptores de NMDA predomina en la capa plexiforme interna (Fletcher et al., 2000) por lo que los transportadores de glicina en este tejido también podrían participar en la neurotransmisión excitadora.

El estudio de los transportadores de Gly en el SNC se ha centrado en los transportadores de alta afinidad por estar relacionados con la modulación de la neurotransmisión. Poco se sabe sobre la función que desempeñan los transportadores de baja afinidad en el sistema nervioso, pero se ha propuesto que, además de tener funciones metabólicas, podrían colaborar con los transportadores de alta afinidad en la recaptura del neurotransmisor poco tiempo después de su liberación, cuando las concentraciones son elevadas (Johnston e Iversen, 1971). Como ya se mencionó, el transporte de baja afinidad que aquí caracterizamos corresponde al sistema A, cuyos sustratos principales son la glutamina y aminoácidos N-metilados. Recientemente se demostró que la principal función de este sistema de transporte en el cerebro es la captura de glutamina en las neuronas para la síntesis de glutamato (Chaudhry et al., 2002). A pesar de que el sistema A también se localizó en astrocitos, su función en estas células se desconoce, pues la liberación de glutamina se lleva a cabo a través de otro sistema de transporte astrocítico denominado N (insensible al MeAIB; Chaudhry et al., 1999). En la retina, se ha propuesto que el sistema N no se encuentra en la glía de Müller sino en las células ganglionares por lo que lleva a cabo la captura de glutamina y no su liberación (Gu et al., 2001). Es posible entonces, que el sistema A en la glía de Müller, además de cumplir con funciones metabólicas y de participar en la recaptura de glicina, sea el responsable de la liberación de glutamina.

REGULACIÓN DEL TRANSPORTE DE GLICINA EN LA GLÍA DE MÜLLER

El segundo objetivo de este trabajo fue el de estudiar las vías de señalamiento intracelular involucradas en la regulación del transporte de glicina en

ESTA TESIS NO SALE
DE LA BIBLIOTECA

la glía de Müller. Cuando se inició el estudio sobre la regulación del transporte, se disponía de muy poca información sobre los mecanismos de regulación de los transportadores de neurotransmisores. Se tenía evidencia de que el ácido araquidónico, liberado por la fosfolipasa A_2, inhibía varios sistemas de transporte dependientes de Na^+, incluyendo los de glicina (Zafra et al., 1990) y los de glutamato (Barbour, 1989); también se había estudiado la modulación de los transportadores de glutamato (Casado et al., 1993) y glicina (Sato, et al., 1995) por la PKC, así como la regulación por AMPc de los transportadores de GABA (Gomeza et al., 1991).

Con el fin de determinar la vía de segundos mensajeros involucrada en la regulación del transporte de glicina en la glía de Müller, se estudió el efecto de diversos fármacos, tanto inhibidores como activadores de distintas vías, sobre la captura de glicina. El transporte de glicina en estas células, no está regulado por procesos de fosforilación a través de la PKC. Los cambios en la concentración de AMPc, que modulan la actividad de los transportadores de GABA, tampoco afectan el transporte de glicina en la glía de Müller. El efecto del MDL12330A, inhibidor de la adenilato ciclasa (AC), resultó ser inespecífico y afectar la viabilidad celular por un mecanismo independiente de la inhibición de esta enzima.

Regulación del Transporte de Glicina por Calmodulina

Los resultados de este trabajo demuestran por primera vez que el Ca^{2+} intracelular y la calmodulina (CaM) regulan el sistema de transporte de glicina aquí caracterizado, pues su actividad se estimula por la liberación de Ca^{2+} de las pozas intracelulares y se inhibe por antagonistas específicos de la calmodulina así como por el quelante de Ca^{2+} intracelular BAPTA-AM.

La vía de regulación a través de la CaM es específica para el transporte de glicina, pues otros sistemas de transporte no se modifican con los inhibidores de la CaM. El complejo Ca^{2+}/calmodulina modula la actividad de diversas enzimas incluyendo cinasas, fosfatasas, adenilato ciclasas y fosfodiesterasas (Van Eldik et al., 1982). La calmodulina también regula la actividad de la sintasa del óxido nítrico

(NOS; Abu-Soud et al., 1994; Stuehr, 1999) sin embargo, en nuestro sistema, el inhibidor de la NOS, L-nitroarginina, no afectó el transporte de glicina (Tabla 2). Se ha reportado que los inhibidores de la calmodulina utilizados en este trabajo inhiben fosfodiesterasas dependientes de Ca^{2+}/calmodulina y por lo tanto, incrementan las concentraciones de nucleótidos cíclicos (VanBelle, 1984; VanStaveren et al., 2001). Nuestro trabajo demuestra que el incremento en las concentraciones de cGMP o cAMP no afecta el transporte de glicina. Nuestros resultados también excluyen a las fosfatasas de proteínas 1, 2A y 2B y a la adenilato ciclasa de estar involucradas en la regulación del transporte, ya que ni el ácido okadaico, la deltametrina ó la tautomicina (Tabla 2), ni los activadores o inhibidores de la adenilato ciclasa afectaron el transporte (figura 6).

Ya que la actividad de la CaM se modula por Ca^{2+}, se estudió el papel del mismo en la regulación del transporte.

Papel del Ca^{2+} en la Regulación del Transporte

Estudios recientes han demostrado que las células gliales, tanto en el SNC como en el SNP, responden a la actividad de neuronas adyacentes con elevaciones en el Ca^{2+} intracelular. Neurotransmisores tan diversos como el glutamato, el GABA, la noradrenalina, la acetilcolina, la dopamina y la adenosina están implicados en la activación de la glía (revisado en: Verkhratsky y Kettenmann, 1996).

Se sabe poco sobre los organelos responsables del almacenamiento de Ca^{2+} en las células gliales. El mejor estudiado es el retículo endoplásmico (RE) el cual se ha propuesto que es el principal componente de las pozas intracelulares que llevan a cabo el intercambio rápido de Ca^{2+} con el citoplasma (Blaustein y Golovina, 2001). La acumulación de Ca^{2+} en el RE de la glía involucra ATPasas que, al igual que en otras células, son sensibles a la tapsigargina (Charles et al., 1993; Kirischuk et al., 1995a;b) y al ácido ciclopiazónico (Golovina et al., 1996). El mecanismo principal de liberación de Ca^{2+} de las pozas intracelulares es la activación de receptores a IP_3 (inositol 1,4,5-trifosfato; Berridge, 1993), cuya producción depende

de la activación de la PLC (fosfolipasa C), acoplada a diversos receptores metabotrópicos a través de proteínas G. En la glía de Müller se ha demostrado la presencia de receptores metabotrópicos de acetilcolina (muscarínicos; Wakakura et al., 1998) y de ATP (P2y; Bringmann et al., 2002) acoplados a la síntesis de IP_3, y la consecuente liberación de Ca^{2+} de las pozas intracelulares. Nuestros resultados demuestran que la activación de estos receptores por carbacol y ATP incrementa el transporte de glicina en la glía de Müller.

Otro tipo de receptores involucrados en la liberación de Ca^{2+} de las pozas intracelulares son receptores activados por Ca^{2+}, cuyo antagonista es la ryanodina, y su agonista la cafeína (Meissner, 1994). La activación fisiológica de la liberación de Ca^{2+} inducida por Ca^{2+} (sensible a ryanodina y cafeína) en células gliales, sólo se ha observado en las células de Schwann (Lev-Ram y Ellisman, 1995) y en células de Müller aisladas de la retina de salamandra (Keirstead y Miller, 1995). Nuestros resultados demuestran que la activación de los receptores de ryanodina modula el transporte de glicina a través de la liberación de Ca^{2+} de las pozas intracelulares, dado que la cafeína estimula el transporte y su efecto se impide por la tapsigargina.

En la retina, las ondas de Ca^{2+} propagadas a través de los astrocitos y células de Müller modifican la actividad de las neuronas adyacentes (Newman y Zahs, 1998). La propagación de las ondas de calcio en la glía se atribuyó inicialmente a la generación y difusión de IP_3 y de Ca^{2+} a través de las uniones comunicantes (Sanderson et al., 1994; Leyerbaert et al., 1998). Estudios más recientes demostraron que una vía extracelular, cuyo mensajero es el ATP (Guan et al., 1997; Cotrina et al., 1998; 2000; Wang et al., 2000), independiente de los contactos intercelulares (Hassinger et al., 1996; Guthrie et al., 1999) y del acoplamiento de uniones comunicantes (Guan et al., 1997; John et al., 1999), está involucrada en la propagación de las ondas. En la retina de la rata, la propagación de las ondas de Ca^{2+} involucra mecanismos tanto intracelulares como extracelulares (Newman, 2001): La propagación de las ondas de Ca^{2+} entre astrocitos está mediada por la difusión de mensajeros intracelulares, mientras que entre astrocitos y células de Müller así como entre células de Müller, las ondas de Ca^{2+} se propagan

principalmente por la liberación de ATP y la subsecuente activación de receptores purinérgicos (Pannicke et al., 2000; Li et al., 2001). Nuestros resultados demuestran que, agentes que generan ondas de Ca^{2+} en la glía de Müller en condiciones fisiológicas, tales como el ATP, el carbacol y la cafeína, estimulan el transporte de glicina.

Newman y Zahs (1998) demostraron en su modelo que la estimulación mecánica de las células de Müller y la subsecuente propagación de ondas de Ca^{2+}, induce la liberación de Glu de las células gliales, el cual actúa sobre las interneuronas inhibidoras de la capa plexiforme interna que, a su vez, liberan GABA y Gly, inhibiendo la tasa de disparo de las células ganglionares. Las células amacrinas poseen receptores de AMPA y NMDA, cuya activación por Glu induce la liberación de GABA y Gly de estas células. Nuestros resultados sugieren que las ondas de Ca^{2+} en las células gliales, además de inducir la liberación de sustancias neuroactivas (Newman y Zahs, 1998; Innocenti et al., 2000), podrían estar modulando tanto la activación de los receptores de NMDA de las células amacrinas, como la inhibición de las células ganglionares mediada por la activación de los receptores de Gly, a través de la regulación de la concentración extracelular de este neurotransmisor.

Se tiene evidencia de que el glutamato aumenta la concentración intracelular de Ca^{2+} en células de Müller de conejo y salamandra (Wakakura y Yamamoto, 1994; Keristead y Miller, 1997); sin embargo, en nuestro sistema, los agonistas de los receptores de Glu no tienen efecto alguno sobre el transporte de glicina. Nuestros resultados concuerdan con los de otros grupos, quienes demostraron que la inducción de las ondas de Ca^{2+} en la glía de Müller de la rata está mediada por ATP, carbacol y fenilefrina, pero no por Glu (Malchow y Ramsey, 1999; Newman y Zahs, 1997; Newman, 2001).

Activación de la Calpaína por la Entrada de Ca $^{2+}$ a través de Ionóforos

A diferencia de la cafeína, el incremento del nivel de Ca^{2+} intracelular inducido por la ionomicina y el A23187, inhibe el transporte de glicina. Como

demostramos a través de la medición intracelular del Ca^{2+}, la cafeína induce una elevación transitoria en la $[Ca^{2+}]$ intracelular, que decae en presencia de la droga debido al vaciamiento de las pozas intracelulares, mientras que la ionomicina induce una elevación sostenida y mucho mayor, que persiste aún cuando se ha retirado el ionóforo del medio. Tomando en cuenta la alta afinidad de la calmodulina por el Ca^{2+} (Kd ≈ 100 nM), es de esperarse que el incremento moderado en la concentración intracelular de Ca^{2+} inducido por la cafeína active a la CaM (Braun y Schulman, 1995). Nuestros experimentos con los inhibidores de proteasas demuestran que el influjo masivo de Ca^{2+} inducido por el ionóforo activa a la calpaína, con mucho menor afinidad por el Ca^{2+} que la calmodulina. En el músculo esquelético del pollo se han descrito tres calpaínas: M-calpaína, m-calpaína y μ-calpaína, cuyo requerimiento de Ca^{2+} para alcanzar la mitad de la actividad máxima es de 3.8 mM, 420 μM y 5 μM, respectivamente (Wolfe et al., 1989).

La activación de proteasas por la entrada masiva de Ca^{2+} en la glía de Müller podría estar más relacionada con condiciones patológicas que fisiológicas. En este sentido, la exposición de astrocitos a condiciones isquémicas eleva la concentración intracelular de Ca^{2+}, debido a la activación de canales de Ca^{2+} sensibles al voltaje así como a la liberación de Ca^{2+} de las pozas intracelulares (Verkhratsky y Kettenmann, 1996). Nuestro estudio demuestra que la inhibición del transporte de glicina por el A23187 y la ionomicina es el resultado de la activación de proteasas dependientes de Ca^{2+}. La proteólisis de elementos del citoesqueleto, activada por el influjo de Ca^{2+} se ha observado en el nervio óptico, en la médula espinal y en las células gliales del sistema periférico (Schlaepfer y Zimmerman, 1981). Más aún, después de una lesión en la médula espinal, la gliosis reactiva consecuente (caracterizada por un aumento en la inmunorreactividad a la GFAP) depende de la activación de la calpaína inducida por la entrada de Ca^{2+} a través de canales de Ca^{2+} sensibles al voltaje (Du et al., 1999). En las neuronas de la retina se ha descrito una activación similar de la calpaína en condiciones de isquemia (Sakamoto et al., 2000). En la glía de Müller podría ocurrir una situación similar, ya

que expresan varios tipos de canales de Ca^{2+} sensibles al voltaje (Puro et al., 1996; Bringmann et al., 2000). La inhibición del transporte de glicina por la activación de la calpaína, cuyos sustratos principales son elementos del citoesqueleto, nos sugirió una relación entre el citoesqueleto y la regulación de los transportadores de glicina. Los resultados de los experimentos con las citocalasinas y la toxina Pet demuestran que existe una interacción funcional entre los transportadores de glicina y el citoesqueleto de actina probablemente a través de la fodrina.

Además de la calpaína, la glía expresa varias proteasas activadas por Ca^{2+}, capaces de alterar irreversiblemente el citoesqueleto de las células gliales (Whitaker et al., 1991; Legrand et al., 1991). Ya que los inhibidores de la calpaína no revirtieron por completo la inhibición del transporte de glicina mediada por ionomicina, es posible la participación de proteasas adicionales en el efecto de la ionomicina.

La CaMKII Regula el Transporte de Glicina de Alta Afinidad en la Glía de Müller

Una vez determinados la participación de la CaM y el papel del calcio en la regulación del transporte, nos enfocamos en el estudio de las enzimas dependientes de Ca^{2+}/CaM involucradas en esta vía. Entre las cinasa y fosfatasas reguladas por Ca^{2+}/calmodulina, la CaMKII (cinasa dependiente de Ca^{2+}/calmodulina II), la MLCK (cinasa de las cadenas ligeras de miosina) y la CaN (calcineurina ó proteína fosfatasa 2B), se expresan en las células gliales y se ha visto que interactúan con el citoesqueleto (Takeuchi et al., 2000; Vallano et al., 2000; Cotrina et al., 1998; Edelman et al., 1992; Matsuda et al., 1998; Vinade et al., 1997). Los resultados obtenidos con los inhibidores específicos de estas enzimas demostraron que la CaMKII participa en la regulación del transporte, no así la MLCK y la CaN. En las neuronas la CaMKII es particularmente importante en la transducción de señales por estar involucrada en la síntesis y liberación de neurotransmisores, en la organización del citoesqueleto, en la regulación de la activación de los receptores de NMDA, así como en el agrupamiento ("clustering") de los receptores en la terminal sináptica y en el desarrollo de la potenciación a largo plazo (Soderling et

al., 1994). En contraste, se dispone de muy poca información sobre el papel y los sustratos de la CaMKII en las células gliales. Se tiene evidencia de que la CaMKII participa en la regulación del citoesqueleto fosforilando selectivamente a las proteínas de los filamentos intermedios GFAP (proteína fibrilar acídica de la glía) y vimentina (Inagaki et al., 1997), en la fosforilación de PEA-15 protegiendo de la apoptosis (Kubes et al., 1998) y en la regulación del metabolismo de los fosfolípidos a través de la activación de la PI3K (cinasa de fosfoinosítidos; Communi et al., 1999). El estudio de la cinética del transporte en presencia de KN62 demostró claramente que el transporte de alta afinidad se inactiva por completo al inhibir a la CaMKII con KN62. Hasta la fecha no se han reportado sitios consenso de fosforilación para la CaMKII en los transportadores de glicina GLYT1 (Liu et al., 1993; Sato et al., 1995), aunque se desconoce la secuencia de estos transportadores en el pollo, es poco probable que la CaMKII fosforile directamente a los transportadores de glicina en la glía de Müller. La regulación del transporte de glicina por la CaMKII podría involucrar un mecanismo indirecto como se ha visto para otros transportadores dependientes de Na^{+}/Cl^{-} (revisado en Gadea y López-Colomé, 2001b), esto es, la activación de un sistema de señalamiento que module subsecuentemente a los transportadores de glicina, o la fosforilación de proteínas del citoesqueleto implicadas en el tráfico y/o estabilización de los transportadores en la membrana.

Como ya se mencionó, los primeros estudios sobre la regulación de los transportadores de neurotransmisores, entre ellos los de glutamato, GABA y glicina, revelaron que la PKC era la principal enzima involucrada en este proceso. Estudios posteriores sugirieron que la PKC modulaba la actividad de los transportadores a través de un mecanismo indirecto, pues el efecto producido por la activación de esta enzima persistía aún cuando se eliminaran todos los sitios posibles de fosforilación en los transportadores. Esto llevó a la búsqueda de proteínas que interactuaran con los transportadores. Actualmente se dispone de muy poca información sobre las proteínas a las que se encuentran asociadas los transportadores. Recientemente se encontró que una de estas proteínas es la

sintaxina 1A, la cual está involucrada en la fusión de las vesículas con la membrana plasmática y forma parte del complejo SNARE junto con la sinaptobrevina (proteína asociada a las vesículas sinápticas) y la proteína SNAP-25 (proteína asociada a los sinaptosomas; Jahn y Sudhof, 1999). La interacción de la sintaxina 1A con los transportadores de GABA aumenta su expresión en la membrana y disminuye los niveles citoplásmicos de estas proteínas, es decir, ocasiona la redistribución subcelular de los transportadores (Horton y Quick, 2001). La interacción de la sintaxina 1A y los transportadores de GABA está modulada por la fosforilación mediada por la PKC de proteínas que se unen a la sintaxina, entre ellas Munc-18 (Beckman et al., 1998). Los transportadores de glicina GLYT1 y GLYT2 también interactúan con la sintaxina 1A. A diferencia de los transportadores de GABA, esta interacción resulta en la inhibición del transporte de glicina por ambos transportadores debido a una disminución en el número de proteínas membranales y a un aumento en las citoplásmicas (Geerlings et al., 2000). Es posible que en la glía de Müller los transportadores de glicina también interactúen con la sintaxina 1A, la cual se ha localizado en células gliales del SNC (Maienschein et al., 1999). Más aún, se ha demostrado que la CaMKII fosforila a la sintaxina 1A y a proteínas asociadas (Risinger and Bennett, 1999; Verona et al., 2000), y que la sintaxina 1A se une a la fodrina (Nakano et al., 2001). Es posible entonces que la regulación de los transportadores de glicina por la CaMKII esté mediada por la sintaxina 1A.

A pesar de que tanto la glía como las neuronas expresan la calmodulina y enzimas dependientes de Ca^{2+}/calmodulina, aquí se demuestra que los inhibidores de la calmodulina así como de la CaMKII carecen de efecto sobre el transporte de glicina neuronal, lo cual podría estar relacionado con la expresión diferencial y/o localización subcelular de las isoformas de la CaMKII en neuronas y glía. En las neuronas, las isoformas predominantes son la α y la β, mientras que la γ y la δ se encuentran en menor cantidad. En astrocitos están presentes únicamente las isoformas δ, γ_B y γ_A. En neuronas las subunidades γ se asocian a la fracción particulada, mientras que en la glía se localizan en la fracción citoplásmica (Vallano

et al., 2000). La discrepancia podría deberse también a diferencias estructurales entre los transportadores y/o las proteínas a las que están acoplados.

Regulación del Transporte de Glicina por el Citoesqueleto: Actina y Fodrina

El mecanismo que subyace a la modulación del transporte de glicina por el citoesqueleto será el objeto de investigaciones futuras; sin embargo, se puede proponer que los transportadores de glicina se unen a los filamentos de actina o a proteínas que se unen a la actina. En consecuencia, la despolimerización de los filamentos de actina ocasionada por la citocalasina o la proteólisis mediada por calpaína de las proteínas que unen a la actina como la fodrina o la ankirina, podrían perturbar la estabilidad de los transportadores de glicina en la membrana plasmática y alterar de esta manera su número o actividad. Nuestros resultados con la toxina Pet sugieren que la fodrina es una de las proteínas responsables de estabilizar los transportadores en la membrana y establecer el vínculo con el citoesqueleto de actina. En apoyo de esta idea, la proteólisis de la fodrina mediada por calpaína inhibe el transporte de L-arginina en células endoteliales de arteria pulmonar (Zharikov y Block, 2000). Otra posibilidad es que la actividad de la CaMKII y su translocación a la membrana se vean afectadas por la despolimerización del citoesqueleto de actina. Se ha demostrado que la localización sináptica de la CaMKII, la cual forma parte de la densidad postsináptica, depende del citoesqueleto de actina (Allison et al., 2000). Por otro lado, el señalamiento por Ca^{2+} en astrocitos, incluyendo la propagación de las ondas de Ca^{2+}, también está relacionado con el citoesqueleto de actina ya que las citocalasinas impiden estos procesos (Cotrina et al., 1998).

Los hallazgos aquí presentados apoyan la participación de la glía de Müller en la regulación de la neurotransmisión en la retina. Las variaciones fisiológicas de la concentración de Ca^{2+} modifican la actividad de los transportadores de glicina, y podrían controlar la concentración de sustancias neuroactivas a través de la regulación diferencial de los sistemas de transporte. La localización de GLYT1 en la capa plexiforme interna (Pow y Hendrickson, 1999) donde las células amacrinas

glicinérgicas hacen sinapsis con las células ganglionares (Lukasiewicz y Roeder, 1995), apoya esta idea. Como ya se mencionó, queda por demostrar la presencia de transportadores de glicina en la glía de Müller in situ.

En la glía de Müller se han descrito sistemas de transporte de alta afinidad para el glutamato. Se ha propuesto que estos transportadores juegan un papel importante en la modulación de la transmisión excitadora de la retina regulando los niveles extracelulares de glutamato. Ya que la glicina actúa como coagonista del glutamato en los receptores de tipo NMDA, y la concentración de glicina en el espacio sináptico puede influir sobre la actividad de estos receptores (Roux y Supplisson, 1998), el transporte de glicina en estas células también podría participar en la modulación de la transmisión en la vía excitadora de la retina, así como contribuir a la regulación de la actividad de los receptores de glicina inhibidores en la capa plexiforme interna (Wu y Maple, 1998).

CONCLUSIONES

1. Estudios previos sobre la localización celular de los transportadores de glicina en la retina, reportaron la ausencia de estos sistemas de transporte en la glía de Müller. Nuestro trabajo demuestra, por primera vez, que la glía de la retina en cultivo es capaz de expresar tanto transportadores de glicina de alta afinidad del tipo GLYT1, como transportadores de baja afinidad.

2. Para el estudio de la regulación del transporte, se exploraron de manera extensa todas las vías de segundos mensajeros que, se sabe, regulan los transportadores de otros aminoácidos relacionados con la neurotransmisión. Ninguna de las vías exploradas resultó estar involucrada en la regulación del transporte de glicina en la glía de Müller.

3. El MDL12230A, reportado como inhibidor de la adenilato ciclasa, tiene un efecto tóxico sobre las células en cultivo, independiente de la inhibición de esta enzima. El tratamiento de los cultivos con este compuesto disminuye de manera considerable la viabilidad celular.

4. Una de las aportaciones más importantes de nuestro trabajo es que, a diferencia de otros sistemas de transporte para aminoácidos neurotransmisores como el glutamato y el GABA, regulados por cAMP o PKC, el sistema de transporte de glicina en la glía de Müller de la retina está regulado por enzimas dependientes de Ca2+/calmodulina, entre ellas la CaMKII.

5. La elevación moderada en los niveles intracelulares de Ca^{2+} inducida por la cafeína, el carbacol y el ATP, tiene como resultado un incremento en el transporte de glicina probablemente a través de la calmodulina y la CaMKII. Estos resultados sugieren que las ondas de Ca^{2+} en las células de Müller, además de inducir la liberación de sustancias neuroactivas, podrían modular la

excitabilidad neuronal a través de la regulación de las concentraciones extracelulares de glicina.

6. La entrada masiva de Ca^{2+} producida por ionóforos, activa un proceso mediado por proteasas que inhibe al transporte. Una de las proteasas involucradas es la calpaína, cuyo sustrato es la fodrina, proteína que forma parte del citoesqueleto. Estos resultados sugieren que la regulación del transporte de glicina en la glía de Müller involucra al citoesqueleto.

7. El transporte de alta afinidad desaparece al inhibir a la CaMKII. El estudio sobre la cinética del transporte sugiere que se trata de un mecanismo indirecto sobre los sistemas de transporte, tal vez a través de elementos del citoesqueleto, como la actina y la fodrina, o de proteínas asociadas encargadas del tráfico y/o la estabilización de los transportadores en la membrana, como la sintaxina 1A.

8. Nuestros resultados abren un campo de exploración sobre la relación entre la regulación de los transportadores y el citoesqueleto.

En la retina, las células de Müller son el tipo más abundante de gliocito. Estas células atraviesan a la retina en todo su espesor desde los fotorreceptores a las células ganglionares. La localización anatómica de estas células, envolviendo a las sinapsis, así como la presencia de un sistema de transporte de glicina, sugiere que juegan un papel importante en la modulación de la neurotransmisión. La regulación de este sistema puede tener implicaciones fisiológicas importantes, pues la concentración de glicina en el espacio sináptico influencia tanto la actividad de los receptores de NMDA, involucrados en la transmisión excitadora por glutamato en las vías verticales de la retina, como los receptores inhibidores de glicina que participan en la inhibición lateral en la capa plexiforme interna.

BIBLIOGRAFÍA

Abu-Soud HM, Yoho LL, Stuehr DJ (1994) Calmodulin controls neuronal nitric-oxide synthase by a dual mechanism. Activation of intra- and interdomain electron transfer. J Biol Chem 269:32047-32050.

Adams RH, Sato K, Shimada S, Tohyama M, Puschel AW, Betz H (1995) Gene structure and glial expression of the glycine transporter GlyT1 in embryonic and adult rodents. J Neurosci 15:2524-2532.

Adler R, Magistretti PJ, Hyndman AG, Shoemaker WJ (1982) Purification and cytochemical identification of neuronal and non- neuronal cells in chick embryo retina cultures. Dev Neurosci 5:27-39.

Allison DW, Chervin AS, Gelfand VI, Craig AM (2000) Postsynaptic scaffolds of excitatory and inhibitory synapses in hippocampal neurons: maintenance of core components independent of actin filaments and microtubules. J Neurosci 20:4545-4554.

Amara SG, Kuhar MJ (1993) Neurotransmitter transporters: recent progress. Annu Rev Neurosci 16:73-93.

Aprison MH, Werman R (1965) The distribution of glycine in cat spinal cord and roots. Life Sci 4:2075-2083.

Aragon MC, Gimenez C, Mayor F (1987) Stoichiometry of sodium- and chloride-coupled glycine transport in synaptic plasma membrane vesicles derived from rat brain. FEBS Lett 212:87-90.

Araque A, Li N, Doyle RT, Haydon PG (2000) SNARE protein-dependent glutamate release from astrocytes. J Neurosci 20:666-673.

Attwell D, Barbour B, Szatkowski M (1993) Nonvesicular release of neurotransmitter. Neuron 11:401-407.

Baker AJ, Zornow MH, Scheller MS, Yaksh TL, Skilling SR, Smullin DH, Larson AA, Kuczenski R (1991) Changes in extracellular concentrations of glutamate, aspartate, glycine, dopamine, serotonin, and dopamine metabolites after transient global ischemia in the rabbit brain. J Neurochem 57:1370-1379.

Barbour B, Szatkowski M, Ingledew N, Attwell D (1989) Arachidonic acid induces a prolonged inhibition of glutamate uptake into glial cells. Nature 342:918-920.

Barnes S, Merchant V, Mahmud F (1993) Modulation of transmission gain by protons at the photoreceptor output synapse. Proc Natl Acad Sci U S A 90:10081-10085.

Barondes SH (1994) Thinking about Prozac. Science 263:1102-1103.

Barres BA (1991) New roles for glia. J Neurosci 11:3685-3694.

Barres BA (1991) Glial ion channels. Curr Opin Neurobiol 1:354-359.

Bartlett PF, Noble MD, Pruss RM, Raff MC, Rattray S, Williams CA (1981) Rat neural antigen-2 (RAN-2): a cell surface antigen on astrocytes, ependymal cells, Muller cells and lepto-meninges defined by a monoclonal antibody. Brain Res 204:339-351.

Beck KA, Nelson WJ (1996) The spectrin-based membrane skeleton as a membrane protein-sorting machine. Am J Physiol 270:C1263-1270.

Beckman ML, Bernstein EM, Quick MW (1998) Protein kinase C regulates the interaction between a GABA transporter and syntaxin 1A. J Neurosci 18:6103-6112.

Bergeron R, Meyer TM, Coyle JT, Greene RW (1998) Modulation of N-methyl-D-aspartate receptor function by glycine transport. Proc Natl Acad Sci U S A 95:15730-15734.

Berridge MJ (1993) Inositol trisphosphate and calcium signalling. Nature 361:315-325.

Betz H, Langosch D, Rundstrom N, Bormann J, Kuryatov A, Kuhse J, Schmieden V, Matzenbach B, Kirsch J (1993) Structure and biology of inhibitory glycine receptors. Ann N Y Acad Sci 707:109-115.

Bezzi P, Carmignoto G, Pasti L, Vesce S, Rossi D, Rizzini BL, Pozzan T, Volterra A (1998) Prostaglandins stimulate calcium-dependent glutamate release in astrocytes. Nature 391:281-285.

Biedermann B, Frohlich E, Grosche J, Wagner HJ, Reichenbach A (1995) Mammalian Muller (glial) cells express functional D2 dopamine receptors. Neuroreport 6:609-612.

Blaustein MP, Golovina VA (2001) Structural complexity and functional diversity of endoplasmic reticulum Ca(2+) stores. Trends Neurosci 24:602-608.

Boje KM, Skolnick P, Raber J, Fletcher RT, Chader G (1992) Strychnine-insensitive glycine receptors in embryonic chick retina: characteristics and modulation of NMDA neurotoxicity. Neurochem Int 20:473-486.

Borowsky B, Hoffman BJ (1998) Analysis of a gene encoding two glycine transporter variants reveals alternative promoter usage and a novel gene structure. J Biol Chem 273:29077-29085.

Borowsky B, Mezey E, Hoffman BJ (1993) Two glycine transporter variants with distinct localization in the CNS and peripheral tissues are encoded by a common gene. Neuron 10:851-863.

Bouvier M, Szatkowski M, Amato A, Attwell D (1992) The glial cell glutamate uptake carrier countertransports pH-changing anions. Nature 360:471-474.

Braun AP, Schulman H (1995) The multifunctional calcium/calmodulin-dependent protein kinase: from form to function. Annu Rev Physiol 57:417-445.

Brecha NC, Weigmann C (1994) Expression of GAT-1, a high-affinity gamma-aminobutyric acid plasma membrane transporter in the rat retina. J Comp Neurol 345:602-611.

Brew H, Attwell D (1987) Electrogenic glutamate uptake is a major current carrier in the membrane of axolotl retinal glial cells. Nature 327:707-709.

Bringmann A, Pannicke T, Weick M, Biedermann B, Uhlmann S, Kohen L, Wiedemann P, Reichenbach A (2002) Activation of P2Y receptors stimulates potassium and cation currents in acutely isolated human Muller (glial) cells. Glia 37:139-152.

Bruun A, Ehinger B (1972) Uptake of the putative neurotransmitter, glycine, into the rabbit retina. Invest Ophthalmol 11:191-198.

Bubb MR, Senderowicz AM, Sausville EA, Duncan KL, Korn ED (1994) Jasplakinolide, a cytotoxic natural product, induces actin polymerization and competitively inhibits the binding of phalloidin to F-actin. J Biol Chem 269:14869-14871.

Bunt-Milam AH, Saari JC (1983) Immunocytochemical localization of two retinoid-binding proteins in vertebrate retina. J Cell Biol 97:703-712.

Calegari F, Coco S, Taverna E, Bassetti M, Verderio C, Corradi N, Matteoli M, Rosa P (1999) A regulated secretory pathway in cultured hippocampal astrocytes. J Biol Chem 274:22539-22547.

Casado M, Bendahan A, Zafra F, Danbolt NC, Aragon C, Gimenez C, Kanner BI (1993) Phosphorylation and modulation of brain glutamate transporters by protein kinase C. J Biol Chem 268:27313-27317.

Communi D, Dewaste V, Erneux C (1999) Calcium-calmodulin-dependent protein kinase II and protein kinase C- mediated phosphorylation and activation of D-myo-inositol 1,4, 5- trisphosphate 3-kinase B in astrocytes. J Biol Chem 274:14734-14742.

Cooper JA (1987) Effects of cytochalasin and phalloidin on actin. J Cell Biol 105:1473-1478.

Copenhagen DR (1991) Synaptic transmission in the retina. Curr Opin Neurobiol 1:258-262.

Cotrina ML, Lin JH, Nedergaard M (1998) Cytoskeletal assembly and ATP release regulate astrocytic calcium signaling. J Neurosci 18:8794-8804.

Chapman AG. 2000. Glutamate and epilepsy. J Nutr 130(4S Suppl):1043-1045.

Charles AC, Dirksen ER, Merrill JE, Sanderson MJ (1993) Mechanisms of intercellular calcium signaling in glial cells studied with dantrolene and thapsigargin. Glia 7:134-145.

Chaudhry FA, Reimer RJ, Bellocchio EE, Danbolt NC, Osen KK, Edwards RH, Storm-Mathisen J (1998) The vesicular GABA transporter, VGAT, localizes to synaptic vesicles in sets of glycinergic as well as GABAergic neurons. J Neurosci 18:9733-9750.

Chaudhry FA, Reimer RJ, Krizaj D, Barber D, Storm-Mathisen J, Copenhagen DR, Edwards RH (1999) Molecular analysis of system N suggests novel physiological roles in nitrogen metabolism and synaptic transmission. Cell 99:769-780.

Chaudhry FA, Schmitz D, Reimer RJ, Larsson P, Gray AT, Nicoll R, Kavanaugh M, Edwards RH (2002) Glutamine uptake by neurons: interaction of protons with system a transporters. J Neurosci 22:62-72.

Choi DW, Maulucci-Gedde M, Kriegstein AR (1987) Glutamate neurotoxicity in cortical cell culture. J Neurosci 7:357-68.

Christensen HN (1984) Organic ion transport during seven decades. The amino acids. Biochim Biophys Acta 779:255-269.

Danysz W, Parsons AC (1998) Glycine and N-methyl-D-aspartate receptors: physiological significance and possible therapeutic applications. Pharmacol Rev 50:597-664.

Daw NW, Brunken WJ, Parkinson D (1989) The function of synaptic transmitters in the retina. Annu Rev Neurosci 12:205-225.

Deitmer JW (2001) Strategies for metabolic exchange between glial cells and neurons. Respir Physiol 129:71-81.

Derouiche A, Rauen T (1995) Coincidence of L-glutamate/L-aspartate transporter (GLAST) and glutamine synthetase (GS) immunoreactions in retinal glia: evidence for coupling of GLAST and GS in transmitter clearance. J Neurosci Res 42:131-143.

Du S, Rubin A, Klepper S, Barrett C, Kim YC, Rhim HW, Lee EB, Park CW, Markelonis GJ, Oh TH (1999) Calcium influx and activation of calpain I mediate acute reactive gliosis in injured spinal cord. Exp Neurol 157:96-105.

Dzubay JA, Jahr CE (1999) The concentration of synaptically released glutamate outside of the climbing fiber-Purkinje cell synaptic cleft. J Neurosci 19:5265-5274.

Ebrey T, Koutalos Y (2001) Vertebrate photoreceptors. Prog Retin Eye Res 20:49-94.

Edelman AM, Higgins DM, Bowman CL, Haber SN, Rabin RA, Cho-Lee J (1992) Myosin light chain kinase is expressed in neurons and glia: immunoblotting and immunocytochemical studies. Brain Res Mol Brain Res 14:27-34.

Edwards RB, Adler AJ, Dev S, Claycomb RC (1992) Synthesis of retinoic acid from retinol by cultured rabbit Muller cells. Exp Eye Res 54:481-490.

Farber D, Adler A (1986) The Retina. A model for cell biology studies. Academic Press. Orlando. 363 pp.

Fawcett JW, Asher RA (1999) The glial scar and central nervous system repair. Brain Res Bull 49:377-391.

Finkbeiner SM (1993) Glial calcium. Glia 9:83-104.

Fletcher EJ, Beart PM, Lodge D (1990) Involvement of glycine in excitatory neuotransmission. En: Glycine Neurotransmission. John Wiley and Sons. New York. 193-218 pp.

Fricke U (1975) Tritosol: a new scintillation cocktail based on Triton X-100. Anal Biochem 63:555-558.

Frishman LJ, Yamamoto F, Bogucka J, Steinberg RH (1992) Light-evoked changes in [K+]o in proximal portion of light-adapted cat retina. J Neurophysiol 67:1201-1212.

Gadea A, Lopez-Colome AM (2001) Glial transporters for glutamate, glycine, and GABA: II. GABA transporters. J Neurosci Res 63:461-468.

Gadea A, Lopez-Colome AM (2001) Glial transporters for glutamate, glycine and GABA I. Glutamate transporters. J Neurosci Res 63:453-460.

Galli A, Mori F, Bargellini M, Coppini L (1993) Sodium-dependent release of exogenous glycine from preloaded rat hippocampal synaptosomes. J Neural Transm Gen Sect 93:167-179.

Geerlings A, Lopez-Corcuera B, Aragon C (2000) Characterization of the interactions between the glycine transporters GLYT1 and GLYT2 and the SNARE protein syntaxin 1A. FEBS Lett 470:51-54.

Giaume C, Venance L (1998) Intercellular calcium signaling and gap junctional communication in astrocytes. Glia 24:50-64.

Gietzen K, Wuthrich A, Bader H (1981) R 24571: a new powerful inhibitor of red blood cell Ca++-transport ATPase and of calmodulin-regulated functions. Biochem Biophys Res Commun 101:418-425.

Goldstein GW (1988) Endothelial cell-astrocyte interactions. A cellular model of the blood- brain barrier. Ann N Y Acad Sci 529:31-39.

Golovina VA, Bambrick LL, Yarowsky PJ, Krueger BK, Blaustein MP (1996) Modulation of two functionally distinct Ca2+ stores in astrocytes: role of the plasmalemmal Na/Ca exchanger. Glia 16:296-305.

Gomeza J, Casado M, Gimenez C, Aragon C (1991) Inhibition of high-affinity gamma-aminobutyric acid uptake in primary astrocyte cultures by phorbol esters and phospholipase C. Biochem J 275:435-439.

Greene JG (1999) Mitochondrial function and NMDA receptor activation: mechanisms of secondary excitotoxicity. Funct Neurol 14(3):171-84.

Grosche J, Matyash V, Moller T, Verkhratsky A, Reichenbach A, Kettenmann H (1999) Microdomains for neuron-glia interaction: parallel fiber signaling to Bergmann glial cells. Nat Neurosci 2:139-143.

Gu S, Roderick HL, Camacho P, Jiang JX (2001) Characterization of an N-system amino acid transporter expressed in retina and its involvement in glutamine transport. J Biol Chem 276:24137-24144.

Guan X, Cravatt BF, Ehring GR, Hall JE, Boger DL, Lerner RA, Gilula NB (1997) The sleep-inducing lipid oleamide deconvolutes gap junction communication and calcium wave transmission in glial cells. J Cell Biol 139:1785-1792.

Guastella J, Brecha N, Weigmann C, Lester HA, Davidson N (1992) Cloning, expression, and localization of a rat brain high-affinity glycine transporter. Proc Natl Acad Sci U S A 89:7189-7193.

Guastella J, Nelson N, Nelson H, Czyzyk L, Keynan S, Miedel MC, Davidson N, Lester HA, Kanner BI (1990) Cloning and expression of a rat brain GABA transporter. Science 249:1303-1306.

Guthrie PB, Knappenberger J, Segal M, Bennett MV, Charles AC, Kater SB (1999) ATP released from astrocytes mediates glial calcium waves. J Neurosci 19:520-528.

Handlogten ME, Dudenhausen EE, Yang W, Kilberg MS (1996) Association of hepatic system A amino acid transporter with the membrane-cytoskeletal proteins ankyrin and fodrin. Biochim Biophys Acta 1282:107-114.

Hanley JG, Jones EM, Moss SJ (2000) GABA receptor rho1 subunit interacts with a novel splice variant of the glycine transporter, GLYT-1. J Biol Chem 275:840-846.

Harada T, Harada C, Watanabe M, Inoue Y, Sakagawa T, Nakayama N, Sasaki S, Okuyama S, Watase K, Wada K, Tanaka K (1998) Functions of the two glutamate transporters GLAST and GLT-1 in the retina. Proc Natl Acad Sci U S A 95:4663-4666.

Hassinger TD, Guthrie PB, Atkinson PB, Bennett MV, Kater SB (1996) An extracellular signaling component in propagation of astrocytic calcium waves. Proc Natl Acad Sci U S A 93:13268-13273.

Hernandez-Cruz A, Escobar AL, Jimenez N (1997) Ca(2+)-induced Ca2+ release phenomena in mammalian sympathetic neurons are critically dependent on the rate of rise of trigger Ca2+. J Gen Physiol 9:147-67.

Hertz L, Dringen R, Schousboe A, Robinson SR (1999) Astrocytes: glutamate producers for neurons. J Neurosci Res 57:417-428.

Heumann R, Villegas J, Herzfeld DW (1981) Acetylcholine synthesis in the Schwann cell and axon in the giant nerve fiber of the squid. J Neurochem 36:765-768.

Holopainen I, Kontro P (1989) Uptake and release of glycine in cerebellar granule cells and astrocytes in primary culture: potassium-stimulated release from granule cells is calcium-dependent. J Neurosci Res 24:374-383.

Horton N, Quick MW (2001) Syntaxin 1A up-regulates GABA transporter expression by subcellular redistribution. Mol Membr Biol 18:39-44.

Huster D, Hjelle OP, Haug FM, Nagelhus EA, Reichelt W, Ottersen OP (1998) Subcellular compartmentation of glutathione and glutathione precursors. A high resolution immunogold analysis of the outer retina of guinea pig. Anat Embryol (Berl) 198:277-287.

Inagaki N, Goto H, Ogawara M, Nishi Y, Ando S, Inagaki M (1997) Spatial patterns of Ca2+ signals define intracellular distribution of a signaling by Ca2+/Calmodulin-dependent protein kinase II. J Biol Chem 272:25195-25199.

Innocenti B, Parpura V, Haydon PG (2000) Imaging extracellular waves of glutamate during calcium signaling in cultured astrocytes. J Neurosci 20:1800-1808.

Ishida A, Kameshita I, Okuno S, Kitani T, Fujisawa H (1995) A novel highly specific and potent inhibitor of calmodulin-dependent protein kinase II. Biochem Biophys Res Commun 212:806-812.

Jager J, Wassle H (1987) Localization of glycine uptake and receptors in the cat retina. Neurosci Lett 75:147-151.

Jahn R, Sudhof TC (1999) Membrane fusion and exocytosis. Annu Rev Biochem 68:863-911.

John GR, Scemes E, Suadicani SO, Liu JS, Charles PC, Lee SC, Spray DC, Brosnan CF (1999) IL-1beta differentially regulates calcium wave propagation between primary human fetal astrocytes via pathways involving P2 receptors and gap junction channels. Proc Natl Acad Sci U S A 96:11613-11618.

Johnston GA, Iversen LL (1971) Glycine uptake in rat central nervous system slices and homogenates: evidence for different uptake systems in spinal cord and cerebral cortex. J Neurochem 18:1951-1961.

Jursky F, Nelson N (1995) Localization of glycine neurotransmitter transporter (GLYT2) reveals correlation with the distribution of glycine receptor. J Neurochem 64:1026-1033.

Jursky F, Tamura S, Tamura A, Mandiyan S, Nelson H, Nelson N (1994) Structure, function and brain localization of neurotransmitter transporters. J Exp Biol 196:283-295.

Kanamori M, Naka M, Asano M, Hidaka H (1981) Effects of N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide and other calmodulin antagonists (calmodulin interacting agents) on calcium- induced contraction of rabbit aortic strips. J Pharmacol Exp Ther 217:494-499.

Kang J, Jiang L, Goldman SA, Nedergaard M (1998) Astrocyte-mediated potentiation of inhibitory synaptic transmission. Nat Neurosci 1:683-692.

Kanner BI (1994) Sodium-coupled neurotransmitter transport: structure, function and regulation. J Exp Biol 196:237-249.

Karowski CJ, Proenza LM (1977) Relationship between Muller cell responses, a local transretinal potential, and potassium flux. J Neurophysiol 40:244-259.

Keirstead SA, Miller RF (1995) Calcium waves in dissociated retinal glial (Muller) cells are evoked by release of calcium from intracellular stores. Glia 14:14-22.

Keirstead SA, Miller RF (1997) Metabotropic glutamate receptor agonists evoke calcium waves in isolated Muller cells. Glia 21:194-203.

Kern TS, Kowluru RA, Engerman RL (1994) Abnormalities of retinal metabolism in diabetes or galactosemia: ATPases and glutathione. Invest Ophthalmol Vis Sci 35:2962-2967.

Kim KM, Kingsmore SF, Han H, Yang-Feng TL, Godinot N, Seldin MF, Caron MG, Giros B (1994) Cloning of the human glycine transporter type 1: molecular and pharmacological characterization of novel isoform variants and chromosomal localization of the gene in the human and mouse genomes. Mol Pharmacol 45:608-617.

Kirischuk S, Scherer J, Kettenmann H, Verkhratsky A (1995) Activation of P2-purinoreceptors triggered Ca2+ release from InsP3- sensitive internal stores in mammalian oligodendrocytes. J Physiol 483:41-57.

Kirischuk S, Moller T, Voitenko N, Kettenmann H, Verkhratsky A (1995) ATP-induced cytoplasmic calcium mobilization in Bergmann glial cells. J Neurosci 15:7861-7871.

Kubes M, Cordier J, Glowinski J, Girault JA, Chneiweiss H (1998) Endothelin induces a calcium-dependent phosphorylation of PEA-15 in intact astrocytes: identification of Ser104 and Ser116 phosphorylated, respectively, by protein kinase C and calcium/calmodulin kinase II in vitro. J Neurochem 71:1307-1314.

Kuhar MJ, Ritz MC, Boja JW (1991) The dopamine hypothesis of the reinforcing properties of cocaine. Trends Neurosci 14:299-302.

Kulik A, Haentzsch A, Luckermann M, Reichelt W, Ballanyi K (1999) Neuron-glia signaling via alpha(1) adrenoceptor-mediated Ca(2+) release in Bergmann glial cells in situ. J Neurosci 19:8401-8408.

Legrand C, Ferraz C, Clavel MC, Rabie A (1991) Distribution of gelsolin in the retina of the developing rabbit. Cell Tissue Res 264:335-338.

Leung PC, Taylor WA, Wang JH, Tipton CL (1984) Ophiobolin A. A natural product inhibitor of calmodulin. J Biol Chem 259:2742-2747.

Lev-Ram V, Ellisman MH (1995) Axonal activation-induced calcium transients in myelinating Schwann cells, sources, and mechanisms. J Neurosci 15:2628-2637.

Li Y, Holtzclaw LA, Russell JT (2001) Muller cell Ca2+ waves evoked by purinergic receptor agonists in slices of rat retina. J Neurophysiol 85:986-994.

Lidsky TI, Yablonsky-Alter E, Zuck LG, Banerjee SP (1997) Antipsychotic drug effects on glutamatergic activity. Brain Res 764:46-52.

Liepe BA, Stone C, Koistinaho J, Copenhagen DR (1994) Nitric oxide synthase in Muller cells and neurons of salamander and fish retina. J Neurosci 14:7641-7654.

Liu QR, Nelson H, Mandiyan S, Lopez-Corcuera B, Nelson N (1992) Cloning and expression of a glycine transporter from mouse brain. FEBS Lett 305:110-114.

Liu QR, Lopez-Corcuera B, Mandiyan S, Nelson H, Nelson N (1993) Cloning and expression of a spinal cord- and brain-specific glycine transporter with novel structural features. J Biol Chem 268:22802-22808.

Lopez-Colome AM, Romo-de-Vivar M (1991) Excitatory amino acid receptors in primary cultures of glial cells from the retina. Glia 4:431-439.

Lopez-Colome AM, Salceda R, Pasantes-Morales H (1978) Potassium-stimulated release of GABA, glycine, and taurine from the chick retina. Neurochem Res 3:431-441.

Lopez-Corcuera B, Martinez-Maza R, Nunez E, Roux M, Supplisson S, Aragon C (1998) Differential properties of two stably expressed brain-specific glycine transporters. J Neurochem 71:2211-2219.

Lukasiewicz PD, Roeder RC (1995) Evidence for glycine modulation of excitatory synaptic inputs to retinal ganglion cells. J Neurosci 15:4592-4601.

Luque JM, Nelson N, Richards JG (1995) Cellular expression of glycine transporter 2 messenger RNA exclusively in rat hindbrain and spinal cord. Neuroscience 64:525-535.

Lloyd KG, De Montis G, Javoy-Agid F, Beaumont K, Lowenthal A, Constantinidis J, Agid Y (1983) Glycine receptors in the human brain: characterization of 3H-strychnine binding and status in pathological conditions. Adv Biochem Psychopharmacol 36:233-238.

Magalhaes MM, Coimbra A (1972) The rabbit retina Muller cell. A fine structural and cytochemical study. J Ultrastruct Res 39:310-326.

Maienschein V, Marxen M, Volknandt W, Zimmermann H (1999) A plethora of presynaptic proteins associated with ATP-storing organelles in cultured astrocytes. Glia 26:233-244.

Malchow RP, Ramsey DJ (1999) Responses of retinal Muller cells to neurotransmitter candidates: a comparative survey. Biol Bull 197:229-230.

Masland RH (1988) Amacrine cells. Trends Neurosci 11:405-410.

Masland RH (2001) Neuronal diversity in the retina. Curr Opin Neurobiol 11:431-436.

Matsuda T, Takuma K, Asano S, Kishida Y, Nakamura H, Mori K, Maeda S, Baba A (1998) Involvement of calcineurin in Ca2+ paradox-like injury of cultured rat astrocytes. J Neurochem 70:2004-2011.

Matute C, Domercq M, Fogarty DJ, Pascual-de-Zulueta M, and Sanchez-Gomez MV. (1999) On how altered glutamate homeostasis may contribute to demyelinating diseases of the CNS. Adv Exp Med Biol 468:97-107.

McNamara D, Dingledine R (1990) Dual effect of glycine on NMDA-induced neurotoxicity in rat cortical cultures. J Neurosci 10:3970-3976.

Meissner G (1994) Ryanodine receptor/Ca2+ release channels and their regulation by endogenous effectors. Annu Rev Physiol 56:485-508.

Mills JW, Mandel LJ (1994) Cytoskeletal regulation of membrane transport events. Faseb J 8:1161-1165.

Moroni F, Alesiani M, Facci L, Fadda E, Skaper SD, Galli A, Lombardi G, Mori F, Ciuffi M, Natalini B, et al. (1992) Thiokynurenates prevent excitotoxic neuronal

death in vitro and in vivo by acting as glycine antagonists and as inhibitors of lipid peroxidation. Eur J Pharmacol 218:145-151.

Moscona AA (1983) On glutamine synthetase, carbonic anhidrase and Müller glia in the retina. En: Preogress in Retinal Research. Pergamon Press. Oxford. 111-135 pp.

Nagelhus EA, Veruki ML, Torp R, Haug FM, Laake JH, Nielsen S, Agre P, Ottersen OP (1998) Aquaporin-4 water channel protein in the rat retina and optic nerve: polarized expression in Muller cells and fibrous astrocytes. J Neurosci 18:2506-2519.

Nakano M, Nogami S, Sato S, Terano A, Shirataki H (2001) Interaction of syntaxin with alpha-fodrin, a major component of the submembranous cytoskeleton. Biochem Biophys Res Commun 288:468-475.

Neal JL (1972) Analysis of Michaelis kinetics for two independent, saturable membrane transport functions. J Theor Biol 35:113-118.

Nelson N (1993) Presynaptic events involved in neurotransmission. J Physiol Paris 87:171-178.

Nelson WJ, Hammerton RW (1989) A membrane-cytoskeletal complex containing Na+,K+-ATPase, ankyrin, and fodrin in Madin-Darby canine kidney (MDCK) cells: implications for the biogenesis of epithelial cell polarity. J Cell Biol 108:893-902.

Nelson N, Lill H (1994) Porters and neurotransmitter transporters. J Exp Biol 196:213-228.

Newman EA (1987) Regulation of potassium levels by Muller cells in the vertebrate retina. Can J Physiol Pharmacol 65:1028-1032.

Newman EA (1993) Inward-rectifying potassium channels in retinal glial (Muller) cells. J Neurosci 13:3333-3345.

Newman EA (1994) A physiological measure of carbonic anhydrase in Muller cells. Glia 11:291-299.

Newman EA (2001) Propagation of intercellular calcium waves in retinal astrocytes and Muller cells. J Neurosci 21:2215-2223.

Newman E, Reichenbach A (1996) The Muller cell: a functional element of the retina. Trends Neurosci 19:307-312.

Newman EA, Zahs KR (1997) Calcium waves in retinal glial cells. Science 275:844-847.

Newman EA, Zahs KR (1998) Modulation of neuronal activity by glial cells in the retina. J Neurosci 18:4022-4028.

Newman EA, Frambach DA, Odette LL (1984) Control of extracellular potassium levels by retinal glial cell K+ siphoning. Science 225:1174-1175.

Nicholls, JG, Martin AR, Wallace BG (1992) From Neuron to Brain, 3rd Edition Edition. Massachusetts: Sinauer Associates. 807 pp.

Nunez E, Lopez-Corcuera B, Vazquez J, Gimenez C, Aragon C (2000) Differential effects of the tricyclic antidepressant amoxapine on glycine uptake mediated by the recombinant GLYT1 and GLYT2 glycine transporters. Br J Pharmacol 129:200-206.

Oakley B, 2nd, Wen R (1989) Extracellular pH in the isolated retina of the toad in darkness and during illumination. J Physiol 419:353-378.

Olney JW, Newcomer JW, Farber NB (1999) NMDA receptor hypofunction model of schizophrenia. J Psychiatr Res 33:523-533.

Pacholczyk T, Blakely RD, Amara SG (1991) Expression cloning of a cocaine- and antidepressant-sensitive human noradrenaline transporter. Nature 350:350-354.

Pannicke T, Fischer W, Biedermann B, Schadlich H, Grosche J, Faude F, Wiedemann P, Allgaier C, Illes P, Burnstock G, Reichenbach A (2000) P2X7 receptors in Muller glial cells from the human retina. J Neurosci 20:5965-5972.

Parnavelas JG, Nadarajah B (2001) Radial glial cells. are they really glia? Neuron 31:881-884.

Parpura V, Haydon PG (2000) Physiological astrocytic calcium levels stimulate glutamate release to modulate adjacent neurons. Proc Natl Acad Sci U S A 97:8629-8634.

Parpura V, Basarsky TA, Liu F, Jeftinija K, Jeftinija S, Haydon PG (1994) Glutamate-mediated astrocyte-neuron signalling. Nature 369:744-747.

Pasantes-Morales H, Klethi J, Ledig M, Mandel P (1972) Free amino acids of chicken and rat retina. Brain Res 41:494-497.

Pasti L, Volterra A, Pozzan T, Carmignoto G (1997) Intracellular calcium oscillations in astrocytes: a highly plastic, bidirectional form of communication between neurons and astrocytes in situ. J Neurosci 17:7817-7830.

Pasti L, Zonta M, Pozzan T, Vicini S, Carmignoto G (2001) Cytosolic calcium oscillations in astrocytes may regulate exocytotic release of glutamate. J Neurosci 21:477-484.

Pfeiffer B, Grosche J, Reichenbach A, Hamprecht B (1994) Immunocytochemical demonstration of glycogen phosphorylase in Muller (glial) cells of the mammalian retina. Glia 12:62-67.

Philippi M, Vyklicky L, Orkand RK (1996) Potassium currents in cultured glia of the frog optic nerve. Glia 17:72-82.

Poitry-Yamate CL, Poitry S, Tsacopoulos M (1995) Lactate released by Muller glial cells is metabolized by photoreceptors from mammalian retina. J Neurosci 15:5179-5191.

Ponce J, Poyatos I, Aragon C, Gimenez C, Zafra F (1998) Characterization of the 5' region of the rat brain glycine transporter GLYT2 gene: identification of a novel isoform. Neurosci Lett 242:25-28.

Pourcho RG, Goebel DJ (1987) A combined Golgi and autoradiographic study of 3H-glycine-accumulating cone bipolar cells in the cat retina. J Neurosci 7:1178-1188.

Pow DV, Crook DK (1995) Immunocytochemical evidence for the presence of high levels of reduced glutathione in radial glial cells and horizontal cells in the rabbit retina. Neurosci Lett 193:25-28.

Pow DV, Hendrickson AE (1999) Distribution of the glycine transporter glyt-1 in mammalian and nonmammalian retinae. Vis Neurosci 16:231-239.

Poyatos I, Ponce J, Aragon C, Gimenez C, Zafra F (1997) The glycine transporter GLYT2 is a reliable marker for glycine- immunoreactive neurons. Brain Res Mol Brain Res 49:63-70.

Puro DG, Stuenkel EL (1995) Thrombin-induced inhibition of potassium currents in human retinal glial (Muller) cells. J Physiol 485:337-348.

Puro DG, Hwang JJ, Kwon OJ, Chin H (1996) Characterization of an L-type calcium channel expressed by human retinal Muller (glial) cells. Brain Res Mol Brain Res 37:41-48.

Rasmussen KE (1974) The Muller cell: a comparative study of rod and cone retinas with and without retinal vessels. Exp Eye Res 19:243-257.

Rauen T, Taylor WR, Kuhlbrodt K, Wiessner M (1998) High-affinity glutamate transporters in the rat retina: a major role of the glial glutamate transporter GLAST-1 in transmitter clearance. Cell Tissue Res 291:19-31.

Reichelt W, Stabel-Burow J, Pannicke T, Weichert H, Heinemann U (1997) The glutathione level of retinal Muller glial cells is dependent on the high-affinity sodium-dependent uptake of glutamate. Neuroscience 77:1213-1224.

Reichenbach A, Henke A, Eberhardt W, Reichelt W, Dettmer D (1992) K+ ion regulation in retina. Can J Physiol Pharmacol 70:S239-247.

Reichenbach A, Stolzenburg JU, Eberhardt W, Chao TI, Dettmer D, Hertz L (1993) What do retinal muller (glial) cells do for their neuronal 'small siblings'? J Chem Neuroanat 6:201-213.

Risau W, Wolburg H (1990) Development of the blood-brain barrier. Trends Neurosci 13:174-178.

Risinger C, Bennett MK (1999) Differential phosphorylation of syntaxin and synaptosome-associated protein of 25 kDa (SNAP-25) isoforms. J Neurochem 72:614-624.

Robinson SR, Dreher Z (1990) Muller cells in adult rabbit retinae: morphology, distribution and implications for function and development. J Comp Neurol 292:178-192.

Rochon D, Rousse I, Robitaille R (2001) Synapse-glia interactions at the mammalian neuromuscular junction. J Neurosci 21:3819-3829.

Rothstein JD, Jin L, Dykes-Hoberg M, Kuncl RW (1993) Chronic inhibition of glutamate uptake produces a model of slow neurotoxicity. Proc Natl Acad Sci USA 90:6591-6595.

Rothstein, JD, Dykes-Hoberg M, Prado CA, Bristol LA, Jin L, Kuncl RW, Kanai Y, Hediger M, Wang Y, Schielke JP, Welty DF (1996) Knockout of glutamate transporters reveals a major role for astroglial transport in excitotoxicity and clearance of glutamate. Neuron 16:675-86.

Roux MJ, Supplisson S (2000) Neuronal and glial glycine transporters have different stoichiometries. Neuron 25:373-383.

Sakamoto YR, Nakajima TR, Fukiage CR, Sakai OR, Yoshida YR, Azuma MR, Shearer TR (2000) Involvement of calpain isoforms in ischemia-reperfusion injury in rat retina. Curr Eye Res 21:571-580.

Sakata K, Sato K, Schloss P, Betz H, Shimada S, Tohyama M (1997) Characterization of glycine release mediated by glycine transporter 1 stably expressed in HEK-293 cells. Brain Res Mol Brain Res 49:89-94.

Sanderson MJ, Charles AC, Boitano S, Dirksen ER (1994) Mechanisms and function of intercellular calcium signaling. Mol Cell Endocrinol 98:173-187.

Saransaari P, Oja SS (1994) Glycine release from hippocampal slices in developing and ageing mice: modulation by glutamatergic receptors. Mech Ageing Dev 76:113-124.

Sarthy PV (1982) The uptake of [3H]gamma-aminobutyric acid by isolated glial (Muller) cells from the mouse retina. J Neurosci Methods 5:77-82.

Sarthy PV (1983) Release of [3H]gamma-aminobutyric acid from glial (Muller) cells of the rat retina: effects of K+, veratridine, and ethylenediamine. J Neurosci 3:2494-2503.

Sato K, Adams R, Betz H, Schloss P (1995) Modulation of a recombinant glycine transporter (GLYT1b) by activation of protein kinase C. J Neurochem 65:1967-1973.

Schlaepfer WW, Zimmerman UP (1981) Calcium-mediated breakdown of glial filaments and neurofilaments in rat optic nerve and spinal cord. Neurochem Res 6:243-255.

Schnitzer J (1987) Retinal astrocytes: their restriction to vascularized parts of the mammalian retina. Neurosci Lett 78:29-34.

Schwartz EA (1993) L-glutamate conditionally modulates the K+ current of Muller glial cells. Neuron 10:1141-1149.

Schwartz EA, Tachibana M (1990) Electrophysiology of glutamate and sodium co-transport in a glial cell of the salamander retina. J Physiol 426:43-80.

Shaw PJ (1999) Calcium, glutamate, and amyotrophic lateral sclerosis: more evidence but no certainties. AnnNeurol 46:803-5.

Shepherd GM (1974) The synaptic Organization of the Brain. An Introduction. New York: Oxford University Press. 364 pp.

Shields DC, Banik NL (1999) Pathophysiological role of calpain in experimental demyelination. J Neurosci Res 55:533-541.

Siliprandi R, Canella R, Carmignoto G, Schiavo N, Zanellato A, Zanoni R, Vantini G (1992) N-methyl-D-aspartate-induced neurotoxicity in the adult rat retina. Vis Neurosci 8:567-573.

Simpson RK, Jr., Gondo M, Robertson CS, Goodman JC (1995) The influence of glycine and related compounds on spinal cord injury- induced spasticity. Neurochem Res 20:1203-1210.

Smith KE, Borden LA, Hartig PR, Branchek T, Weinshank RL (1992) Cloning and expression of a glycine transporter reveal colocalization with NMDA receptors. Neuron 8:927-935.

Soderling TR, Tan SE, McGlade-McCulloh E, Yamamoto H, Fukunaga K (1994) Excitatory interactions between glutamate receptors and protein kinases. J Neurobiol 25:304-311.

Somohano F, Lopez-Colome AM (1991) Characteristics of excitatory amino acid uptake in cultures from neurons and glia from the retina. J Neurosci Res 28:556-562.

Sontheimer H (1994) Voltage-dependent ion channels in glial cells. Glia 11:156-172.

Spike RC, Watt C, Zafra F, Todd AJ (1997) An ultrastructural study of the glycine transporter GLYT2 and its association with glycine in the superficial laminae of the rat spinal dorsal horn. Neuroscience 77:543-551.

Stuehr DJ (1999) Mammalian nitric oxide synthases. Biochim Biophys Acta 1411:217-230.

Supplisson S, Bergman C (1997) Control of NMDA receptor activation by a glycine transporter co- expressed in Xenopus oocytes. J Neurosci 17:4580-4590.

Sykova E (1983) Extracellular K+ accumulation in the central nervous system. Prog Biophys Mol Biol 42:135-189.

Szatkowski M, Barbour B, Attwell D (1990) Non-vesicular release of glutamate from glial cells by reversed electrogenic glutamate uptake. Nature 348:443-446.

Takeuchi Y, Yamamoto H, Fukunaga K, Miyakawa T, Miyamoto E (2000) Identification of the isoforms of Ca(2+)/Calmodulin-dependent protein kinase II in rat astrocytes and their subcellular localization. J Neurochem 74:2557-2567.

Tanaka K, Watase K, Manabe T, Yamada K, Watanabe M, Takahashi K, Iwama H, Nishikawa T, Ichihara N, Kikuchi T, Okuyama S, Kawashima N, Hori S, Takimoto

M, Wada K (1997) Epilepsy and exacerbation of brain injury in mice lacking the glutamate transporter GLT-1. Science 276:1699-702.

Tout S, Chan-Ling T, Hollander H, Stone J (1993) The role of Muller cells in the formation of the blood-retinal barrier. Neuroscience 55:291-301.

Tsai G, van Kammen DP, Chen S, Kelley ME, Grier A, Coyle JT (1998) Glutamatergic neurotransmission involves structural and clinical deficits of schizophrenia. Biol Psychiatry 44:667-674.

Uhl GR, Hartig PR (1992) Transporter explosion: update on uptake. Trends Pharmacol Sci 13:421-425.

Uhl GR, Johnson PS (1994) Neurotransmitter transporters: three important gene families for neuronal function. J Exp Biol 196:229-236.

Vallano ML, Beaman-Hall CM, Mathur A, Chen Q (2000) Astrocytes express specific variants of CaM KII delta and gamma, but not alpha and beta, that determine their cellular localizations. Glia 30:154-164.

Van Belle H (1984) The effect of drugs on calmodulin and its interaction with phosphodiesterase. Adv Cyclic Nucleotide Protein Phosphorylation Res 17:557-567.

Van Eldik LJ, Zendegui JG, Marshak DR, Watterson DM (1982) Calcium-binding proteins and the molecular basis of calcium action. Int Rev Cytol 77:1-61.

van Staveren WC, Markerink-van Ittersum M, Steinbusch HW, de Vente J (2001) The effects of phosphodiesterase inhibition on cyclic GMP and cyclic AMP accumulation in the hippocampus of the rat. Brain Res 888:275-286.

Verkhratsky A, Kettenmann H (1996) Calcium signalling in glial cells. Trends Neurosci 19:346-352.

Verkhratsky A, Steinhauser C (2000) Ion channels in glial cells. Brain Res Brain Res Rev 32:380-412.

Verona M, Zanotti S, Schafer T, Racagni G, Popoli M (2000) Changes of synaptotagmin interaction with t-SNARE proteins in vitro after calcium/calmodulin-dependent phosphorylation. J Neurochem 74:209-221.

Villa PG, Henzel WJ, Sensenbrenner M, Henderson CE, Pettmann B (1998) Calpain inhibitors, but not caspase inhibitors, prevent actin proteolysis and DNA fragmentation during apoptosis. J Cell Sci 111:713-722.

Villaseca JM, Navarro-Garcia F, Mendoza-Hernandez G, Nataro JP, Cravioto A, Eslava C (2000) Pet toxin from enteroaggregative Escherichia coli produces cellular damage associated with fodrin disruption. Infect Immun 68:5920-5927.

Vinade L, Goncalves CA, Wofchuk S, Gottfried C, Rodnight R (1997) Evidence for a role for calcium ions in the dephosphorylation of glial fibrillary acidic protein (GFAP) in immature hippocampal slices and in astrocyte cultures from the rat. Brain Res Dev Brain Res 104:11-17.

Virgo L, de Belleroche J (1995) Induction of the immediate early gene c-jun in human spinal cord in amyotrophic lateral sclerosis with concomitant loss of NMDA receptor NR-1 and glycine transporter mRNA. Brain Res 676:196-204.

Wakakura M, Yamamoto N (1994) Cytosolic calcium transient increase through the AMPA/kainate receptor in cultured Muller cells. Vision Res 34:1105-1109.

Wakakura M, Utsunomiya-Kawasaki I, Ishikawa S (1998) Rapid increase in cytosolic calcium ion concentration mediated by acetylcholine receptors in cultured retinal neurons and Muller cells. Graefes Arch Clin Exp Ophthalmol 236:934-939.

Wang Z, Haydon PG, Yeung ES (2000) Direct observation of calcium-independent intercellular ATP signaling in astrocytes. Anal Chem 72:2001-2007.

Weiss B, Prozialeck W, Cimino M, Barnette MS, Wallace TL (1980) Pharmacological regulation of calmodulin. Ann N Y Acad Sci 356:319-345.

Wheater PR, Burkitt HG, Daniels VG (1987) Functional Histology. Chrchill Livingstone. London. 348 pp.

Whitaker JN, Herman PK, Sparacio SM, Zhou SR, Benveniste EN (1991) Changes induced in astrocyte cathepsin D by cytokines and leupeptin. J Neurochem 57:406-414.

Wilson JM, Sanyal S, Van Tol HH (1998) Dopamine D2 and D4 receptor ligands: relation to antipsychotic action. Eur J Pharmacol 351:273-286.

Willbold E, Rothermel A, Tomlinson S, Layer PG (2000) Muller glia cells reorganize reaggregating chicken retinal cells into correctly laminated in vitro retinae. Glia 29:45-57.

Witkovsky P, Stone S, Ripps H (1985) Pharmacological modification of the light-induced responses of Muller (glial) cells in the amphibian retina. Brain Res 328:111-120.

Witt A, Brady ST (2000) Unwrapping new layers of complexity in axon/glial relationships. Glia 29:112-117.

Wolfe FH, Sathe SK, Goll DE, Kleese WC, Edmunds T, Duperret SM (1989) Chicken skeletal muscle has three Ca2+-dependent proteinases. Biochim Biophys Acta 998:236-250.

Wu SM, Maple BR (1998) Amino acid neurotransmitters in the retina: a functional overview. Vision Res 38:1371-1384.

Zafra F, Poyatos I, Gimenez C (1997) Neuronal dependency of the glycine transporter GLYT1 expression in glial cells. Glia 20:155-162.

Zafra F, Alcantara R, Gomeza J, Aragon C, Gimenez C (1990) Arachidonic acid inhibits glycine transport in cultured glial cells. Biochem J 271:237-242.

Zafra F, Aragon C, Olivares L, Danbolt NC, Gimenez C, Storm-Mathisen J (1995) Glycine transporters are differentially expressed among CNS cells. J Neurosci 15:3952-3969.

Zharikov SI, Block ER (2000) Association of L-arginine transporters with fodrin: implications for hypoxic inhibition of arginine uptake. Am J Physiol Lung Cell Mol Physiol 278:L111-117.

TESIS CON
FALLA DE ORIGEN

APÉNDICE I

BAPTA-AM	Quelante de calcio intracelular	5-10 μM
Cafeína	En la glía de Müller estimula la liberación de calcio de pozas intracelulares	5-10 mM
Caliculina A	Inhibidor de fosfatasas PP1 y PP2A	10 nM
Ciclosporina A	Inhibidor de la Calcineurina	10 μM
Citocalasina B	Impide la polimerización de la actina	10-50 μM
Citocalasina D	Impide la polimerización de la actina	10-50 μM
Colchicina	Impide la polimerazación de la tubulina	25 μM
Dantroleno	Bloqueador de la liberación de Ca^{2+} de pozas intracelulares	30 μM
Deltametrina	Inhibidor de la calcineurina	20 μM
DOG	Agonista del DAG (activa PKC)	100 μg/ml
EGTA	Quelante de Ca2+ extracelular	1 mM
Estaurosporina	Inhibidor de la PKC	100 nM
Forskolina	Activador de la Adenilato ciclasa	5 μM
Genisteina	Inhibidor de cinasas de tirosina	0.3-0.5 mM
H-7	Inhibidor de la PKC	50 μM
KN-62	Inhibidor de la cinasa dependiente de Ca2+-Calmodulin II (CaMKII).	20-40 μM
MDL-12330A	"Inhibidor de la Adenilato Ciclasa"	5-100 μM
Mepacrina	Inhibidor de la fosfolipasa A2	50 μM
ML-7	Inihibidor de la MLCK	20 μM
ML-9	Inhibidor de la MLCK	20 μM
Neomicina	Inihibidor de la fosfolipasa C	110 μM
Ofiobolina A	Toxina de helmintosporium inhibidor de la calmodulina	50 μM
PMA	Agonista del DAG (PKC)	1 μM
Polimixina B	Inihibidor de la PKC	400 μM
Queleritrina	Inhibidor de la PKC	1 μM
R-24571	Inhibidor de la calmodulina	1-5 μM
Ryanodina	Inihibdor de la liberación de Ca2+ de las pozas intracelulares	1 μM
SQ-22536	Inihibidor de la Adenilato ciclasa	20-100 μM
Tapsigargina	Inhibidor de la ATPasa de Ca2+ del retículo endoplásmico	1 μM
Tautomicina	Inhibidor de fosfatasas PP1 y PP2A	300 nM- 1 μM
Taxol (Paclitaxel)	Impide la despolimerización de la tubulina	1 μM
Trifluoperazina	Inhibidor de la calmodulina	10-25 μM
W-7	Anticalmodulínico	5-20 μM
Wortmanina	Inhibidor de la PI3-cinasa	100nM-0.5 μM

GLIA 26:273–279 (1999)

Characterization of Glycine Transport in Cultured Müller Glial Cells From the Retina

ANA GADEA,[1] EDITH LÓPEZ,[1] AND ANA MARÍA LÓPEZ-COLOMÉ[1,2]*
[1]*Instituto de Fisiología Celular, Departamento de Neurociencias, UNAM, México, D.F., México*
[2]*Facultad de Medicina, Departamento de Bioquímica, UNAM, México, D.F., México*

KEY WORDS radial glia; chick retina; NMDA receptors; neurotransmitter uptake

ABSTRACT Rapid termination of the synaptic action of glutamate (Glu) and glycine (Gly) is achieved by uptake into the presynaptic terminal and glial cells. In the vertebrate CNS, Gly acts both as an inhibitory neurotransmitter and as a Glu modulator or coagonist at postsynaptic N-methyl-D-aspartate (NMDA) receptors. We have previously described NMDA receptors in Müller cells of chick retina coupled to the phosphoinositide cascade, the entry of calcium, and the activation of protein kinase C (PKC; López-Colomé et al. Glia 9:127–135, 1993). A colocalization of Gly transporters and NMDA receptors has been reported in brain tissue (Smith et al. Neuron 8:927–936, 1992); since the concentration of Gly could participate in the modulation of Glu excitatory transmission in the vertical pathways of the retina, transport of Gly in monolayer cultures of Müller cells was studied. Gly transport was found pH-sensitive with an optimum at pH 7.4. Kinetic analysis of the saturation curve for Gly within a concentration range of 0.01–2 mM, revealed two components of transport: a low-affinity system with K_m = 1.7 mM, V_{max} = 30 nmol/10 min/mg protein, and a high-affinity one with a K_m = 27 µM, V_{max} = 3 nmol/10 min/mg protein. Both systems were Na^+-dependent; the high-affinity system proved also dependent on external Cl^- and was inhibited by sarcosine, characteristic of GLYT1 transporters. The inhibition of low-affinity uptake by 2-(methylamino)isobutyric acid (MeAIB) and 2-aminoisobutyric acid (AIB) suggests the presence of transport system A in Müller cells. The process is energy-requiring, since Gly transport was decreased by metabolic inhibitors. Data obtained are in keeping with a modulatory role for Müller glia on excitatory transmission in the retina. *GLIA 26:273–279, 1999.* © 1999 Wiley-Liss, Inc.

TESIS CON FALLA DE ORIGEN

INTRODUCTION

The amino acid glycine functions as a classical inhibitory neurotransmitter in the spinal cord, the brain stem, and retina (Daly, 1990), exerting its effects through the glycine inhibitory receptor, a ligand-gated chloride channel, competitively antagonized by strychnine (Betz et al., 1993). Furthermore, glycine also modulates excitatory neurotransmission as an obligatory coagonist of glutamate at N-methyl-D-aspartate (NMDA)-activated glutamate receptors via a binding site, distinct from that on the strychnine-sensitive glycine receptor (Fletcher et al., 1990).

The termination of amino acid neurotransmission in the CNS involves the rapid removal of neurotransmitter from synapses by reuptake either into the presynaptic terminal or surrounding glia through specialized transport systems (Kanner, 1989) driven by the electrochemical Na^+ potential (Kanner, 1983). Inhibition or stimulation of uptake could modulate the strength of synaptic action by regulating the available levels of endogenous transmitters. The development of selective inhibitors may therefore represent a novel therapeutic approach to the treatment of neurological disorders (Kanner, 1994).

The presence of high-affinity and low-affinity transport systems for several neurotransmitter amino acids,

Grant sponsor: CONACyT; Grant number: 3286P-N; Grant sponsor: DGAPA; Grant number: IN210998.

*Correspondence to: Ana María López-Colomé, Instituto de Fisiología Celular, UNAM, Apartado Postal 70-253, 04510, México, D.F. México.

Received 28 December 1997; Accepted 17 December 1998

© 1999 Wiley-Liss, Inc.

TESIS CON
FALLA DE ORIGEN

including glycine, in different areas of the CNS has been shown. High-affinity Na^+-dependent systems have been related to the termination of transmitter action (Iversen, 1971), whereas low-affinity systems would play a metabolic role (Johnston and Iversen, 1971). Among the low-affinity transport systems, the A system accepts most zwitterionic amino acids, such as alanine, glycine, 2-aminoisobutyric acid (AIB), and its derivative 2-(methylamino)isobutyric acid (MeAIB; Christensen, 1984). Another Na^+-dependent transport system for glycine is system ASC, transporting neutral amino acids without branched side chains such as alanine, serine and cysteine (Shotwell and Oxender, 1983).

Cloning of GABA (Guastella et al., 1990) and norepinephrine (Pacholczyk et al., 1991) transporters allowed the subsequent isolation of a number of cDNAs encoding homologous Na^+/Cl^--dependent neurotransmitter transporters, including those for glycine. Two different glycine transporters have been cloned, GLYT1 (Guastella et al., 1992; Liu et al., 1992; Smith et al., 1992), of which three isoforms derived from alternative splicing and/or promoter usage exist (Borowsky et al., 1993; Kim et al., 1994; Adams et al., 1995): GLYT1a, GLYT1b, and GLYT1c, which differ in the amino-terminal region (Kim et al., 1994), and GLYT2, encoded by a different gene. GLYT1 and GLYT2 show distinct anatomical distribution within the CNS, and are pharmacologically distinguishable, GLYT1 being sensitive and GLYT2 insensitive to sarcosine (n-methylglycine; Liu et al., 1993).

The expression of GLYT1a and GLYT1b has been demonstrated by in situ hybridization in several regions of the CNS of adult rats and mice (Guastella et al., 1992; Liu et al., 1992; Smith et al., 1992; Borowsky et al., 1993). GLYT1a mRNA is present in regions rich in neuronal cell bodies and its colocalization with mRNA for inhibitory glycine receptor subunits has been proposed (Borowsky et al., 1993); this receptor is also expressed in several peripheral tissues such as liver, lung, and stomach. In contrast, GLYT1b mRNA in the brain colocalizes with the NMDA subtype of glutamate receptors (Smith et al., 1992). Glycine transporters could modulate NMDA receptor activity, regulating its availability at the coagonist site of this receptor (Johnson and Ascher, 1987), through uptake into neighboring glial cells and/or reverse transporter-mediated release (Attwell et al., 1993). GLYT2 mRNA expression seems to be restricted to the brain stem and spinal chord, parallel to that of inhibitory strychnine-sensitive glycine receptors (Jursky and Nelson, 1995).

In the retina, Müller cells are the most abundant glial cell type, its processes ensheathing excitatory synapses at the plexiform layers. In addition to their structural and nutritional roles, the precise anatomical localization of Müller cells has suggested a role in the modulation of neurotransmission (Newman and Reichenbach, 1996). We have previously characterized NMDA receptors in whole chick retina (López-Colomé and Somohano, 1992) as well as in Müller cells from this tissue, coupled to the phosphoinositide cascade, the entry of calcium and the activation of protein kinase C (PKC; López-Colomé et al., 1993). In order to provide evidence supporting the participation of glycine transporters in the modulation of glutamate excitatory transmission in the vertical pathways of the retina, glycine uptake was characterized in confluent monolayer cultures of Müller cells from 7-day-old chick embryos.

MATERIALS AND METHODS

Chemicals

[^{3}H]Glycine was purchased from Amersham, Buckinghamshire, U.K. (Sp. Act. 17.5 Ci/mmol) or Dupont-New England Nuclear, Boston, MA (Sp. Act. 42–43.8 Ci/mmol). Tissue culture reagents and plastics were from GIBCO (Grand Island, NY). All other chemicals and reagents were from Sigma Chemical Co. (St. Louis, MO), except potassium cyanide (Baker, Xalostoc, México).

Cell Culture

Primary cultures of Müller glia cells were obtained as described previously (López-Colomé and Romo-De-Vivar, 1991). Retinas from 7-day-old embryos were dissected and washed in Hanks' solution free from Ca^{2+} and Mg^{2+} (g/100 ml): NaCl 0.8, KCl 0.04, KH_2PO_4 0.006, Na_2HPO_4 0.0125, phenol red 0.002, and glucose 0.1. Tissue was dissociated in 0.25% trypsin followed by filtration through a 50 µm mesh nylon net, resuspended in Minimum Essential Medium (MEM) containing 0.05% glucose, 25 mM $NaHCO_3$, 0.0125% gentamycin, 0.0125% penicillin, 0.0125% streptomycin, 0.025% neomycin, and 10% fetal bovine serum (FBS). Cells were seeded onto 24-well tissue-culture plates at a density of 2.5×10^5 cells per well, and maintained at 37°C in a humidified atmosphere of 5% CO_2:95% air. The purity of the culture was assessed by glial fibrillary acidic protein (GFAP; Björklund et al., 1985) and neuron-specific enolase (NSE) antibodies (Schmechel et al., 1980); as previously described, 95% of the cells were $GFAP^+$ and NSE^- at day 12 in vitro, in which cultures formed a confluent monolayer (López-Colomé and Romo-de-Vivar, 1991). Medium was changed every other day. Confluent cell cultures were used for all experiments.

Uptake Experiments

Assays were performed at 37°C in 0.5 ml of Krebs-Ringer bicarbonate buffer (KRB), containing (in mM) NaCl, 118; KH_2PO_4, 2.0; KCl, 4.7; $CaCl_2$, 2.5; $MgSO_4$, 1.4; $NaHCO_3$, 25; and glucose, 5.6, pH 7.4. All solutions were prepared in double distilled water. Prior to the experiment, the growth medium was removed, and cultures were rinsed twice with 0.5 ml of prewarmed KRB (37°C). After 5 min, medium was replaced by 0.5 ml of KRB containing [^{3}H]-Gly/Gly 1:25,000. Cultures

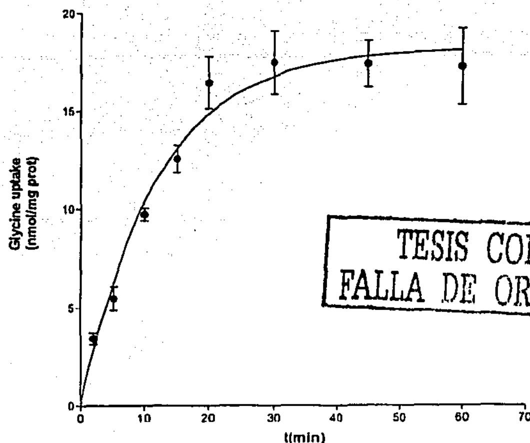
were incubated for the indicated period of time in the presence of different drugs in order to study their effect on glycine transport. At the end of the assay, cells were rinsed with 3 × 0.5 ml of fresh KRB (2–4°C), dissolved in 1 ml of 1 M HCl and counted for radioactivity after the addition of 1 ml 1 M NaOH and 5 ml of Tritosol (Fricke, 1975) in a liquid scintillation counter (Beckman Instruments, Palo Alto, CA).

Experiments in order to evaluate the effect of pH on transport activity were performed in Krebs-Ringer solution in which $NaHCO_3$ was isosmotically replaced by sodium acetate for pH values < 7.0 or sodium borate for pH values ≥ 8.0.

Experiments were carried out in triplicate, and replicated at least three times with different cell cultures. Results are expressed as the mean ± S.E.M. of three independent experiments performed in triplicate. Corrections were made for specific activity.

The protein content was determined for two wells of each plate by the method of Lowry et al. (1951).

Data were analyzed using the INPLOT (version 3.1) program from GraphPad software (San Diego, CA).



TESIS CON FALLA DE ORIGEN

Fig. 1. Time course of glycine transport. Müller cells were incubated in the presence of 1 mM glycine ([^{3}H]-Gly/Gly 1:25,000) for the indicated time period. Each point represents the mean ± S.E.M. of three experiments performed in triplicate.

RESULTS

Time-Course and pH Dependence of Uptake

Glycine transport (1 mM final concentration, [^{3}H]-Gly/Gly 1:25,000) was found saturable; no further increase in accumulation was seen after 30 min (Fig. 1). As shown in Figure 2, glycine uptake in Müller cells was sensitive to changes in pH. Uptake experiments were performed at pH values from pH 5 to 8.9. The results show that the optimum pH for transport was 7.4.

Kinetics of Glycine Uptake

Saturation curves for glycine transport in Müller cells were obtained by measuring the rate of glycine incorporation over a wide range of substrate concentrations (10 µM-2 mM, [^{3}H]-Gly/Gly 1:5,000). Eadie-Hofstee kinetic analysis of the data revealed two saturable components of the system (Fig. 3), a high-affinity component and a low-affinity one. In such system, the high-affinity component contributes to the low-affinity component, which results in an apparent increase in the affinity of the latter. In order to account for overlapping, modified constants were calculated by the method of Neal (1972). Values for K_m and V_{max} for the high- and low-affinity components were K_m = 27 ± 7 µM, V_{max} = 3 ± 0.8 nmol/10 min/mg protein, and K_m = 1.7 ± 0.4 mM, V_{max} = 30 ± 5 nmol/10 min/mg protein, respectively.

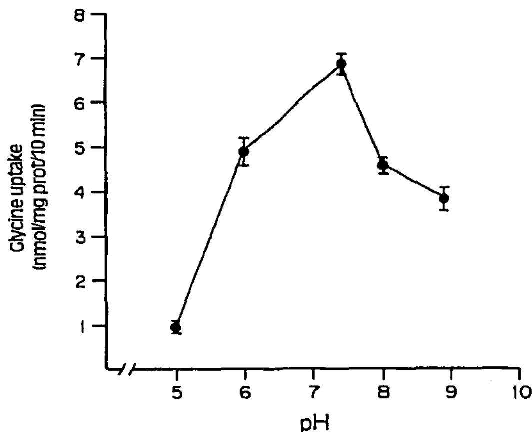


Fig. 2. pH-dependence of glycine uptake. Confluent cultures were incubated for 10 min in the presence of 1 mM glycine ([^{3}H]-Gly/Gly 1:25,000) at the indicated pH values, as described in Materials and Methods. Each point represents the mean ± S.E.M. of four experiments performed in triplicate.

Ionic Dependence of Glycine Transport

Na^+- and Cl^--dependent glycine transport in brain slices and membrane vesicles has been reported (López-Corcuera and Aragón, 1989; López-Corcuera et al., 1989), whereas low-affinity transport systems for amino acids other than glycine have been shown to depend exclusively on the presence of Na^+ (Christensen, 1984). The ionic dependence for both components of the transport system was analyzed. Isosmotical concentration of choline or lithium chloride substituted for NaCl in

TESIS CON
FALLA DE ORIGEN

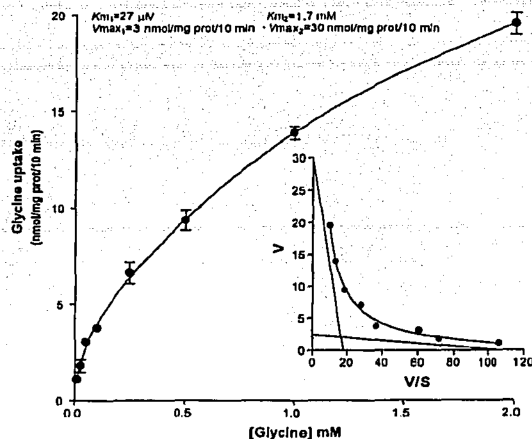


Fig. 3. Kinetics of glycine uptake in Müller cells. Müller cells grown to confluence were incubated for 10 min in the presence of glycine at concentrations ranging from 0.01 mM to 2 mM ([³H]-Gly/Gly 1:5,000). Eadie-Hofstee analysis is depicted in the inset. Values are the mean ± S.E.M. of two to four triplicate determinations.

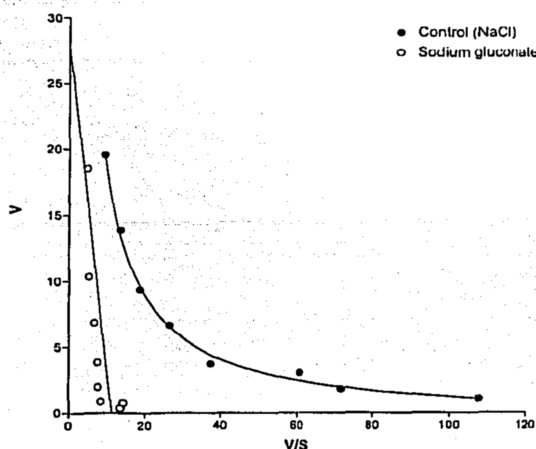


Fig. 5. Chloride-dependence of glycine uptake. Müller cells were incubated for 10 min in the presence of Gly within a concentration range of 0.01–2 mM ([³H]-Gly/Gly 1:25,000) in the following media: KRB control (with 118 mM NaCl) (closed circle) or KRB in which NaCl has been isosmotically replaced by sodium gluconate (open circle). Data were analyzed as an Eadie-Hofstee plot. Values are the mean ± S.E.M. of three determinations performed in triplicate.

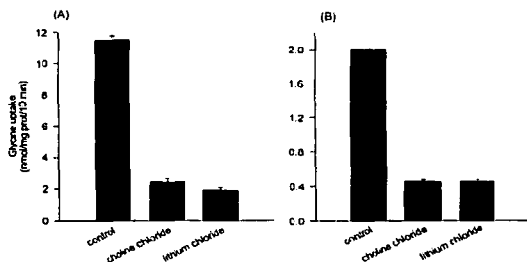


Fig. 4. Sodium-dependence of glycine uptake. Müller cells were incubated for 10 min with 1 mM glycine ([³H]-Gly/Gly 1:25,000) (A) or 10 µM ([³H]-Gly/Gly 1:5,000) (B) in KRB containing 118 mM NaCl, or Na^+-free; Na^+ was replaced by 118 mM lithium chloride, or choline chloride. Values are the mean ± S.E.M. of three determinations in triplicate.

Na^+-free conditions; sodium gluconate substituted for NaCl in Cl^--free experiments, and two different concentrations of glycine were used in order to distinguish between the high- and the low-affinity components (10 µM and 1 mM glycine, respectively). As depicted in Figure 4, glycine uptake was strictly Na^+-dependent for both high- and low-affinity systems, whereas the absence of Cl^- ions specifically abolished high-affinity uptake (Fig. 5).

Preincubation of the cultures with nigericin (5 µM), an ionophore capable of collapsing Na^+ gradient (Rodríguez and Sitges, 1996), inhibits glycine transport 52 ± 4% in Na^+-containing medium (Fig. 6). In Na^+-free medium, inhibition of transport by nigericin was reduced to 12 ± 2.1%.

Energy-Dependence of Glycine Uptake

The energetic requirement for glycine transport in Müller cells was studied for each component, in the presence of energy production inhibitors at 10 µM and 1 mM glycine. Table 1 shows that transport at 1 mM glycine concentration is energy-requiring, since potassium cyanide (1 mM), 2,4-dinitrophenol (200 µM) and iodoacetate (1 mM) decreased transport by 13 ± 0.4%, 18 ± 0.7%, and 24 ± 0.2%, respectively. Ouabain (200 µM), potently inhibited low-affinity glycine uptake (65 ± 4%). At low concentrations of glycine (10 µM), potassium cyanide, 2,4-dinitrophenol, and iodoacetate inhibited transport by 11 ± 1.2%, 36 ± 0.8%, and 29 ± 1.4%, respectively; ouabain also had a strong inhibitory effect on transport (59 ± 2%). When tested together, ouabain and iodoacetate inhibited low-affinity uptake by 64 ± 1.8% and the high-affinity one by 75 ± 2%.

Pharmacological Characteristics of Glycine Uptake

In order to determine the pharmacological specificity of both systems, the effect of various amino acids and derivatives on glycine transport (25 µM and 1 mM) by Müller cells was tested. Glycine is a substrate for several amino acid transport systems in various tissues, therefore the relationship of the transport system in Müller cells with previously identified systems was examined. Results in Table 2 show that 100 µM sarco-

TESIS CON
FALLA DE ORIGEN

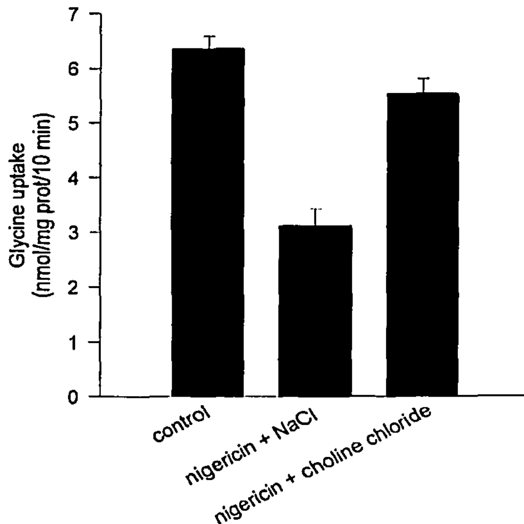


Fig. 6. Effect of nigericin on glycine transport. Müller cells were preincubated for 15 min in the presence of 5 μM nigericin in control KRB or Na^+-free KRB. The buffer was removed and the transport assay was carried out in KRB containing 1mM glycine ([^{3}H]-Gly/Gly 1:25,000). Values are expressed as the mean ± S.E.M. of three triplicate determinations.

TABLE 1. Effect of metabolic inhibitors on glycine transport†

	nmol/10 min/mg protein	
Tested compound	High affinity uptake (I)	Low affinity uptake (II)
None	1.68 ± 0.083 (100)	10.58 ± 0.42 (100)
Potassium cyanide (KCN)	1.49 ± 0.057* (89)	9.2 ± 0.52* (87)
2,4-dinitrophenol (DNP)	1.07 ± 0.064** (64)	8.67 ± 0.74** (82)
Iodoacetate	1.20 ± .04** (71.5)	8.0 ± 0.32** (76)
Ouabain	0.58 ± 0.02*** (35)	4.3 ± 0.20*** (41)
Iodoacetate + DNP	0.93 ± 0.052*** (55)	7.72 ± 0.46** (73)
Iodoacetate + KCN	1.04 ± 0.08** (62)	7.93 ± 0.59** (75)
Iodoacetate + ouabain	0.42 ± 0.071*** (25)	3.82 ± 0.21*** (36)

†Müller cells were preincubated with 1 mM KCN, 200 μM DNP, 1mM iodoacetate, or 200 μM ouabain for 15 min. Uptake experiments were performed as described in Materials and Methods in the presence of 10 μM (I) or 1 mM (II) glycine. Values are the mean ± S.E.M. of at least three experiments performed in triplicate. Values in parentheses represent % of control values. Statistical significance of differences from control: *$P < 0.05$, **$P < 0.02$, ***$P < 0.001$.

sine inhibited by 76 ± 7% under high-affinity transport conditions, and 1 mM sarcosine inhibited by 38 ± 2% under low-affinity transport conditions. Methylaminoisobutyric acid (MeAIB) and aminoisobutyric acid (AIB), substrates for transport system A (Shotwell and Oxender, 1983), inhibited low-affinity uptake by ~ 50%; the same results were obtained in experiments performed in the absence of Cl^- (data not shown). Neither alanine or serine, substrates for system ASC (Shotwell and Oxender, 1983), inhibited glycine uptake. Taurine or GABA did not compete for glycine uptake.

High-affinity transport accounts for 20% of total uptake under low-affinity (1 mM Gly) conditions (Neal,

TABLE 2. Pharmacological specificity of glycine transport†

	nmol/10 min/mg protein	
Tested compound	High affinity uptake (I)	Low affinity uptake (II)
None	2.12 ± 0.24 (100)	12.11 ± 0.37 (100)
D-serine	2.14 ± 0.16 (101)	12.17 ± 0.74 (100.4)
GABA	2.31 ± 0.32 (109)	11.87 ± 0.49 (98)
Taurine	2.13 ± 0.15 (100)	12.19 ± 0.59 (100.6)
β-alanine	2.07 ± 0.05 (98)	13.14 ± 1.04 (108)
Sarcosine	0.51 ± 0.05*** (24)	7.52 ± 0.38** (62)
MeAIB	2.08 ± 0.09 (98)	5.81 ± 0.48*** (48)
AIB	2.22 ± 0.10 (104)	6.19 ± 0.53*** (51)

†Uptake experiments were performed as described in Materials and Methods, in the presence of the different amino acids and amino acid derivatives (5 mM); sarcosine was used at concentrations of 100 μM in I, and 1 mM in II; glycine 25 μM (I) or 1 mM (II). Values are the mean ± S.E.M. of three experiments performed in triplicate. Values in parentheses represent % of control values. Statistical significance of differences from control: **$P < 0.02$, ***$P < 0.001$.

1972). Since low-affinity glycine transport was 38% inhibited by 1 mM sarcosine (Table 2), the effect of 0.1, 0.25, 0.5, and 1 mM sarcosine on the kinetics of glycine uptake was tested. As shown in Figure 7, high-affinity uptake was completely abolished at 0.1 mM sarcosine, whereas the low-affinity component remained unchanged. At higher concentrations (1 mM), sarcosine slightly inhibited low-affinity uptake due to an increase in K_m value which in this condition was 2.5 ± 0.6 mM.

DISCUSSION

We have demonstrated the presence of specific glycine uptake in Müller cells from chick retina, bearing pharmacological and kinetic characteristics described for GLYT1 transport system (Liu et al., 1993). Previous studies have demonstrated the expression of GLYT1 in amacrine cells, as well as in the outer plexiform layer of the retina, and suggested its absence in retinal glia (Zafra et al., 1995). The lack of agreement with our results could relate to differences in techniques or, alternatively, to species differences, since those studies were performed in rat retina.

The system we here characterized in Müller cells can be resolved into two components, a high-affinity one which could be identified as a GLYT1 type of transporter by its requirement for Na^+ as well as Cl^-, narrow substrate specificity and acceptance of sarcosine as a high-affinity substrate. The low-affinity one shares characteristics with described system A, since it is inhibited by MeAIB and AIB, shows Na^+- but not Cl^--dependence, exhibits a low K_m (Christensen, 1984; Zafra and Giménez, 1989), and is highly insensitive to sarcosine.

The study of neurotransmitter transporters has revealed that the main driving force for this process is the electrochemical gradient of sodium, maintained by the Na^+/K^+-ATPase (Kanner, 1994). Glycine transport in Müller cells was shown to be energy dependent since metabolic inhibitors such as iodoacetate, potassium cyanide, and dinitrophenol, which inhibit ATP production at different levels, diminished transport by lowering the activity of the Na^+/K^+-ATPase. This idea is

TESIS CON
FALLA DE ORIGEN

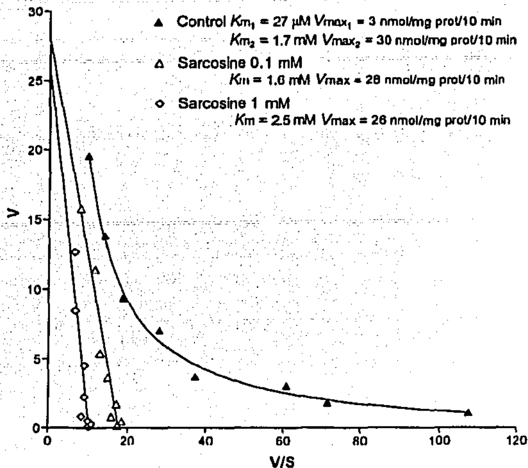


Fig. 7. Effect of sarcosine on the kinetics of glycine uptake. Müller cells were incubated for 10 min with 0.01–2 mM glycine ([^{3}H]-Gly/Gly 1:25,000), in the absence (control, closed triangle) or presence of sarcosine (0.1 mM, open triangle, or 1 mM, open diamond). Data were analyzed as an Eadie-Hofstee plot. Values are expressed as the mean ± S.E.M. of two determinations performed in triplicate.

supported by the fact that ouabain, which directly inhibits Na^+/K^+-ATPase activity, potently inhibited transport. Evidence has accumulated supporting the regulation of neurotransmitter transporters' activity by phosphorylation (Casado et al., 1993; Gomeza et al., 1991); if such were the case for glycine transport in Müller cells, a decrease in ATP production would directly affect the activity of this system. It is worth mentioning that high-affinity uptake showed to be more sensitive to changes in the electrochemical gradient of Na^+ than low-affinity transport, which could be indicative of different regulatory mechanisms for the two components of this process. On this line, nigericin, shown to collapse the Na^+-gradient (Rodríguez and Sitges, 1996), inhibited glycine transport in the presence of Na^+ (Fig. 6). The slight inhibition of transport by the ionophore in the absence of Na^+ could be due to the transport of K^+, which in turn could induce depolarization and the release of glycine by an inverse activity of the transporter (Attwell et al., 1993).

In addition to energy-dependent high-affinity uptake, exogenously added neurotransmitters can accumulate in brain tissue through homoexchange with endogenous material (Raiteri et al., 1975). A partial contribution of this process to the present results cannot be ruled-out under our conditions.

Glutamate, acting at ionotropic and metabotropic receptors, is widely accepted as the main excitatory transmitter in the vertical pathway of the retina (Daw et al., 1989). Müller radial glial cells closely ensheathe the excitatory synapses and are exposed to glutamate released by neurons. We have previously characterized NMDA-sensitive glutamate receptors in these cells and demonstrated the activation of the phosphoinositide cascade (López-Colomé et al., 1993), the increase in AP-1-DNA binding (López-Colomé et al., 1994), and the regulation of glutamate receptors expression by glutamate (López et al., 1998). GLYT1, specifically GLYT1b, has been related to NMDA receptors in the CNS, based on colocalization. The present data demonstrating the presence of a glycine transporter bearing the properties of GLYT1 open the possibility of Müller glia participating in the modulation of glutamate neurotransmission in the retina through the regulation of glycine concentration.

On this matter, it has been suggested that the glycine coagonist site at the NMDA receptors is saturated under physiological conditions (Thomson et al., 1989); however, in cerebellar granule cells, where GLYT1 is abundant, the activation of synaptic currents mediated by NMDA receptors can be evoked only in the presence of added glycine (D'Angelo et al., 1990), which supports an influence of glycine transport on the activity of NMDA receptors. Additionally, in the retina, the possible contribution of GLYT1 to the regulation of the inhibitory glycine receptors receiving input from amacrine cells at the inner plexiform layer should also be considered (Iuvone, 1986).

The role of the low-affinity uptake systems in the removal of glycine from the synaptic cleft is still unclear. This system may participate, jointly with high-affinity uptake, in the elimination of glycine shortly after its release, when the concentration is high, in addition to serving general metabolic functions similar to those proposed in neurons (Johnston and Iversen, 1971).

Glycine could participate in excitotoxic processes potentiating the effect of glutamate on NMDA receptors as well as in the pathogenesis of diseases related to inhibitory glycine receptors (Simpson et al., 1995; Lloyd et al., 1983). On this line, the pharmacological characterization of glycine transporters provides a tool for the modification of glycine concentrations through specific drugs affecting glycine reuptake.

Together with our previous results, the present data support a modulatory role for Müller glia in glutamate-mediated neurotransmission in the retina, possibly through an influence on NMDA receptor activity.

ACKNOWLEDGMENTS

This work was supported in part by grants 3286P-N from CONACyT and IN210998 from DGAPA to A.M.L.-C.

REFERENCES

Adams RH, Sato K, Shimada S, Tohyama M, Püschel AW, Betz H. 1995. Gene structure and glial expression of the glycine transporter GLYT1 in embryonic and adult rodents. J Neurosci 15:2524–2532.

Attwell D, Barbour B, Szatkowsky M. 1993. Nonvesicular release of neurotransmitter. Neuron 11:401–407.
Betz H, Langosch D, Rundstrom N, Bormann J, Kuryatov A, Kuhse J, Schmieden V, Matzenbach B, Kirsch J. 1993. Structure and biology of inhibitory glycine receptors. Ann NY Acad Sci 707:109–115
Björklund H, Bignami A, Dahl D. 1985. Immunohistochemical demonstration of glial fibrillary acidic protein in normal rat Müller glia and retinal astrocytes. Neurosci Lett 54:363–368.
Borowsky B, Mezey E, Hoffman BJ. 1993. Two glycine transporter variants with distinct localization in the CNS and peripheral tissues are encoded by a common gene. Neuron 10:851–863.
Casado M, Bendahan A, Zafra F, Danbolt NC, Aragón C, Giménez C,. Kanner B. 1993. Phosphorylation and modulation of brain glutamate transporters by protein kinase C. J Biol. Chem 268:27313–27317.
Christensen HN. 1984. Organic ion transport during seven decades: the amino acids. Biochim Biophys Acta 799:255–269.
Daly EC. 1990. The biochemistry of glycinergic neurons. In: Glycine neurotransmission. New York: John Wiley and Sons, Inc. p 25–67.
D'Angelo E, Rossi P, Garthwaite J. 1990. Dual-component NMDA receptor currents at a single central synapse. Nature 346:467–470
Daw NW, Brunken WJ, Parkinson D. 1989. The function of synaptic transmitters in the retina. Annu Rev Neurosci 12:205–225.
Fletcher EJ, Beart PM, Lodge D. 1990. Involvement of glycine in excitatory neurotransmission. In: Glycine neurotransmission. New York: John Wiley and Sons, Inc. p. 193–218.
Fricke V. 1975. A new scintillation cocktail based on Triton-X-100. Anal Biochem 63:555–558.
Gomeza J, Casado M, Giménez C, Aragón C. 1991. Inhibition of high-affinity γ-aminobutyric acid uptake in primary astrocyte cultures by phorbol esters and phospholipase C. Biochem J 275:435–439.
Guastella J, Nelson N, Nelson H, Czyzyk L, Keynan S, Miedel MC, Davidson N, Lester HA, Kanner BI. 1990. Cloning and expression of a rat brain GABA transporter. Science 249:1303–1306.
Guastella J, Brecha N, Weigmann C, Lester HA, Davidson N. 1992. Cloning, expression and localization of a rat brain high-affinity glycine transporter. Proc Natl Acad Sci USA 89:7189–7193.
Iuvone MP. 1986. Neurotransmitters and neuromodulators in the retina: regulation, interactions and cellular effects. In: The retina: a model for cell biology studies. Part II. Orlando, FL: Academic Press, Inc. 348 p.
Iversen LL. 1971. Role of transmitter uptake mechanisms in synaptic neurotransmission. Br J Pharmacol 41:571–591.
Johnson JW, Ascher P. 1987. Glycine potentiates the NMDA response in cultured mouse brain neurons. Nature 325:529–533.
Johnston GAR, Iversen LL. 1971. Glycine uptake in rat central nervous system slices and homogenates: evidence for different uptake systems in spinal cord and cerebral cortex. J Neurochem 18:1951–1961.
Jursky F, Nelson N. 1995. Localization of glycine neurotransmitter transporter (GLYT2) reveals correlation with the distribution of glycine receptor. J Neurochem 64:1026–1033.
Kanner BI. 1983. Bioenergetics of neurotransmitter transport. Biochim Biophys Acta 726:293–316.
Kanner BI. 1989. Ion-coupled neurotransmitter transport. Curr Opin Cell Biol 1:735–738.
Kanner BI. 1994. Sodium-coupled neurotransmitter transport: structure, function and regulation. J Exp Biol 196:237–249.
Kim K-M, Kingsmore SF, Han H,. Yang-Fen TL, Godinot N, Seldin MF, Caron MG, Giros B. 1994. Cloning of the human glycine transporter type 1: molecular and pharmacological characterization of novel isoform variants and chromosomal localization of the gene in the human and mouse genomes. Mol Pharmacol 45:608–617.
Liu Q-R, Nelson H, Mandiyan S, López-Corcuera B, Nelson N. 1992. Cloning and expression of a glycine transporter from mouse brain. FEBS Lett 305:110–114.
Liu Q-R, López-Corcuera B, Mandiyan S, Nelson H, Nelson N. 1993. Cloning and expression of a spinal cord and brain-specific glycine transporter with novel structural features. J Biol Chem 268:22802–22808.
Lloyd KG, De-Montis G, Javoy-Agid F, Beaumont K, Lowenthal A, Constantinidis J, Agid Y. 1983. Glycine receptors in the human brain: characterization of 3H-strychnine binding and status in pathological conditions. Adv Biochem Psychopharmacol 36:233–238.
López-Colomé AM, Romo-de-Vivar M. 1991. Excitatory amino acid receptors in primary cultures of glial cells from the retina. Glia 4:431–439
López-Colomé AM, Ortega A, Romo-de-Vivar M. 1993. Excitatory amino acid-induced phosphoinositide hydrolysis in Müller glia. Glia 9:127–135.
López-Colomé AM, Somohano F. 1992. N-methyl-D-aspartate receptors in the retina: 3-[(+/-)-2-carboxypiperazin-4-yl]-propyl-1-phosphonic acid (CPP) binding studies. Neuropharmacology 31(6):577–584.
López-Colomé AM, Murbatián J, Ortega A. 1994. Excitatory amino acid-induced AP-1 DNA binding activity in Müller glia. J Neurosci Res 41:179–184.
López T, López-Colomé AM, Ortega A. 1998. Changes in GluR4 expression induced by metabotropic receptor activation in radial glia cultures. Mol Brain Res 58:40–46.
López-Corcuera B, Aragón C. 1989. Solubilization and reconstitution of the sodium-and-chloride-coupled glycine transporter from rat spinal cord. Eur J Biochem 181:519–524.
López-Corcuera B, Kanner BI, Aragón C. 1989. Reconstitution and partial purification of the sodium and chloride-coupled glycine transporter from rat spinal cord. Biochim Biophys Acta 983(2):247–252.
Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ. 1951. Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275.
Neal JL. 1972. Analysis of Michaelis-Menten kinetics for two independent saturable membrane transport functions. J Theor Biol 35:113–118.
Newman E, Reichenbach A. 1996. The Müller cell: a functional element of the retina. TINS 19:307–312.
Pacholczyk T, Blakely RD, Amara SG. 1991. Expression cloning of a cocaine- and antidepressant-sensitive human noradrenaline transporter. Nature 350:350–354.
Raiteri M, Federico R, Coletti A, Levi G. 1975. Release and exchange studies relating to the synaptosomal uptake of GABA. J Neurochem 24:1243–1250.
Rodríguez R, Sitges M. 1996. Nigericin-induced Na+/H+ and K+/H+ exchange in synaptosomes: effect on GABA release. Neurochem Res 21:889–895.
Schmechel DE, Brightman MW, Marangos PJ. 1980. Neurons switch from non-neuronal to neuron-specific enolase during differentiation. Brain Res 190:195–214.
Shotwell MA, Oxender DL. 1983. The regulation of neutral amino acid transport in mammalian cells. Biochim Biophys Acta 737:267–284
Simpson RK, Gondo M, Robertson CS, Goodman JC. 1995. The influence of glycine and related compounds on spinal cord injury-induced spasticity. Neurochem Res 20:1203–1210.
Smith KE, Borden LA, Hartig PA, Branchek T, Weinshank RL. 1992. Cloning and expression of a glycine transporter reveal colocalization with NMDA receptors. Neuron 8:927–936.
Thomson AM, Walker VE, Flynn DM. 1989. Glycine enhances NMDA-receptor mediated synaptic potentials in neocortical slices. Nature 338:422–424.
Zafra F, Giménez C. 1989. Characteristics and adaptive regulation of glycine transport in cultured glial cells. Biochem J 258:403–408.
Zafra F, Aragón C, Olivares L, Danbolt NC, Giménez C, Storm-Mathisen J. 1995. Glycine transporters are differentially expressed among CNS cells. J Neurosci 15:3952–3969.

ELSEVIER

Brain Research 838 (1999) 200–204

BRAIN RESEARCH

www.elsevier.com/locate/bres

Short communication

The adenylate cyclase inhibitor MDL-12330A has a non-specific effect on glycine transport in Müller cells from the retina

Ana Gadea [a], Edith López [a], Ana María López-Colomé [a,b,*]

[a] *Instituto de Fisiología Celular, Departamento de Neurociencias, UNAM, Apartado Postal 70-253, 04510, D.F., Mexico*
[b] *Facultad de Medicina, Departamento de Bioquímica, UNAM, 04510, D.F., Mexico*

Accepted 25 May 1999

Abstract

Müller glial cells express two transport systems for glycine (Gly): one with low affinity and another identified as GLYT1 with high affinity. The latter colocalizes with NMDA receptors in the CNS. Gly is considered as an obligatory coagonist at NMDA receptors, and, hence, the Gly transport system could contribute to the modulation of glutamate (Glu) excitatory transmission in the vertical pathways of the retina. For this reason, the regulation of Gly transport by cAMP was studied. We report here a non-specific effect of MDL-12330A, a compound reported to inhibit adenylate cyclase (AC), on Gly transport in Müller glia. This effect might be due to a toxic action on the cells, decreasing cell viability, and not to a specific inhibition of the adenylate cyclase. Non-specific effects of this drug should be considered when the participation of cAMP in any biological process is studied. We have clearly demonstrated that cAMP does not participate in the regulation of Gly transport in Müller glia. © 1999 Published by Elsevier Science B.V. All rights reserved.

Keywords: Radial glia; Chick retina; Cyclic AMP; Neurotransmitter uptake

The fast removal of neurotransmitters from the synaptic cleft carried out by Na^+-dependent high-affinity transporter proteins located on neuronal and glial cells, is considered as a major mechanism for the termination of synaptic transmission [8]. Although the reuptake process is under physiological control, very little information is currently available regarding the possibility of the regulation of these proteins by second messengers. In this regard, arachidonic acid, released via the activation of phospholipase A_2, has been shown to inhibit several sodium-coupled uptake systems, including those for glycine (Gly) [18] and glutamate (Glu) [1]. The modulation of Glu transporters by protein kinase C (PKC) [2,6], and the regulation of GABA transporters by cAMP, have also been demonstrated [5].

To date, two different Gly transporters have been cloned: GLYT1, which seems to colocalize mainly with NMDA receptors [15], and GLYT2, colocalized with the inhibitory Gly receptors [9]. Recent results from our laboratory have demonstrated the presence in Müller cells of a Gly transport system showing two components, one of high affinity for Gly and another of lower affinity [4]. The high affinity system was characterized as GLYT1, since it is inhibited by sarcosine. In order to explore a possible role for glial Gly transport in modulating Glu excitatory transmission in the vertical pathways of the retina, through the regulation of the extracellular concentration of the coagonist at NMDA receptors, the regulation of this transport system by cAMP was studied.

The compound *N*-(*cis*-2-phenyl-cyclopentyl)azacyclotridecan-2-imine-hydrochloride [MDL-12330A] has been shown to inhibit specifically adenylate cyclase activity in different tissues [14,7], thus decreasing cellular cAMP concentrations [13]. We report here a non-specific effect of this compound on Gly transport in Müller glia, probably through a toxic action on the cells and not through the inhibition of adenylate cyclase.

Primary cultures of Müller glia were obtained as described previously [11] and confluent cell cultures were used for all experiments.

The uptake assays were performed at 37°C in 0.5 ml of Krebs–Ringer bicarbonate buffer (KRB), containing (in mM): NaCl, 118; KH_2PO_4, 2.0; KCl, 4.7; $CaCl_2$, 2.5;

* Corresponding author. Fax: +52-56-22-56-07; E-mail: acolome@ifisiol.unam.mx

0006-8993/99/$ - see front matter © 1999 Published by Elsevier Science B.V. All rights reserved.
PII: S0006-8993(99)01683-2

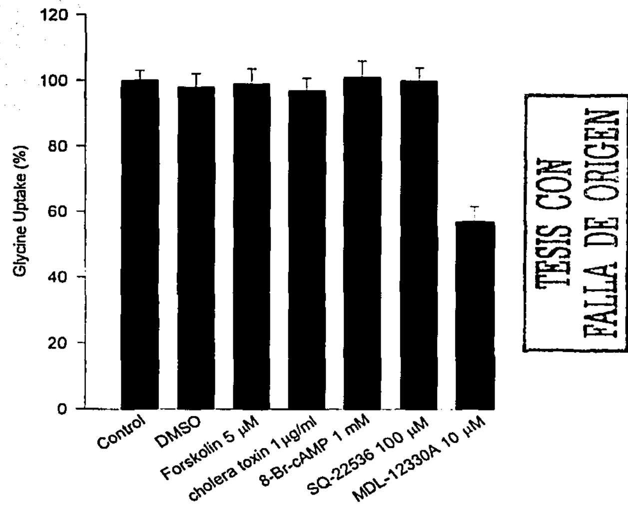
$MgSO_4$, 1.4; $NaHCO_3$, 25; glucose, 5.6; pH 7.4. All solutions were prepared in double distilled water. Previous to the experiment, the growth medium was removed, and cultures were rinsed twice with 0.5 ml of prewarmed KRB (37°C). After 5 min, medium was replaced by 0.5 ml of KRB containing [^{3}H]-Gly/Gly 1:25000 (Sp. Act. 51.1 Ci/mmol, Dupont-New England Nuclear), [^{3}H]-Leu/Leu 1:25000 (Sp. Act. 47 Ci/mmol, Amersham, Bucks., UK), or [^{3}H]D-Asp/D-Asp 1:50 (Sp. Act. 27 Ci/mmol, Amersham). Cultures were incubated for the indicated periods of time in the presence of different drugs in order to study their effect on transport. At the end of the assay, cells were rinsed with 3 × 0.5 ml of fresh KRB (2–4°C), dissolved in 1 ml of 1 N HCl and counted for radioactivity after the addition of 1 ml 1 N NaOH and 5 ml of Tritosol [3] in a liquid scintillation counter (Beckman). Cell viability was assessed by means of the trypan blue exclusion technique [17].

Experiments were carried out in triplicate, and replicated at least three times with different cell cultures. Corrections were made for specific activity. The protein content was determined for two wells of each plate by the method of Lowry et al. [12]. Data were analyzed using the INPLOT (version 3.1) program from Graph PAD software, San Diego, CA.

In order to determine the intracellular pathways involved in the regulation of Gly transport in Müller cells, the participation of cAMP in this process was studied. Fig. 1 shows the effect of agents known to increase intracellular cAMP directly, such as forskolin and cholera toxin and also of 8-Br-cAMP (Sigma, St. Louis, MO): none of these drugs had any effect on Gly transport. The adenylate cyclase inhibitors SQ-22536 and MDL-12330A (Research Biochemicals International) were also tested, and only the later showed an inhibitory effect on Gly transport (40% inhibition with respect to control). Müller cells were then incubated in the presence of increasing concentrations of MDL-12330A and, as shown in Fig. 2, an apparent dose-dependent decrease of Gly transport was observed, with an IC_{50} of 50 μM.

In order to assess the specificity of this effect, the uptake of other amino acids by Müller cells was measured in the presence of forskolin, SQ-22536 and MDL-12330A. The Na^+-independent uptake of leucine has been shown to be regulated by Ca^{2+} and calmodulin [16], whereas the Na^+-dependent uptake of D-aspartate is regulated through the activation of PKC [2]. In both cases, transport was inhibited by MDL-12330A (60% and 80%, respectively) while forskolin and SQ-22536 had no effect (Fig. 3).

As MDL-12330A seemed to have a non-specific effect on amino acid transport, cell viability after the treatment with this compound (50 μM) was quantified. The inhibitory effect of MDL-12330A at 20 min was not accompanied by a change in cell viability, however, following 40 min incubation, inhibition by the drug was associated with a marked decline in cell viability (30% with respect to control), which clearly demonstrates a toxic effect of this compound (Fig. 4). Cell viability was not affected by 20 or 40 min incubation with SQ-22536 (100 μM).



TESIS CON FALLA DE ORIGEN

Fig. 1. Participation of cAMP in the regulation of Gly transport. Müller cells were preincubated with the indicated compounds for 20 min in KRB. Since MDL-12330A was dissolved in DMSO, a control was added with 10 μl of DMSO in 0.5 ml of KRB. Gly transport assay was then carried out for 10 min in KRB with 1 mM Gly ([^{3}H]-Gly/Gly 1:25000). Values are expressed as the mean ± standard error of three experiments performed in triplicate. *Significantly different from control ($p < 0.001$, Student's "*t*" test).

Our results suggest that the inhibition of Gly transport by MDL-12330A is not due to its reported specific inhibition of AMP cyclase, but to a non-specific effect since transporters shown to be regulated by enzymatic pathways unrelated to cAMP were also inhibited by the drug. In support to this assumption, compounds which stimulate cAMP synthesis, such as forskolin and cholera toxin, or the non-hydrolyzable cAMP analog 8-Br-cAMP did not modify Gly uptake. Moreover, SQ-22536, which has been proposed as a more specific adenylate cyclase inhibitor than MDL-12330A [10] did not affect Gly (Fig. 1), leucine or D-aspartate transport (Figs. 3 and 4). Furthermore, at longer periods of incubation, MDL-12330A affects cell viability, due to a toxic effect on the cells. Segal and Ingbar [13] had previously reported a decrease in cell viability when incubating rat thymocytes with 50 μM MDL-12330A for 40 min, but still reported its inhibitory effects on adenylate cyclase at a 25 μM concentration.

The nature of the inhibitory action of MDL-12330A on adenylate cyclase activity is not clear. Guellaen et al. [7] studied adenylate cyclase activity in rat liver plasma membranes and suggested that MDL-12330A inhibition of adenylate cyclase is irreversible, since the effect remains in

TESIS CON
FALLA DE ORIGEN

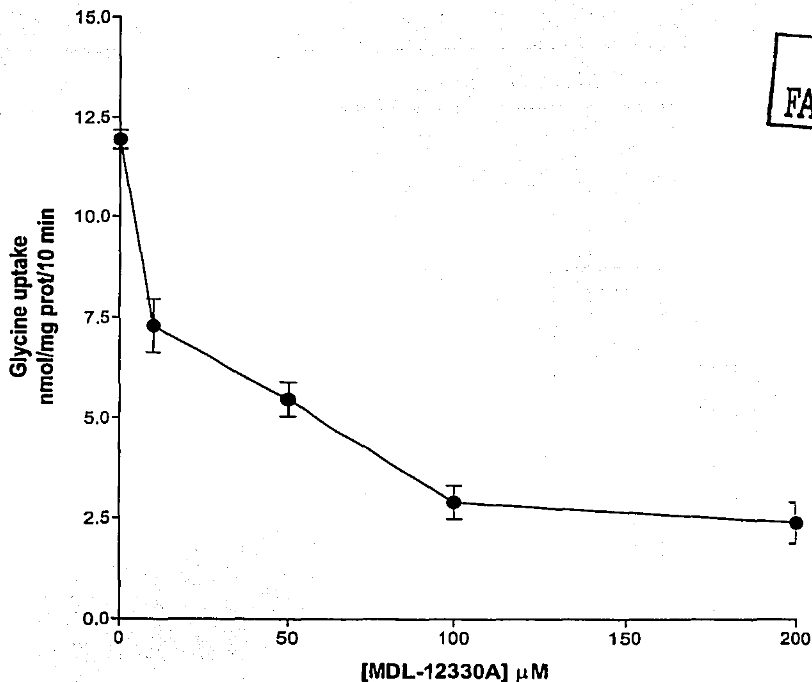


Fig. 2. Effect of increasing concentrations of MDL-12330A on Gly transport. Cells were preincubated with 10, 50, 100 and 200 μM MDL-12330A for 20 min. Gly transport assay was then carried out for 10 min in KRB with 1 mM Gly ([^{3}H]-Gly/Gly 1:25000). Values are expressed as means ± standard error of three experiments performed in triplicate.

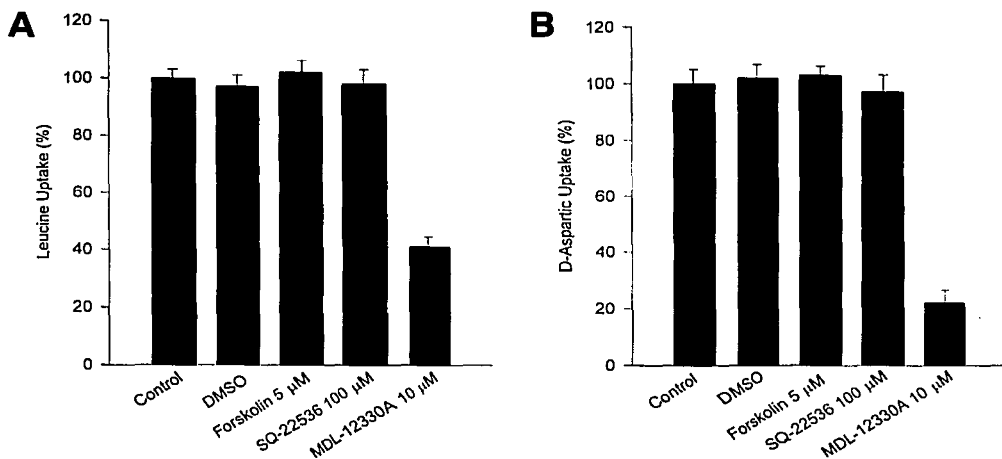
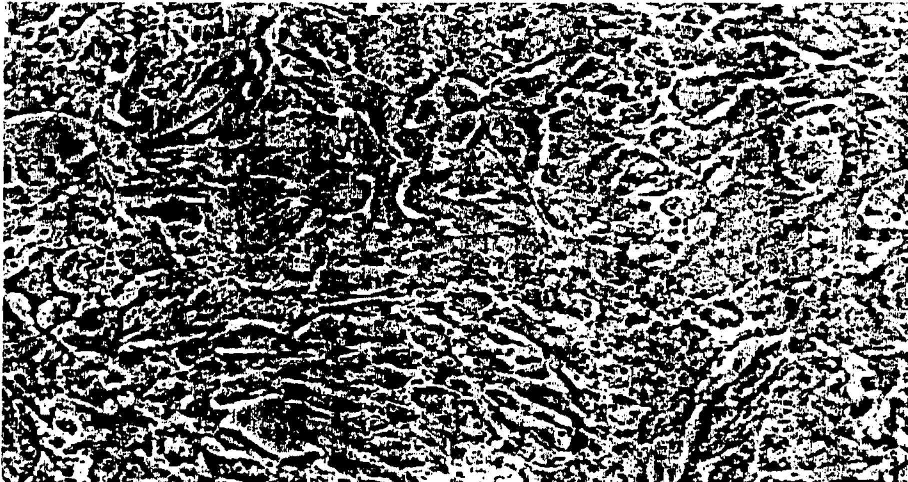


Fig. 3. Effect of MDL-12330A on leucine and D-aspartate transport. Müller cells were preincubated with the indicated compounds for 20 min in KRB. Since MDL-12330A was dissolved in DMSO, a control was added with 10 μl of DMSO in 0.5 ml of KRB. transport assays were then carried out for 10 min in KRB with (A) 250 nM D-aspartate ([^{3}H]-D-Asp/D-Asp 1:50) or (B) 1 mM Leucine ([^{3}H]-Leu/Leu 1:25000). Values are expressed as means ± standard error of three experiments performed in triplicate. * Significantly different from control ($p < 0.001$, Student's "t" test).

TESIS CON
FALLA DE ORIGEN

A



B

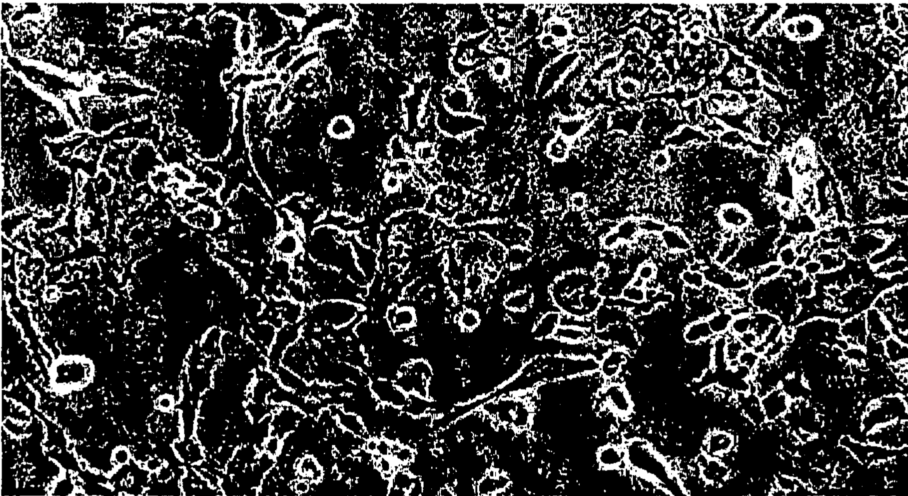


Fig. 4. Effect of MDL-12330A on cell viability. (A) Control culture of Müller cells. (B) Müller glia culture after treatment with MDL-12330A 50 μM, 40 min. Cell viability percent was then assessed by means of the trypan blue exclusion technique [17].

spite of removal of the agent and further washing of the membranes. Since this compound is highly hydrophobic, these authors suggested that its irreversible action could be due to a tight binding to hydrophobic components of the adenylate cyclase membrane complex, acting at its catalytic subunit.

Elucidation of the mechanism through which MDL-12330A affects adenylate cyclase activity awaits further investigation; nevertheless, non-specific effects of this drug on all processes unrelated to the cyclase should be considered when the participation of cAMP in any biological process is studied. In the present case, it seems clear that

cAMP does not participate in the regulation of Gly transport in Müller glia.

Acknowledgements

This work was supported in part by grant IN-210998 from DGAPA to A.M.L.C.

References

[1] B. Barbour, Modulation of glutamate transporters by arachidonic acid, Nature 342 (1989) 918–920.
[2] M. Casado, A. Bendahan, F. Zafra, N.C. Danbolt, C. Aragón, C. Giménez, B. Kanner, phosphorylation and modulation of brain glutamate transporters by protein kinase C, J. Biol. Chem. 268 (36) (1993) 27313–27317.
[3] V. Fricke, A new scintillation cocktail based on Triton-X-100, Anal. Biochem. 63 (1975) 555–558.
[4] A. Gadea, A.M. López-Colomé. Characterization of glycine transport in cultured Müller glial cells from the retina. Glia (1999) (in press).
[5] J. Gomeza, M. Casado, C. Giménez, C. Aragón, Inhibition of high-affinity γ-aminobutyric acid uptake in primary astrocyte cultures by phorbol esters and phospholipase C, Biochem. J. 275 (1991) 435–439.
[6] M.I. Gonzalez, A. Ortega, Regulation of the Na+-dependent high affinity glutamate/aspartate transporter in cultured Bergmann glia by phorbol esters, J. Neurosci. Res. 50 (4) (1997) 585–590.
[7] G. Guellaen, J.L. Mahu, P. Mavier, P. Berthelot, J. Hanoune, RMI 12330A, an inhibitor of adenylate cyclase in rat liver, Biochim. Biophys. Acta 484 (1977) 465.
[8] L.L. Iversen, Role of transmitter uptake mechanism in synaptic transmission, Br. J. Pharmacol. 41 (1971) 571–579.
[9] F. Jursky, N. Nelson, Localization of glycine neurotransmitter transporter (GLYT2) reveals correlation with the distribution of glycine receptor, J. Neurochem. 64 (3) (1995) 1026–1033.
[10] Lippe, Aridizzone (1991).
[11] A.M. López-Colomé, M. Romo-de-Vivar, Excitatory amino acid receptors in primary cultures of glial cells from the retina, Glia 4 (5) (1991) 431–439.
[12] O.H. Lowry, N.J. Rosenbrough, A.L. Farr, R.J. Randall, Protein measurement with the Folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.
[13] J. Segal, S.H. Ingbar, 3,5,3'-Triiodothyronine increases cellular adenosine 3',5'-monophosphate concentration and sugar uptake in rat thymocytes by stimulating adenylate cyclase activity: studies with the adenylate cyclase inhibitor MDL 12330A (1989).
[14] B.W. Siegel, N.L. Wiech, RMI 12330A: an inhibitor of cholera toxin induced intestinal hypersecretion which also inhibits adenylate cyclase activity, Gastroenerology 70 (1976) 976, (Abstract A-79).
[15] K.E. Smith, L.A. Borden, P.A. Hartig, T. Branchek, R.L. Weinshank, Cloning and expression of a glycine transporter reveal colocalization with NMDA receptors, Neuron 8 (1992) 927–936.
[16] T. Takadera, T. Mohri, Effect of intracellular free Ca2+ on leucine transport in Chang liver cells, Cell Struct. Funct. 10 (4) (1985) 349–359.
[17] M.C. Trachtenberg, D.J. Packey, Rapid isolation of mammalian Müller cells, Brain Res. 261 (1) (1983) 43–52.
[18] F. Zafra et al., Arachidonic acid inhibits glycine transport in cultured glial cells, Biochem. J. 271 (1990) 237–242.

TESIS CON
FALLA DE ORIGEN

Journal of Neurochemistry, 2002, **80**, 634–645

TESIS CON
FALLA DE ORIGEN

Role of Ca^{2+} and calmodulin-dependent enzymes in the regulation of glycine transport in Müller glia

Ana Gadea,* Edith López,* Arturo Hernández-Cruz† and Ana María López-Colomé*'‡

*Instituto de Fisiología Celular, Departamentos de *Neurociencias and †Biofísica and ‡Facultad de Medicina, Departamento de Bioquímica, Apartado, México*

Abstract

Glycine (Gly) is considered an obligatory co-agonist at NMDA receptors. Müller glia from the retina harbor functional NMDA receptors, as well as low and high affinity Gly transporters, the later identified as GLYT1. We here studied the regulation of Gly transport in primary cultures of Müller glia, as this process could contribute to the modulation of NMDA receptor activity at glutamatergic synapses in the retina. We demonstrate that neither glutamate stimulation nor the activation or inhibition of protein kinases A or C modify transport. In order to assess a function for Ca^{2+} and calmodulin (CaM)-dependent processes in the regulation of Gly transport, we explored the participation of Ca^{2+} concentration, CaM and Ca^{2+}/CaM-dependent enzymes on Gly transporter activity. ATP and carbachol, known to induce Ca^{2+} waves in Müller cells, as well as caffeine-induced Ca^{2+} release from intracellular stores stimulated transport, whereas Ca^{2+} chelation by BAPTA-AM markedly reduced transport. CaM inhibitors W-7, ophiobolin A, R-24571 and trifluoperazine, induced a specific dose-dependent inhibition of transport. The inhibition of CaMKII by the autocamtide-2-related inhibitory peptide or by KN62 caused a decrease in transport which, in the case of KN62, was due to the abolition of the high affinity component, ascribed to GLYT1. Our results further suggest that Gly transport is under cytoskeletal control, as activation of calpain by major increases in $[Ca^{2+}]i$ induced by ionophores, as well as actin destabilization clearly inhibit uptake. We here demonstrate for the first time the participation of CaM, CaMKII and the actin cytoskeleton in the regulation of Gly transport in glia. Ca^{2+} waves are induced in Müller cells by distinct neuroactive compounds released by neurons and glia, hence the regulation of [Gly] by this system may be of physiological relevance in the control of retinal excitability.

Keywords: CaMKII, chick retina, neurotransmitter uptake, NMDA receptors, radial glia.

J. Neurochem. (2002) **80**, 634–645.

The termination of chemical neurotransmission in the CNS involves the rapid removal of neurotransmitter from synapses by re-uptake into either the pre-synaptic terminal or the surrounding glia through specific transport systems. Drugs blocking transporter function can influence neural activity by increasing the duration of neurotransmitter action (Kanner 1994).

Glycine (Gly) plays a double role in the control of neuronal excitability. The function of Gly as a classical inhibitory neurotransmitter in the spinal cord, the brain stem and the retina, interacting with a chloride-permeable ligand-gated receptor competitively antagonized by strychnine is well established (Aprison 1990). More recently, Gly has been shown to function as an obligatory coagonist of glutamate (glu) at *N*-methyl-D-aspartate (NMDA) receptors through a strychnine-insensitive binding site (Fletcher *et al.* 1990), thus contributing to the modulation of excitatory neurotransmission.

An overactivation of NMDA glu receptors leads to excitotoxicity, and has been shown to participate in neurodegenerative and convulsive processes in the CNS (Rothman and Olney 1995; Chapman 1998; Dannhardt and Kohl 1998). As Gly could contribute to the overactivation of NMDA

Received July 23, 2001; revised manuscript received October 16, 2001; accepted November 19, 2001.

Address correspondence and reprint requests to A. María López-Colomé, Instituto de Fisiología Celular, UNAM, Apartado Postal 70–253, 04510 D. F., México. E-mail: acolome@ifisiol.unam.mx

Preliminary results were presented at the Conference 'Molecular and Functional Diversity of Ion Channels and Receptors', New York Academy of Sciences, May 1998, New York, USA.

Abbreviations used: CaM, calmodulin; CNS, central nervous system; glu, glutamate; Gly, glycine; NMDA, *N*-methyl-D-aspartate; PKC, protein kianse C; R-24571, calmidazoluim; TFP, trifluoperazine; W-7, *N*-(6-aminohexyl)-5-chloro-1-naphtalene-sulfonamide.

© 2002 International Society for Neurochemistry,

receptors, its concentration at glutamatergic synapses should be tightly regulated.

In brain stem and spinal cord, Gly uptake does not influence the time course of inhibitory post-synaptic currents (Singer and Berger 1999; Titmus *et al.* 1996), indicating the replenishing of neurotransmitter pre-synaptic pool as the function of Gly transporters. As a glu co-agonist, the role of Gly transporters in keeping local extracellular Gly concentration below saturating levels for NMDARs which would allow potentiation of receptors by sudden increases of extracellular Gly, has been demonstrated both in heterologous systems, and in brain stem (Supplisson and Bergman 1997; Bergeron *et al.* 1998). Such an increase could originate either by diffusion from nearby synapses, which is Ca^{2+}-dependent, or from the Ca^{2+}-independent reversal of transport, as observed in ischemic condition (Baker *et al.* 1991). Ca^{2+}-independent release has been ascribed to glial Gly transporters (reviewed in Gadea and López-Colomé 2001a), which have a stoichiometry of $2/Na^{+}/Cl^{-}/Gly$, versus neuronal ones, with $3Na^{+}/Cl^{-}/Gly$ stoichiometry (Roux and Supplisson 2000).

Although re-uptake is subject to physiological regulation, very little information is currently available regarding the role of second messengers in this process. Arachidonic acid, which may be released via phospholipase A_2 activation, has been shown to inhibit several sodium-coupled uptake systems, including those for glycine (Zafra *et al.* 1990) and glutamate (Barbour 1989); also, the regulation of glutamate transporters by protein kinase C (PKC; Casado *et al.* 1993), as well as that of GABA by cAMP has been demonstrated (Gomeza *et al.* 1991).

Two different glycine transporters have been cloned: GLYT1, with three isoforms derived from alternative splicing and/or promoter usage termed GLYT1a, GLYT1b and GLYT1c, inhibited by sarcosine and expressed in glial cells throughout the CNS, and GLYT2, expressed predominantly in brain stem and spinal cord, where glycine is considered the major inhibitory neurotransmitter (reviewed in Gadea and López-Colomé 2001a). GLYT1 thus, shows the expected properties for controlling extracellular Gly concentration, tonically modulating NMDA receptors, whereas GLYT2 could function in Gly accumulation at inhibitory glycinergic synapses.

In the retina, Müller cells are the most abundant glial cell type, its processes ensheathing excitatory synapses at the plexiform layers. In addition to their structural and nutritional functions, the precise anatomical localization of Müller cells has suggested a role in the modulation of neurotransmission (Newman and Reichenbach 1996). In Müller cells we have previously characterized two Gly transport systems showing high (K_m = 27 μM) and low affinity (K_m = 1.7 mM); the high affinity transporter was identified as GLYT1, inhibited by sarcosine (Gadea *et al.* 1999a). As Gly transport system in these cells could participate in the modulation of glutamate excitatory transmission in the vertical pathways of the retina as well as in the termination of inhibitory glycinergic transmission in this tissue, its regulation by second messengers in confluent monolayer cultures of Müller cells was studied. Our data show that glycine transport in these cells is under the regulation of calcium- and calmodulin- dependent processes.

Materials and methods

Chemicals

[^{3}H]Glycine was purchased from Dupont-New England Nuclear (Boston, MA, USA; Sp. Act. 42–43.8 Ci/mmol). Tissue culture reagents and plastics were from Gibco (Grand Island, NY, USA). Jasplakinolide was purchased from Molecular Probes (Eugene, OR, USA), the myristoylated autocamtide-2 related inhibitory peptide (AIP) and Calpain inhibitors III and V were from Calbiochem (La Jolla, CA, USA). All other chemicals and reagents were from Sigma (St Louis, MO, USA).

Cell culture

Primary cultures of Müller glia were obtained as described previously (López-Colomé and Romo-De-Vivar 1991). Retinas from 7-day-old chick embryos (Alpes, Puebla, México) were dissected and washed in Hanks solution free from Ca^{2+} and Mg^{2+} (g/100 mL): NaCl 0.8, KCl 0.04, KH_2PO_4 0.006, Na_2HPO_4 0.0125, phenol red 0.002, glucose 0.1. Tissue was dissociated in 0.25% trypsin, followed by filtration through a 50-μm mesh nylon net, resuspended in minimum essential medium (MEM) containing 0.05% glucose, 25 mM $NaHCO_3$, 0.0125% gentamycin, 0.0125% penicillin, 0.0125% streptomycin, 0.025% neomycin and 10% fetal bovine serum (FBS). Cells were seeded onto 24-well plates at a density of 2.5×10^5 cells per well, and maintained at 37°C in a humidified atmosphere of 5% CO_2 : 95% air. For intracellular [Ca^{2+}] measurements, cells were seeded on #1 round glass coverslips (1.75×10^5 cells per well). The purity of the culture was assessed by glial fibrillary acidic protein (GFAP; Björklund *et al.* 1985) and neuron-specific enolase (NSE) antibodies (Schmechel *et al.* 1980): 95% of the cells were $GFAP^+$ and NSE^- at day 12 *in vitro*, at which cultures formed a confluent monolayer. Medium was changed every other day. Confluent cell cultures were used for all experiments.

Primary cultures of neurons were prepared from retinas obtained from 7-day-old chick embryos. Retinas were incubated for 35 min at 37°C in 0.5% trypsin in Ca^{2+}- and Mg^{2+}-free Hanks' solution, and mechanically dissociated. Cells were plated at low density (0.6×10^6 cells/dish) on poly-D,L-ornithin-coated 12-well plates, grown in OPTI-MEM-I (reduced serum medium, modification of Eagle's MEM) plus 3% FBS, and maintained at 37°C in a humidified atmosphere of 5% CO_2 : 95% air. The purity of the culture was determined as described for glia; as previously reported (Somohano and López-Colomé 1991), more than 90% of the cells were NSE^+ and $GFAP^-$ at day 5 *in vitro*, when cultures were used for uptake experiments.

Uptake experiments

Assays were performed at 37°C in 0.5 mL of Krebs–Ringer bicarbonate buffer (KRB) containing: NaCl 118 mM; KH_2PO_4 2.0 mM; KCl 4.7 mM; $CaCl_2$ 2.5 mM; $MgSO_4$ 1.4 mM; $NaHCO_3$

25 mM; glucose 5.6 mM; pH 7.4. All solutions were prepared in double-distilled water. Previous to the experiment, the growth medium was removed, and cultures were rinsed twice with 0.5 mL of pre-warmed KRB (37°C). After 5 min, medium was replaced by 0.5 mL of KRB containing [^{3}H]Gly/Gly 1 : 25000 (41.1 Ci/mmol). Cultures were incubated for the indicated period of time in the presence of different drugs in order to study their effect on glycine transport.

At the end of the assay, cells were rinsed with 3 × 0.5 mL of fresh KRB (2–4°C), dissolved in 1 mL of 1 M HCl and counted for radioactivity after the addition of 1 mL 1 M NaOH and 5 mL of Tritosol (Fricke 1975) in a liquid scintillation counter (Beckman).

Experiments were carried out in triplicate, and replicated at least three times with different cell cultures. Results are expressed as the mean ± SEM of three independent experiments performed in triplicate. Corrections were made for specific activity.

The protein content was determined for two wells of each plate by the method of Lowry *et al.* (1951).

Data were analyzed using the INPLOT (version 3.1) program from GraphPad Software (San Diego, CA, USA). Student's *t*-test was applied in most cases, comparing each condition with control.

Measurement of intracellular Ca^{2+} concentration

Methods are described in detail elsewhere (Hernandez-Cruz *et al.* 1997). Briefly, cells were loaded with fura-2 by incubation with the acetoxymethyl (AM) ester form of the dye (fura-2/AM; Molecular Probes, Eugene, OR, USA) at a final concentration of 2 μM, with no dispersing agents added. Cells were allowed to load for 30–45 min at 37°C and then rinsed continuously for 5 min before the beginning of the experiment. Coverslips containing Müller glia were placed in a recording chamber (Mod. RC-25; Warner Instruments, Hamden, CT, USA) on an inverted microscope (Nikon Diaphot TMD; Nikon Corp., Tokyo, Japan).

Ca^{2+} levels were determined by recording pairs of images with an UV objective (Nikon UV-F 100X, 1.3 NA.) and an intensified CCD camera (c2400–87, Hamamatsu, Bridgewater, NJ, USA), using alternating illumination by two nitrogen pulsed lasers (Laser Science, Inc), tuned at 340 nm and 380 nm excitation, respectively (BioLase Imaging, Newton, MA, USA). The sampling rate (340/380 ratioing) was 2.5 Hz. Background images taken at 340 and 380 nm illumination were used for on-line background subtraction. Image acquisition and processing was controlled with BioLase's FL-2 software. $[Ca^{2+}]_i$ determinations from the soma of individual cells were calculated using the formula:

$$[Ca^{2+}] = K_D(F_f/F_b)(R - R_{min})/(R_{max}/R)$$

where the dissociation constant (K_D) of fura-2 for Ca^{2+} is 300 nM, F_f/F_b is the ratio of fluorescence values for Ca^{2+}-free/Ca^{2+}-bound indicator at 380 nm excitation, R is the fluorescence ratio at 340/380 nm for the unknown $[Ca^{2+}]$, and R_{min}, R_{max} are the ratio of fura-2 fluorescence at 340/380 nm of Ca^{2+}-free and Ca^{2+}-bound fura-2. The values of F_f/F_b, R_{min}, and R_{max} for Ca^{2+} were empirically determined using calibration solutions containing 50 μM fura-2 pentapotassium salt (Molecular Probes), and $[Ca^{2+}]$ in the range 0–40 μM. Baseline $[Ca^{2+}]_i$ was defined as the time-averaged $[Ca^{2+}]_i$ measured in the absence of stimulation over a period of 3 min. The peak amplitude of the Ca^{2+} transients was measured as the difference between the absolute peak $[Ca^{2+}]_i$ and the resting baseline $[Ca^{2+}]_i$. Data processing was accomplished with routines contained in ORIGIN 3.78 (Microcal Software, Northampton, MA, USA).

Solutions

Cells were continuously superfused (~1 mL/min) with KRB. Test solutions were pressure-applied (10 psi) via independent glass puffer pipettes (tip diameter ~ 2 μm), placed within 100 μm from the cell(s) under examination. Application of test solutions and drugs was controlled by a Picospritzer II device (General Valve, Fairfield, NJ, USA). Test solutions used were: (i) caffeine 5 mM dissolved in normal saline, and (ii) ionomycin 10 μM dissolved in normal saline. Experiments were carried out at 22–23°C.

Results

Glycine transport is not regulated by PKC or PKA

In order to determine the intracellular pathways involved in the regulation of glycine transport in Müller cells, different drugs related to second messenger cascades were tested. Results in Table 1 show that specific PKA and PKC activators or inhibitors had no effect on glycine transport. The participation of cAMP in this process was examined by testing the effect of agents shown to directly increase intracellular cAMP, such as forskolin and cholera toxin, as well as 8-Br-cAMP and the adenylate cyclase selective inhibitor SQ-22536: none of these drugs had an effect on glycine transport. The effect of MDL-12330 A was also tried; although 40% inhibition of transport was observed, the effect of the drug showed to be non-specific (Gadea *et al.* 1999b).

Incubation of Müller cells with the PKC activators phorbol-12-myristate-3-acetate, 1,2-dioctanoyl-rac-glycerol, or the PKC inhibitors staurosporine, H7, chelerytrine and polymyxin B had no effect on transport. Neither did the PLC inhibitor neomycin (Table 1).

Regulation of glycine transport by calmodulin

The participation of CaM and Ca^{2+}/CaM-dependent enzymes in the regulation of glycine transport in Müller glia was studied. The antipsychotic phenotiazine trifluoperazine (TFP; Weiss *et al.* 1980), *N*-(6-aminohexyl)-5-chloro-1-naphtalene-sulfonamide (W-7; Kanamori *et al.* 1981), the antimycotic miconazole derivative calmidazoluim (R-24571; Gietzen *et al.* 1981) and the phytotoxic fungal metabolite ophiobolin A (Leung *et al.* 1984) have all been reported to inhibit a variety of enzyme reactions and biological processes stimulated by Ca^{2+}/CaM. All these anticalmodulin drugs inhibited glycine transport. Figure 1 shows dose–response curves for W-7 (maximal inhibition 40%), ophiobolin A (maximal inhibition 45%), trifluoperazine (maximal inhibition 80%), and R-24571 (maximal inhibition 82%). The IC_{50}s calculated for each case were 40 ± 2.5 μM, 50 ± 1.2 μM, 2.5 ± 0.5 μM and 1 ± 0.2 μM, respectively.

Table 1 Effect of intracellular messenger-related drugs on glycine transport in cultured Müller glia[a]

Tested compound	Concentration	t (min) incubation	Glycine uptake (% of control)
Forskolin	7.5 μM	30 and 60 min	99 ± 4
8-Br-cAMP	1 mM	30 min	98 ± 4
8-Br-cGMP	1 mM	30 and 60 min	100 ± 5
SQ-22536	100 μM	30 and 60 min	99 ± 6
HA-1004	100 μM	60 min	98 ± 5
Cholera toxin	1 μg/mL	30 and 60 min	100 ± 3
Pertussis toxin	1 μg/mL	30 and 60 min	101 ± 6
PMA	0.5 μM	30 and 60 min	100 ± 8
DOG	100 μg/mL	30 and 60 min	101 ± 4
Chelerytrine	1 μM	30 and 60 min	100 ± 4
Staursporine	100 nM	45 min	97 ± 5
H-7	50 μM	30 min	100 ± 6
Polymyxin B	400 μM	30 min	99 ± 6
Neomycin	110 μM	30 min	99 ± 8
Mepacrine	50 μM	20 min	98 ± 8
Okadaic acid	100 nM	30 and 60 min	98 ± 7
Deltamethrin	20 μM	30 and 60 min	98 ± 7
Cyclosporin A	50 nM-20 μM	30 and 60 min	99 ± 6
Tautomycin	300 nM^{-1} μM	30 and 60 min	97 ± 8
ML-9	5 μM-200 μM	60 min	100 ± 4
Wortmannin	1 μM	30 and 60 min	101 ± 7
Genistein	50 μM	60 min	100 ± 5

[a]Müller cells were pre-incubated in the presence of the indicated compounds. Uptake experiments were performed as described in Materials and methods in the presence of 1 mM glycine. HA-1004, *N*-(2-Guanidinoethyl)-5-isoquinolinesulfonamide hydrochloride; SQ-22536, 9-(tetrahydro-2-furanyl)-9*H*-purin-6-amine; PMA, Phorbol 12-myristate 13-acetate; DOG, 1,2-dioctanoyl-rac-glycerol; H-7,1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride; ML-9, 1-(5-chloronaphtalene-1-sulfonyl)-1*H*-hexahydro-1,4-diazepine. Values are expressed as percentage of glycine uptake with respect to control and are the mean ± SEM of at least three independent experiments performed in triplicate. No significant differences from control were observed.

Anticalmodulin drugs have been shown to interact in a calmodulin-independent fashion, with several other proteins such as dopamine D2 receptors (Wilson *et al.* 1998) and NMDA receptors (Lidsky *et al.* 1997). In order to assess the specificity of these drugs on glycine transport in Müller cells, the uptake of distinct transmitter and non-transmitter amino acids was measured after treatment with calmodulin-related drugs. The regulation of the Na^+-dependent high affinity glutamate/aspartate transporter system in Müller glia by PKC has been demonstrated (González *et al.* 1999) however, as shown in Table 2, pre-incubation of Müller cells with the calmodulin inhibitors R24571, TFP and W-7 decreased D-aspartate uptake 49%, 57% and 50%, respectively. The Na^+-independent uptake of leucine has been shown to be regulated by Ca^{2+} and calmodulin in Chang liver cells

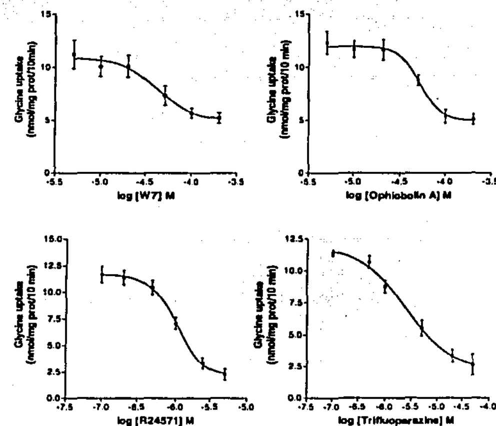


Fig. 1 Involvement of calmodulin in the regulation of glycine transport in Müller cells. Confluent cultures were pre-incubated with increasing concentrations of R-24571 (maximal inhibition 82%, IC_{50} 1 ± 0.2 μM), trifluoperazine (TFP; maximal inhibition 80%, IC_{50} 2.5 ± 0.5 μM), W-7 (maximal inhibition 40%, IC_{50} 40 ± 2.5 μM) or ophiobolin A (maximal inhibition 45%, IC_{50} 50 ± 1.2 μM) for 20 min The transport assay was carried out in the presence of 1 mM glycine for 10 min as described in Materials and methods. Values are expressed as the mean ± SEM of three experiments performed in triplicate. IC_{50}s were calculated in each case using the INPLOT (version 3.1) program from GraphPad software.

(Takadera and Mohri 1985), whereas the Na^+- and Cl^--dependent uptake of GABA in astrocytes is regulated through the activation of PKC (Gomeza *et al.* 1991). Pre-incubation of Müller cells with the inhibitors did not affect GABA or leucine uptake.

The re-uptake of Gly from the synaptic cleft is undertaken by glia as well as the neuronal pre-synaptic terminals, hence, the involvement of calmodulin in the regulation of glycine transport in cultured neurons from the retina was also evaluated. Pre-incubation of neurons with calmodulin-related drugs had no effect on glycine transport (Table 2).

Regulation of glycine transport by Ca^{2+}

As calmodulin is activated by calcium, we tested the effect of dantrolene (1 mM), which blocks intracellular calcium release in some preparations, thapsigargin (2 μM), inhibitor of the endoplasmic reticulum Ca^{2+}-ATPases, the extracellular Ca^{2+} chelator EGTA (1 mM in nominally Ca^{2+}-free buffer), and the intracellular Ca^{2+} chelator BAPTA-AM (10 μM in nominally Ca^{2+}-free buffer plus 0.5 mM EGTA), on glycine transport in Müller glia. As shown in Table 3, only BAPTA-AM inhibited glycine transport (40%).

In order to activate calmodulin and presumably stimulate uptake, cells were incubated with the calcium ionophores

TESIS CON FALLA DE ORIGEN

TESIS CON
FALLA DE ORIGEN

Table 2 Effect of calmodulin and CaMKII inhibitors on the uptake of D-aspartate, leucine and GABA in Müller cells and on glycine uptake in retinal neurons[a]

	Control	R24571	TFP	W-7
D-aspartate uptake (% of control)	100 ± 9	50.2 ± 8.7	43.02 ± 3.2[b]	50.0 ± 2.0[b]
Leucine uptake (% of control)	100 ± 8.9	71.6 ± 14.1	81.3 ± 10.0	83.5 ± 8.7
GABA uptake (% of control)	100 ± 5.4	87.0 ± 12.8	97.2 ± 7.4	99.1 ± 4.8
Neuronal Gly uptake (% of control)	100 ± 17	95.9 ± 22	86.1 ± 16	96.8 ± 15

[a]Müller cells were pre-incubated with 5 μM R-24571, 25 μM trifluoperazine (TFP), or 10 μM W-7 for 20 min The transport assay was then carried out for 10 min in KRB containing 1 mM leucine, 5 mM D-aspartate or 1 mM GABA. Cultured neurons were pre-incubated with these compounds for 20 min, Gly transport assay was carried out as described in Materials and methods in the presence of 1 mM glycine for 10 min. Values are expressed as percentage of control ± SEM of three independent experiments performed in triplicate. [b]Significantly different from control ($p < 0.001$, Student's *t*-test).

Table 3 Role of intracellular and extracellular Ca^{2+} in the regulation of glycine transport in Müller cells[a]

	Glycine uptake (% of control)
Control	100 ± 4
EGTA	101 ± 5
BAPTA-AM	59 ± 7[b]
Dantrolene	95 ± 4
Thapsigargin	104 ± 7

[a]Müller glia cultures were pre-incubated in nominally Ca^{2+}-free KRB with 1 mM EGTA or 10 μM BAPTA-AM plus 0.5 mM EGTA for 30 min. 30 μM dantrolene or 2 mM thapsigargin were tested in normal KRB for 30 min. Uptake experiments were performed as described in Materials and methods in the presence of 1 mM glycine for 10 min. Values are the mean ± SEM of three triplicate experiments. [b]Significantly different from control ($p < 0.001$, Student's *t*-test).

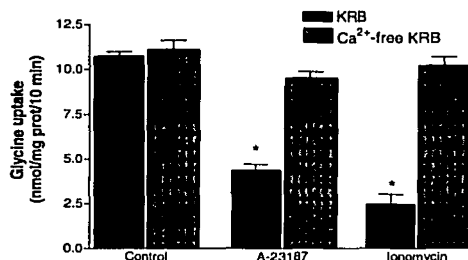


Fig. 2 Effect of the Ca^{2+} ionophores A-23187 and ionomycin on glycine uptake in Müller cells. Müller cells were incubated for 15 min with 10 μM A-23187 or 10 μM ionomycin in KRB containing 2.5 mM $CaCl_2$ (solid bars) or in nominally Ca^{2+}-free KRB (dotted bars). Uptake experiments were performed as described in Materials and methods in the presence of 1 mM glycine for 10 min. Values are the mean ± SEM of four triplicate determinations. *Significantly different from control ($p < 0.001$, Student's *t*-test).

A23187 (10 μM) and ionomycin (10 μM). Contrary to the expected result, both ionophores strongly inhibited glycine transport in the presence of Ca^{2+} (60% and 78%, respectively; Fig. 2).

Elevation of intracellular Ca^{2+} by high K^+, glutamate, carbachol or ATP stimulation, as well as by caffeine has been demonstrated in Müller cells (Wakakura and Yamamoto 1994; Keirstead and Miller 1995, 1997; Newman and Zahs 1997). In order to explore the effect of a moderate increase in $[Ca^{2+}]_i$ on glycine transport, 5 mM caffeine, 2 mM carbachol or 1 mM ATP were tested. As seen in Table 4, in this condition caffeine, carbachol and ATP increased glycine transport by 50, 65 and 30%, respectively. Caffeine-induced stimulation of glycine transport was prevented by prior incubation with 2 μM thapsigargin. The possibility of Gly transport regulation by glutamate receptor activity was also explored. Glutamate or the glu receptor agonists NMDA, KA, AMPA, L-AP4 and t-ACPD had no effect on Gly transport (Table 4).

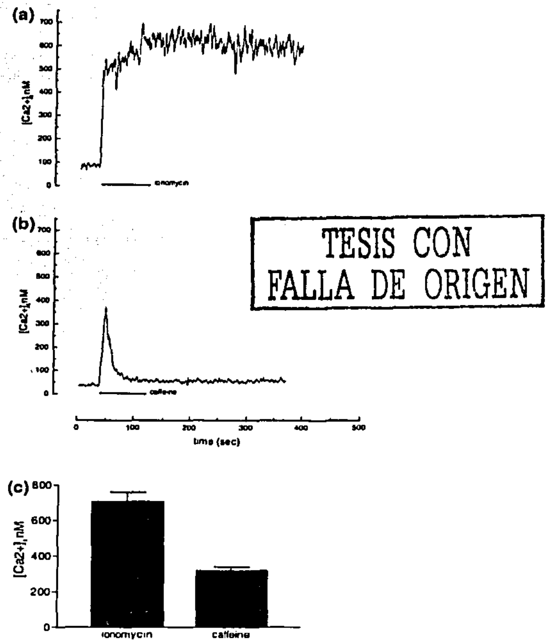
The opposite effects of caffeine and ionomycin on glycine transport could be due to differences in the intracellular $[Ca^{2+}]$ increase induced by these agents. As shown in Fig. 3, ionomycin elicited a pronounced and sustained intracellular Ca^{2+} increase in all the cells examined ($n = 60$). Caffeine was less consistent in raising internal calcium, producing a smaller and transient calcium increase in 45% of the cells tested ($n = 155$). The peak amplitude of the Ca^{2+} increase elicited by ionomycin was 703 ± 55 nM, whereas that for caffeine was 315 ± 23 nM (Fig. 7c).

Huge increases in $[Ca^{2+}]_i$ such as those induced by ionomycin may be more related to pathological than to

Table 4 Effect of glial calcium wave-inducing agents on glycine transport in Müller cells[a]

	Glycine uptake (% of control)
Control	100 ± 8
Caffeine	150 ± 14[b]
Thapsi + Caffeine	101 ± 10[c]
ATP	165 ± 28[b]
Carbachol	130 ± 7[b]
Glu	106 ± 9
NMDA	93 ± 10
AMPA	102 ± 9
KA	104 ± 13
L-AP4	98 ± 8
t-ACPD	95 ± 14

[a]Müller cells were pre-incubated with 5 mM caffeine, 1 mM ATP or 2 mM carbachol for 15 min in nominally Ca^{2+}-free KRB plus 0.5 mM EGTA. When tested together, 5 mM caffeine was added following 15 min pre-incubation with 2 μM thapsigargin in Ca^{2+}-free KRB plus 0.5 mM EGTA. In another set of experiments, Müller cells were pre-incubated with 1 mM glutamate (Glu), *N*-methyl-D-aspartate (NMDA), alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA), kainate (KA), L(+)-2-amino-4-phosphonobutyric acid (L-AP4), or *trans*-aminocyclopentane-1,3-dicarboxylic acid (t-ACPD) for 1 h in KRB. Uptake experiments were performed as described in Materials and methods in the presence of 1 mM glycine. Values are the mean ± SEM of at least three experiments performed in triplicate. [b]Significantly different from control ($p < 0.005$, Student's *t*-test). [c]Significantly different from caffeine ($p < 0.005$, Student's *t*-test).



TESIS CON
FALLA DE ORIGEN

Fig. 3 Intracellular $[Ca^{2+}]$ increase elicited by caffeine and ionomycin in Müller cells. Intracellular Ca^{2+} concentration measurements were performed as described in Materials and methods. Traces obtained from two representative cells are shown. (a) Ionomycin-induced Ca^{2+} increase in one Müller cell. (b) Caffeine-induced Ca^{2+} increase in one Müller cell. (c) Average peak amplitude of the Ca^{2+} increase induced by ionomycin and caffeine. Data represent the mean response ± SEM of 60 cells treated with ionomycin and 62 cells responsive to caffeine.

physiological conditions. On this line, elevated $[Ca^{2+}]i$ associated with specific brain pathologies has been shown to activate the intracellular cysteine protease calpain in glial cells (Du *et al.* 1999; Shields and Banik 1999). Figure 4 shows that while the serine protease inhibitor phenylmethylsulfonyl fluoride (PMSF) has no effect on transport, pre-incubation of Müller cells with a protease inhibitor cocktail (PIC) or with the cell permeable calpain inhibitors III and V, decreases by 30% and 37%, respectively, the ionomycin-induced inhibition.

Regulation of glycine transport by the actin cytoskeleton

Several calpain-mediated processes such as the proteolysis of cytoskeletal components have been described in glial cells (Finkbeiner 1993). Actin as well as the actin-binding cytoskeletal proteins fodrin (spectrin) and ankyrin are major substrates for calpain (Villa *et al.* 1998). Additionally, recent studies have shown ankyrin and fodrin to possess high-affinity binding sites for integral membrane proteins and to be involved in the regulation of membrane protein functions (Mills and Mandel 1994; Beck and Nelson 1996), including transport (Nelson and Hammerton 1989; Handlogten *et al.* 1996; Zharikov and Block 2000). Destabilization of actin in Müller cells by pre-treatment with 10 μM cytochalasin B or D, alkaloids which promote actin disassembly by inhibiting new filament growth (Cooper 1987), had an inhibitory effect on glycine transport (41% and 50%, respectively). This effect was decreased by prior incubation with the cell permeable actin-stabilizing peptide jasplakinolide (1 μM; Bubb *et al.* 1994; Fig. 5). Colchicine (25 μM), a microtubule depolymerizing agent, did not modify glycine transport.

Participation of CaM-dependent enzymes in the regulation of glycine transport

Among the Ca^{2+}/CaM-regulated kinases and phosphatases, CaMKII, myosin light chain kinase (MLCK) and calcineurin (CaN, protein phosphatase-2B) are expressed in glial cells and have been shown to interact with the cytoskeleton (Edelman *et al.* 1992; Vinade *et al.* 1997; Cotrina *et al.* 1998; Matsuda *et al.* 1998; Takeuchi *et al.* 2000; Vallano *et al.* 2000). The MLCK inhibitor ML-9 and the CaN inhibitors deltamethrin and cyclosporin A had no effect on

TESIS CON
FALLA DE ORIGEN

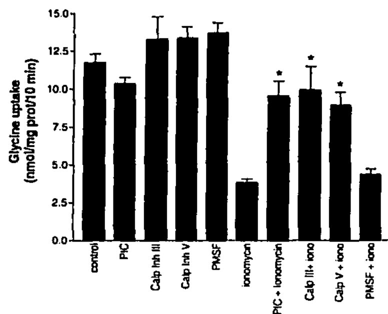
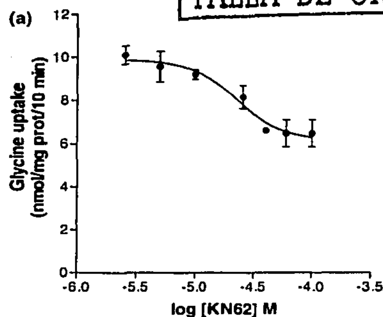


Fig. 4 The inhibitory effect of ionomycin on glycine transport is prevented by calpain inhibitors III and V. Cultures were incubated for 90 min with 50 μM Calpain inhibitor III, V or phenylmethylsulfonyl fluoride (PMSF) 0.5 mM before 10 μM ionomycin was added for further 15 min. In control experiments, cells were incubated for 90 min with the inhibitors. The transport assay was carried out as described in Materials and methods in the presence of 1 mM glycine for 10 min Values are the mean ± SEM of three determinations performed in triplicate. *Significantly different from ionomycin ($p < 0.001$, Student's *t*-test).



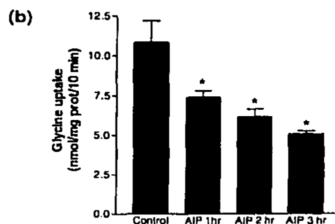


Fig. 6 Regulation of glycine transport by CaMKII in Müller cells. (a) Confluent cultures were pre-incubated with increasing concentrations of KN62 (maximal inhibition 40%, IC_{50} 20 ± 1.3 μM). The transport assay was carried out as described in Materials and methods in the presence of 1 mM glycine for 10 min. Values are expressed as the mean ± SEM of three experiments performed in triplicate. IC_{50}s were calculated in each case using the INPLOT (version 3.1) program from GraphPad software. (b) Confluent cultures were pre-incubated with 50 μM AIP (myristoylated autocamtide-2 related inhibitory peptide) for the indicated time. Values are the mean ± SEM of two triplicate determinations. *Significantly different from control ($p < 0.001$, Student's *t*-test).

glycine transport (Table 1). The CaMKII inhibitor KN62 inhibited glycine transport (maximal inhibition 40%) with a calculated IC_{50} of 20 ± 1.3 μM (Fig. 6a). KN62 has been reported to inhibit also CaMKIV hence, the effect of the myristoylated autocamtide-2 related inhibitory peptide (AIP), a highly specific inhibitor of CaMKII (Ishida *et al.* 1995) was tested. As shown in Fig. 6(b), the AIP had a similar effect to KN62 on Gly transport.

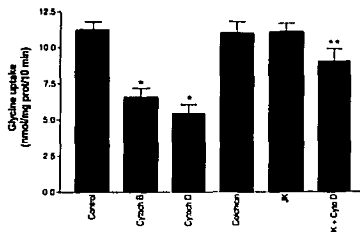


Fig. 5 Involvement of the actin cytoskeleton in the regulation of glycine transport in Müller glia. Cultures were pre-incubated for 2 h with 10 μM cytochalasin B, 10 μM cytochalasin D or 25 μM colchicine, or 1 h with 1 μM jasplakinolide (JK). When tested together, jasplakinolide was added 30 min prior to cytochalasin D. The transport assay was carried out as described in Materials and methods in the presence of 1 mM glycine for 10 min. Values are the mean ± SEM of three determinations performed in triplicate. *Significantly different from control ($p < 0.001$, Student's *t*-test). **Significantly different from cytochalasin D ($p < 0.001$, Student's *t*-test).

In order to explore the mechanism by which CaMKII inhibits Gly transport, we studied the effect of KN62 on Gly transport kinetics. Eadie–Hofstee analysis of the saturation curve revealed that 40 μM KN62 abolishes the high affinity component attributed to GLYT1 (Fig. 7). KN62 did not affect leucine, GABA or D-aspartate uptake in Müller glia, nor glycine uptake by retinal neurons (data not shown).

Discussion

The main finding of this work is that, unlike transport systems for the neurotransmitter amino acids GABA and glutamate which are regulated by cAMP and/or PKC in CNS neurons and glia (Casado *et al.* 1991, 1993; Gomeza *et al.* 1994; Dowd and Robinson 1996; Conradt and Stoffel 1997; Davis *et al.* 1998; González *et al.* 1999), glycine transport in Müller cells is regulated by Ca^{2+}/CaM activation of CaMKII.

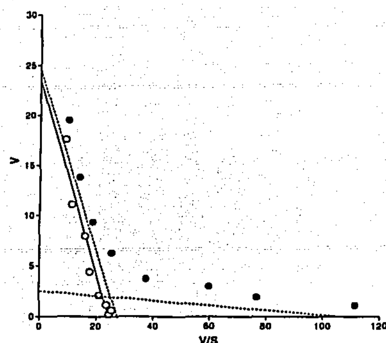


Fig. 7 Abolition of high affinity Gly transport by the CaMKII inhibitor KN62. Confluent cultures were pre-incubated with different concentrations of glycine at concentrations ranging from 0.01 mM to 2 mM ([^{3}H]-Gly/Gly 1 : 5000). When treated with KN62 40 μM, cells were pre-incubated with the compound for 20 min. Eadie–Hofstee analysis (control = dashed line; KN62 = solid line) and kinetic parameters were calculated in each case using the INPLOT (version 3.1) program from Graph PAD Software. Values are the mean ± SEM of two triplicate determinations. ●, Control: K_{m1} = 27 μM, K_{m2} = 1.7 mM, V_{max1} = 3 nmol/mg prot/10 min, V_{max2} = 30 nmol/mg prot/10 min; ○, KN62 40 mm: K_m = 1.5 mM, V_{max} = 30 nmol/mg prot/10 min.

Moreover, although the activity of recombinant GLYT1b expressed in HEK239 cells is decreased by PKC activation (Sato *et al.* 1995), neither the activation nor the inhibition of this enzyme or changes in cAMP concentration modify Gly transport in retinal Müller cells.

We previously characterized specific high affinity glycine transporters in Müller cells from the chick retina, identified as GLYT1 (Gadea *et al.* 1999a). The results from this work demonstrate for the first time that Gly transport in Müller cells is regulated by intracellular Ca^{2+} and calmodulin, as activity is stimulated by Ca^{2+} release from intracellular stores and decreased by specific calmodulin antagonists as well as by the intracellular calcium chelator BAPTA-AM. Ca^{2+}/calmodulin complex modulates the activity of several enzymes including CaM-dependent protein kinases, protein phosphatases, adenylyl cyclases and phosphodiesterases (Van Eldik *et al.* 1982). CaM also regulates the activity of nitric oxide synthase (Abu-Soud *et al.* 1994; Stuehr 1999); however, in our system, the NOS inhibitor, L-nitroarginine did not affect glycine transport (data not shown). The calmodulin inhibitors used in this work have often been reported as potent inhibitors of Ca^{2+}/calmodulin phosphodiesterases, therefore increasing cyclic nucleotide concentrations (Van Belle 1984; Van Staveren *et al.* 2001). We here demonstrate that raising cGMP or cAMP concentrations does not modify transport. Our results also indicate that protein phosphatases 1, 2A and 2B, as well as adenylyl cyclases are not involved in the regulation of glycine transport, as okadaic acid, deltamethrin, tautomycin and AC activators or inhibitors failed to affect uptake (Table 1).

CaMKII is particularly important in the brain due to its involvement in the development of LTP as well as in NMDA-activation of AMPA receptors (Soderling *et al.* 1994). In contrast, although its activity has been detected in astrocytes (Babcock-Atkinson *et al.* 1989), very little evidence on the role of CaMKII in glial cells is available. Evidence exists for CaMKII participation in glial processes including the regulation of the cytoskeleton by selective phosphorylation of the intermediate filament proteins GFAP and vimentin (Yano *et al.* 1994; Inagaki *et al.* 1997), protection from apoptosis, phosphorylation of PEA-15 (Kubes *et al.* 1998), and the regulation of phospholipid metabolism through the activation of PI_3K (Communi *et al.* 1999). Our data clearly demonstrate that high affinity transport is abolished upon inhibition of CaMKII by KN62. As consensus motifs for CaMKII phosphorylation have not been found in GLYT1 (Liu *et al.* 1993; Sato *et al.* 1995), direct phosphorylation of the transporter seems unlikely. The modulation of transport by CaMKII might therefore involve an indirect mechanism as shown for other Na^+/Cl^--dependent transporters (reviewed in Gadea and López-Colomé 2001b), e.g. activation of a signaling system which subsequently modulates glycine transporter, or the phosphorylation of cytoskeletal proteins implicated in the trafficking and/or clustering of the transporters.

Although both neurons and glia express calmodulin and calmodulin-dependent enzymes, we here show the lack of effect of calmodulin (Table 2) and CaMKII inhibitors on neuronal glycine transport, which could relate to the differential expression and subcellular location of CaMKII isoforms in neurons and glia. In neurons, the predominant forms are α and β, with minor amounts of δ and γ, whereas in astrocytes only δ_2, γ_B and γ_A are present. Additionally, the γ subunits associate to the particulate fraction in neurons, whereas in glia they locate to the cytoplasmic fraction (Vallano *et al.* 2000). Discrepancy could also arise from structural differences between transporters and/or their coupling to distinct membrane proteins.

The generation of Ca^{2+} waves by a number of stimuli has been demonstrated in glial cells. In the retina, Ca^{2+} waves propagated through astrocytes and Müller cells modify the light-evoked spike activity in nearby neurons (Newman and Zahs 1998). The propagation of glial calcium waves was initially ascribed to the generation and further diffusion of inositol trisphosphate (IP3) through gap junctions (Sanderson *et al.* 1994; Leybaert *et al.* 1998). More recent evidence shows the involvement of an extracellular pathway in wave propagation, independent from cell/cell contact (Hassinger *et al.* 1996; Guthrie *et al.* 1999) or gap junctional coupling (Guan *et al.* 1997; John *et al.* 1999). The messenger has been shown to be ATP, as Ca^{2+} wave propagation releases

TESIS CON FALLA DE ORIGEN

ATP from glial cells (Cotrina *et al.* 1998; Wang *et al.* 2000), and ATP receptor antagonists and apyrase block this process in cultured astrocytes (Guan *et al.* 1997; Cotrina *et al.* 1998, 2000). In the rat retina, wave propagation involves both intracellular and extracellular mechanisms (Newman 2001): astrocytes mainly use intracellular messenger diffusion, whereas from astrocytes to Müller cells and from Müller cells to other Müller cells, waves are propagated primarily by the release of ATP acting on purinergic receptors (Li *et al.* 2000; Pannicke *et al.* 2000). We demonstrate here that agents shown to trigger calcium waves in Müller glia under physiological conditions (through Ca^{2+} release from intracellular pools), such as ATP and carbachol, stimulate glycine transport; this effect was also induced by caffeine (Table 4). These results suggest that glial calcium waves could modulate retinal excitability by regulating extracellular glycine concentration, in addition to the release of neuroactive compounds (Newman and Zahs 1998; Innocenti *et al.* 2000).

The stimulation of Gly transport by caffeine, carbachol and ATP and its inhibition by BAPTA-AM revealed the requirement of intracellular Ca^{2+} for activating CaM, as inhibiting CaM also decreases transport. A glutamate-induced increase in intracellular $[Ca^{2+}]$ has been reported in rabbit and salamander Müller cells (Wakakura and Yamamoto 1994; Keirstead and Miller 1997). Our data showing the lack of effect of GluR agonists on Gly transport (Table 4) and intracellular $[Ca^{2+}]$ (data not shown) are in agreement with those from Newman and Zahs (1997), which demonstrate the induction of Ca^{2+} waves in rat Müller glial cells by ATP, carbachol and phenylephrine, but not by glutamate (Malchow and Ramsey 1999; Newman 2001).

In contrast with caffeine, increasing internal calcium with A-23187 or ionomycin inhibits transport (Fig. 2). As shown by intracellular $[Ca^{2+}]$ measurements, caffeine induces a transient elevation in $[Ca^{2+}]_i$ which declines in the presence of the drug, due to the depletion of the intracellular store, whereas ionomycin evokes a greater and sustained elevation that persists even upon withdrawal of the ionophore (Fig. 3). Based on the high-affinity of CaM for Ca^{2+} ($K_d \approx 100$ nM), the moderate increase in intracellular $[Ca^{2+}]$ induced by caffeine, may activate the membrane-bound inducible form of CaMKII (Braun and Schulman 1995) which in turn activates the transporter (directly or indirectly). The higher increase in $[Ca^{2+}]_i$ by ionophores activates proteases including calpain, with lower affinity for Ca^{2+}. In chicken skeletal muscle, three calpains have been described: high-m-calpain, m-calpain and μ-calpain, whose calcium requirements for half maximal activity are 3.8 mM, 420 μM and 5 μM, respectively (Wolfe *et al.* 1989).

The activation of proteases by raises in $[Ca^{2+}]_i$ may be more related to pathological than to physiological conditions. On this matter, exposure of astrocytes to ischaemic conditions has been shown to trigger an $[Ca^{2+}]_i$ elevation due to the activation of voltage-gated Ca^{2+} channels as well as to Ca^{2+} release form intracellular pools (Verkhratsky and Kettenmann 1996). In the present study, we demonstrate that the inhibition of Gly transport by A23187 and ionomycin, is a result of Ca^{2+}-activation of proteases. Proteolysis due to Ca^{2+} influx has been observed in rat optic nerve, spinal cord and peripheral nerve glial cells (Schlaepfer and Zimmerman 1981). Moreover, an activation of calpain due to Ca^{2+} entry through voltage-gated Ca^{2+} channels has been shown to increase GFAP immunoreactivity in astrocytes, following spinal cord injury (Du *et al.* 1999). Similar activation of calpain by ischemic insults has been described in retinal neurons (Sakamoto *et al.* 2000), and could also occur in Müller glia, which bares distinct types of voltage-gated Ca^{2+} channels (Puro *et al.* 1996; Bringmann *et al.* 2000). Our results demonstrate that glycine transport is inhibited by the activation of calpain, whose major substrates are cytoskeletal elements, suggesting a link between the cytoskeleton and the regulation of glycine transporters.

In addition to calpain (Banik *et al.* 1991; Perlmutter *et al.* 1990), glia contains several Ca^{2+}-activated proteases, capable of irreversibly altering glial cytoskeleton (Legrand *et al.* 1991; Whitaker *et al.* 1991). As the addition of calpain inhibitors did not prevent completely the inhibition of Gly transport by ionomycin, the involvement of additional proteases must be considered.

The mechanism underlying the modulation of Gly transport by the actin cytoskeleton remains to be established; however, it can be proposed that glycine transporters bind to actin filaments or to actin binding proteins. Consequently, disruption of actin filaments by cytochalasin or proteolysis of the actin binding proteins such as fodrin or ankyrin by calpain might decrease the stability of glycine transporters in the cell membrane and alter their number or activity. In support of this assumption, calpain-mediated proteolysis of fodrin inhibits L-arginine transport in pulmonary artery endothelial cells (Zharikov and Block 2000). Alternatively, CaMKII activity and translocation to the membrane could be affected by the disruption of the actin cytoskeleton. On this regard, the synaptic clustering of CaMKII, a core component of the post-synaptic density (PSD), is completely dependent on an intact actin cytoskeleton (Allison *et al.* 2000). Ca^{2+} signaling in astrocytes, including the propagation of Ca^{2+} waves, is also related to actin dynamics as cytochalasins have been shown to impair these processes (Cotrina *et al.* 1998). In this context, it is interesting to speculate that as calmodulin inhibitors also affected D-aspartate but not GABA or leucine uptake (Table 2), glycine and D-aspartate transport systems, although specifically regulated by CaMKII and PKC, respectively, could share a common cytoskeletal link under the control of CaM-dependent enzymes.

Findings from this work further support an active participation of Müller glia in the regulation of retinal neurotransmission, as it demonstrates that physiological variations in Ca^{2+} concentration due to calcium-waves in these cells

(Finkbeiner 1993) may control the concentration of neuroactive compounds at synaptic sites through the differential regulation of transporter systems. Localization of GLYT1 at the inner plexiform layer (Pow and Hendrickson 1999) where retinal ganglion cells receive glycinergic synaptic inputs from amacrine cells supports this idea (Lukasiewicz and Roeder 1995), although immunocytochemical studies have failed to identify Gly transporters in Müller glia *in situ* (Reye *et al.* 2001). Importantly, Gly concentration at the synaptic cleft might influence the activity of NMDA receptors within the excitatory vertical pathways of the retina, and might also contribute to regulate the activity of inhibitory glycine receptors at the inner plexiform layer (Wu and Maple 1998). Studies under way are aimed to determine the molecular mechanism relating calmodulin, CaMKII and cytoskeletal elements in the control of Gly transport in Müller cells from the retina.

Acknowledgement

This work was partially supported by grants from DGAPA-UNAM, IN-210998 to AMLC and PAEP 201332 to AG.

References

Abu-Soud H. M., Yoho L. L. and Stuehr D. J. (1994) Calmodulin controls neuronal nitric-oxide synthase by a dual mechanism. Activation of intra- and interdomain electron transfer. *J. Biol. Chem.* **269**, 32047–32050.

Allison D. W., Chervin A. S., Gelfand V. I. and Craig A. M. (2000) Postsynaptic scaffolds of excitatory and inhibitory synapses in hippocampal neurons: maintenance of core components independent of actin filaments and microtubules. *J. Neurosci.* **20**, 4545–4554.

Aprison M. H. (1990) The discovery of the neurotransmitter role of glycine, in *Glycine Neurotransmission* (Ottersen O. P. and Storm-Mathisen J., eds), pp. 1–23. John Wiley and Sons, Ltd, New York.

Babcock-Atkinson E., Norenberg M. D., Norenberg L. O. and Neary J. T. (1989) Calcium/calmodulin-dependent protein kinase activity in primary astrocyte cultures. *Glia* **2**, 112–118.

Baker A. J., Zornow M. H., Scheller M. S., Yaksh T. L., Skilling S. R., Smullin D. H., Larson A. A. and Kuczenski R. (1991) Changes in extracelluar concentrations of glutamate, aspartate, glycine, dopamine, serotonin and dopamine metabolites after transient global ischemia in the rabbit brain. *J. Neurochem.* **57**, 1370–1379.

Banik N. L., DeVries G. H., Neuberger T., Russell T., Chakrabarti A. K. and Hogan E. L. (1991) Calcium-activated neutral proteinase (CANP; calpain) activity in Schwann cells: immunofluorescence localization and compartmentation of mu- and mCANP. *J. Neurosci. Res.* **29**, 346–354.

Barbour B. (1989) Modulation of glutamate transporters by arachidonic acid. *Nature* **342**, 918–920.

Beck K. A. and Nelson W. J. (1996) The spectrin-based membrane skeleton as a membrane protein-sorting machine. *Am. J. Physiol.* **270**, C1263–C1270.

Bergeron R., Meyer T. M., Coyle J. T. and Greene R. W. (1998) Modulation of *N*-methyl-D-aspartate receptor function by glycine transport. *Proc. Natl. Acad. Sci. USA* **95**, 15730–15734.

Björklund H., Bignami A. and Dahl D. (1985) Immunohistochemical demonstration of glial fibrillary acidic protein in normal rat Müller glia and astrocytes. *Neurosci. Lett.* **54**, 363–368.

Braun A. P. and Schulman H. (1995) The multifunctional calcium/calmodulin-dependent protein kinase: from form to function. *Annu. Rev. Physiol.* **57**, 417–445.

Bringmann A., Schopf S. and Reichenbach A. (2000) Developmental regulation of calcium channel-mediated currents in retinal glial (Müller) cells. *J. Neurophysiol.* **84**, 2975–2983.

Bubb M. R., Senderowicz A. M., Sausville E. A., Duncan K. L. and Korn E. D. (1994) Jasplakinolide, a cytotoxic natural product, induces actin polymerization and competitively inhibits the binding of phalloidin to F-actin. *J. Biol. Chem.* **269**, 14869–14871.

Casado M., Zafra F., Aragon C. and Gimenez C. (1991) Activation of high-affinity uptake of glutamate by phorbol esters in primary glial cell cultures. *J. Neurochem.* **57**, 1185–1190.

Casado M., Bendahan A., Zafra F., Danbolt N. C., Aragón C., Giménez C. and Kanner B. (1993) Phosphorylation and modulation of brain glutamate transporters by protein kinase C. *J. Biol. Chem.* **268**, 27313–27317.

Chapman A. G. (1998) Glutamate receptors in epilepsy. *Prog. Brain Res.* **116**, 371–383.

Communi D., Dewaste V. and Erneux C. (1999) Calcium-calmodulin-dependent protein kinase II and protein kinase C-mediated phosphorylation and activation of D-myo-inositol 1,4,5-triphosphate 3-kinase B in astrocytes. *J. Biol. Chem.* **274**, 14734–14742.

Conradt M. and Stoffel W. (1997) Inhibition of the high-affinity brain glutamate transporter GLAST-1 via direct phosphorylation. *J. Neurochem.* **68**, 1244–1251.

Cooper J. A. (1987) Effects of cytochalasin and phalloidin on actin. *J. Cell Biol.* **105**, 1473–1478.

Cotrina M. L., Lin J. H. and Nedergaard M. (1998) Cytoskeletal assembly and ATP release regulate astrocytic calcium signaling. *J. Neurosci.* **18**, 8794–8804.

Cotrina M. L., Lin J. H., Lopez-Garcia J. C., Naus C. C. and Nedergaard M. (2000) ATP-mediated glia signaling. *J. Neurosci.* **20**, 2835–2844.

Dannhardt G. and Kohl B. K. (1998) The glycine site on the NMDA receptor: structure-activity relationships and possible therapeutic applications. *Curr. Med. Chem.* **5**, 253–263.

Davis K. E., Straff D. J., Weinstein E. A., Bannerman P. G., Correale D. M., Rothstein J. D. and Robinson M. B. (1998) Multiple signaling pathways regulate cell surface expression and activity of the excitatory amino acid carrier 1 subtype of Glu transporter in C6 glioma. *J. Neurosci.* **18**, 2475–2485.

Dowd L. A. and Robinson M. B. (1996) Rapid stimulation of EAAC1-mediated Na^+-dependent l-glutamate transport activity in C6 glioma cells by phorbol ester. *J. Neurochem.* **67**, 508–516.

Du S., Rubin A., Klepper S., Barret C., Kim Y. C., Rhim H. W., Lee E. B., Park C. W., Markelonis G. J. and Oh T. H. (1999) Calcium influx and activation of calpain I mediated acute reactive gliosis in injured spinal cord. *Exp. Neurol.* **157**, 96–105.

Edelman A. M., Higgins D. M., Bowman C. L., Haber S. N., Rabin R. A. and Cho-Lee J. (1992) Myosin light chain kinase is expressed in neurons and glia: immunoblotting and immunocytochemical studies. *Brain Res. Mol. Brain Res.* **14**, 27–34.

Finkbeiner S. M. (1993) Glial calcium. *Glia.* **9**, 83–104.

Fletcher E. J., Beart P. M. and Lodge D. (1990) Involvement of glycine in excitatory neurotransmission, in *Glycine Neurotransmission* (Ottersen O. P. and Storm-Mathisen J., eds), pp. 193–218. John Wiley and Sons, Ltd, New York.

Fricke V. (1975) A new scintillation cocktail based on Triton-X-100. *Anal. Biochem.* **63**, 555–558.

Gadea A. and López-Colomé A. M. (2001a) Glial transporters for glutamate, glycine, and GABA III. Glycine transporters. *J. Neurosci. Res.* **64**, 218–222.

Gadea A. and López-Colomé A. M. (2001b) Glial transporters for glutamate, glycine, and GABA. II. GABA transporters. *J. Neurosci. Res.* **63**, 461–468.

Gadea A., López E. and López-Colomé A. M. (1999a) Characterization of glycine transport in cultured Müller cells. *Glia*. **26**, 273–279.

Gadea A., López E. and López-Colomé A. M. (1999b) The adenylate cyclase inhibitor MDL-12330A has a non-specific effect on glycine transport in Müller cells from the retina. *Brain Res.* **838**, 200–204.

Gietzen K., Wuthrich A. and Bader H. (1981) R. 24571: a new powerful inhibitor of red blood cell Ca^{2+}-transport ATPase and of calmodulin-regulated functions. *Biochem. Biophys. Res. Commun.* **101**, 418–425.

Gomeza J., Casado M., Giménez C. and Aragón C. (1991) Inhibition of high-affinity γ-aminobutyric acid uptake in primary astrocyte cultures by phorbol esters and phospholipase C. *Biochem. J.* **275**, 435–439.

Gomeza J., Casado M., Giménez C. and Aragón C. (1994) Cellular distribution and regulation by cAMP of the GABA transporter (GAT-1) mRNA. *Brain Res. Mol. Brain Res.* **21**, 150–156.

González M. I., López-Colomé A. M. and Ortega A. (1999) Sodium-dependent glutamate transport in Müller glial cells: regulation by phorbol esters. *Brain Res.* **831**, 140–145.

Guan X., Cravatt B. F., Ehring G. R., Hall J. E., Boger D. L., Lerner R. A. and Gilula N. B. (1997) The sleep-inducing lipid oleamide deconvolutes gap junction communication and calcium wave transmission in glial cells. *J. Cell Biol.* **139**, 1785–1792.

Guthrie P. B., Knappenberger J., Segal M., Bennett M. V., Charles A. C. and Kater S. B. (1999) ATP released from astrocytes mediates glial calcium waves. *J. Neurosci.* **19**, 520–528.

Handlogten M. E., Dudenhausen E. E., Yang W. and Kilberg M. S. (1996) Association of hepatic system A amino acid transporter with the membrane-cytoskeletal proteins ankyrin and fodrin. *Biochim. Biophys. Acta.* **1282**, 107–114.

Hassinger T. D., Guthrie P. B., Atkinson P. B., Bennett M. V. and Kater S. B. (1996) An extracellular signaling component in propagation of astrocytic calcium waves. *Proc. Natl Acad. Sci. USA* **93**, 13268–13273.

Hernandez-Cruz A., Escobar A. L. and Jimenez N. (1997) Ca (2+)-induced Ca^{2+} release phenomena in mammalian sympathetic neurons are critically dependent on the rate of rise of trigger Ca^{2+}. *J. General Physiol.* **109**, 147–167.

Inagaki N., Goto H., Ogawara M., Nishi Y., Ando S. and Inagaki M. (1997) Spatial patterns of Ca^{2+} signals define intracellular distribution of a signaling by Ca^{2+}/calmodulin-dependent protein kinase II. *J. Biol. Chem.* **272**, 25195–25199.

Innocenti B., Parpura V. and Haydon P. G. (2000) Imaging extracellular waves of glutamate during calcium signaling in cultured astrocytes. *J. Neurosci.* **20**, 1800–1808.

Ishida A., Kameshita I., Okuno S., Kitani T. and Fujisawa H. (1995) A novel highly specific and potent inhibitor of calmodulin-dependent protein kinase II. *Biochem. Biophys. Res. Commun.* **212**, 806–812.

John G. R., Scemes E., Suadicani S. O., Liu J. S., Charles P. C., Lee S. C., Spray D. C. and Brosnan C. F. (1999) IL-1β differentially regulates calcium wave propagation between primary human fetal astrocytes via pathways involving P2 receptors and gap junction channels. *Proc. Natl Acad. Sci. USA* **96**, 11613–11618.

Kanamori M., Naka M., Asano M. and Hidaka H. (1981) Effects of N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide and other calmodulin antagonists (calmodulin interacting agents) on calcium-induced contraction of rabbit aortic strips. *J. Pharmacol. Exp Ther.* **217**, 494–499.

Kanner B. I. (1994) Sodium-coupled neurotransmitter transport: structure, function and regulation. *J. Exp. Biol.* **196**, 237–249.

Keirstead S. A. and Miller R. F. (1995) Calcium waves in dissociated retinal glial (Müller) cells are evoked by release of calcium from stores. *Glia* **14**, 14–22.

Keirstead S. A. and Miller R. F. (1997) Metabotropic glutamate receptor agonists evoke calcium waves in isolated Müller cells. *Glia* **21**, 194–203.

Kubes M., Cordier J., Glowinski J., Girault J. A. and Chneiweiss H. (1998) Endothelin induces a calcium-dependent phosphorylation of PEA-15 in intact astrocytes: identification of Ser104 and Ser116 phosphorylated, respectively, by protein kinase C and calcium/calmodulin kinase II *in vitro*. *J. Neurochem.* **71**, 1307–1314.

Legrand C., Ferraz C., Clavel M. C. and Rabie A. (1991) Distribution of gelsolin in the retina of the developing rabbit. *Cell Tissue Res.* **264**, 335–338.

Leung P. C., Taylor W. A., Wang J. H. and Tipton C. L. (1984) Ophiobolin A: a natural product inhibitor of calmodulin. *J. Biol. Chem.* **259**, 2742–2747.

Leybaert L., Paemeleire K., Strahonja A. and Sanderson M. J. (1998) Inositol-trisphosphate-dependent intercellular calcium signaling in and between astrocytes and endothelial cells. *Glia* **24**, 398–407.

Li Y., Holtzclaw L. A. and Russell J. T. (2000) Muller cell Ca2+ waves evoked by purinergic receptor agonists in slices of rat retina. *J. Neurophysiol.* **85**, 986–994.

Lidsky T. I., Yablonsky-Alter E., Zuck L. G. and Banerjee S. P. (1997) Antipsychotic drug effects on glutamatergic activity. *Brain Res.* **764**, 46–52.

Liu Q. R., López-Corcuera B., Mandiyan S., Nelson H. and Nelson N. (1993) Cloning and expression of a spinal cord and brain-specific glycine transporter with novel structural features. *J. Biol. Chem.* **268**, 22802–22808.

López-Colomé A. M. and Romo-De-Vivar M. (1991) Excitatory amino acid receptors in primary cultures of glial cells from the retina. *Glia* **4**, 431–439.

Lowry O. H., Rosenbrough N. J., Farr A. L. and Randall R. J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265–275.

Lukasiewicz P. D. and Roeder R. C. (1995) Evidence for glycine modulation of excitatory synaptic inputs to retinal ganglion cells. *J. Neurosci.* **15**, 4592–4601.

Malchow R. P. and Ramsey D. J. (1999) Responses of retinal Müller cells to neurotransmitter candidates: a comparative survey. *Biol. Bull.* **197**, 229–230.

Matsuda T., Takuma K., Asano S., Kishida Y., Nakamura H., Mori K., Maeda S. and Baba A. (1998) Involvement of calcineurin in Ca^{2+} paradox-like injury of cultured rat astrocytes. *J. Neurochem.* **70**, 2004–2011.

Mills J. W. and Mandel L. J. (1994) Cytoskeletal regulation of membrane transport events. *FASEB J.* **8**, 1161–1165.

Nelson W. J. and Hammerton R. W. (1989) A membrane-cytoskeletal complex containing Na+,K+-ATPase, ankyrin, and fodrin in Madin-Darby canine kidney (MDCK) cells: implications for the biogenesis of epithelial cell polarity. *J. Cell Biol.* **108**, 893–902.

Newman E. (2001) Propagation of intercellular calcium waves in retinal astrocytes and Müller cells. *J. Neurosci.* **21**, 2215–2223.

Newman E. and Reichenbach A. (1996) The Müller cell: a functional element of the retina. *TINS* **19**, 307–312.

Newman E. and Zahs K. R. (1997) Calcium waves in retinal glial cells. *Science* **275**, 844–847.

Newman E. and Zahs K. R. (1998) Modulation of neuronal activity by glial cells in the retina. *J. Neurosci.* **18**, 4022–4028.

Pannicke T., Fischer W., Biedermann B., Schadlich H., Grosche J., Faude F., Wiedemann P., Allgaier C., Illes P., Burnstock G. and Reichenbach A. (2000) P2X7 receptors in Müller glial cells from the human retina. *J. Neurosci.* **20**, 5965–5972.

Perlmutter L. S., Gall C., Baudry M. and Lynch G. (1990) Distribution of calcium-activated protease calpain in the rat brain. *J. Comp. Neurol.* **296**, 269–276.

Pow D. and Hendrickson A. E. (1999) Distribution of the glycine transporter glyt-1 in mammalian and nonmammalian retinae. *Vis. Neurosci.* **16**, 231–239.

Puro D. G., Hwang J. J., Kwon O. J. and Chin H. (1996) Characterization of an 1-type calcium channel expressed by human retinal Müller (glial) cells. *Brain Res. Mol. Brain Res.* **37**, 41–48.

Reye P., Penfold P. and Pow D. V. (2001) Glyt-1 expression in cultured human Muller cells and intact retinae. *Glia* **34**, 311–315.

Rothman S. M. and Olney J. W. (1995) Excitotoxicity and the NMDA receptor: still lethal after 8 years. *Trends Neurosci.* **18**, 57–58.

Roux M. J. and Supplisson S. (2000) Neuronal and glial glycine transporters have different stoichiometrics. *Neuron* **25**, 373–383.

Sakamoto Y. R., Nakajima T. R., Fukiage C. R., Sakai O. R., Yoshida Y. R., Azuma M. R. and Shearer T. R. (2000) Involvement of calpain isoforms in ischemia-reperfusion injury in rat retina. *Curr. Eye Res.* **21**, 571–580.

Sanderson M. J., Charles A. C., Boitano S. and Dirksen E. R. (1994) Mechanisms and function of intercellular calcium signaling. *Mol. Cell. Endocrinol.* **98**, 173–187.

Sato K., Adams R., Betz H. and Schloss P. (1995) Modulation of a recombinant glycine transporter (GLYT1b) by activation of protein kinase C. *J. Neurochem.* **65**, 1967–1973.

Schlaepfer W. W. and Zimmerman U. P. (1981) Calcium-mediated breakdown of glial filaments and neurofilaments in rat optic nerve and spinal cord. *Neurochem. Res.* **6**, 243–255.

Schmechel D. E., Brightman M. W. and Marangos P. J. (1980) Neurons switch from non-neuronal enolase to neuron-specific enolase during differentiation. *Brain Res.* **190**, 195–214.

Shields D. C. and Banik N. L. (1999) Pathophysiological role of calpain in experimental demyelination. *J. Neurosci. Res.* **55**, 533–541.

Singer J. H. and Berger A. J. (1999) Contribution of single channel properties to the time course and the amplitude variance of quantal glycine currents recorded in rat motoneurons. *J. Neurophysiol.* **81**, 1608–1616.

Soderling T. R., Tan S. E., McGlade-McCulloh E., Yamamoto H. and Fukunaga K. (1994) Excitatory interactions between glutamate receptors and protein kinases. *J. Neurobiol.* **25**, 304–311.

Somohano F. and López-Colomé A. M. (1991) Characteristics of excitatory amino acid uptake in cultures from neurons and glia from the retina. *J. Neurosci. Res.* **28**, 556–562.

Stuehr D. J. (1999) Mammalian nitric oxide synthases. *Biochim. Biophys. Acta.* **1114**, 217–230.

Supplisson S. and Bergman C. (1997) Control of NMDA receptor activation by a glycine transporter co-expressed in *Xenopus* oocytes. *J. Neurosci.* **17**, 4580–4590.

Takadera T. and Mohri T. (1985) Effect of intracellular free Ca^{2+} on leucine transport in Chang liver cells. *Cell Struc. Funct.* **10**, 349–359.

Takeuchi Y., Yamamoto H., Fukunaga K., Miyakawa T. and Miyamoto E. (2000) Identification of the isoforms of $Ca(^{2+})$ /calmodulin-dependent protein kinase II in rat astrocytes and their subcellular localization. *J. Neurochem.* **74**, 2557–2567.

Titmus M. J., Korn H. and Farber D. S. (1996) Diffusion, not uptake, limits glycine concentration in the synaptic cleft. *J. Neurophysiol.* **75**, 1738–1752.

Vallano M. L., Beaman-Hall C. M., Mathur A. and Chen Q. (2000) Astrocytes express specific variants of CaM KII delta and gamma, but not alpha and beta, that determine their cellular localizations. *Glia.* **30**, 154–164.

Van Belle H. (1984) The effect of drugs on calmodulin and its interaction with phosphodiesterase. *Adv. Cyclic Nucleotide Protein Phosphorylation Res.* **17**, 557–567.

Van Eldik L. J., Zendegui J. G., Marshak D. R. and Watterson D. M. (1982) Calcium-binding proteins and the molecular basis of calcium action. *Int. Rev. Cytol.* **77**, 1–61.

Van Staveren W. C., Markerink-van Ittersum M., Steinbusch H. W. and de Vente J. (2001) The effects of phosphodiesterase inhibition on cyclic GMP and cyclic AMP accumulation in the hippocampus of the rat. *Brain Res.* **888**, 275–286.

Verkhratsky A. and Kettenmann H. (1996) Calcium signalling in glial cells. *Trends Neurosci.* **19**, 346–352.

Villa P. G., Henzel W. J., Sensenbrenner M., Henderson C. E. and Pettmann B. (1998) Calpain inhibitors, but not caspase inhibitors, prevent actin proteolysis and DNA fragmentation during apoptosis. *J. Cell Sci.* **111**, 713–722.

Vinade L., Goncalves C. A., Wofchuk S., Gottfried C. and Rodnight R. (1997) Evidence for a role for calcium ions in the dephosphorylation of glial fibrillary acidic protein (GFAP) in immature hippocampal slices and in astrocyte cultures from the rat. *Brain Res. Dev. Brain Res.* **104**, 11–17.

Wakakura M. and Yamamoto N. (1994) Cytosolic calcium transient increase through the AMPA/kainate receptor in cultured Müller cells. *Vis. Res.* **34**, 1105–1109.

Wang Z., Haydon P. G. and Yeung E. S. (2000) Direct observation of calcium-independent intercellular ATP signaling in astrocytes. *Anal. Chem.* **72**, 2001–2007.

Weiss B., Prozialeck W., Cimino M., Barnette M. S. and Wallace T. L. (1980) Pharmacological regulation of calmodulin. *Ann. NY Acad. Sci.* **356**, 319–345.

Whitaker J. N., Herman P. K., Sparacio S. M., Zhou S. R. and Benveniste E. N. (1991) Changes induced in astrocyte cathepsin D by cytokines and leupeptin. *J. Neurochem.* **57**, 406–414.

Wilson J. M., Sanyal S. and Van Tol H. H. (1998) Dopamine D2 and D4 receptor ligands: relation to antipsychotic action. *Eur J. Pharmacol.* **351**, 273–286.

Wolfe F. H., Sathe S. K., Goll D. E., Kleese W. C., Edmunds T. and Duperret S. M. (1989) Chicken skeletal muscle has three Ca^{2+}-dependent proteinases. *Biochim. Biophys. Acta.* **998**, 236–250.

Wu S. M. and Maple B. R. (1998) Amino acid neurotransmitters in the retina: a functional overview. *Vision Res.* **38**, 1371–1384.

Yano S., Fukunaga K., Ushio Y. and Miyamoto E. (1994) Activation of Ca^{2+}/calmodulin-dependent protein kinase II and phosphorylation of intermediate filament proteins by stimulation of glutamate receptors in cultured rat cortical astrocytes. *J. Biol. Chem.* **269**, 5428–5439.

Zafra F., Aragón C., Olivares L., Danbolt N. C., Giménez C. and Storm-Mathisen J. (1990) Arachidonic acid inhibits glycine transport in cultured glial cells. *Biochem. J.* **271**, 237–242.

Zharikov S. I. and Block E. R. (2000) Association of l-arginine transporters with fodrin: implications for hypoxic inhibition of arginine uptake. *Am. J. Physiol. Lung Cell Mol. Physiol.* **278**, 111–117.

Journal of Neuroscience Research 63:453–460 (2001)

Mini-Review

Glial Transporters for Glutamate, Glycine and GABA I. Glutamate Transporters

Ana Gadea[1] and Ana María López-Colomé[1,2]*
[1]Instituto e Fisiología Celular, Departamento de Neurociencias, UNAM, Mexico
[2]Facultad de Medicina, Departamento de Bioquímica, UNAM, Mexico

The termination of chemical neurotransmission in the CNS involves the rapid removal of neurotransmitter from synapses by specific transport systems. Such mechanism operates for the three major amino acid neurotransmitters glutamate, γ-aminobutyric acid (GABA) and glycine. To date, five different high-affinity Na^+-dependent glutamate (Glu) transporters have been cloned: GLT1, GLAST, EAAC1, EAAT4 and EAAT5. The first two are expressed mainly by glial cells, and seem to be the predominant Glu transporters in the brain. A major function of Glu uptake in the nervous system is to prevent extracellular Glu concentrations from raising to neurotoxic levels in which glial transporters seem to play a critical role in protecting neurons from glutamate-induced excitotoxicity. Under particular conditions, glial GluTs have been shown to release Glu by reversal of activity, in a Ca^{2+}- and energy-independent fashion. Furthermore, an activity of these transporters as ion channels or transducing units coupled to G-proteins has recently been reported. The localization, stoichiometry, and regulation of glial GluTs are outlined, as well as their possible contributions to nervous system diseases as ALS, AD and ischemic damage. J. Neurosci. Res. 63: 453–460, 2001. © 2001 Wiley-Liss, Inc.

Key words: neurotransmitter uptake; neuron-glia interaction; second messengers; membrane protein; synaptic modulation

Neurons and glia accumulate neurotransmitters by a sodium dependent cotransport process. The Na^+/K^+-ATPase generates an inwardly directed electrochemical sodium gradient, used by several neurotransmitter carriers to drive the transport of these compounds against their concentration gradient (Kanner, 1983, 1989). The uptake of neurotransmitters by their transporters located in the plasma membrane of nerve terminals and glial cells plays an important role in the termination of synaptic transmission, and is also thought to provide synaptic insulation by preventing neurotransmitter spread to nearby synapses.

The most important amino acids subserving neurotransmitter functions in the CNS are γ-aminobutyric acid (GABA) and glycine acting as inhibitory neurotransmitters, and glutamic acid acting as an excitatory neurotransmitter. Glycine also participates in excitatory neurotransmission, as a coagonist of glutamate at N-methyl-D-aspartate (NMDA) receptors.

This review, divided in three sections, gathers experimental evidence for the presence of glial transporters for these neurotransmitters, and discusses the possible physiological implications of uptake systems in the modulation of normal and pathological neurotransmission.

Glutamate Transporters

Glutamate (Glu) is the main excitatory neurotransmitter in the brain and the retina, and has been shown to exert its action through the activation of specific ionotropic and metabotropic receptors (Meldrum, 2000). The excessive release of glutamate is an early and critical event in the Ca^{2+}-mediated death of neurons, and has been implicated in neurodegenerative processes associated with ischemia, epilepsy, and other neuropathological conditions (Matute et al., 1999; Shaw, 1999; Greene, 1999; Nicoletti et al., 1999; Chapman 2000). The postsynaptic actions of glutamate are rapidly terminated by high affinity glutamate uptake into neurons and glial cells (Kanner, 1993; Kanai et al., 1993; Danbolt, 1994) that is a Na^+- and K^+-coupled process (Nicholls and Atwell, 1990). The high affinity, Na^+-dependent excitatory amino acid (EAA) transporters constitute a heterogeneous group initially defined by pharmacological profiles (Robinson et al., 1991). To date, five different high-affinity Na^+- dependent Glu transporters have been cloned: EAAC1 (Kanai and Heideger, 1992), GLT1 (Pines et al., 1992), GLAST (Storck et al., 1992), EAAT4 (Fairman et al., 1995) and EAAT5 (Arriza et al., 1997). Several studies on their localization and functional roles have been done to un-

Contract grant sponsor: DGAPA; Contract grant numbers: IN-210998, PAEP-201332.

*Correspondence to: Ana María López-Colomé, Instituto de Fisologia Celular, UNAM, Apartado Postal 70-253, 04510, D.F., Mexico. E-mail: acolome@ifisiol.unam.mx

Received 10 November 2000; Accepted 17 November 2000

© 2001 Wiley-Liss, Inc.

derstand their possible differential contributions to synaptic transmission in normal and pathological conditions, as will be discussed.

Tissue and Cell Localization

The EAAC1 carrier, first cloned from the rabbit intestine, seemed to be quite abundant in brain, although relatively high levels of expression were also detected outside the nervous system in kidney, heart, muscle, lung, placenta and liver (Kanai and Heideger, 1992; Danbolt et al., 1992; Asta et al., 1983; Tanaka, 1993; Meister et al., 1993; Dall'Arriza et al., 1994; Kanai et al., 1994; Torp et al., 1994; Mukainaka et al., 1995; Nakayama et al., 1996; Arriza et al., 1997). In contrast, GLT1 and GLAST were considered as nervous system-specific proteins based on immunocytochemical and histochemical localization (Rothstein et al., 1994). The cognate mRNAs, however, were also found in peripheral tissues (Arriza et al., 1994; Krischner et al., 1994; Manfras et al., 1994; Tanaka, 1994; Nakayama et al., 1996). In the brain, the mRNA encoding EAAT4 is mainly confined to the cerebellar Purkinje dendrites (Yamada et al., 1996; Dehnes et al., 1998), and EAAT5 mRNA is selectively expressed by the retina, mainly localized to Müller cells (Arriza et al., 1997). The functional relevance of these transporters is presently unclear.

In addition to the five Na^+-dependent Glu transporters, evidence exists for two chloride-dependent transport processes for Glu in glioma cells and astrocytes in primary culture (Kimelberg, 1979), and in partially purified rat brain synaptosomes (Zaczec et al., 1987).

Within the brain, in situ hybridization showed localization of EAAC1 to glutamatergic neurons, of GLAST to cerebellar Bergmann glia and of GLT1 to astrocytes. More recent inmunocytochemical studies, using antipeptide antibodies directed to the C-terminal domain of each transporter, have demonstrated a more complex pattern.

In addition to glutamatergic neurons, EAAC1 is also present in GABAergic neurons such as cerebellar Purkinje cells and spinal cord ventral horn cells (Rothstein et al., 1994; Velaz-Faircloth, 1996). It is also expressed in some brain astroglial cells (Conti et al., 1998). In the retina, this transporter is located to horizontal, amacrine, and ganglion cells (Rauen and Kanner, 1994; Schultz and Stell, 1996).

Immunocytochemically, GLT1 and GLAST were detected exclusively in glial cells (Danbolt et al., 1992; Levy et al., 1993; Lehre et al., 1995). GLT1 was found restricted to astrocytes throughout the central nervous system, hippocampus and cerebral cortex showing the highest concentration. These results were confirmed, using antibodies to synthetic peptides corresponding to GLT1 amino acid residues 12–26, 493–508 and 559–573, and GLAST amino acid residues 522–541 and 504–518 (Rothstein et al., 1994; Lehre et al., 1995; Schmitt et al., 1997). The highest concentrations of GLAST are found in the Bergmann glia of the cerebellum. In the adult brain, astrocytic membranes facing nerve terminals, axons and spines, show higher density of GLT1 and GLAST than those facing capillaries, pia or stem dendrites, which is consistent with the importance of astrocytic transporters for Glu clearance from the extracellular space after synaptic transmission (Chaudhry et al., 1995). In the retina, GLAST immunoreactivity and the corresponding mRNA were detected in astrocytes, Müller cells and pigment epithelium, but not in neurons or microglia (Otori et al., 1994; Derouiche and Rauen, 1995; Lehre et al., 1997); in contrast, GLT1 is expressed in different types of bipolar cells and in some amacrine cells, but not in Müller cells or other retinal glia (Rauen and Kanner, 1994; Rauen et al., 1996). Moreover, GLT1 mRNA expression (but not the protein) in hippocampal pyramidal cells (Schmitt et al., 1997; Torp et al., 1997), as well as neuronal expression of GLT1, have been demonstrated after ischemic insult (Martin et al., 1997). GLT1 is also expressed in microcultures of hippocampal neurons (Mennerick et al., 1998). Recently, a transient expression of GLT1 by neurons in the developing brain was demonstrated, suggesting the participation of this transporter in the topographic organization of the brain (Northington et al., 1999).

Oligodendroglia is devoid of GLAST or GLT1 in rat brain (Chaudhry et al., 1995), although mRNAs for GLAST and to a smaller extent for GLT1, were expressed in all types of rat cultured glia including oligodendrocytes (Kondo et al., 1995).

GLT1 and GLAST proteins are coexpressed in the same astrocytes, the ratio of expression varying in different regions, depending on the particular type of adjacent glutamatergic synapse (Chaudhry et al., 1995; Lehre et al., 1995; Haugeto et al., 1996). Selective in vivo knockout of individual Glu transporters has provided additional proof supporting astroglial uptake of Glu by GLT1 and GLAST as the main mechanism in the clearance of this neurotransmitter and therefore, in protecting neurons from Glu excitotoxicity (Rothstein et al., 1996; see below).

Stoichiometry and Channel Properties

The uptake of Glu is coupled to the inward movement of two or three Na^+ plus one H^+, and the outward transport of one K^+ (Zerangue and Kavanaugh, 1996). Although neurotransmitter transporters and ligand-gated ion channels are generally considered structurally and functionally distinct, the association of channel-like ion fluxes with neurotransmitter transport indicates that carriers may be more similar to channels than was thought previously (reviewed in Lester et al., 1994; Sonders and Amara, 1996; DeFelice et al., 1996).

Recent studies in human cortex and cerebellum cloned transporters demonstrate that the transport of Glu by all the five known subtypes of GluTs is associated with a Cl^-conductance (revised in Seal and Amara, 1999) rapidly activated by Glu and dependent on Na^+, but not thermodynamically coupled to the transport process (Wadiche et al., 1995). The extent of Cl^- conductance varies dramatically within different transporter subtypes: for GLAST, GLT1 and EAAC1 the chloride flux is a relatively small component of the current, whereas for neuronal transporters EAAT4 and EAAT5, the anion flux is almost completely responsible for the current elicited by

the substrate: For EAAT4, 95% of the current generated by the transporter arises from Cl^- movement (Fairman et al., 1995). The anion conductance associated to Glu transport in glial cells could prevent reduction in the rate of transport due to the depolarization resulting from electrogenic Glu uptake, thus having an influence in the regulation of general excitability.

An interesting feature correlating transporters and channels is the presence in EAAT5, of a PDZ binding motif at the C-terminus domain, identified previously in NMDARs and K^+ channels, which has been implicated in receptor and ion channel clustering at the synapse, thus suggesting EAAT5 participation in signal transduction activation (Arriza et al., 1997).

In retinal glial (Müller) cells, Glu activates an outward current at positive potentials, which results from an anionic conductance associated with GluTs (Eliasof and Jahr, 1996). Such current is activated by extracellular Glu during uptake and by intracellular Glu during release by reversed operation of the carrier, unlike Glu receptor-gated channels (Billups et al., 1996). Also in Müller cells, Na^+ carries a current in the absence of Glu, which has been interpreted as an uncoupled movement of Na^+ through the transporter (Schwartz and Tachibana, 1990).

From the first report on the purification of a Glu transporter (Danbolt et al., 1990), an oligomeric structure for these proteins has been suggested. Cross-linking of Glu transporter proteins in intact membranes, detergent extracts, liposomes containing active reconstituted Glu transporters, and transfected HeLa cells, clearly showed the presence of GLAST, GLT1, and EAAC1 multimers (Haugeto et al., 1996). Trimers are predominant in the case of GLT1, whereas GLAST and EAAT4 (Dehnes et al., 1998), may also exist as dimers. In vivo, GLT1 and GLAST seem to exist as homo-oligomers, because they do not associate despite their common location. A very recent work has shown a pentameric structure for human neuronal Glu transporter EAAT3, the human homolg of EAAC1 (Eskandari et al., 2000). It is yet unclear whether substrate-driving ions share a single permeation pathway with the anion current, or if a single transporter protein has multiple ion permeation pathways. On this matter, agents affecting the translocation of the substrate do not alter the anion conductance, suggesting that transport and anion-permeation are separate processes that undergo independent regulation (Trotti et al., 1998). Future studies on the oligomeric structure of these transporters may reveal the answer.

Regulation of Glutamate Transporters

The modulation of Glu transporters activity has been demonstrated in different model systems. A series of reports (Drejer et al., 1983; Voisin et al., 1993; Gegelashvili et al., 1997) suggest that a molecule(s) secreted by neurons, Glu included, could upregulate Glu transporter activity in glial cells. A 70% decrease in D-aspartate uptake in striatum 10 days after cortical lesions was reversed by the application of gangliosides, despite the dramatic loss of glutamatergic synapses; reduction in uptake capacity was ascribed to astrocytes due to a chronic deficit of Glu or other factors normally secreted by neurons (Shifman, 1991). Later results have shown glutamatergic deafferentation to reduce glial GLT1 and GLAST but not neuronal EAAC1 (Levy et al., 1995; Ginsberg et al., 1995). In agreement with these results, in the absence of neurons, cultured astrocytes express only GLAST, whereas in the presence of neurons GLT1 is also expressed and the expression of GLAST is increased (Swanson et al., 1997; Schlag et al., 1998). Moreover, the exposure of cortical astroglial cultures to pituitary adenylate cyclase-activating polypeptide (PACAP) or to neuron-conditioned medium (NCM) increased the expression of GLT1, GLAST and glutamine synthetase (GS); the effect was inhibited by PACAP-inactivating antibodies or by PACAP receptor antagonists (Figiel and Engele, 2000). These results imply that PACAP exerts its actions on glial Glu turnover through PAC1 receptors, inducing the expression of GLAST via activation of PKA signaling pathways, and of GLT1 via both, PKA and PKC pathways. Further evidence has shown that neuronal soluble factors determine the induction of GLT1 in cortical astrocytes through a signaling pathway involving the activation of p42/44 MAPK (mitogen activated protein kinase) and CREM (cyclic adenosine monophosphate responsive element modulator) and ATF-1 (activating transcription factor-1) by tyrphostin-sensitive receptor tyrosine kinases (RTK; Gegelashvili et al., 2000). These studies suggest that GLT1 and GLAST expression is regulated independently by diffusible molecules secreted by neurons.

Glutamate has been shown to regulate Glu transport. Expression of GLAST protein in cultured astrocytes is increased upon prolonged exposure to Glu or kainate. In dBcAMP-treated astrocytes, characterized by increased Glu uptake capacity, alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and (±)-1-aminocyclopentane-trans-1,3-dicarboxylic acid (t-ACPD) induce a further upregulation of GLAST and the corresponding mRNA, respectively (Gegelashvili et al., 1996). Recent studies demonstrate that glutamate-induced upregulation of Glu uptake by astrocytes is a result of increased cell-surface expression of GLAST, mediated by actin cytoskeleton-dependent translocation of the transporter to (or decreased removal from) the cell membrane. The effect of Glu was mimicked by D-aspartate, inhibited by cytochalasins, blocked in Na^+-free medium, but not affected by Glu receptor antagonists (Duan et al., 1999). Preincubation of cultured astrocytes with Glu has also been reported to reduce extracellular Glu concentrations (Ye and Sontheimer, 1999); interestingly, this effect is mimicked by metabotropic Glu receptor agonists, but is not blocked by metabotropic receptor antagonists. This effect could be mediated by a substrate-stimulated increase in transporter activity, because several metabotropic agonists are substrates for the transporters (Ye and Sontheimer, 1998). In C6 glioma cells expressing EAAC1, activation of protein kinase C (PKC) leads to the translocation of the transporter from the intracellular compartment to

the cell surface, whereas the PI3K inhibitor wortmannin decreases membrane EAAC1 in C6 glioma cells (Davis et al., 1998). Also, in BTC4 glioma cells, which intrinsically express EAAT4, incubation with Glu or other transportable substrates induces the redistribution of the protein from the cytoplasm to the plasmamembrane (Gegelashvili et al., 2000). Marie and Attwell (1999) have recently demonstrated an increase in GLAST affinity for Glu by disrupting the interaction between an intracellular protein and the last eight amino acids of the GLAST C-terminus, which bares similarity with the PDZ binding domain of ion channels C-termini. It is tempting to speculate that the interaction of GluTs with membrane proteins of the SNARE system, could control the subcellular localization of Glu transporters and modulate their activity.

Signaling molecules including PKC, arachidonic acid and cytokines have been shown to regulate GluTs activity. An increase in brain GLT1 activity upon activation of PKC (Casado et al., 1993), as well as a decrease in the activity of the human homologue of GLT1 have been demonstrated (Ganel and Crosson, 1998). In C6 glioma cells, a rapid stimulation of Glu transport by phorbol esters, attributed to EAAC1, has been observed (Dowd and Robinson, 1996). GLAST activity, however, is inhibited by phosphorylation at a non-PKC consensus site (Conradt and Stoeffel, 1997). Arachidonic acid has also been reported to inhibit several sodium-dependent amino acid transporters including those for Glu, glycine and GABA (Rhoads et al., 1983; Barbour et al., 1989; Zafra et al., 1990). These results indicate that transporter subtypes are regulated by distinct cell-type specific mechanisms.

An interesting question to be solved by future research is whether Glu transporters themselves may serve as signal transducing units as proposed (Gegelashvili and Schousboe, 1998), based on the observation that the third intracellular domain of GLAST, GLT-1, and EAAC1 contain a similar motif to that in IGF-II and α-adrenergic receptors that binds Gα subunits of G-proteins.

Glial Glutamate Transporters in Disease

Protection of neurons from Glu-induced excitotoxicity by GluTs was first demonstrated in organotypic rat spinal cord cultures (Rothstein et al., 1993), in which the identity of the Glu transporter involved was determined using antisense oligonucleotides directed to the N-terminal segment of the cloned Glu transporters (Rothstein et al., 1996). Oligonucleotides directed to glial transporters GLT1 and GLAST but not to neuronal EAAC1 induced specific degeneration of motor neurons; thus glial, and not neuronal Glu transporters seem to protect neurons from Glu excitotoxicity. Recent studies demonstrated that GLT1 knockout mice, show lethal spontaneous seizures, selective neuronal degeneration in hippocampus, and increased susceptibility to acute cortical injury (Tanaka et al., 1997). In contrast, EAAC1 knockout mice differ from controls only in a reduction in locomotor activity (Peghini et al., 1997). Hence EAAC1 might have a function other than the stringent regulation of synaptic Glu concentration. In fact, glial transporters GLT1 and to a lesser extent GLAST, contribute largely to the maintenance of the tonic cerebrospinal Glu concentration, whereas the contribution of neuronal EAAC1 is negligible (Rothstein et al., 1996).

Due to the critical role of the high-affinity GluTs in the maintenance of $[Glu]_o$ below neurotoxic levels, alteration of their function or expression levels may contribute to the postischemic vulnerability of neurons. A decrease in GLT1 mRNA and protein was observed in rat hippocampal CA1 region after transient forebrain ischemia (Torp et al., 1995), whereas the expression of GLAST and EAAC1 were unaltered, hence, the high sensitivity of neurons in this condition could relate to the decrease in the glial transporter GLT1. Further on, the expression of GLT1 protein in CA1 was unchanged during the immediate postischemic period, decreasing markedly from Days 2 to 4, whereas in CA3, a progressive increase in GLT1 protein was observed (Bruhn et al., 2000). Reduction in the expression of GLT1 in CA1 shown in this study, was ascribed to degeneration of CA1 pyramidal neurons, whereas the concomitant postischemic upregulation of Glu uptake in CA3 astrocytes could explain the relative resistance of pyramidal neurons from this region to ischemic damage. These findings are in keeping with previous work demonstrating simultaneous stimulation of ATPase activity and Glu uptake in cultured rat cortical astrocytes after sublethal ischemic insult (Stanimirovic et al., 1997) that clearly shows that glial cells undergo adaptive-protective responses to ischemia (for review see Ottersen et al., 1996).

Oxidative stress is associated with several brain pathologies leading to acute or chronic neurodegeneration. Increasing evidence indicates that distinct Glu transporters are targets for biological oxidants and share one or more oxidant-vulnerable sites (Trotti et al., 1998). The activity of GLT1, GLAST and EAAC1 are equally inhibited by oxidants via a direct action on the transporter protein, consistent with the regulation of Glu uptake by the redox state of reactive cysteine residues in the transporter molecule (Trotti et al., 1997).

Glial GLT1 malfunction has been implicated in the pathogenesis of amyotrophic lateral sclerosis (ALS; Bristol and Rothstein, 1996). A reduction in Glu uptake was found in synaptosomes obtained from postmortem ALS material, ascribed to a major and specific decrease in GLT1 protein from the motor cortex (>70%) and spinal cord (57% Rothstein et al., 1995), due to defective translational or post-translational mechanisms. In keeping with this idea, transgenic mice overexpressing mutant human Cu^{2+}/Zn^{2+} superoxide dismutase (SOD1), implied in some familial forms of ALS, develop ALS symptoms parallel to loss of activity of GLT1 by excessive nitration (Rothstein et al., 1996). The presence of abnormal GLT1 mRNA in ALS postmortem material, however, has recently been reported (Lin et al., 1998).

Several studies have demonstrated that Na^+-dependent Glu uptake is decreased in tissues obtained from patients suffering from Alzheimer disease (AD; Masliah et al., 1996). The participation of β-amyloid peptide (βA) in

TESIS CON
FALLA DE ORIGEN

the neuropathology of AD, including free radical injury and excitotoxicity is widely documented. Treatment of neuron/astrocyte co-cultures with βA has been shown to increase the vulnerability of neurons to Glu-induced cell death (Koh et al., 1990), probably due to inhibition of astrocyte Glu transporters by βA (Harris et al., 1996). A more recent study shows significant inhibition of L-Glu uptake by βA (25–35) in rat hippocampal astrocyte cultures, prevented by the antioxidant Trolox, as well as by the β-amyloid peptide precursor (APP; Masliah et al., 2000), ascribed to astroglial GLAST and GLT1 decrease. The levels of mRNA expression were not altered, however, suggesting an effect related to post-transcriptional mechanisms or to the trafficking of the transporter to the membrane. Secreted products resulting from APP proteolysis have been shown to alter Glu transporter function via protein kinase A (PKA)- and PKC-dependent pathways. Astroglial Glu transporters are inihibited by βA but stimulated by αA (Masliah et al., 1998). Although this finding suggests that aberrantly processed APP might impair astroglial Glu uptake contributing to the neurodegenerative process in AD (Li et al., 1997), recent data demonstrate individual variations in the expression of GLT1 and GLAST protein in human autopsy samples, with no significant correlation to AD (Beckstrom et al., 1999).

Glutamate taken up by glial cells is converted by glutamine synthetase (GS), an enzyme confined to glial cells, into glutamine that is released, taken up by neurons and converted to Glu by phosphate-activated glutaminase, allowing the recycling of this transmitter. Additionally, glial uptake of Glu has been shown to stimulate glycolysis and to trigger the export of lactate, subserving a detoxification pathway for Glu in astrocytes and an efficient energy source for neurons respectively (Hertz et al., 1999), thus protecting neurons from glucose deprivation and hypoxic episodes (Izumi et al., 1997). Recently, a common transcriptional regulation of GLAST and GS inducible by cortisol has been demonstrated, which could reflect a requirement for coordinated regulation of uptake and degradation systems for Glu in glia (Rauen and Wiessner, 2000).

As mentioned above, GLAST is the predominant transporter expressed by retinal Müller cells, and has been shown to play an important role in excitatory transmission in the retina, because Glu uptake by these cells dominates total retinal Glu uptake (Rauen et al., 1998). On this line, although GLAST mutant mice electroretinogram shows normal a-wave (that reflects the activity of photoreceptors), the b-wave originated mainly in Müller cells is attenuated by more than 50% as compared to wild-type mice (Harada et al., 1998). Also in these mutants, ischemic retinal damage is exacerbated. Signaling form photoreceptors to bipolar cells through graded potentials (Juusola, 1996), requires clearance of Glu from the synaptic cleft, which clearly underlines the essential role of GLAST in shaping the time course of synaptic transmission at the photoreceptor-bipolar synapse.

Evidence for Glu release by the reversal of uptake under physiological conditions comes from studies in salamander Müller cells, showing the stimulation of Glu uptake after a raise in intracellular K^+ concentration, due to the coupling of K^+ extrusion to the inward cotransport of Glu and Na^+ (Barbour et al., 1988; Szatkowski et al., 1990; Holopainen and Kontro, 1990). The reversal of Glu uptake could contribute to excitotoxicity under conditions of energy failure, such as ischemia (Atwell et al., 1993; Kanai et al., 1995) due to a significant local increase in extracellular Glu concentration.

Studies reviewed here, clearly underline a prevalent role for glial Glu transporters in the maintenance and modulation of synaptic transmission.

ACKNOWLEDGMENTS

This work was supported in part by grant IN-210998 from DGAPA to A.M.L.C. and PAEP 201332 to A.G.

REFERENCES

Arriza JL, Fairman WA, Wadiche JI, Murdoch GH, Kavanaugh MP, Amara SG. 1994. Function comparisons of three glutamate transport subtypes cloned from the human motor cortex. J Neurosci 14:5559–5569.

Arriza JL, Eliasof S, Kavanaugh MP, Amara SG. 1997. Excitatory amino acid transporter 5, a retinal glutamate transporter coupled to a chloride conductance. Proc Natl Acad Sci USA 94:4155–4160.

Attwell D, Barbour B, Szatkowsky M. 1993. Nonvesicular release of neurotransmitter. Neuron 11:401–407.

Barbour B, Brew H, Attwell D. 1988. Electrogenic glutamate uptake in glial cells is activated by intracellular potassium. Nature 335:433–435.

Barbour B. 1989. Modulation of glutamate transporters by arachidonic acid. Nature 342:918–920.

Beckstrom H, Julsrud L, Haugeto O, Dewar D, Graham DI, Lehre KP, Storm-Mathisen J, Danbolt NC. 1999. Interindividual differences in the levels of the glutamate transporters GLAST and GLT, but no clear correlation with Alzheimer disease. J Neurosci Res 55:218–229.

Billups B, Rossi D, Attwell D. 1996. Anion conductance behavior of the glutamate uptake carrier in salamander retinal glial cells. J Neurosci 16:6722–6731.

Bristol LA, Rothstein JD. 1996. Glutamate transporter gene expression in amyotrophic lateral sclerosis motor cortex. Ann Neurol 39:676–679.

Bruhn T, Levy LM, Nielsen M, Christensen T, Johansen FF, Diemer NH. 2000. Ischemia induced changes in expression of the astrocyte glutamate transporter GLT1 in hippocampus of the rat. Neurochem Int 37:277–285.

Casado M, Bendahan A, Zafra F, Danbolt NC, Aragón C, Giménez C, Kanner B. 1993. Phosphorylation and modulation of brain glutamate transporters by protein kinase C. J Biol Chem 268:27313–27317.

Chapman AG. 2000. Glutamate and epilepsy. J Nutr 130:1043–1045.

Chaudhry FA, Lehre KP, vanLookeren M, Campagne M, Ottersen OP, Danbolt NC, Storm-Mathisen J. 1995. Glutamate transporters in glial plasmamembranes: highly differentiated localizations revealed by quantitative ultrastructural immunocytochemistry. Neuron 15:710–720.

Conradt M, Stoffel W. 1997. Inhibition of the high-affinity brain glutamate transporter GLAST-1 via direct phosphorylation. J Neurochem 68:1244–1251.

Conti F, DeBiasi S, Minelli A, Rothstein JD, Melone M. 1998. EAAC1, a high-affinity glutamate tranporter, is localized to astrocytes and gabaergic neurons besides pyramidal cells in the rat cerebral cortex. Cereb Cortex 8:108–116.

Dall'Asta V, Gazzola GC, Franchi-Gazzola R, Bussolati O, Longo N, Guidotti GG. 1983. Pathways of L-glutamic acid transport in cultured human fibroblasts. J Biol Chem 258:6371–6379.

TESIS CON
FALLA DE ORIGEN

Danbolt NC, Pines G, Kanner BI. 1990. Purification and reconstitution of the sodium- and potassium-coupled glutamate transport glycoprotein from rat brain. Biochemistry 29:6734–6740.

Danbolt NC, Storm-Mathisen J, Kanner BA. 1992. A [Na^+ + K^+]-coupled L-glutamate transporter purified from rat brain is located in glial cell processes. Neuroscience 51:295–310.

Danbolt NC. 1994. The high affinity uptake system for excitatory amino acids in the brain. Prog Neurobiol 44:377–396.

Davis KE, Straff DJ, Weinstein EA, Bannerman PG, Correale DM, Rothstein JD, Robinson MB. 1998. Multiple signaling pathways regulate cell surface expression and activity of the excitatory amino acid carrier 1 subtype of Glu transporter in C6 glioma. J Neurosci 18:2475–2485

Dehnes Y, Chaudhry FA, Ullensvang K, Lehre KP, Storm-Mathisen J, Danbolt NC. 1998. The glutamate transporter EAAT4 in rat cerebellar Purkinje cells: a glutamate-gated chloride channel concentrated near the synapse in parts of the dendritic membrane facing astroglia. J Neurosci 18:3606–3619.

Derouiche A, Rauen T. 1995. Coincidence of L-glutamate/L-aspartate transporter (GLAST) and glutamine synthetase (GS) immunoreactions in retinal glia: evidence for coupling of GLAST and GS in transmitter clearance. J Neurosci Res 42:131–43.

DeFelice LJ, Blakely RD. 1996. Pore models for transporters? Biophys J 70:579–580.

Dowd LA, Robinson MB. 1996. Rapid stimulation of EAAC1-mediated Na^+-dependent L-glutamate transport activity in C6 glioma cells by phorbol ester. J Neurochem 67:508–516.

Drejer J, Meier E, Schousboe A. 1983. Novel neuron-related regulatory mechanisms for astrocytic glutamate and GABA high affinity uptake. Neurosci Lett 37:301–306.

Duan S, Anderson CM, Stein BA, Swanson RA. 1999. Glutamate induces rapid upregulation of astrocyte glutamate transport and cell-surface expression of GLAST. J Neurosci 19:10193–10200.

Eliasof S, Jahr CE. 1996. Retinal glial cell glutamate transporter is coupled to an anionic conductance. Proc Natl Acad Sci USA 93:4153–4158.

Eskandari S, Kreman M, Kavanaugh MP, Wright EM, Zampighi GA. 2000. Pentameric assembly of a neuronal glutamate transporter. Proc Natl Acad Sci USA 97:8641–8646.

Fairman WA, Vandenberg RJ, Arriza JL, Kavanaugh MP, Amara SG. 1995. An excitatory amino-acid transporter with properties of a ligand-gated chloride channel. Nature 375:599–603.

Figiel M, Engele J. 2000. Pituitary adenylate cyclase-activating polypeptide (PACAP), a neuron-derived peptide regulating glial glutamate transport and metabolism. J Neurosci 20:3596–3605.

Ganel R, Crosson CE. 1998. Modulation of human glutamate transporter activity by phorbol ester. J Neurochem 70:993–1000.

Gegelashvili G, Civenni G, Racagni G, Danbolt NC, Schousboe I, Schousboe A. 1996. Glutamate receptor agonists up-regulate glutamate transporter GLAST in astrocytes. Neuroreport 8:261–265.

Gegelashvili G, Danbolt NC, Schousboe A. 1997. Neuronal soluble factors differentially regulate the expression of the GLT1 and GLAST glutamate transporters in cultured astroglia. J Neurochem 69:2612–2615.

Gegelashvili G, Schousboe A. 1998. Cellular distribution and kinetic properties of high-affinity glutamate transporters. Brain Res Bull 45:233–238

Gegelashvili G, Dehnes Y, Danbolt NC, Schousboe A. 2000. The high-affinity glutamate transporters GLT1, GLAST, and EAAT4 are regulated via different signalling mechanisms. Neurochem Int 37:163–170.

Greene JG. 1999. Mitochondrial function and NMDA receptor activation: mechanisms of secondary excitotoxicity. Funct Neurol 14:171–184.

Ginsberg SD, Martin LJ, Rothstein JD. 1995. Regional deafferentation down-regulates subtypes of glutamate transporter proteins. J Neurochem 65:2800–2803.

Harada T, Harada C, Watanabe M, Inoue Y, Sakagawa T, Nakayama N, Sasaki S, Okuyama S, Watase K, Wada K, Tanaka K. 1998. Functions of the two glutamate transporters GLAST and GLT-1 in the retina. Proc Natl Acad Sci USA 95:4663–4666.

Harris ME, Wang Y, Pedigo NW Jr, Hensley K, Butterfield DA, Carney JM. 1996. Amyloid beta peptide (25–35) inhibits Na^+-dependent glutamate uptake in rat hippocampal astrocyte cultures. J Neurochem 67:277–286.

Haugeto O, Ullensvang K, Levy I, Chaudhry FA, Honoré T, Nielsen M, Lehre KP, Danbolt NC. 1996. Brain Glutamate Transporter Proteins Form Homomultimers. J Biol Chem 271:27715–27722.

Hertz L, Dringen R, Schousboe A, Robinson SR. 1999. Astrocytes: glutamate producers for neurons. J Neurosci Res 57:417–428.

Holopainen I., Kontro P. 1990. D-Aspartate release from cerebellar astrocytes: modulation of the high K-induced release by neurotransmitter amino acids. Neuroscience 36:115–120.

Izumi Y, Benz AM, Katsuki H, Zorumsky CF. 1997. Endogenous monocarboxylates sustain hippocampal synaptic function and morphological integrity during energy deprivation. J Neurosci 17:9448–9457.

Juusola M, French AS, Uusitalo RO, Weckstrom M. 1996. Information processing by graded-potential transmission through tonically active synapses. Trends Neurosci 19:292–297.

Kanai Y, Hediger MA. 1992. Primary structure and functional characterization of a high affinity glutamate transporter. Nature 360:467–471

Kanai Y, Smith CP, Hediger MA. 1993. The elusive transporters with a high affinity for glutamate. Trends Neurosci 16:365–370.

Kanai Y, Stelzner M, Nussberger S, Khawaja S, Hebert SC, Smith CO, Hediger MA. 1994. The neuronal and epithelial human high affinity glutamate transporter. Insights into structure and mechanism of transport. J Biol Chem 269:20599–20606.

Kanai Y, Nussberger S, Romero MF, Boron WF, Hebert SC, Hediger MA. 1995. Electrogenic properties of the epithelial and neuronal high affinity glutamate transporter. J Biol Chem 270:16561–16568

Kanner BI. 1983. Bioenergetics of neurotransmitter transport. Biochem Biophys Acta 726:293–316.

Kanner BI. 1989. Ion-coupled neurotransmitter transport. Curr Opin Cell Biol 1:735–738.

Kanner BI. 1993. Glutamate transporters from brain. A novel neurotransmitter transporter family. FEBS Lett 325:95–99.

Kimelberg HK, Biddlecomb S, Bourke RS. 1979. SITS inhibitable Cl^- transport and Na^+-dependent H^+ production in primary astroglial functions. Brain Res 173:111–124.

Koh JY, Yang LL, Cotman CW. 1990. Beta-amyloid protein increases the vulnerability of cultured cortical neurons to excitotoxic damage. Brain Res 533:315–320.

Kondo K, Hashimoto H, Kitanaka J, Sawada M, Suzumura A, Marunouchi T, Baba A. 1995. Expression of glutamate transporters in cultured glial cells. Neurosci Lett 24:188:140–142.

Krischner MA, Copeland NG, Gilbert DJ, Jenkins NA, Amara SG. 1994. Mouse excitatory amino acid transporter EAAT2: isolation characterization, and proximity to neuroexcitability loci on mouse chromosome 2. Genomics 24:218–224.

Lehre KP, Levy LM, Ottersen OP, Storm-Mathisen J, Danbolt NC. 1995. Differential expression of two glial glutamate transporters in the rat brain: quantitative and immunocytochemical observations. J Neurosci 15:1835–1853.

Lehre KP, Davanger S, Danbolt NC. 1997. Localization of the glutamate transporter protein GLAST in rat retina. Brain Res 744:129–137.

Lester HA, Mager S, Quick MW, Corey JL. 1994. Permeation properties of neurotransmitter transporters. Annu Rev Pharmacol Toxicol 34:219–249.

Levy L, Lehre KP, Rolstad B, Danbolt DC. 1993. A monoclonal antibody raised against a [Na^+ + K^+]coupled L-glutamate transporter purified from rat brain confirms glial cell localization. FEBS Lett 317:79–84.

TESIS CON
FALLA DE ORIGEN

Levy LM, Lehre KP, Walaas SI, Storm-Mathisen J, Danbolt NC. 1995. Down-regulation of glial glutamate transporters after glutamatergic denervation in the rat brain. Eur J Neurosci 7:2036–2041.
Li S, Mallory M, Alford M, Tanaka S, Masliah E. 1997. Glutamate transporter alterations in Alzheimer's disease are possibly associated with abnormal APP expression. J Neuropathol Exp Neurol 56:901–911.
Lin CL, Bristol LA, Jin L, Dykes-Hoberg M, Crawford T, Clawson L, Rothstein JD. 1998. Aberrant RNA processing in a neurodegenerative disease: the cause for absent EAAT2, a glutamate transporter, in amyotrophic lateral sclerosis. Neuron 20:589–602.
Manfras BJ, Rudert WA, Trucco M, Boehm BO. 1994. cloning and characterization of a glutamate transporter cDNA from human brain and pancreas. Biochim Biophys Acta 1195:185–188
Marie H, Attwell D. 1999. C-terminal interactions modulate the affinity of GLAST glutamate transporters in salamander retinal glial cells. J Physiol 520:393–397.
Martin LJ, Brambrink AM, Lehmann C, Portera-Cailliau C, Koehler R, Rothstein J, Traystman RJ. 1997. Hypoxia-ischemia causes abnormalities in glutamate transporters and death of astroglia and neurons in newborn striatum. Ann Neurol 42:335–348.
Masliah E, Alford M, DeTeresa R, Mallory M, Hansen L. 1996. Deficient glutamate transport is associated with neurodegeneration in Alzheimer's disease. Ann Neurol 40:759–766.
Masliah E, Raber J, Alford M, Mallory M, Mattson MP, Yang D, Wong D, Mucke L. 1998. Amyloid protein precursor stimulates excitatory amino acid transport. Implications for roles in neuroprotection and pathogenesis. J Biol Chem 273:12548–12554.
Masliah E, Alford M, Mallory M, Rockenstein E, Moechars D, Van Leuven F. 2000. Abnormal glutamate transport function in mutant amyloid precursor protein transgenic mice. Exp Neurol 163:381–387.
Matute C, Domercq M, Fogarty DJ, Pascual-de-Zulueta M, and Sanchez-Gomez MV. 1999. On how altered glutamate homeostasis may contribute to demyelinating diseases of the CNS. Adv Exp Med Biol 468:97–107.
Meister B, Arvidsson U, Zhang X, Jacobson G, Villar MJ, Hokfelt T. 1993. Glutamate transporter mRNA and glutamate-like immunoreactivity in spinal motoneurons. Neuroreport 5:337–340.
Meldrum BS. 2000. Glutamate as a neurotransmitter in the brain: review of physiology and pathology. J Nutr 130:1007–1115.
Mennerick S, Dhond RP, Benz A, Xu W, Rothstein JD, Danbolt NC, Isenberg KE, Zorumski CF. 1998. Neuronal expression of the glutamate transporter GLT-1 in hippocampal microcultures. J Neurosci 18:4490–4499.
Mukainaka Y, Tanaka K, Hagiwara T, Wada K. 1995. molecular cloning of two glutamate transporters subtypes from mouse brain. Biochim Biophys Acta 1244:233–237.
Nakayama T, Kawakami H, Tanaka K, Nakamura S. 1996. Expression of three glutamate transporter subtype mRNAs in human brain regions and peripheral tissues. Mol Brain Res 36:189–192.
Nicoletti F, Bruno V, Catania MV, Battaglia G, Copani A, Barbagallo G, Cena V, Sanchez-Prieto J, Spano PF, Pizzi M. 1999. Group-I metabotropic glutamate receptors: hypotheses to explain their dual role in neurotoxicity and neuroprotection. Neuropharmacology 38:1477–1484.
Nicholls DG, Attwell D. 1990. The release and uptake of excitatory amino acids. Trends Pharmacol Sci 11:462–468.
Northington FJ. 1999. GLT1, glial glutamate transporter, is transiently expressed in neurons and develops astrocyte specificity only after midgestation in the ovine fetal brain. J Neurobiol 39:515–526.
Otori Y, Shimada S, Tanaka K, Ishimoto I, Tano Y, Tohyama M. 1994. Marked increase in glutamate-aspartate transporter (GLAST/GluT1) mRNA after transient retinal ischemia. Mol Brain Res 27:310–314.
Ottersen OP, Laake JH, Reichelt W, Haug FM, Torp R. 1996. Ischemic disruption of glutamate homeostasis in brain: quantitative immunocytochemical analyses. J Chem Neuroanat 12:1–14.
Peghini P, Janzen J, Stoffel W. 1997. Glutamate transporter EAAC-1-deficient mice develop dicarboxylic aminoaciduria and behavioral abnormalities but no neurodegeneration. EMBO J 16:3822–3832.
Pines G, Danbolt NC, Bj/rås M, Zhang Y, Bendahan A, Eide L, Koepsell H, Storm-Mathisen J, Seeberg E, Kanner BI. 1992. Cloning and expression of a rat brain L-glutamate transporter. Nature 360:464–467.
Rauen T, Kanner BI. 1994. Localization of the glutamate transporter GLT1 in rat and macaque monkey retina. Neurosci Lett 169:137–140.
Rauen T, Rothstein JD, Wassle H. 1996. Differential expression of three glutamate transporter subtypes in the rat retina. Cell Tissue Res 286:325–336.
Rauen T, Taylor WR, Kuhlbrodt K, Wiessner M. 1998. High-affinity glutamate transporters in the rat retina: a major role of the glial glutamate transporter GLAST-1 in transmitter clearance. Cell Tissue Res 291:19–31.
Rauen T, Wiessner M. 2000. Fine tuning of glutamate uptake and degradation in glial cells: common transcriptional regulation of GLAST1 and GS. Neurochem Int 37:179–189.
Rhoads DE, Ockner RK, Peterson NA, Raghupathy E. 1983. Modulation of membrane transport by free fatty acids: inhibition of synaptosomal sodium-dependent amino acid uptake. Biochemistry 22:1965–1970.
Robinson MB, Hunter-Ensor M, Sinor J. 1991. Pharmacologically distinct sodium-dependent L-[3H]glutamate transport processes in rat brain. Brain Res 544:196–202.
Rothstein JD, Jin L, Dykes-Hoberg M, Kuncl RW. 1993. Chronic inhibition of glutamate uptake produces a model of slow neurotoxicity. Proc Natl Acad Sci USA 90:6591–6595.
Rothstein JD, Martin L, Levey A, Dykes-Hoberg M, Jin L, Wu D, Nash N, Kuncl RW. 1994. Localization of neuronal and glial glutamate transporters. Neuron 13:713–25.
Rothstein JD, Van Kammen M, Levey AI, Martin LJ, Kuncl RW. 1995. Selective loss of glial glutamate transporter GLT-1 in amyotrophic lateral sclerosis. Ann Neurol 38:73–84.
Rothstein, JD, Dykes-Hoberg M, Prado CA, Bristol LA, Jin L, Kuncl RW, Kanai Y, Hediger M, Wang Y, Schielke JP, Welty DF. 1996. Knockout of glutamate transporters reveals a major role for astroglial transport in excitotoxicity and clearance of glutamate. Neuron 16:675–686.
Schlag BD, Vondrasek JR, Munir M, Kalandadze A, Zelenaia OA, Rothstein JD, Robinson MB. 1998. Regulation of the glial Na^{+}-dependent glutamate transporters by cyclic AMP analogs and neurons. Mol Pharmacol 53:355–369.
Schmitt A, Asan E, Puschel B, Kugler P. 1997. Cellular and regional distribution of the glutamate transporter GLAST in the CNS of rats: nonradioactive in situ hybridization and comparative immunocytochemistry. J Neurosci 17:1–10.
Schultz K, Stell WK. 1996. Immunocytochemical localization of the high-affinity glutamate transporter, EAAC1, in the retina of representative vertebrate species. Neurosci Lett 211:191–194.
Schwartz EA, Tachibana M. 1990. Electrophysiology of glutamate and sodium co-transport in a glial cell of the salamander retina. J Physiol 426:43–80.
Seal RP, Amara SG. 1999. Excitatory amino acid transporters: a family in flux. Annu Rev Pharmacol Toxicol 39:431–456.
Shaw PJ. 1999. Calcium, glutamate, and amyotrophic lateral sclerosis: more evidence but no certainties. AnnNeurol 46:803–805.
Shifman M. 1991. The effect of gangliosides upon recovery of aspartate/glutamatergic synapses in striatum after lesions of the rat sensorimotor cortex. Brain Res 568:323–324.
Sonders MS, Amara SG. 1996. Channels in transporters. Curr Opin Neurobiol 6:294–302.

TESIS CON
FALLA DE ORIGEN

Stanimirovic DB, Ball R, Durkin JP. 1997. Stimulation of glutamate uptake and Na,K-ATPase activity in rat astrocytes exposed to ischemia-like insults. Glia 19:123–134.

Storck T, Schulte S, Hoffman K, Stuffel W. 1992. Structure, expression and functional analysis of a Na^+-dependent glutamate/aspartate transporter form rat brain. Proc Natl Acad Sci USA 89:10955–10959.

Swanson RA, Farrell K, Stein BA. 1997. Astrocyte energetics, function, and death under conditions of incomplete ischemia: a mechanism of glial death in the penumbra. Glia 21:142–153.

Szatkowski M, Barbour B, Attwell D. 1990. Non-vesicular release of glutamate from glial cells by reversed electrogenic glutamate uptake. Nature 348:443–446.

Tanaka K. 1993. Expression cloning of a rat glutamate transporter. Neurosci Res 16:149–153.

Tanaka K. 1994. Pharmacological characterization of a cloned rat glutamate transporter (GluT-1). Brain Res Mol Brain Res 21:167–170.

Tanaka K, Watase K, Manabe T, Yamada K, Watanabe M, Takahashi K, Iwama H, Nishikawa T, Ichihara N, Kikuchi T, Okuyama S, Kawashima N, Hori S, Takimoto M, Wada K. 1997. Epilepsy and exacerbation of brain injury in mice lacking the glutamate transporter GLT-1. Science 276:1699–1702.

Torp R, Danbolt NC, Babaie E, Bjorås M, Seeberg E, Storm-Mathisen J, Ottersen OP. 1994. Differential expression of two glial glutamate transporters in the rat brain: an in situ hybridization study. Eur J Neurosci 6:936–942

Torp R, Lekieffre D, Levy L, Haug FM, Danbolt NC, Meldrum BS, Ottersen OP. 1995. Reduced postischemic expression of a glial glutamate transporter, GLT1, in the rat hippocampus. Exp Brain Res 103:51–58.

Torp R, Hoover F, Danbolt NC, Storm-Mathisen J, Ottersen OP. 1997. Differential distribution of the glutamate transporters GLT1 and rEAAC1 in rat cerebral cortex and thalamus: an in situ hybridization analysis. Anat Embryol 195:317–326.

Trotti D, Rizzini BL, Rossi D, Haugeto O, Racagni G, Danbolt NC, Volterra A. 1997. Neuronal and glial glutamate transporters possess an SH-based redox regulatory mechanism. Eur J Neurosci 9:1236–1243.

Trotti D, Danbolt NC, Volterra A. 1998. Glutamate transporters are oxidant-vulnerable: a molecular link between oxidative and excitotoxic neurodegeneration? Trends Pharmacol Sci 19:328–334.

Velaz-Faircloth M, McGraw TS, Malandro MS, Fremeau RT Jr, Kilberg MS, Anderson KJ. 1996. Characterization and distribution of the neuronal glutamate transporter EAAC1 in rat brain. Am J Physiol 270:C67–C75.

Voisin P, Viratelle O, Girault JM, Morrison-Bogorad M, Labouesse J. 1993. Plasticity of astroglial glutamate and gamma-aminobutyric acid uptake in cell cultures derived from postnatal mouse cerebellum. J Neurochem 60:114–127.

Wadiche JI, Amara SG, Kavanaugh MP. 1995. Ion fluxes associated with excitatory amino acid transport. Neuron 15:721–728.

Yamada K, Watanabe M, Shibata T, Tanaka K, Wada K, Inoue Y. 1996. EAAT4 is a post-synaptic glutamate transporter at Purkinje cell synapses. Neuroreport 7:2013–2017.

Ye ZC, Sontheimer H. 1999. Metabotropic glutamate receptor agonists reduce glutamate release from cultured astrocytes. Glia 25:270–281

Ye ZC, Sontheimer H. 1998. Stereospecific transport of trans-ACPD and its impact on intracellular glutamate levels in astrocytes. Soc Neurosci Abstr 24:2070.

Zafra F, Alcantara R, Gomeza J, Aragon C, Gimenez C. 1990. Arachidonic acid inhibits glycine transport in cultured glial cells. Biochem J 271:237–242.

Zaczek R, Balm M, Arlis S, Drucker H, Coyle JT. 1987. Quisqualate-sensititive, chloride-dependent transport of glutamate into rat brain synaptosomes. J Neurosci Res 18:425–431.

Zerangue N, Kavanaugh MP. 1996. Flux coupling in a neuronal glutamate transporter. Nature 383:634–637

Journal of Neuroscience Research 63:461–468 (2001)

Mini-Review

Glial Transporters for Glutamate, Glycine, and GABA: II. GABA Transporters

Ana Gadea[1] **and Ana María López-Colomé**[1,2]*
[1]Instituto de Fisiología Celular, Departamento de Neurociencias, UNAM, México, D.F., México
[2]Facultad de Medicina, Departamento de Bioquímica, UNAM, México, D.F., México

The termination of chemical neurotransmission in the central nervous system (CNS) involves the rapid removal of neurotransmitter from synapses. This is fulfilled by specific transport systems in neurons and glia, including those for γ-aminobutyric acid (GABA), the main inhibitory neurotransmitter in the brain. Glial cells express the cloned Na^+/Cl^--dependent, high-affinity GABA transporters (GATs) GAT1, GAT2, and GAT3, as well as the low-affinity transporter BGT1. In situ hybridization and immunocytochemistry have revealed that each transporter shows distinct regional distribution in the brain and the retina. The neuronal vs. glial localization of the different transporters is not clear-cut, and variations according to species, neighboring excitatory synapses, and developmental stage have been reported. The localization, stoichiometry, and regulation of glial GATs are outlined, and the participation of these structures in development, osmoregulation, and neuroprotection are discussed. A decrease in GABAergic neurotransmission has been implicated in the pathophysiology of several CNS disorders, particularly in epilepsy. Since drugs which selectively inhibit glial but not neuronal GABA uptake exert anticonvulsant activity, clearly the establishment of the molecular mechanisms controlling GATs in glial cells will be an aid in the chemical treatment of several CNS-related diseases. J. Neurosci. Res. 63: 461–468, 2001. © 2001 Wiley-Liss, Inc.

Key words: neurotransmitter uptake; neuron-glia interaction; second messengers; membrane protein; synaptic modulation

GABA TRANSPORTERS

γ-aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the central nervous system and has a widespread distribution in the adult brain. The rapid termination of GABA transmission is achieved through high-affinity GABA transport into both GABAergic neurons and glial cells (Schousboe et al., 1983; Schousboe and Westergaard, 1995).

The cloning of GABA (Guastella et al., 1990) and norepinephrine (Pacholczyk et al., 1991) transporters allowed the subsequent isolation of related cDNAs which constitute the Na^+/Cl^--dependent neurotransmitter transporter superfamily. The members of this superfamily share a similar membrane topology arranged in 12 transmembrane domains, N- and C-termini on the cytoplasmic side, and a potential glycosylation sequence between transmembrane helices III and IV (for review, see Kanner, 1994; Nelson and Lill, 1994; Uhl and Johnson, 1994). Molecular cloning studies have revealed the existence of three high-affinity subtypes of GABA transporters in the rat and human brain: GAT1, GAT2, and GAT3 (Borden et al., 1992, 1995a; Guastella et al., 1990), and one of lower affinity, BGT-1 (Yamauchi et al., 1992).

Tissue and Cell Localization

Several studies have addressed the tissue and cellular distribution of GABA transporters. The GABA transporters (GATs) GAT1 and GAT3 are expressed exclusively in the central nervous system (CNS), whereas GAT2 and BGT1 are also present in peripheral tissues, mainly liver and kidney (Clark et al., 1992; Borden et al., 1994; Guastella et al., 1990; Jursky et al., 1994; Liu et al., 1993; Nelson et al., 1990; Rasola et al., 1995; Yamauchi et al., 1992). The presence of GABA uptake systems in glial cells was first demonstrated by autoradiographic studies showing $[^3H]\beta$-alanine uptake in cortical slices and synaptosome preparations (Schon and Kelly, 1975), as well as in astrocyte primary cultures (Hertz et al., 1978; Balcar et al., 1979); pharmacological studies also showed that GABA transport in cultured astrocytes from different brain regions (Hosli and Hosli, 1979) as well as to membrane vesicles derived from cortical astrocytes, is highly sensitive to β-alanine (Mabjeesh et al., 1992) and other GABA analogues (Schousboe et al., 1978).

GABA transporters do not follow a specific cell type expression pattern. Although GAT1 has long been con-

Contract grant sponsor: DGAPA; Contract grant number: IN-210998; Contract grant sponsor: PAEP; Contract grant number: 102322.

*Correspondence to: Ana María López-Colomé, Ph.D., Instituto de Fisiología Celular, UNAM, Apartado Postal 70-253, 04510, D.F., México. E-mail: acolome@ifisiol.unam.mx

Received 28 November 2000; Accepted 4 December 2000

© 2001 Wiley-Liss, Inc.

sidered a neuronal GABA transporter (Iversen and Kelly, 1975; Mabjeesh et al., 1992), recent studies have clearly shown its presence in distal astrocytic processes of all the rat brain regions examined so far (cerebral cortex: Minelli et al., 1995; retina: Johnson et al., 1996; hippocampus: Ribak et al., 1996a; cerebellum: Rattray and Priestley, 1993; Ribak et al., 1996b; thalamus: DeBiasi et al., 1998). GAT1 has been also localized to astrocytic processes in human and in monkey cerebral cortex (Conti et al., 1998); therefore, the astrocytic localization of GAT1 seems to be a consistent feature in the mammalian cortex. GAT3 is mainly expressed by glial cells in the brain and the retina, but neuronal expression has also been demonstrated, particularly in the latter (Clark et al., 1992; Durkin et al., 1995; Yang et al., 1997; Johnson et al., 1996; Ribak et al., 1996a; Durkin et al., 1995; Borden et al., 1995a). GAT2 was proposed to have a nutritional role and to perform non-neuronal functions since it was first localized to cells of the leptomeninges, choroid plexus, and ependyma in the brain (Ikegaki et al., 1994; Durkin et al., 1995), and to the pigment and ciliary body epithelia in the retina (Honda et al., 1995; Johnson et al., 1996), but its presence in neurons and glial cells has been further demonstrated (Borden et al., 1995a; Conti et al., 1999; Voutsinos et al., 1998; Obata et al., 1997; Zhao et al., 2000; Redecker, 1999). A role for GAT2 in development has also been proposed, since mRNA for this protein is more abundant in neonatal than in adult mouse brain (Liu et al., 1993). BGT1 is most probably a glial transporter, since its transcripts were observed in type 1 and type 2 astrocyte cultures, but not in neuronal cell cultures (Borden et al., 1995a); its presence in the brain has been related with osmoregulation (Borden et al., 1995b; Lopez-Corcuera et al., 1992; Rasola et al., 1995; Bitoun and Tappaz, 2000).

Presynaptic localization of GAT1 by in situ hybridization and immunocytochemistry in GABAergic neurons has been shown, correlated or not with the presence of GABA and GAD67 (glutamic acid decarboxylase; Radian et al., 1990; Durkin et al., 1995; Augood et al., 1995).

In the cerebral cortex of adult rats, GAT1 exhibits the highest level of expression, followed by GAT3 and GAT2. In addition to neurons, GAT1 mRNA and GAT1 immunoreactivity are also localized to some distal astrocytic processes (Minelli et al., 1995). GAT3 localizes exclusively in astrocytic processes (Minelli et al., 1996), and GAT2 is expressed by epithelial, glial, and neuronal cells (Conti et al., 1999). GAT1 is also expressed robustly in the human and monkey cerebral cortex, where it localizes to both neurons and astrocytic processes near axon terminals forming GABAergic synapses, and scattered in the neuropil (Conti et al., 1998). Morphology and distribution of distal astrocytic processes labeled for GAT2 are similar to those labeled for GAT1 and GAT3 (Minelli et al., 1995, 1996); however, only GAT2 immunoreactivity is present in astrocytic cell bodies and their proximal processes (Conti et al., 1999). GAT2 mRNA is also expressed in vitro by O-2A/type 2 astrocytes, supporting the idea that cortical glial GABA transport is mediated also by GAT2. Although the role of BGT1 in the cortex remains to be established, its presence has been reported in type 1 astrocytic cultures derived from rat brain (Borden et al., 1995a). Rat cortical astrocytes, therefore, express GAT1, GAT2, GAT3, and BGT1. These observations raise several issues regarding the relative contribution of each of these transporters to overall GABA uptake by glial cells in the cortex, and of the functional significance of multiple GABA uptake systems. Based on the distinct distribution and degree of expression of GATs abovementioned, although glial GABA uptake in the cortex seems to be mediated largely by GAT3, GAT1, and GAT2 playing a minor role in the removal of extrasynaptic GABA, because of the differential ionic dependency, inhibitor sensitivity (Guastella et al., 1990; Borden et al., 1992; Clark et al., 1992; Keynan et al., 1992), and modulation of the three high-affinity GATs (Gomeza et al., 1991; Corey et al., 1994; Quick et al., 1997), it is reasonable to think that their relative contribution to glial GABA uptake is dynamically regulated, providing for a great adaptability in the control of extracellular GABA levels.

Astrocytic GATs neighboring GABAergic synapses are placed strategically to take up locally released GABA, thus contributing to the termination of GABA-mediated inhibitory synaptic transmission and to the modulation of the synaptic action of GABA. In contrast, the function of astrocytic GATs, such as GAT1, located extrasynaptically, may be the control of the paracrine spread of GABA to excitatory and inhibitory neighboring synapses (Isaacson et al., 1993; Thomson and Gahwiler, 1992; Rossi and Hamann, 1998). Regarding this suggestion, the label for GAT1 and GAT3 was detected in astrocytic processes enveloping several axon terminals, together with their postsynaptic dendrites in the thalamus (DeBiasi et al., 1998), but not in cerebral cortex (Minelli et al., 1995, 1996) or hippocampus (Ribak et al., 1996a), although in the cerebellum a dense glial labeling for GAT3 envelops Purkinje axon terminals (Itouji et al., 1996; Ribak et al., 1996b). A role for astrocytes in the insulation of synapses is supported by the expression of GABA transporters in thalamic and cerebellar glial processes, but not in GABAergic terminals, indicating the participation of astrocytes in the modulation of GABA transmission in these areas. Astrocytic processes labeled for GAT1 and GAT3 are also found scattered in the neuropil, distant from GABAergic terminals and occasionally neighboring axon terminals from excitatory synapses, where GABA uptake by glia could limit GABA action on distant nonsynaptic $GABA_A$ receptors (Spreafico et al., 1993; Alvarez et al., 1996). Moreover, GABA uptake by astrocytes has been proposed to regulate the action of GABA at $GABA_B$ presynaptic receptors located on excitatory terminals (Soltesz and Crunelli, 1992; Ulrich and Huguenard, 1996), aimed to inhibit synaptic transmission via G protein-mediated modulation of presynaptic Ca^{2+} channels (Isaacson, 1998; reviewed in Isaacson, 2000). GABA taken up by glial cells is rapidly metabolized by GABA transaminase (Iversen and Kelly, 1975) which displays high

activity in astrocytes. Additionally, GABA transporters in astrocytes can also mediate GABA release (see below; Gallo et al., 1991).

In situ hybridization studies in the cerebellum revealed GAT1 mRNA predominantly localized to the molecular and Purkinje cell layers, whereas GAT3 is found in the deep cerebellar nuclei (Clark et al., 1992; Durkin et al., 1995; Rattray and Priestly, 1993). GAT2 mRNA, first reported exclusively in the leptomeninges, was later found in the cerebellum, predominantly in the granular layer (Voutsinos et al., 1998), in agreement with the localization of its cognate protein (Ikegaki et al., 1994). At the cellular level, GAT1 mRNA was found predominantly in cell bodies of GABAergic basket and stellate neurons; GAT1 mRNA and immunoreactivity were also detected in cell bodies and glial processes ensheathing Purkinje cells somata and dendrites, respectively, (Voutsinos et al., 1998; Morara et al., 1996), which most likely correspond to Bergmann glia (Rattray and Priestly, 1993; Ribak et al., 1996b). GAT3 mRNA is predominantly confined to glial cells (Voutsinos et al., 1998), consistent with immunocytochemical studies localizing this transporter to cerebellar glial processes (Itouji et al., 1996). The dense glial labeling for GAT3 surrounding GABAergic Purkinje axon terminals seems to compensate for the apparent lack of GABA transporters in these neurons (Ribak et al., 1996b). In the developing rat cerebellar cortex, GAT3 immunoreactivity appears initially in somata and primary processes of postatal day (P)7–21 astrocytes, and is later identified in distal processes in the adult (Yan and Ribak, 1998). GABA acting as an excitatory transmitter plays an important neurotrophic role in brain development (Cherubini et al., 1991; Staley et al., 1995); hence, the early expression of GATs in astrocytes suggests their involvement in the differentiation and maturation of developing neurons, probably through the release of GABA.

BGT1 was originally cloned from Madin-Darby canine kidney cells (Yamauchi et al., 1992), and subsequently its expression has been demonstrated in most mammalian tissues including the CNS (Rasola et al., 1995; Borden et al., 1995b, 1996). BGT1 mRNA has been detected in all mouse and human brain regions (López-Corcuera et al., 1992; Rasola et al., 1995). Since BGT1 transcripts were identified in type 1 and 2 astrocytes, but not in neurons in culture, BGT1 was proposed to be mainly glial, although its presence in glial cells from rat brain slices has not been shown (Borden et al., 1995b), and pharmacological data indicate that BGT1 makes only a minor contribution to GABA transport in these cells. The distribution of BGT1 mRNA in the brain does not correlate with GABAergic pathways, discarding a role in the termination of GABA transmission; BGT1 could, however, be involved in the removal of GABA diffused from synaptic regions. On the other hand, BGT1 might contribute to volume regulation in the CNS (Yamauchi et al., 1992; Borden et al., 1995b) since betaine, which has been assigned a role in osmoregulation, is a substrate for BGT1 (Heilig et al., 1989). A recent study on this matter (Bitoun and Tappaz, 2000) demonstrates a significant increase in mRNA levels of BGT1 in cultured cortical astrocytes exposed to hyperosmotic conditions; increased transcription of BGT1 gene is likely mediated by the osmotic responsive element (ORE) recently identified in the 5' flanking region of the BGT1 gene (Miyakawa et al., 1998).

Immunocytochemical studies in the mammalian retina using specific antibodies for the distinct GABA transporters have shown GAT1 localized mainly to neurons including amacrine, displaced amacrine, interplexiform, and ganglion cell processes throughout the inner plexiform layer (IPL), and to lower extent in Müller cells (Brecha and Weigmann, 1994; Ruiz et al., 1994; Durkin et al., 1995; Johnson et al., 1996). Immunoreactivity for GAT3 is expressed mainly in Müller cells (Brecha and Weigmann, 1994; Brecha et al., 1995; Honda et al., 1995) and amacrine cells at the inner nuclear layer (INL; Brecha et al., 1995; Johnson et al., 1996), whereas GAT2 immunostaining was found in the pigment and ciliary epithelia in the mammalian retina (Honda et al., 1995; Johnson et al., 1996).

In contrast to mammalian retinae, Müller cells in salamander and in most of the ectotherms do not take up GABA (for review see Marc, 1992). In the tiger salamander retina, GAT1 immunoreactivity was found in bipolar, amacrine, and interplexiform cells as well as in the ganglion cell layer. No detectable staining was found in horizontal cells or in structures resembling Müller cells. GAT3 antibodies labeled fewer cells and cell types than GAT1 antibodies, localized to amacrine cells and cells in the ganglion cell layer, but not horizontal cells, bipolar cells, or Müller cell like-structures (Yang et al., 1997). Retinal horizontal cells of ectotherms are GABAergic (for review see Marc, 1992; Wu, 1992) and bear electrogenic GABA transporters (Malchow and Ripps, 1990; Cammack and Schwartz, 1993; Takahashi et al., 1995). Since GAT1 and GAT3 antisera did not detectably label horizontal cells, the presence of an unidentified GABA transporter in these species is suggested. Similar results were obtained in the salmon retina, where GAT1 immunoreactivity was present in amacrine cells and the IPL, but not in Müller cells; as opposed to the salamander retina, however, bipolar cells were not labeled in this species (Ekström and Anzelius, 1998).

Not all nonmammalian Müller cells lack the ability to take up GABA. In contrast with the abovementioned species, bullfrog Müller cells strongly express GAT1 and GAT2, but not GAT3. Somata, major processes, endfeet, and branchlets of most Müller cells expressed GAT1, whereas a moderate labeling for GAT2 was observed in main trunks and endfeet of 80–90% of these cells (Zhao et al., 2000).

Available evidence suggests that GABAergic transmission in the retina could be modulated through the uptake of GABA, not only by retinal neuronal elements but also by Müller cells in both mammalians and nonmammalians. Moreover, GATs in Müller cells could play

a protective role from excessive GABA inhibition during physiological and/or pathological events.

Also regarding the visual system, the mRNAs encoding for GAT1, GAT2, and GAT3 are expressed in the optic nerve of both neonatal and adult rats (Howd et al., 1997). Since optic nerves contain mainly axons and glia, GAT1 and GAT3 mRNAs identified are likely of glial origin. It is possible, however, that axons may contain GAT1 mRNA (Gioio et al., 1994), which is predominantly expressed by neurons, while the expression of GAT2 mRNA probably corresponds to fragments of pia-arachnoid present in the tissue.

Stoichiometry and Channel Properties

GABA transporters belong to the family of Na^+- and Cl^--coupled neurotransmitter transporters (for reviews, see Nelson and Lill, 1994; Uhl and Johnson, 1994). Studies on GABA transport in diverse expression systems revealed GAT1 to be strictly Na^+-dependent but only partially Cl^--dependent (Mager et al., 1993; Lu et al., 1995). GAT1 expressed in mouse LtK^- cells shows a calculated stoichiometry of two Na^+, one Cl^-, and one GABA molecule per cycle (Keynan et al., 1992), in agreement with previous data obtained in synaptic plasma membrane vesicles and for the purified transporter (Kanner, 1983; Keynan and Kanner, 1988; Radian and Kanner, 1983), as well as in electrophysiological studies (Kavanaugh et al., 1992; Mager et al., 1993, 1996; Risso et al., 1996). The cotransport of GABA with three Na^+ and one or two Cl^- by BGT1 has been demonstrated recently (Matskevitch et al., 1999). Importantly, the predicted amount of charge crossing the membrane during the transport cycle, based on stoichiometric ion fluxes, is smaller than the charge movement experimentally determined; thus, GABA transporters such as those for glutamate (Glu), exhibit ligand-gated ion channel properties (Mager et al., 1996; Cammack and Schwartz, 1994, 1996; reviewed in Sonders and Amara, 1996). GABA transporters also exhibit substrate-independent leak currents carried by Li^+ and K^+ (Mager et al., 1993; Cammack and Schwartz, 1996), blocked by the substrate and by transport inhibitors (reviewed in Sonders and Amara, 1996). These characteristics suggest that GABA transporters, in addition to a role in the termination of synaptic GABA transmission, can also influence neuronal and glial excitability. On this line, a raise in intracellular calcium due to the opening of L-type calcium channels, and a subsequent calcium-induced calcium release from intracellular stores induced by GAT-mediated depolarization, has been documented (Haugh-Scheidt et al., 1995).

Reverse operation of GATs can result in the nonvesicular release of GABA by breakdown of the sodium/chloride/GABA gradient or by cell depolarization (Attwell et al., 1993). Such nonvesicular release has been identified in both neurons (Schwartz, 1987; Yang et al., 1999) and glia (Gallo et al., 1991). GABA is released from cultured striatal neurons by high $[K^+]$, veratridine, and Glu receptor agonists in the absence of calcium, or in the presence of tetanus toxin (Pin and Bockaert, 1989). In cultured hippocampal neurons, large $GABA_A$ receptor-mediated responses have been observed as a result of carrier-mediated GABA release from neuronal and/or glial neighboring cells, induced by brief alteration of Na^+ or K^+ electrochemical gradient (Gaspary et al., 1998). Since carrier-mediated release of GABA does not rely on ATP but on ion gradients, it may offer advantages during periods of high energy utilization, such as burst firing or seizures.

Regulation of GABA Transporters

The existence of several types of GABA transporters opens the possibility of distinct regulatory systems according to the phenotype and/or the anatomical localization of the cells expressing these transporters. Short-term regulation of neurotransmitter transporters involving phosphorylation/dephosphorylation has been studied. The inhibition of high-affinity GABA uptake in glial cells by phorbol ester activation of PKC (protein kinase C), due to a decrease in affinity, has been reported (Gomeza et al., 1991). Supported by the presence of multiple consensus sites for PKC phosphorylation on GABA transporters, these data suggested the regulation of GABA uptake by direct transporter phosphorylation, although removal of the PKC consensus sites from GAT1 failed to eliminate PKC-induced inhibition in oocytes (Corey et al., 1994). More recent studies on this matter suggest that GABA transporter function could be regulated by components of the vesicle docking and fusion machinery. In support of this idea, injection of antisense oligonucleotides directed to synaptophysin or syntaxin into oocytes expressing total rat brain mRNA, as well as inactivation of these proteins by botulinum toxins (BTXs), eliminates the regulation of GAT1 by PKC (Quick et al., 1997). Later studies have shown PKC to regulate the interaction between GAT1 and syntaxin 1A in neurons endogenously expressing all three proteins, provided that Munc18, a substrate for PKC phosphorylation, is also present (Beckman et al., 1998). Although modulation of GABA transport by PMA (phorbol 12-myristate 13-acetate) and BTX was not observed in cultured astrocytes, the presence of SNARE (soluble NSF receptors) complex proteins in glial cells including syntaxin, synaptobrevin, and SNAP-23 has recently been reported (Araque et al., 2000; Hepp et al., 1999; Madison et al., 1999). The signal which triggers PKC-mediated regulation of GAT1 is still unknown; however, specific agonists of G-protein-coupled receptors for serotonin, acetylcholine, and Glu downregulate GAT1 function in neurons (Beckman et al., 1999). Such functional inhibition has been ascribed to the redistribution of the transporter from the plasmamembrane to intracellular locations; moreover, BTX prevents the receptor-mediated inhibition, suggesting the involvement of syntaxin 1A. Extracellular GABA also regulates GAT1 by increasing transporter expression in the plasmamembrane of hippocampal neurons. Hippocampal astrocyte cultures exposed to GABA also show a marked increase in GABA uptake, although the mechanism underlying this effect has not been studied (Bernstein and Quick, 1999).

GABA uptake by cerebellar glia is stimulated in vitro by adrenaline (Hansson and Ronnback, 1991) or by GABA-CIP (GABA-carrier inducing protein), a protein released by granule cells (Nissen et al., 1992), and inhibitory control of glial GABA uptake by serotonin has been demonstrated in ependymocytes of the subcomissural organ in vivo (SCO; Didier-Bazes et al., 1989, 1992). A direct serotonergic control of glial GABA uptake has been further demonstrated in vitro, since serotonin stimulated the activity and mRNA expression of GABA transporters in cerebellar astrocyte cultures. Serotonin regulation of glial GABA transport might be involved in some pharmacological effects of serotonergic drugs, i.e., antidepressants, known to increase extracellular serotonin levels (Voutsinos et al., 1998).

Glial GABA Transporters in Disease

A decrease in GABAergic neurotransmission has been implicated in the pathophysiology of several CNS disorders, particularly in epilepsy. Consequently, research in this area has focused on the development of pharmacological agents capable of increasing GABAergic function. Intracerebroventricular application of the glia-selective GABA transport inhibitors 4,5,6,7-tetrahydroisoxazole [4,5-c]pyridin-3-ol (THPO), and 5,6,7,8-tetrahydro-4H-isoxazolo(4,5-c)azepin-3-ol (THAO), has been shown to protect against seizures induced by drugs known to impair GABAergic neurotransmission (Krogsgaard-Larsen et al., 1987); in contrast, the inhibition of neuronal GABA uptake by L-DABA (L-2,4-diamino butyric acid) results in proconvulsant behavior (Gonsalves et al., 1989). These data have led to the proposal that selective block of glial GABA transport elevates GABA concentration in nerve terminals, whereas the selective blockage of the neuronal transporter depletes the releasable neurotransmitter pool (Wood et al., 1980; Schousboe et al., 1983). However, the lipophilic derivatives of piperidencarboxylic acid (tiagabine, SKF-89976A, CI-966, and NNC-711), highly selective for GAT1, have been reported to exhibit anticonvulsant properties (Borden et al., 1994; Clark et al., 1992; Suzdak et al., 1992). It is evident that no simple correlation exists between the pharmacological characteristics of GABA transport mediated by the cloned carriers and that of neuronal and glial GABA uptake (Schousboe and Westergaard, 1993; Borden, 1996). Although the participation of neuronal and glial GABA transporters in seizure activity has not been fully elucidated, a role for GABA has been proposed, and impaired GABA release due to a decrease in GATs has been observed (During et al., 1995). However, increases in extracellular GABA concentration during kindling (Ueda and Tsuru, 1995) and human seizures (During et al., 1995) could relate to nonvesicular GABA release due to the upregulation of GABA transporters (Hirao et al., 1998). Increases in the expression of GAT1 and GAT3 following mechanical and chemical lesions in rat cerebral cortex and $FeCl_3$ treatment in amygdala have been shown to propagate to the contralateral side, suggesting the existence of transcellular signaling mechanisms regulating GATs expression in the cerebral cortex, probably as a protective mechanism (Yan and Ribak, 1999; Ueda and Willmore, 2000a). On the other hand, the upregulation of GATs expression in epilepsy may result in lowered GABAergic transmission and therefore contribute to the generation of seizures. Actually, one of the effects of the anitiepileptic drug, valproate, is the downregulation of neuronal and glial GAT1 and GAT3 protein expression, which results in an increased extracelluar GABA concentration (Ueda and Willmore, 2000b).

In addition to epilepsy, existing evidence supports a role for GABA transporters in several clinically related processes. Demonstration of the inhibitory action of some anesthetics on GABA uptake into striatal synaptosomes, suggests the involvement of this mechanism in the effect shown by these agents (Mantz et al., 1995). Also, postmortem analysis of schizophrenic brains has revealed a decreased number of GABA uptake sites in subcortical regions, which reveals GABAergic mechanisms are abnormal in schizophrenia (Simpson et al., 1992). Further studies are required to elucidate the participation of glial GABA uptake in these clinical conditions.

Recent work demonstrated the modulation of seizure development in a kindling model of epilepsy by transplantation of immortalized mouse neurons and glial cells genetically engineered to produce GABA by driving GAD (glutamic acid decarboxylase) expression (Thomson et al., 2000). Although the mechanism of GABA release was not investigated, it is interesting to speculate that GABA transporters could be involved.

Although recent information supports a role for glial GATs in the modulation of inhibitory neurotransmission in the CNS and the retina, further research is needed in order to establish their precise function.

ACKNOWLEDGMENTS

This work was supported in part by grant IN-210998 from DGAPA to A.M.L.-C., and PAEP 102322 to A.G.

REFERENCES

Alvarez FJ, Taylor-Blake B, Fyffe RE, De Blas AL, Light AR. 1996. Distribution of immunoreactivity for the beta 2 and beta 3 subunits of the GABAA receptor in the mammalian spinal cord. J Comp Neurol 365: 392–412.

Araque A, Li N, Doyle RT, Haydon PG. 2000. SNARE protein-dependent glutamate release from astrocytes. J Neurosci 20:666–673.

Attwell D, Barbour B, Szatkowsky M. 1993. Nonvesicular release of neurotransmitter. Neuron 11:401–407.

Augood SJ, Herbison AE, Emson PC. 1995. Localization of GAT-1 GABA transporter mRNA in rat striatum: cellular coexpression with GAD67 mRNA, GAD67 immunoreactivity, and parvalbumin mRNA. J Neurosci 15:865–874.

Balcar VJ, Mark J, Borg J, Mandel P. 1979. High-affinity uptake of gamma-aminobutyric acid in cultured glial and neuronal cells. Neurochem Res 4:339–354.

Beckman ML, Bernstein EM, Quick MW. 1998. Protein kinase C regulates the interaction between a GABA transporter and syntaxin 1A. J Neurosci 18:6103–6112.

Beckman ML, Bernstein EM, Quick MW. 1999. Multiple G protein-coupled receptors initiate protein kinase C redistribution of GABA transporters in hippocampal neurons. J Neurosci 19:RC9.

TESIS CON
FALLA DE ORIGEN

Bernstein EM, Quick MW. 1999. Regulation of gamma-aminobutyric acid (GABA) transporters by extracellular GABA. J Biol Chem 274:889–895.

Bitoun M, Tappaz M. 2000. Gene expression of the transporters and biosynthetic enzymes of the osmolytes in astrocyte primary cultures exposed to hyperosmotic conditions.Glia 32:165–176.

Borden LA. 1996. GABA transporter heterogeneity: pharmacology and cellular localization. Neurochem Int 29:335–356.

Borden LA, Smith KE, Hartig PR, Branchek TA, Weinshank RL. 1992. Molecular heterogeneity of the gamma-aminobutyric acid (GABA) transport system. Cloning of two novel high affinity GABA transporters from rat brain. J Biol Chem 267:21098–21104.

Borden LA, Dhar TG, Smith KE, Branchek TA, Gluchowski C, Weinshank RL. 1994. Cloning of the human homologue of the GABA transporter GAT-3 and identification of a novel inhibitor with selectivity for this site. Receptors Channels 2:207–213.

Borden LA, Smith KE, Vaysse PJ, Gustafson EL, Weinshank RL, Branchek TA. 1995a. Re-evaluation of GABA transport in neuronal and glial cell cultures: correlation of pharmacology and mRNA localization. Receptors Channels 3:129–146.

Borden LA, Smith KE, Gustafson EL, Branchek TA, Weinshank RL. 1995b. Cloning and expression of a betaine/GABA transporter from human brain. J Neurochem 64:977–984.

Brecha N, Weigmann C. 1994. Expression of GAT-1, a high-affinity gamma-aminobutyric acid plasma membrane transporter in the rat retina. J Comp Neurol 345:602–611.

Brecha N, Johnson J, Khan T, Evens C, Rican D. 1995. Multiple GABA plasma membrane transporters are expressed in the vertebrate retina. Invest Ophtalmol Vis Sci 36:S214.

Cammack JN, Schwartz EA. 1993. Ions required for the electrogenic transport of GABA by horizontal cells of the catfish retina. J Physiol 472:81–102.

Cammack JN, Schwartz EA. 1994. A GABA transporter operates asymmetrically and with variable stoichiometry. Neuron 13:949–960.

Cammack JN, Schwartz EA. 1996. Channel behavior in a gamma-aminobutyrate transporter. Proc Natl Acad Sci U S A 93:723–727.

Cherubini E, Gaiarsa JL, Ben-Ari Y. 1991. GABA: an excitatory transmitter in early postnatal life. Trends Neurosci 14:515–519.

Clark JA, Deutch AY, Gallipoli PZ, Amara SG. 1992. Functional expression and CNS distribution of a beta-alanine-sensitive neuronal GABA transporter. Neuron 9:337–348.

Conti F, Melone M, De Biasi S, Minelli A, Brecha NC, Ducati A. 1998. Neuronal and glial localization of GAT-1, a high-affinity gamma-aminobutyric acid plasma membrane transporter, in human cerebral cortex: with a note on its distribution in monkey cortex. J Comp Neurol 396:51–63.

Conti F, Zucarello LV, Barbaresi P, Minelli A, Brecha NC, Melone M. 1999. Neuronal, glial, and epithelial localization of gamma-aminobutyric acid transporter 2, a high-affinity gamma-aminobutyric acid plasma membrane transporter, in the cerebral cortex and neighboring structures. J Comp Neurol 409:482–494.

Corey JL, Davidson N, Lester HA, Brecha N, Quick MW. 1994. Protein kinase C modulates the activity of a cloned gamma-aminobutyric acid transporter expressed in Xenopus oocytes via regulated subcellular redistribution of the transporter. J Biol Chem 269:14759–14767.

DeBiasi S, Vitellaro-Zucarello L, Brecha NC. 1998. Immunoreactivity for the GABA transporter-1 and GABA transporter-3 is restricted to astrocytes in the rat thalamus. A light and electron-microscopic immunolocalization. Neuroscience 83:815–828.

Didier-Bazes M, Aguera M, Chouaf L, Harandi H, Calas A, Meiniel A, Belin MF. 1989. Neuronal control of [3H]GABA uptake in the ependymocytes of the subcommissural organ: an in vivo model of neuron-glia interaction. Brain Res 489:137–145.

Didier-Bazes M, Chouaf L, Hardin H, Aguera M, Voutsinos B, Belin MF. 1992. Developmental neuron-glia interactions: role of serotonin innervation upon the differentiation of the ependymocytes of the rat subcommissural organ. Prog Brain Res 91:343–347.

Durkin MM, Smith KE, Borden LA, Weinshank RL, Branchek TA, Gustafson EL. 1995. Localization of messenger RNAs encoding three GABA transporters in rat brain: an in situ hybridization study. Brain Res Mol Brain Res 33:7–21.

During MJ, Ryder KM, Spencer DD. 1995. Hippocampal GABA transporter function in temporal-lobe epilepsy. Nature 376:174–177.

Ekstrom P, Anzelius M. 1998. GABA and GABA-transporter (GAT-1) immunoreactivities in the retina of the salmon (Salmo salar L.). Brain Res 812:179–185.

Gallo V, Patrizio M, Levi G. 1991. GABA release triggered by the activation of neuron-like non-NMDA receptors in cultured type 2 astrocytes is carrier-mediated. Glia 4:245–255.

Gaspary HL, Wang W, Richerson GB. 1998. Carrier-mediated GABA release activates GABA receptors on hippocampal neurons. J Neurophysiol 80:270–281.

Gioio AE, Chun JT, Crispino M, Capano CP, Giuditta A, Kaplan BB. 1994. Kinesin mRNA is present in the squid giant axon. J Neurochem 63:13–18.

Gomeza J, Casado M, Giménez C, Aragón C. 1991. Inhibition of high-affinity γ-aminobutyric acid uptake in primary astrocyte cultures by phorbol esters and phospholipase C. Biochem J 275:435–439.

Gonsalves SF, Twitchell B, Harbaugh RE, Krogsgaard-Larsen P, Schousboe A. 1989. Anticonvulsant activity of intracerebroventricularly administered glial GABA uptake inhibitors and other GABAmimetics in chemical seizure models. Epilepsy Res 4:34–41.

Guastella J, Nelson N, Nelson H, Czyzyk L, Keynan S, Miedel MC, Davidson N, Lester HA, Kanner BI. 1990. Cloning and expression of a rat brain GABA transporter. Science 249:1303–1306.

Hansson E, Ronnback L. 1991. Receptor regulation of the glutamate, GABA and taurine high-affinity uptake into astrocytes in primary culture. Brain Res 548:215–221.

Haugh-Scheidt L, Malchow RP, Ripps H. 1995. GABA transport and calcium dynamics in horizontal cells from the skate retina. J Physiol 488:565–576.

Heilig CW, Stromski ME, Blumenfeld JD, Lee JP, Gullans SR. 1989. Characterization of the major brain osmolytes that accumulate in salt-loaded rats. Am J Physiol 257(Renal Fluid Electrolyte Physiol 26):1108–1116.

Hepp R, Perraut M, Chasserot-Golaz S, Galli T, Aunis D, Langley K, Grant NJ. 1999. Cultured glial cells express the SNAP-25 analogue SNAP-23. Glia 27:181–187.

Hertz L, Wu PH, Schousboe A. 1978. Evidence for net uptake of GABA into mouse astrocytes in primary cultures—its sodium dependence and potassium independence. Neurochem Res 3:313–323.

Hirao T, Morimoto K, Yamamoto Y, Watanabe T, Sato H, Sato K, Sato S, Yamada N, Tanaka K, Suwaki H. 1998. Time-dependent and regional expression of GABA transporter mRNAs following amygdala-kindled seizures in rats. Brain Res Mol Brain Res 54:49–55.

Honda S, Yamamoto M, Saito N. 1995. Immunocytochemical localization of three subtypes of GABA transporter in rat retina. Brain Res Mol Brain Res 33:319–325.

Hosli L, Hosli E. 1979. Autoradiographic studies on the cellular localization of GABA and beta-alanine uptake by neurones and glia in tissue culture. Adv Exp Med Biol 123:205–218.

Howd AG, Rattray M, Butt AM. 1997. Expression of GABA transporter mRNAs in the developing and adult rat optic nerve. Neurosci Lett 235:98–100.

Ikegaki N, Saito N, Hashima M, Tanaka C. 1994. Production of specific antibodies against GABA transporter subtypes (GAT1, GAT2, GAT3) and their application to immunocytochemistry. Brain Res Mol Brain Res 26:47–54.

TESIS CON
FALLA DE ORIGEN

Isaacson JS. 1998. GABAB receptor-mediated modulation of presynaptic currents and excitatory transmission at a fast central synapse. J Neurophysiol 80:1571–1576.
Isaacson JS. 2000. Spillover in the spotlight. Curr Biol 10:R475–477.
Isaacson JS, Solis JM, Nicoll RA. 1993. Local and diffuse synaptic actions of GABA in the hippocampus. Neuron 10:165–175.
Itouji A, Sakai N, Tanaka C, Saito N. 1996. Neuronal and glial localization of two GABA transporters (GAT1 and GAT3) in the rat cerebellum. Brain Res Mol Brain Res 37:309–316.
Iversen LL, Kelly JS. 1975. Uptake and metabolism of gamma-aminobutyric acid by neurons and glial cells. Biochem Pharmacol 24:933–938.
Johnson J, Chen TK, Rickman DW, Evans C, Brecha NC. 1996. Multiple gamma-Aminobutyric acid plasma membrane transporters (GAT-1, GAT-2, GAT-3) in the rat retina. J Comp Neurol 375:212–224.
Jursky F, Tamura S, Tamura A, Mandiyan S, Nelson H, Nelson N. 1994. Structure, function and brain localization of neurotransmitter transporters. J Exp Biol 196:283–295.
Kanner BI. 1983. Bioenergetics of neurotransmitter transport. Biochim Biophys Acta 726:293–316.
Kanner BI. 1994. Sodium-coupled neurotransmitter transport: structure, function and regulation. J Exp Biol 196:237–249.
Kavanaugh MP, Arriza JL, North RA, Amara SG. 1992. Electrogenic uptake of gamma-aminobutyric acid by a cloned transporter expressed in Xenopus oocytes. J Biol Chem 267:22007–22009.
Keynan S, Kanner BI. 1988. gamma-Aminobutyric acid transport in reconstituted preparations from rat brain: coupled sodium and chloride fluxes. Biochemistry 27:12–17.
Keynan S, Suh YJ, Kanner BI, Rudnick G. 1992. Expression of a cloned gamma-aminobutyric acid transporter in mammalian cells. Biochemistry 31:1974–1979.
Krogsgaard-Larsen P, Falch E, Larsson OM, Schousboe A. 1987. GABA uptake inhibitors: relevance to antiepileptic drug research. Epilepsy Res 1:77–93.
Liu QR, Lopez-Corcuera B, Mandiyan S, Nelson H, Nelson S. 1993. Molecular characterization of four pharmacologically distinct gamma-aminobutyric acid transporters in mouse brain. J Biol Chem 268:2106–2112.
Lopez-Corcuera B, Liu QR, Mandiyan S, Nelson H, Nelson N. 1992. Expression of a mouse brain cDNA encoding novel gamma-aminobutyric acid transporter. J Biol Chem 267:17491–17493.
Lu CC, Kabakov A, Markin VS, Mager S, Frazier GA, Hilgemann DW. 1995. Membrane transport mechanisms probed by capacitance measurements with megahertz voltage clamp. Proc Natl Acad Sci USA 92:11220–11224.
Mabjeesh NJ, Frese M, Rauen T, Jeserich G, Kanner BI. 1992. Neuronal and glial gamma-aminobutyric acid+ transporters are distinct proteins. FEBS Lett 299:99–102.
Madison DL, Krueger WH, Cheng D, Trapp BD, Pfeiffer SE. 1999. SNARE complex proteins, including the cognate pair VAMP-2 and syntaxin-4, are expressed in cultured oligodendrocytes. J Neurochem 72:988–998.
Mager S, Naeve J, Quick M, Labarca C, Davidson N, Lester HA. 1993. Steady states, charge movements, and rates for a cloned GABA transporter expressed in Xenopus oocytes. Neuron 10:177–188.
Mager S, Kleinberger-Doron N, Keshet GI, Davidson N, Kanner BI, Lester HA. 1996 .Ion binding and permeation at the GABA transporter GAT1. J Neurosci 16:5405–5414.
Malchow RP, Ripps H. 1990. Effects of gamma-aminobutyric acid on skate retinal horizontal cells: evidence for an electrogenic uptake mechanism. Proc Natl Acad Sci USA 87:8945–8949.
Mantz J, Lecharny JB, Laudenbach V, Henzel D, Peytavin G, Desmonts JM. 1995. Anesthetics affect the uptake but not the depolarization-evoked release of GABA in rat striatal synaptosomes. Anesthesiology 82:502–511.
Marc RE. 1992. Structural organization of GABAergic circuitry in ectotherm retinas. Prog Brain Res 90:61–92.
Matskevitch I, Wagner CA, Stegen C, Broer S, Noll B, Risler T, Kwon HM, Handler JS, Waldegger S, Busch AE, Lang F. 1999. Functional characterization of the Betaine/gamma-aminobutyric acid transporter BGT-1 expressed in Xenopus oocytes. J Biol Chem 274:16709–16716.
Minelli A, Brecha NC, Karschin C, DeBiasi S, Conti F. 1995. GAT-1, a high-affinity GABA plasma membrane transporter, is localized to neurons and astroglia in the cerebral cortex. J Neurosci 15:7734–7746.
Minelli A, DeBiasi S, Brecha NC, Zuccarello LV, Conti F. 1996. GAT-3, a high-affinity GABA plasma membrane transporter, is localized to astrocytic processes, and it is not confined to the vicinity of GABAergic synapses in the cerebral cortex. J Neurosci 16:6255–264.
Miyakawa H, Woo SK, Chen CP, Dahl SC, Handler JS, Kwon HM. 1998. Cis- and trans-acting factors regulating transcription of the BGT1 gene in response to hypertonicity. Am J Physiol 274:753–761.
Morara S, Brecha NC, Marcotti W, Provini L, Rosina A. 1996. Neuronal and glial localization of the GABA transporter GAT1 in the cerebellar cortex. Neuroreport 7:2993–2996.
Nelson H, Mandiyan S, Nelson N. 1990. Cloning of the human brain GABA transporter. FEBS Lett 269:181–184.
Nelson H, Lill H. 1994. Porters and neurotransmitter transporters. J Exp Biol 196:213–228.
Nissen J, Schousboe A, Halkier T, Schousboe I. 1992. Purification and characterization of an astrocyte GABA-carrier inducing protein (GABA-CIP) released from cerebellar granule cells in culture. Glia 6:236–243.
Obata H, Shimada K, Sakai N, Saito N. 1997. GABAergic neurotransmission in rat taste buds: immunocytochemical study for GABA and GABA transporter subtypes. Brain Res Mol Brain Res 49:29–36.
Pacholczyk T, Blakely RD, Amara SG. 1991. Expression cloning of a cocaine- and antidepressant-sensitive human noradrenaline transporter. Nature 350:350–354.
Pin JP, Bockaert J. 1989. Two distinct mechanisms, differentially affected by excitatory amino acids, trigger GABA release from fetal mouse striatal neurons in primary culture. J Neurosci 9:648–656.
Quick MW, Corey JL, Davidson N, Lester HA. 1997. Second messengers, trafficking-related proteins, and amino acid residues that contribute to the functional regulation of the rat brain GABA transporter GAT1. J Neurosci 17:2967–2979.
Radian R, Kanner BI. 1983. Stoichiometry of sodium- and chloride-coupled gamma-aminobutyric acid transport by synaptic plasma membrane vesicles isolated from rat brain. Biochemistry 22:1236–1241.
Radian R, Ottersen OP, Storm-Mathisen J, Castel M, Kanner BI. 1990. Immunocytochemical localization of the GABA transporter in rat brain. J Neurosci 10:1319–1330.
Rasola A, Galietta LJV, Barone V, Romeo G, Bagnasco S. 1995. Molecular cloning and functional characterization of a GABA/betaine transporter from human kidney. FEBS Lett 373:229–233.
Rattray M, Priestley JV. 1993. Differential expression of GABA transporter-1 messenger RNA in subpopulations of GABA neurons. Neurosci Lett 156:163–166.
Redecker P. 1999. Immunoreactivity for multiple GABA transporters (GAT-1, GAT-2, GAT-3) in the gerbil pineal gland. Neurosci Lett 266:117–120.
Ribak CE, Tong WM, Brecha NC. 1996a. GABA plasma membrane transporters, GAT-1 and GAT-3, display different distributions in the rat hippocampus. J Comp Neurol 367:595–606.
Ribak CE, Tong WM, Brecha NC. 1996b. Astrocytic processes compensate for the apparent lack of GABA transporters in the axon terminals of cerebellar Purkinje cells. Anat Embryol 194:379–390.
Risso S, DeFelice LJ, Blakely RD. 1996. Sodium-dependent GABA-induced currents in GAT1-transfected HeLa cells. J Physiol 490:691–702.

Rossi DJ, Hamann M. 1998. Spillover-mediated transmission at inhibitory synapses promoted by high affinity alpha6 subunit GABA(A) receptors and glomerular geometry. Neuron 20:783–795.

Ruiz M, Egal H, Sarthy V, Qian X, Sarkar HK. 1994. Cloning, expression, and localization of a mouse retinal gamma-aminobutyric acid transporter. Invest Ophthalmol Vis Sci 35:4039–4048.

Schon F, Kelly JS. 1975. Selective uptake of (3H)beta-alanine by glia: association with glial uptake system for GABA. Brain Res 86:243–257.

Schousboe A, Westergaard N. 1995. Transport of neuroactive amino acids in astrocytes. In: Kettenmann H, Ransom BR, editors. Neuroglia. New York: Oxford University Press. p 246–258.

Schousboe A, Krogsgaard-Larsen P, Svenneby G, Hertz L. 1978. Inhibition of the high-affinity, net uptake of GABA into cultured astrocytes by beta-proline, nipecotic acid and other compounds. Brain Res 153:623–626.

Schousboe A, Larsson OM, Wood JD, Krogsgaard-Larsen P. 1983. Transport and metabolism of gamma-aminobutyric acid in neurons and glia: implications for epilepsy. Epilepsia 24:531–538.

Schwartz EA. 1987. Depolarization without calcium can release gamma-aminobutyric acid from a retinal neuron. Science 238:350–355.

Simpson MD, Slater P, Royston MC, Deakin JF. 1992. Regionally selective deficits in uptake sites for glutamate and gamma-aminobutyric acid in the basal ganglia in schizophrenia. Psychiatry Res 42:273–282.

Soltesz I, Crunelli V. 1992. GABAA and pre- and post-synaptic GABAB receptor-mediated responses in the lateral geniculate nucleus. Prog Brain Res 90:151–169.

Sonders MS, Amara SG. 1996. Channels in transporters. Curr Opin Neurobiol 6:294–302.

Spreafico R, De Biasi S, Amadeo A, De Blas AL. 1993. GABAA-receptor immunoreactivity in the rat dorsal thalamus: an ultrastructural investigation. Neurosci Lett 158:232–236.

Staley KJ, Soldo BL, Proctor WR. 1995. Ionic mechanisms of neuronal excitation by inhibitory GABAA receptors. Science 269:977–981.

Suzdak PD, Frederiksen K, Andersen KE, Sorensen PO, Knutsen LJ, Nielsen EB. 1992. NNC-711, a novel potent and selective gamma-aminobutyric acid uptake inhibitor: pharmacological characterization. Eur J Pharmacol. 224:189–198.

Takahashi K, Miyoshi S, Kaneko A, Copenhagen DR. 1995. Actions of nipecotic acid and SKF89976A on GABA transporter in cone-driven horizontal cells dissociated from the catfish retina. Jpn J Physiol 45:457–473.

Thompson K, Anantharam V, Behrstock S, Bongarzone E, Campagnoni A, Tobin AJ. 2000. Conditionally immortalized cell lines, engineered to produce and release GABA, modulate the development of behavioral seizures. Exp Neurol 161:481–489.

Thompson SM, Gahwiler BH. 1992. Effects of the GABA uptake inhibitor tiagabine on inhibitory synaptic potentials in rat hippocampal slice cultures. J Neurophysiol 67:1698–1701.

Ueda Y, Tsuru N. 1995. Simultaneous monitoring of the seizure-related changes in extracellular glutamate and gamma-aminobutyric acid concentration in bilateral hippocampi following development of amygdaloid kindling. Epilepsy Res 20:213–219.

Ueda Y, Willmore LJ. 2000a. Hippocampal gamma-aminobutyric acid transporter alterations following focal epileptogenesis induced in rat amygdala. Brain Res Bull 52:357–361.

Ueda Y, Willmore LJ. 2000b. Molecular regulation of glutamate and GABA transporter proteins by valproic acid in rat hippocampus during epileptogenesis. Exp Brain Res 133:334–339.

Uhl GR, Johnson PS. 1994. Neurotransmitter transporters: three important gene families for neuronal function. J Exp Biol 196:229–236.

Ulrich D, Huguenard JR. 1996. GABAB receptor-mediated responses in GABAergic projection neurones of rat nucleus reticularis thalami in vitro. J Physiol 493:845–854.

Voutsinos B, Dutuit M, Reboul A, Fevre-Montange M, Bernard A, Trouillas P, Akaoka H, Belin MF, Didier-Bazes M. 1998. Serotoninergic control of the activity and expression of glial GABA transporters in the rat cerebellum. Glia 23:45–60.

Wood JD, Schousboe A, Krogsgaard-Larsen P. 1980. In vivo changes in the GABA content of nerve endings (synaptosomes) induced by inhibitors of GABA uptake. Neuropharmacology 19:1149–1152.

Wu SM. 1992. Feedback connections and operation of the outer plexiform layer of the retina. Curr Opin Neurobiol 2:462–468.

Yamauchi AS, Uchida S, Kwon HM, Preston AS, Brooks-Robey R, García-Pérez A, Burg MB, Handler JS. 1992. Cloning of a Na+ and Cl− dependent betaine transporter that is regulated by hypertonicity. J Biol Chem 267:649–652.

Yan XX, Ribak CE. 1998. Developmental expression of gamma-aminobutyric acid transporters (GAT-1 and GAT-3) in the rat cerebellum: evidence for a transient presence of GAT-1 in Purkinje cells. Brain Res Dev Brain Res 111:253–269.

Yan XX, Ribak CE. 1999. Alteration of GABA transporter expression in the rat cerebral cortex following needle puncture and colchicine injection. Brain Res 816:317–328.

Yang CY, Brecha NC, Tsao E. 1997. Immunocytochemical localization of gamma-aminobutyric acid plasma membrane transporters in the tiger salamander retina. J Comp Neurol 389:117–126.

Yang XL, Gao F, Wu SM. 1999. Modulation of horizontal cell function by GABA(A) and GABA(C) receptors in dark- and light-adapted tiger salamander retina. Vis Neurosci 16:967–979.

Zhao JW, Du JL, Li JS, Yang XL. 2000. Expression of GABA transporters on bullfrog retinal Muller cells. Glia 31:104–117.

TESIS CON
FALLA DE ORIGEN

Journal of Neuroscience Research 64:218–222 (2001)

TESIS CON
FALLA DE ORIGEN

Mini-Review

Glial Transporters for Glutamate, Glycine, and GABA III. Glycine Transporters

Ana Gadea[1] and Ana María López-Colomé[1,2]*
[1]Instituto de Fisiología Celular, Departamento de Neurociencias, UNAM, México
[2]Facultad de Medicina, Departamento de Bioquímica, UNAM, México

Glial cells possess transport systems for the three major amino acid neurotransmitters glutamate, γ-aminobutyric acid (GABA) and glycine, involved in the arrest of neurotransmission mediated by these compounds. Two glycine transporters have been cloned: GLYT1, mainly expressed by glial cells and shown to colocalize with NMDA receptors, and GLYT2, exclusively expressed by neurons and colocalized with the inhibitory glycine receptors. The way in which the regulation of extracellular glycine concentration by glial glycine transporters affects physiological and pathological conditions is discussed. The presence, differential pharmacology and specific regulation of glycine transporters in glial cells strongly support an important role for glia in the modulation of both, excitatory and inhibitory neurotransmission. J. Neurosci. Res. 64:218–222, 2001. © 2001 Wiley-Liss, Inc.

Key words: neurotransmitter uptake; neuron-glia interaction; second messengers; membrane protein; synaptic modulation

Glycine subserves two important roles as a neurotransmitter in the CNS. It acts as an inhibitory neurotransmitter in the spinal cord, brain stem, and the retina (Daly, 1990). Such inhibitory action is mediated by a glycine receptor that functions as a ligand-gated Cl^- channel activated by glycine and competitively antagonized by strychnine (Betz et al., 1993). By blocking the action of glycine, strychnine causes severe seizures. Glycine also participates in excitatory neurotransmission, acting as an obligatory coagonist of Glu at *N*-methyl-D-aspartate (NMDA)-activated Glu receptors via a strychnine insensitive binding site on this receptor (Fletcher et al., 1990).

Cloning of GABA (Guastella et al., 1990) and norepinephrine (Pacholczyk et al., 1991) transporters allowed the subsequent isolation of a number of cDNAs encoding homologous neurotransmitter transporters, including those for glycine. Two different genes encoding glycine transporters have been identified and termed GLYT1 and GLYT2. As a result of alternative splicing or promoter usage, transcription of the GLYT1 gene results in three different mRNA isoforms, namely GLYT1a, GLYT1b and GLYT1c, which differ in the amino-terminal region (Kim et al., 1994). It has recently been demonstrated that transcription of the rat variants GLYT1a and GLYT1b is mediated by alternative promoter usage rather than alternative splicing of a single promoter (Borowsky and Hoffman, 1998). GLYT1a and 1b have been cloned from rat and mouse (Guastella et al., 1992; Liu et al., 1992; Smith et al., 1992; Borowsky et al., 1993), and the GLYT1b and 1c homologues have been isolated from human (Kim et al., 1994). GLYT2 gene also codifies for two isoforms termed GLYT2a and GLY2b generated by alternative splicing of the 5′ flanking region (Ponce et al., 1999). Both isoforms display similar regional distribution and kinetic characteristics, however, GLYT2a accumulates glycine into transfected COS cells, whereas GLYT2b seems only to exchange or release glycine. GLYT1 and GLYT2 show distinct anatomical distribution within the CNS and are highly specific for glycine. Although they show relevant differences related to their structure and localization, there are no known pharmacological differences between their transport activities except for the sensitivity of the GLYT1 isoforms to the inhibition by sarcosine (*n*-methylglycine; Liu et al., 1993). It was recently reported that the tricyclic antidepressant amoxapine inhibits glycine transport by GLYT2a, whereas it has little effect on the activity of GLYT1 (Nuñez et al., 2000).

Tissue and Cell Localization

Expression of GLYT1 isoforms in tissues other than the brain seems to be isoform- and species-specific. No detectable GLYT1b mRNA in rat kidney, spleen and aorta, but a low expression in liver have been reported (Smith et al., 1992). In other studies low levels of GLYT1a mRNA in rat liver and no detectable signals in the kidney were observed (Guastella et al., 1992), whereas the oppo-

Contract grant sponsor: DGAPA; Contract grant number: IN-210998; Contract grant sponsor: PAEP; Contract grant number: 102322.

*Correspondence to: Ana María López-Colomé, PhD, Instituto de Fisiología Celular, UNAM, Aparatado Postal 70-253, 04510, México D.F., México. E-mail: acolome@ifisiol.unam.mx

Received 19 December 2000; Accepted 21 December 2000

© 2001 Wiley-Liss, Inc.

TESIS CON
FALLA DE ORIGEN

site has been reported in mouse (Liu et al., 1992). Borowsky et al. (1993) detected GLYT1a (GLYT1 in this paper) in lung, liver, spleen, stomach and uterus, but were unable to demonstrate significant hybridization in tissues other than brain for GLYT1b (GLYT2 in this paper). A strong signal for human GLYT1c mRNA has been shown in the brain, but little or no signals in other tissues (Kim et al., 1994). The expression of GLYT2 has been found exclusively in the brain and spinal cord of the rat (Liu et al., 1993); no other species have been examined.

In situ hybridization, northern blotting and immunoassay have been used to study the distribution of GLYT2 and GLYT1 isoforms in specific regions of the brain. Probes that did not distinguish between the GLYT1 isoforms revealed the greatest expression in the cerebellum, brain stem, olfactory bulb, and spinal cord (Guastella et al., 1992, 1993; Liu et al., 1992; Smith et al., 1992; Jursky et al., 1994). As for cellular localization, it has been suggested that GLYT1 is exclusively expressed by glia (Adams et al., 1995), although neuronal GLYT1 expression has also been reported (Borowsky et al., 1993). More recent data further support some neuronal expression. Immunoreactivity for GLYT1 has been shown in amacrine cells of rat, macaque, cat, rabbit, and chick retinae (Zafra et al., 1995; Pow and Hendrickson, 1999). No labeling in glial cells was found in these studies. In contrast, a high affinity glycine transport system in cultured Müller cells from the chick retina, identified as GLYT1 has been recently characterized (Gadea et al., 1999). Taking into account all available data, it can be concluded that GLYT1 expression is predominantly glial, whereas that of GLYT2 is restricted to neurons in the pons-medulla, cerebellum and spinal cord, excepting cerebellar Golgi cells.

Localization of GLYT subtypes in the brain has suggested specific functional roles related to neurotransmission. High levels of GLYT2 have been detected in glycinergic nerve terminals (Zafra et al., 1995): indeed, it has been considered as a reliable marker for glycine-immunoreactive neurons (Poyatos et al., 1997). Also, colocalization of GLYT2 expression and strychnine-sensitive glycine receptors in the spinal cord and brain have been shown (Luque et al., 1995; Jursky and Nelson, 1995), strongly suggesting a role for GLYT2 in the termination of glycinergic neurotransmission. As mentioned before, it was recently found that GLYT2 gene gives rise to two isoforms: GLYT2a and GLYT2b, both expressed in the same anatomical regions (Ponce et al., 1998). Because GLYT2b mRNA is identifiable only by RT-PCR but not by Northern blot nor by RNase protection due to its low concentration, it has been suggested that previous studies on the localization of GLYT2 using Northern blot and in situ hybridization techniques correspond to the distribution of GLYT2a mRNA. Colocalization of GLYT1 and NMDA receptors (Smith et al., 1992) suggest an involvement in both, glycinergic and glutamatergic neurotransmission.. Additionally, GLYT1 locates to areas not associated with glycinergic pathways (Borowsky et al., 1992; Liu et al., 1992; 1993; Luque et al., 1995; Zafra et al., 1995). GLYT1 expression, however, has been detected in glial cells surrounding putative glycinergic synapses (Zafra et al., 1995); it seems possible that particular isoforms of GLYT1 are associated with NMDA receptors whereas others could relate to inhibitory glycine receptors.

STOICHIOMETRY AND CHANNEL PROPERTIES

It is generally assumed that most Na^+/Cl^--coupled transporters have a stoichiometry of $2Na^+/Cl^-$ per substrate molecule (Nelson and Lill, 1994). Early studies of the glycine transport system in synaptic plasma membrane vesicles showed that two Na^+ ions and one Cl^- ion are cotransported with the substrate in each transport cycle (Aragón et al., 1987). In this preparation, however, both glycine transporters, GLYT1 and GLYT2 probably coexist, and the particular properties of each isoform could not be determined. A later study suggested that two Na^+ ions with equal affinities were required for glycine transport by GLYT1b and three by GLYT2 stably expressed in HEK cells (López-Corcuera et al., 1998). These results were recently corroborated by Roux and Supplisson (2000), who also demonstrated that the neuronal and glial glycine transporters have different reverse transport capabilities: GLYT2 has a kinetic constraint for reverse transport, thus limiting glycine release. The authors proposed that this asymmetry in glycine fluxes may be essential to maintain a high amount of neurotransmitter inside the presynaptic neurons during periods of electrical activity. In contrast, GLYT1 exhibits no such limitation for reverse transport: in oocytes expressing GLYT1b, glycine accumulation evokes a net outward current upon glycine removal. Such transient release could be of physiological relevance after synaptic release of glycine and its reuptake to neuronal and glial cells, when its extracellular concentration is decreased by GLYT2.

In contrast to glutamate and GABA transporters that exhibit ligand-gated ion channel properties (Gadea and López-Colomé, 2001a,b), GLYTs exhibit a tight coupling between ionic and substrate fluxes. This holds particularly true for GLYT1b, which exhibits no leak current and strictly voltage-independent net number of charges translocated with each glycine molecule (Roux and Supplisson, 2000).

Attwell et al. (1993) have suggested that activation of AMPARs in glial cells by glutamate may produce a depolarization and a raise in $[Na^+]_i$ that could be sufficient to reverse GLYT1b transport, thus increasing [Gly]o and potentiating NMDA receptors. On this line, glycine release from glial cells has been reported in different systems, and has led to the idea that in the vicinity of glutamatergic synapses, glial Ca^{2+}-independent glycine release contributes to excitatory neurotransmission (Holopainen and Kontro, 1989; Galli et al., 1993; Saransaari and Oja, 1994; Sakata et al., 1997).

REGULATION OF GLYCINE TRANSPORTERS

Currently very little information is available regarding the role of second messengers in the regulation of

TESIS CON
FALLA DE ORIGEN

glycine transport. Arachidonic acid, which may be released via phospholipase A_2, has been shown to inhibit several sodium-coupled uptake systems, including those for glycine: high-affinity glycine transport in C6 glioma cells is inhibited by arachidonic acid due to the perturbation of the lipid domain surrounding the transporters (Zafra et al., 1990). In Müller cells from the retina, the involvement of Ca^{2+}/calmodulin-dependent enzymes in the regulation of glycine transport has been demonstrated (López-Colomé and Gadea, 1999).

Sato et al. (1995) reported that the activity of recombinant mouse GLYT1b expressed in HEK-239 cells is decreased upon PKC activation by phorbol esters due to a reduction in the V_{max} of glycine uptake. Substitution of all potential PKC phosphorylation consensus sites did not abolish the effect of phorbol ester treatment, however, suggesting that transport modulation by PKC might involve an indirect mechanism. On this line, Geerlings et al. (2000) have recently demonstrated a functional and physical interaction between both glycine transporters, GLYT1 and GLYT2, and the SNARE protein syntaxin 1A. Cotransfection of syntaxin 1A and GLYT1 or GLY2 in COS cells decreases the number of transporter proteins on the plasma membrane, but not their overall expression. Immunoprecipitation studies have shown a physical interaction of GLYTs with syntaxin 1A not only in COS cells, but also in brain tissue. Therefore it is possible that, as for GABA transporters, PKC could regulate the interaction between GLYTs and syntaxin 1A.

Neuronal regulation of glial GLYT1 expression has also been reported (Zafra et al., 1997): rat spinal cord glial cells in purified cultures do not express GLYT1, but they do so in mixed neuronal-glial cultures in which the elimination of neurons by cytotoxic treatments reduces GLYT1 glial expression. These findings support the possibility of a regulatory interaction between neurons and glia for GLYT1 glial expression.

GLIAL GLYCINE TRANSPORTERS IN DISEASE

Glycine could participate in excitotoxic processes potentiating the effect of Glu at NMDA receptors, as well as in the pathogenesis of diseases related to inhibitory glycine receptors (Lloyd et al., 1983; Simpson et al., 1995). In this respect, the role of gly transporters in keeping local extracellular gly concentration below saturating levels for NMDARs, which would allow potentiation of receptors by sudden increases of extracellular gly, has been demonstrated in an heterologous system, as well as in brainstem slices (Supplisson and Bergman, 1997; Bergeron et al., 1998). Such rises could derive from Ca^{2+}-dependent release and diffusion from nearby synapses, or from a Ca^{2+}-independent mechanism due to reverse transport, as in ischemic condition, in which a reduction of Na^+ and Cl^- electrochemical gradient occurs (Baker et al., 1991). Such Ca^{2+}-independent release most probably comes from glial gly transporters which, as mentioned above, have a stoichiometry of 2/Na+/Cl^-/gly, vs. neuronal ones with 3/Na+/Cl^-/gly stoichiometry (Roux and Supplisson, 2000). The loss of human spinal cord GLYT1 in amyotrophic lateral sclerosis (ALS), has been reported (Virgo and Belleroche, 1995); the authors suggest that the deficit in GLYT1 mRNA likely affects synaptic inactivation of glycine and potentiates the action of glutamate at the NMDA receptors in motor neurons, therefore contributing to the neurotoxic condition.

Because high affinity NMDA channel blockers, such as phenylcyclidine (PCP), mimic both positive and negative symptoms of schizophrenia in humans, it has been suggested that hypofunction of the glutamatergic system might occur in this disease; in fact, there are some indications of glutamatergic hypofunction in schizophrenic patients, such as decreased NMDA receptor-stimulated glutamate release in synaptosomal preparations (Danysz and Parsons, 1998; Olney et al., 1999). It would be difficult to consider the clinical use of direct NMDAR agonists, because of their neurotoxic and convulsive potential, favoring blockade of glial GLYT1 glycine transporters as a suitable approach. The pharmacological study of glycine transporters provides a tool for the manipulation of glycine concentrations by means of specific drugs affecting its reuptake.

Because glycine has a variety of functions in the CNS, the regulation of extracellular glycine concentration by glycine transporters may be of physiological relevance for effective neuronal signaling. The way in which the regulation of extracellular glycine concentration by glial glycine transporters affects physiological and pathological conditions, however, remains to be established.

CONCLUSIONS

Numerous studies have shown the presence of transport systems in glia for the three major amino acid neurotransmitters in the CNS: Glu, GABA, and Glycine. The precise localization of neurotransmitter transporters and their involvement in the regulation of synaptic activity is an important issue to understand the role of glial cells in the overall processes of neurotransmission.

Although the reuptake process is under physiological control, very little information is currently available regarding the possibility of the regulation of these proteins by second messengers. In this regard, arachidonic acid has been shown to inhibit several sodium-coupled uptake systems, including those for glycine (Zafra et al., 1990) and Glu (Barbour, 1989). The modulation of Glu transporters by PKC (Casado et al., 1993; Gonzalez and Ortega, 1997), the regulation of GABA transporters by cAMP (Gomeza et al., 1991), as well as the regulation of glycine transport in Müller cells by Ca^{2+}/calmodulin-dependent enzymes (López-Colomé and Gadea, 1999) has also been demonstrated. Future studies on this issue might shed light on the interactive relationship of neurons and glia, involved in the regulation of neurotransmission.

It has been recognized for many years that the membrane transport systems for neuroactive compounds are capable of carrying a net outward flux as well as net inward movement of the substrate. It is very likely that glial cells could release such compounds through this mechanism. The operation of transport systems is electrogenic and

TESIS CON
FALLA DE ORIGEN

strongly dependent on transmembrane ion gradients and membrane potential. The stoichiometry of the transport reaction determines the substrate concentration gradient that the system can generate, as well as the sensitivity of the system to changes in membrane potential and ion concentrations. In ionic conditions that fail to support the existing substrate gradient, neurotransmitter transporters run backward, acting as a calcium independent, non-vesicular mechanism for transmitter release (Attwell et al., 1993). Glial transporters may play an important role not only in the clearance of neurotransmitters released by neurons, but also in releasing neuroactive compounds in response to multiple stimuli, through the activation of specific neurotransmitter receptors (Shao and McCarthy, 1994; Gallo and Russell, 1995; Porter and McCarthy, 1997).

Several examples illustrate that the electrical responses elicited by Glu and GABA application to glial cells can be attributed to transporters rather than receptors on the basis of their pharmacology and ion dependency. Early work showed that GABA iontophoresis depolarizes cortical glial cells in a Na^+-dependent fashion (Krnjevic and Schwartz, 1967). On this line, electrical responses have been recorded in glial cells co-cultured with hippocampal neurons, attributable to electrogenic Glu uptake after single stimulations of the neuron (Mennerick et al., 1994, 1996). Although the mechanism has not been established, the depolarization of Bergmann glia after parallel fiber stimulation has also been reported (Clark and Barbour, 1997). Despite the poor understanding regarding signaling mechanisms between neurons and glial cells, one interesting possibility is that Na^+-dependent Glu uptake stimulates glucose uptake and the consequent release of lactate in astrocytes, which in turn, may serve as neuronal energy source (Pellerin and Magistretti, 1994; Takahashi et al., 1995).

It is also possible that glial cell depolarization resulting from electrogenic Glu, GABA and Gly uptake may play an initiatory role in intracellular signaling. Three recent studies suggest the interesting possibility that the opening of voltage-gated Ca^{2+} channels (VGCC) may result from transporter-mediated depolarization. GABA applied to isolated skate retinal horizontal cells, while fluorometrically monitoring intracellular Ca^{2+} concentration, depolarizes Müller cells in a concentration-dependent manner, thus leading to nifedipine-blockable increases in the concentration of intracellular Ca^{2+}. The pharmacology and ionic dependence of these responses were those for GABA transporters and not for $GABA_A$ or $GABA_B$ receptors (Haugh-Scheidt et al., 1995). An analogous elevation of intracellular Ca^{2+} after application of EAAT substrates, but not of GluR agonists has also been found in pituitary cells (Villalobos et al., 1995). Because Ca^{2+} elevation was blocked by nisoldipine, an opening of VGCC by EAAT-mediated depolarization has been proposed. In support of this idea, glycine has been shown to induce an increase in intracellular Ca^{2+} in cortical oligodendrocyte progenitor (OP) cells by Ca^{2+} entry through VGCCs activated both, by Gly transporter- and Gly receptor-induced depolarization (Belachew et al., 2000).

The evidence contained in this review, clearly emphasizes the active participation of glial cells in the regulation of neurotransmission.

ACKNOWLEDGMENTS

This work was supported in part by grant IN-210998 from DGAPA to A.M.L.C. and PAEP 102322 to A.G.

REFERENCES

Adams RH, Sato K, Shimada S, Tohyama M, Püschel AW, Betz H. 1995. Gene structure and glial expression of the glycine transporter GLYT1 in embryonic and adult rodents. J Neurosci 15:2524–2532.

Aragón MC, Giménez C, Mayor F. 1987. Stoichiometry of sodium- and chloride-coupled glycine transport in synaptic plasma membrane vesicles derived from rat brain. FEBS Lett 212:87–90.

Attwell D, Barbour B, Szatkowsky M. 1993. Nonvesicular release of neurotransmitter. Neuron 11:401–407.

Baker AJ, Zornow MH, Scheller MS, Yaksh TL, Skilling SR, Smullin DH, Larson AA, Kuczenski R. 1991. Changes in extracellular concentrations of glutamate, aspartate, glycine, dopamine, serotonin, and dopamine metabolites after transient global ischemia in the rabbit brain. J Neurochem 57:1370–1379.

Barbour B. 1989. Modulation of glutamate transporters by arachidonic acid. Nature 342:918–920.

Belachew S, Malgrange B, Rigo JM, Rogister B, Leprince P, Hans G, Nguyen L, Moonen G. 2000. Glycine triggers an intracellular calcium influx in oligodendrocyte progenitor cells that is mediated by the activation of both the ionotropic glycine receptor and Na^+-dependent transporters. Eur J Neurosci 12:1924–1930.

Bergeron R, Meyer TM, Coyle JT, Greene RW. 1998. Modulation of *N*-methyl-D-aspartate receptor function by glycine transport. Proc Natl Acad Sci USA 95:15730–15734.

Betz H, Langosch D, Rundstrom N, Bormann J, Kuryatov A, Kuhse J, Schmieden V, Matzenbach B, Kirsch J. 1993. Structure and biology of inhibitory glycine receptors. Ann N Y Acad Sci 707:109–115.

Borowsky B, Mezey E, Hoffman BJ. 1993. Two glycine transporter variants with distinct localization in the CNS and peripheral tissues are encoded by a common gene. Neuron 10:851–863.

Borowsky B, Hoffman BJ. 1998. Analysis of a gene encoding two glycine transporter variants reveals alternative promoter usage and a novel gene structure. J Biol Chem 273:29077–29085.

Casado M, Bendahan A, Zafra F, Danbolt NC, Aragón C, Giménez C, Kanner B. 1993. phosphorylation and modulation of brain glutamate transporters by protein kinase C. J Biol Chem 268:27313–27317.

Clark BA, Barbour B. 1997. Currents evoked in Bergmann glial cells by parallel fiber stimulation in rat cerebellar slices. J Physiol 502:335–350.

Daly EC. 1990. The biochemistry of glycinergic neurons. In: Ottersen OP, Storm-Mathisen J, editors. Glycine neurotransmission. New York: John Wiley and Sons, Ltd. p 25–67.

Danysz W, Parsons ACG. 1998. Glycine and *N*-methyl-D-aspartate receptors: physiological significance and possible therapeutic applications. Pharmacol Rev 50:597–664.

Fletcher EJ, Beart PM, Lodge D. 1990. Involvement of glycine in excitatory neurotransmission. In: Ottersen OP, Storm-Mathisen J, editors. Glycine neurotransmission. New York: John Wiley & Sons. p 193–218.

Gadea A, López E, López-Colomé AM. 1999. Characterization of glycine transport in cultured Müller glial cells from the retina. Glia 26:273–279.

Gadea A, López-Colomé AM. 2001a. Glial transporters for glutamate, glycine and GABA. II. Glutamate transporters. J Neurosci Res 63:453–460.

Gadea A, López-Colomé AM. 2001b. Glial transporters for glutamate, glycine and GABA. II. GABA transporters. J Neurosci Res 63:461–468.

TESIS CON
FALLA DE ORIGEN

Galli A, Mori F, Bargellini M, Coppini L. 1993. Sodium-dependent release of exogenous glycine from preloaded rat hippocampal synaptosomes. J Neural Transm Gen Sect 93:167–179.

Gallo V, Russell JT. 1995. Excitatory amino acid receptors in glia: different subtypes for distinct functions? J Neurosci Res 42:1–8.

Geerlings A, López-Corcuera B, Aragón C. 2000. Characterization of the interactions between the glycine transporters GLYT1 and GLYT2 and the SNARE protein syntaxin 1A. FEBS Lett 470:51–54.

Gomeza J, Casado M, Giménez C, Aragón C. 1991. Inhibition of high-affinity γ-aminobutyric acid uptake in primary astrocyte cultures by phorbol esters and phospholipase C. J Biochem 275:435–439.

Gonzalez MI, Ortega A. 1997. Regulation of the Na^+-dependent high affinity glutamate/aspartate transporter in cultured Bergmann glia by phorbol esters. J Neurosci Res 50:585–590.

Guastella J, Nelson N, Nelson H, Czyzyk L, Keynan S, Miedel MC, Davidson N, Lester HA, Kanner BI. 1990. Cloning and expression of a rat brain GABA transporter. Science 249:1303–1306.

Guastella J, Brecha N, Weigmann C, Lester A, Davidson N. 1992. Cloning, expression and localization of a rat brain high-affinity glycine transporter. Proc Natl Acad Sci USA 89:7189–7193.

Haugh-Scheidt L, Malchow RP, Ripps H. 1995. GABA transport and calcium dynamics in horizontal cells from the skate retina. J Physiol 488:565–576.

Holopainen I, Kontro P. 1989. Uptake and release of glycine in cerebellar granule cells and astrocytes in primary culture: potassium-stimulated release from granule cells is calcium-dependent. J Neurosci Res 24:374–383.

Jursky F, Tamura S, Tamura A, Mandiyan S, Nelson H, Nelson N. 1994. Structure, function and brain localization of neurotransmitter transporters. J Exp Biol 196:283–295.

Jursky F, Nelson N. 1995. Localization of glycine neurotransmitter transporter (GLYT2) reveals correlation with the distribution of glycine receptor. J Neurochem 64:1026–1033.

Kim KM, Kingsmore SF, Han H, Yang-Fen TL, Godinot N, Seldin MF, Caron MG, Giros B. 1994. Cloning of the human glycine transporter type 1: molecular and pharmacological characterization of novel isoform variants and chromosomal localization of the gene in the human and mouse genomes. Mol Pharmacol 45:608–617.

Krnjevic K, Schwartz S. 1967. The action of γ-aminobutyric acid on cortical neurones. Exp Brain Res 3:320–336.

Liu QR, Nelson H, Mandiyan S, López-Corcuera B, Nelson N. 1992. Cloning and expression of a glycine transporter from mouse brain. FEBS Lett 305:110–114.

Liu QR, López-Corcuera B, Mandiyan S, Nelson H, Nelson N. 1993. Cloning and expression of a spinal cord and brain-specific glycine transporter with novel structural features. J Biol Chem 268:22802–22808.

Lloyd KG, De-Montis G, Javoy-Agid F, Beaumont K, Lowenthal A, Constantinidis J, Agid Y. 1983. Glycine receptors in the human brain: characterization of ^{3}H-strychnine binding and status in pathological conditions. Adv Biochem Psychopharmacol 36:233–238.

López-Colomé AM, Gadea A. 1999. Regulation of glycine transport in cultured Müller cells by Ca^{2+}/calmodulin-dependent enzymes. Ann NY Acad Sci 868:685–688.

López-Corcuera B, Martinez-Maza R, Nuñez E, Roux M, Supplisson S, Aragón C. 1998. Differential properties of two stably expressed brain-specific glycine transporters. J Neurochem 71:2211–2219.

Luque JM, Nelson N, Richards JG. 1995. Cellular expression of glycine transporter 2 messenger RNA exclusively in rat hindbrain and spinal cord. Neuroscience 64:525–535.

Mennerick S, Zorumski CF. 1994. Glial contributions to excitatory neurotransmission in cultured hippocampal cells. Nature 368:59–62.

Mennerick S, Benz A, Zorumski CF. 1996. Components of glial responses to exogenous and synaptic glutamate in rat hippocampal microcultures. J Neurosci 16:55–64.

Nelson H, Lill H. 1994. Porters and neurotransmitter transporters. J Exp Biol 196:213–228.

Nuñez E, López-Corcuera B, Vazquez J, Giménez C, Aragón C. 2000. Differential effects of the tricyclic antidepressant amoxapine on glycine uptake mediated by the recombinant GLYT1 and GLYT2 glycine transporters. Br J Pharmacol 129:200–206.

Olney JW, Newcomer JW, Farber NB. 1999. NMDA receptor hypofunction model of schizophrenia. J Psychiatr Res 33:523–533.

Pacholczyk T, Blakely RD, Amara SG. 1991. Expression cloning of a cocaine- and antidepressant-sensitive human noradrenaline transporter. Nature 350:350–354.

Pellerin L, Magistretti PJ. 1994. Glutamate uptake into astrocytes stimulates aerobic glycolysis: a mechanism coupling neuronal activity to glucose utilization. Proc Natl Acad Sci USA 91:10625–10629.

Ponce J, Poyatos I, Aragón C, Giménez C, Zafra F. 1998. Characterization of the 5′ region of the rat brain glycine transporter GLYT2 gene: identification of a novel isoform. Neurosci Lett 242:25–28.

Porter JT, McCarthy KD. 1997. Astrocytic neurotransmitter receptors in situ and in vivo. Prog Neurobiol 51:439–455.

Pow DV, Hendrickson AE. 1999. Distribution of the glycine transporter glyt-1 in mammalian and nonmammalian retinae. Vis Neurosci 16:231–239.

Poyatos I, Ponce J, Aragón C, Giménez C, Zafra F. 1997. The glycine transporter GLYT2 is a reliable marker for glycine-immunoreactive neurons. Brain Res Mol Brain Res 49:63–70.

Roux MJ, Supplisson S. 2000. Neuronal and glial glycine transporters have different stoichiometries. Neuron 25:373–383.

Sakata K, Sato K, Schloss P, Betz H, Shimada S, Tohyama M. 1997. Characterization of glycine release mediated by glycine transporter 1 stably expressed in HEK-293 cells. Brain Res Mol Brain Res 49:89–94.

Saransaari P, Oja SS. 1994. Glycine release from hippocampal slices in developing and ageing mice: modulation by glutamatergic receptors. Mech Aging Dev 76:113–124.

Sato K, Adams R, Betz H, Schloss P. 1995. Modulation of a recombinant glycine transporter (GLYT1b) by activation of protein kinase C. J Neurochem 65:1967–1973.

Shao Y, McCarthy KD. 1994. Plasticity of astrocytes. Glia 11:147–155.

Simpson RK, Gondo M, Robertson CS, Goodman JC. 1995. The influence of glycine and related compounds on spinal cord injury-induced spasticity. Neurochem Res 20:1203–1210.

Smith KE, Borden LA, Hartig PA, Branchek T, Weinshank RL. 1992. Cloning and expression of a glycine transporter reveal colocalization with NMDA receptors. Neuron 8:927–936.

Supplisson S, Bergman C. 1997. Control of NMDA receptor activation by a glycine transporter co-expressed in Xenopus oocytes. J Neurosci 17:4580–4590.

Takahashi S, Driscoll BF, Law MJ, Sokoloff L. 1995. Role of sodium and potassium ions in regulation of glucose metabolism in cultured astroglia. Proc Natl Acad Sci USA 92:4616–4620.

Villalobos C, Garcia-Sancho J. 1995. Glutamate increases cytosolic calcium in GH3 pituitary cells acting via a high-affinity glutamate transporter. FASEB J 9:815–819.

Virgo L, de Belleroche J. 1995. Induction of the immediate early gene *c-jun* in human spinal cord in amyotrophic lateral sclerosis with concomitant loss of NMDA receptor NR-1 and glycine transporter mRNA. Brain Res 676:196–204.

Zafra F, Alcantara R, Gomeza J, Aragón C, Giménez C. 1990. Arachidonic acid inhibits glycine transport in cultured glial cells. Biochem J 271:237–242.

Zafra F, Aragón C, Olivares L, Danbolt NC, Giménez C, Storm-Mathisen J. 1995. Glycine transporters are differentially expressed among CNS cells. J Neurosci 15:3952–3969.

Zafra F, Poyatos I, Giménez C. 1997. Neuronal dependency of the glycine transporter GLYT1 expression in glial cells. Glia 20:155–162.