# Comparative analysis of sodium-dependent L-glutamate transport of synaptosomal and astroglial membrane vesicles from mouse cortex

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Uptake of [³H]L-glutamate into membrane vesicles prepared from either mouse cortical astrocyte cultures or synaptosomes was found to be an electrogenic sodium- and potassium-dependent transport process with saturable uptake kinetics. Pharmacological differences were revealed by using a variety of substrate analogues. L-*trans*-PDC inhibited the synaptosomal glutamate transport 2–4-fold stronger than the astroglial uptake. The substrate analogues DL-threo-β-hydroxy-aspartate, DL-aspartate-β-hydroxamate, L-aspartate and D-aspartate inhibited glutamate transport of astroglial and neuronal membrane vesicles in a distinctive manner, whereas D-glutamate, quisqualate and dihydrokainate had no effect in either case. Immunoblotting and immunocytochemical labeling with antibodies against the rat brain glutamate transporter revealed the selective reaction of a band at about 75 kDa mol. wt. and a specific pattern of astrocyte immunostaining.

Astroglial membrane vesicle; L-Glutamate transport; Synaptosome; Immunoblotting; Immunocytochemistry; Glutamate analogue

## 1. INTRODUCTION

The major part of the excitatory neurotransmission process in the mammalian central nervous system is mediated by the amino acid L-glutamate. Termination of such neuronal activity is brought about by a sodium-dependent high affinity uptake system. Although isolated nerve ending fractions are known to accumulate glutamate via a high affinity transport system [1–3], other biochemical evidence [4,5] suggests that uptake into astroglial cells may be of even greater importance in terminating transmitter action in the brain. Whilst in the past L-glutamate transport of neurons and astrocytes for itself has been studied in brain slices, cell cultures or rat brain membrane preparations [6–8], a detailed comparative biochemical analysis of the synaptosomal and astroglial glutamate transport of the same brain region under identical conditions is still lacking. In particular, a closer pharmacological characterization of the respective substrate binding sites would be desirable to obtain a better insight into the specific structural requirements and the basic features of either L-glutamate transport system. A most suitable experimental paradigm for a comparative approach of this kind is

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*Abbreviations:* CCCP, carbonyl-cyanide-*m*-chlorophenylhydrazone; Tris, Tris(hydroxymethyl)-aminomethane; L-*trans*-PDC, L-transpyrrolidine-2,4-dicarboxylate.

membrane vesicle preparations derived either from cultured cortical astrocytes or synaptosomal fractions, which allow one to individually analyse each of the components involved in the transport process, including ionic dependence and pharmacological specificity, without metabolic interference from the cellular cytoplasm. In addition to this in the present study an immunochemical approach was made using antibodies recently generated against a rat brain glutamate transporter [9].

## 2. MATERIALS AND METHODS

### 2.1. *Materials*
L-[³H]Glutamate (21–56 Ci/mmol), D-[³H]glucose (20–30 Ci/mmol), ECL (enhanced chemiluminescence)-Western Blotting Detection System and ECL-Hyperfilm were obtained from Amersham Buchler. Reagents for cell cultures, including Dulbecco's Modified Eagle's Medium, MEM HEPES, and fetal calf serum were from Gibco/BRL. PDC (L-*trans*-pyrrolidine-2,4-dicarboxylic acid) was from Tocris Neuramin Ltd. D- and L-aspartic acid, dihydrokainic acid, D- and L-glutamic acid, DL-threo-β-hydroxyaspartic acid, quisqualic acid, valinomycin, nigericin, FITC- and TRITC-conjugated secondary antibodies, anti-rabbit IgG-peroxidase conjugate, streptavidin-peroxidase conjugate and WGA-Sepharose were from Sigma. Monoclonal antibodies against GFAP were purchased from Boehringer. Antiserum against the purified rat brain glutamate transporter molecule was generated in rabbits as previously described [9]. Membrane filters OE 67 were from Schleicher and Schuell. All other reagents were of the purest grade commercially available.

### 2.2. *Methods*

#### 2.2.1 Cell culture
Primary cultures of mouse cortical astrocytes were obtained from two-day-old BALB/c mice essentially as described [10]. Cells were
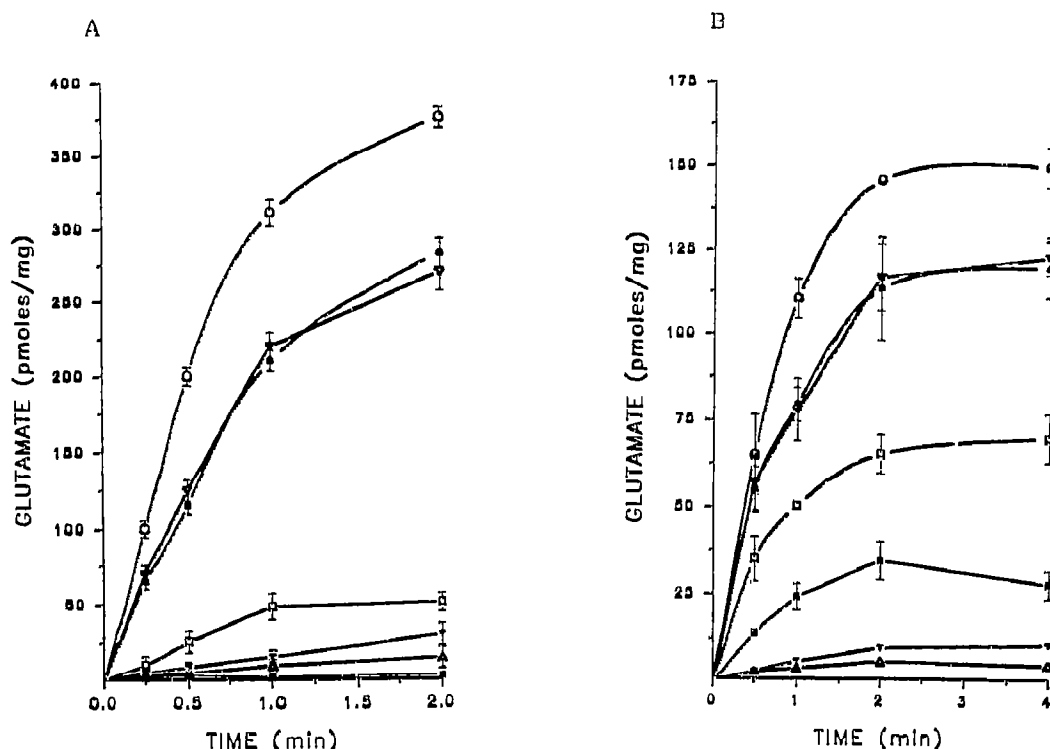
Fig. 1. Comparison of ion-dependence and effect of ionophores on glutamate transport of astroglial (A) and synaptosomal (B) membrane vesicles. The transport of 1 $\mu$Ci L-[$^3$H]glutamate was measured for the indicated time period, using membrane vesicles at 20 $\mu$g protein per time point. Membrane vesicles were preloaded with internal medium (0.32 M mannitol, 0.12 M KP$_i$, pH 6.8, 0.5 mM K-EDTA, 1 mM MgSO$_4$) and incubated in external solution containing: 0.15 M LiCl ($\triangle$), 0.15 M NaNO$_3$ ($\triangledown$ or 0.15 M NaCl ($\bullet$). Internal potassium was substituted by 0.1 M Tris-HCl, pH 6.8 ($\blacktriangledown$). The following ionophores were added immediately before starting the experiment by addition of the vesicles to the external medium (0.15 M NaCl, 0.32 M mannitol): none ($\bullet$), 2.5 $\mu$M valinomycin (o), 5 $\mu$M nigericin ($\blacksquare$), 5 $\mu$M CCCP ($\square$). Error bars indicate $\pm$ SD ($n \geq 4$ sets of triplicate).

grown on poly-D-lysine (50 mg/l) coated 250 ml culture flasks at 37°C in a moist atmosphere containing 5% CO$_2$. Cultures were fed twice a week with Dulbecco's Modified Eagle's Medium (high glucose formulation) supplemented with 10% fetal calf serum, 2 mM glutamine, 2.4 g/l sodium bicarbonate and 100 mg/l gentamycin. In these cell cultures flat polyclonal GFAP-positive cells described as type I astrocytes [11] represented more than 95% of the cell population.

2.2.2. Preparation of astroglial membrane vesicles

After 3 weeks in culture cells were washed with 0.32 M mannitol and 1 mM K-EDTA pH 7.4 and harvested with a rubber policeman. Subsequently they were homogenized on ice in 0.12 M potassium phosphate buffer, pH 6.8, containing 0.32 M mannitol, 1 mM MgSO$_4$ and 0.5 mM K-EDTA (homogenization buffer) and centrifuged for 10 min at 1,000 × g to remove nuclei and undisrupted cells. The supernatant was centrifuged for 20 min at 27,000 × g at 4°C. The resulting pellet was suspended and diluted in 5 mM Tris-HCl and 1 mM K- EDTA, pH 7.4. After the osmotic shock, the membranes were collected by centrifuging at 27,000 × g for 20 min. The pellet was resuspended in homogenization buffer, divided into aliquots and frozen in liquid nitrogen. Under these conditions transport activity of the membrane vesicles was stable at least 3 months.

2.2.3. Preparation of synaptosomes

Synaptosomes were prepared essentially as described by Booth and Clark [12] with the following modifications. After removal from the discontinous Ficoll/sucrose gradient, synaptosomes were diluted in

0.32 M mannitol, 1 mM K-EDTA and 10 mM Tris/HCl, pH 7.4, and centrifuged at 5,500 × g for 10 min to remove Ficoll. The washed synaptosomes were osmotically shocked as in the case of astroglial membrane vesicles, subsequently resuspended in homogenization buffer, frozen in liquid nitrogen and stored in aliquots at −70°C.

2.2.4. Transport assay

Astroglial or synaptosomal membrane vesicles were loaded with the desired internal medium during vesicle preparation. Subsequently, 20 $\mu$l (1–2 mg/ml) of membrane vesicles were diluted at room temperature into the respective external solution supplemented with 1 $\mu$Ci of L-[$^3$H]glutamic acid. The composition of internal and external media varying for each experiment are given in the legends to the figures and tables. In the inhibition experiments L-[$^3$H]glutamate and inhibitors were added simultaneously. The uptake assay was terminated by rapidly filtering on cellulose acetate filters (0.45 $\mu$m), which were quickly rinsed with 20 vols. of ice-cold stopping solution (0.32 M mannitol and 0.15 M NaCl). Uptake rates were corrected for background and leakage (i.e. uptake at 0°C). Experiments were done at least in triplicate.

2.2.5 Immunofluorescence

Cultures were fixed and permeabilized in ethanol/acetic acid (19:1 v/v) for 5 min at −20°C. After several washes with MEM-HEPES and MEM-HEPES containing 1% bovine serum albumin, cells were sequentially incubated for 30 min at room temperature with the antibody against the purified L-glutamate transporter from rat brain (diluted 1:20 in MEM-BSA) and with a monoclonal anti-GFAP antibody
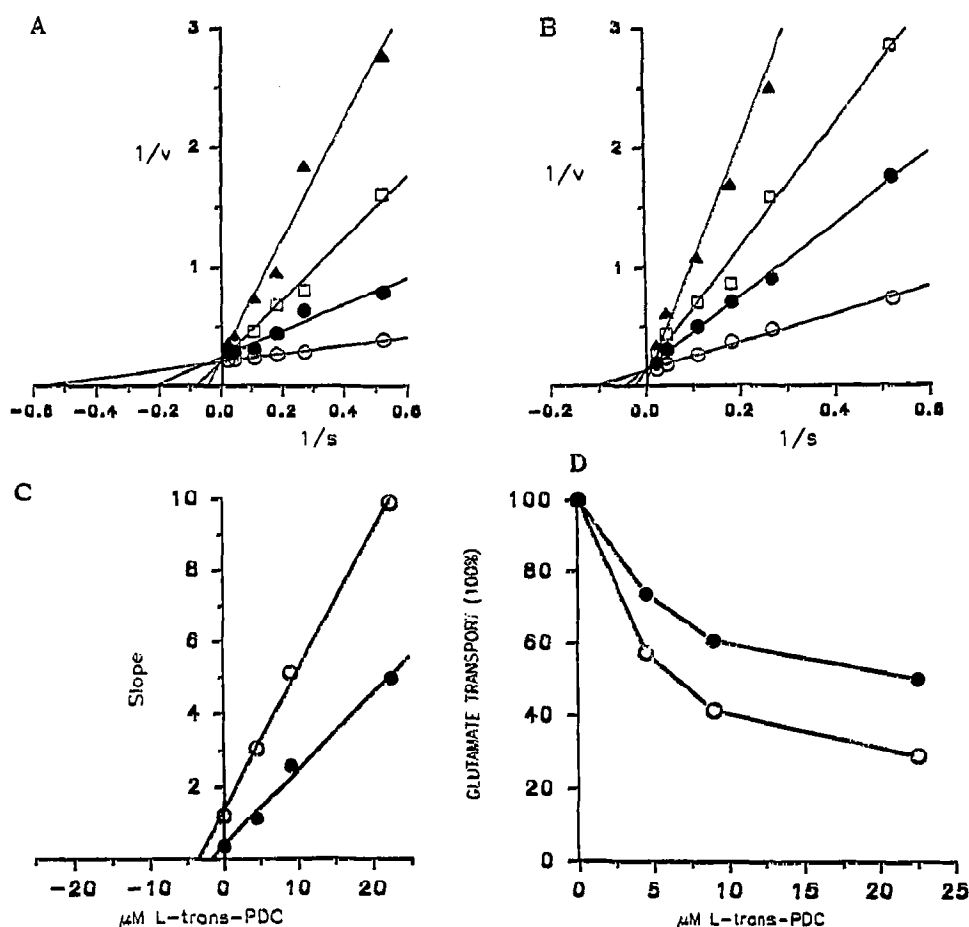
Fig. 2. Competitive inhibition of astroglial (A) and synaptosomal (B) L-[$^3$H]glutamate transport by L-trans-PDC. Lineweaver-Burk plot of L-glutamate (V = nmol L-glutamate/min · mg of protein; S = $\mu$M glutamate). Transport was measured by using 30 $\mu$g of membrane protein per time point with 1 $\mu$Ci of L-[$^3$H]glutamate and unlabeled L-glutamate between 2 and 100 $\mu$M. Triplicate of measurements for astroglial or synaptosomal membrane vesicles were taken in the linear range of uptake (0.5 min) at 25°C in the absence and presence (o = none, ● = 5 $\mu$M, □ = 10 $\mu$M, ▲ = 25 $\mu$M) of L-trans-PDC. Lines were determined by linear regression and yielded $K_m$ and $V_{max}$ for astrological membrane vesicles (1.8 $\mu$M and 4.9 nmol/min·mg of protein) and synaptosomal membrane vesicles (10 $\mu$M and 7.2 nmol/min·mg of protein). (C) The $K_i$ values for L-trans-PDC of astroglial (●, 1.6 $\mu$M) and synaptosomal (o, 3.5 $\mu$M) membrane vesicles were determined by a replot of the slopes. (D) The L-trans-PDC effect on astroglial (●) and synaptosomal (o) glutamate transport was compared. The transport reaction was terminated after 1 min in the presence of the indicated final concentration of the substrate analogue, using 50 $\mu$g of membrane vesicle protein per time point and 1 $\mu$Ci L-[$^3$H]glutamate.

(diluted 1:5). After four washes in MEM-BSA the secondary antibody mixture (FITC-conjugated goat anti-mouse IgG and TRITC-conjugated goat anti-rabbit IgG, both diluted 1:50 in MEM-BSA) was added for 30 min. After rinsing in MEM-BSA preparations were mounted in gevatol and examined in an Olympus IMT2 inverted microscope.

2.2.6. Chromatography on wheat germ agglutinin-Sepharose (WGA)
WGA-chromatography was done essentially as described [13].

2.2.7 Other methods
SDS/PAGE [14] was done with 4% stacking and 10% separating gel (0.5 mm thick). Immunoblotting was done essentially as described [15]. For immunoblot analysis separated proteins were transferred to nitrocellulose membranes (Western blot technique). After incubation with 0.3% gelatine and 0.05% Tween 20 (v/v) at room temperature for 30 min to block nonspecific binding sites the nitrocellulose strips were treated (overnight, 4°C) with anti-glutamate transporter antibodies (1:3,000). After the next blocking step the secondary antibody (perox-

idase conjugated goat anti-rabbit IgG, diluted 1:3,000) was added for 30 min at room temperature. The thoroughly rinsed blots were developed with the Amersham ECL-Detection kit and the antibody reaction detected by chemiluminescence using Hyperfilm-ECL. Protein determination was done according to the Bradford method [16].

2.2.8. Internal volume of the membrane vesicles
Volume determination of membrane vesicles was done essentially as described [17]. The internal volume for [$^3$H]L-glutamate was found to be 2.3 ± 0.29 $\mu$l/mg of protein for astroglial and 2.5 ± 0.24 $\mu$l/mg of protein for synaptosomal membrane vesicles. If the determination was done with [$^3$H]D-glucose a internal volume of 3.7 ± 0.4 $\mu$l/mg of protein for astroglial and 3.3 ± 0.36 $\mu$l/mg of protein for synaptosomal membrane vesicles were determined.

## 3. RESULTS AND DISCUSSION

The uptake characteristics of L-[$^3$H]glutamate into membrane vesicles derived from cultured astrocytes or

cortical mouse synaptosomes are illustrated by Fig. 1. Both astroglial as well as synaptosomal membrane vesicles, loaded with potassium phosphate and diluted into NaCl containing medium accumulated glutamate in a time-dependent and saturable manner. Replacement of either external sodium by lithium or internal potassium by Tris⁺ ions led to a complete inhibition of uptake in both cases. When external chloride was substituted by nitrate, on the other hand, no change in transport activity was observed. In the presence of the ionophore nigericin, which collapses Na⁺- and K⁺-ion gradients, the astroglial and synaptosomal glutamate uptake was strongly diminished. With the potassium-specific ionophore valinomycin present in the external medium, an approximately 50% stimulation was noted. Since under the experimental conditions chosen here valinomycin is expected to enhance the membrane potential, the astroglial as well as the synaptosomal transport appear to be electrogenic processes. This is in confirmation with the inhibitory effect observed after addition of the proton conductor CCCP which in turn will diminish the membrane potential.

Furthermore Lineweaver-Burk analysis revealed $K_m$ and $V_{max}$ values of 1.8 $\mu$M and 4.9 nmol/min·mg) for the astroglial glutamate uptake, whereas for the synaptosomal membrane preparation a $K_m$ of 7.5 $\mu$M and a
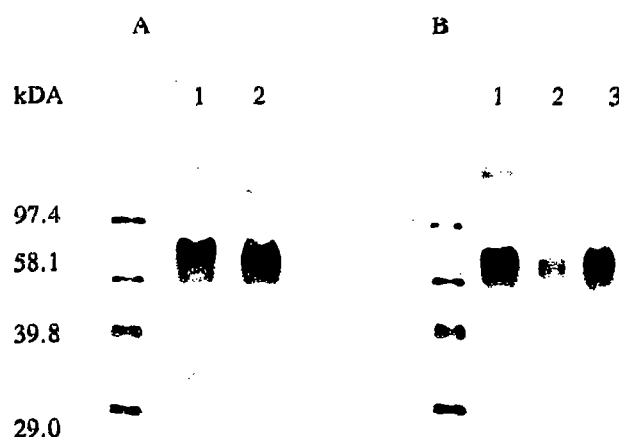
A             B



Fig. 3. Immunoreactivity of total astroglial and synaptosomal membrane proteins and $N$-acetyl-D-glucosamine (NAG) eluate from the WGA column. (A) Membrane vesicles of comparable transport activity from cultured astrocytes (lane 1) and synaptosomes (lane 2) were subjected to SDS-PAGE and immunoblotted with anti-rat glutamate transporter antiserum. (B) NAG eluate of the WGA column of astrocytes (lane 1), synaptosomes (lane 3) and astroglial non-WGA binding proteins ('flow-through fraction') (lane 2) were loaded per each lane with 0.2 $\mu$g of protein and subsequently immunoblotted with anti-rat glutamate transporter antiserum.

## Table I

Inhibition of L-glutamate uptake by substrate analogues

| Compound (25 $\mu$M) | L-[³H]glutamate uptake (% of control) | |
|---|---|---|
| | Astroglial membrane vesicles | Synaptosomal membrane vesicles |
| Control | 100 | 100 |
| | ($K_m$ 1.8 $\mu$M ± 0.4) | ($K_m$ 7.5 $\mu$M ± 0.8) |
| D-Aspartate | 52 ± 4 | 62 ± 5 |
| | ($K_i$ 2.9 ± 0.3)* | ($K_i$ 3.6 ± 0.6)⁺ |
| L-Aspartate | 36 ± 3 | 53 ± 4 |
| | ($K_i$ 0.55 ± 0.09)⁺ | ($K_i$ 3.4 ± 0.3)⁺ |
| DL-Threo-β-hydroxy-aspartate | 49 ± 2 | 25 ± 4 |
| | ($K_i$ 1.4 ± 0.2)⁺ | ($K_i$ 1.9 ± 0.4)⁺ |
| DL-Aspartate-β-hyroxamate | 89 ± 3 | 82 ± 5 |
| | ($K_i$ 87.8 ± 1.4)* | ($K_i$ 18.9 ± 0.5)⁺ |
| Dihydrokainate | 105 ± 3 | 102 ± 6 |
| D-Glutamate | 98 ± 4 | 97 ± 5 |
| Quisqualate | 104 ± 3 | 100 ± 4 |

Preloaded (0.32 M mannitol, 0.12 M KP$_i$, pH 6.8, 0.5 mM K-EDTA, 1 mM MgSO₄) astroglial and synaptosomal membrane vesicles were simultaneously incubated with 20 $\mu$M unlabeled L-glutamate (in addition with 1 $\mu$Ci L-[³H]glutamate) and substrate analogues (25 $\mu$M) for 1 min at 25°C in external solution (0.32 M mannitol, 0.15 M NaCl). The uptake data presented is reported as mean % of control ± S.D. ($n \geq$ 4 sets of triplicate). The values ($\mu$M) for $K_i$ (slope inhibition constants) were computed from experimental data similar to that in Fig. 2, fitted to the equation for linear competitive inhibition (Lineweaver-Burk plot). Symbols indicate: + = competitive or * = noncompetitive inhibition of L-glutamate uptake.

$V_{max}$ value of 6.7 nmol/min·mg were determined, indicating a 4-fold higher affinity of the astrocytic L-glutamate transporter for its physiological substrate (Fig. 2A and B). The reported $K_m$ and $V_{max}$ values were determined from the average of 4–6 experiments, while the plots shown in Fig. 2 are from a single representative experiment.

Hence in their basic features the glutamate uptake systems described here are quite similar to those previously found in astrocyte cultures [7], brain slices [18] or crude rat brain membrane preparations [6].

In order to allow for a more precise pharmacological comparison between the neuronal and glial glutamate transporter, a variety of synthetic glutamate analogues were tested in parallel. Each of the compounds was employed without preincubation and at low concentrations (<25 $\mu$M), to ensure physiological competition with the glutamate binding site. L-trans-Pyrrolidine-2,4-dicarboxylate (L-trans- PDC) a recently synthesized glutamate analogue, which is known to selectively inhibit synaptosomal glutamate transport but not to interfere with glutamate receptor binding [19], inhibited the astroglial and synaptosomal glutamate uptake in a distinctive manner (Fig. 2). In the case of synaptosomal membrane fractions a half-maximal inhibition was already achieved by about 5 $\mu$M L-trans-PDC whereas in astroglial membrane vesicles as much as 25 $\mu$M of this glutamate analogue was necessary to yield the same effect (Fig. 2D). Lineweaver-Burk plots (shown in Fig. 2A and B) clearly demonstrate the concentration-de-
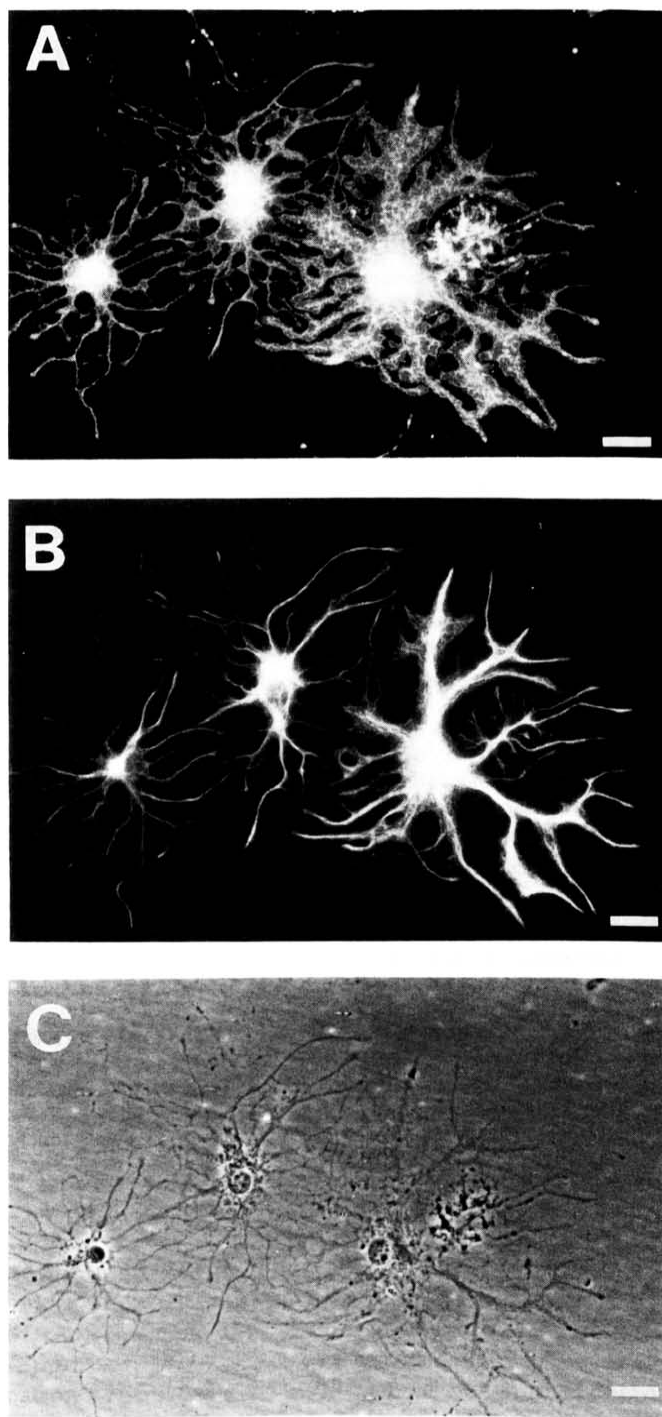
Fig. 4. Double labeling immunostaining of cultured mouse astrocytes using anti-rat glutamate transporter antiserum (A) in conjunction with monoclonal anti-GFAP antibodies (B) and phase contrast views (C). Scale bars 18 $\mu$m.

pendent effect of L-trans-PDC on either transporter, reflecting competitive inhibition in both cases. The $K_i$ values for L-trans-PDC determined by a replot of the slopes gave a value of 1.6 $\mu$M for the astroglial glutamate transport and of 3.3 $\mu$M for the synaptosomal

glutamate transport, respectively (Fig. 2C). The corresponding ratios of $K_m/K_i$ demonstrate a 2–4-fold higher affinity of L-trans-PDC for the synaptosomal than for the astroglial glutamate transporter. Interestingly dihydrokainate, which differs from L-trans-PDC only by the 4-isopropyl and the 3-carboxymethyl side chain, neither showed an inhibitory effect on the astroglial nor on the synaptosomal glutamate transport at these low levels of concentration. The inhibitory effect of dihydrokainate previously reported by others, was measured only in the presence of excessively high amount of this agent [20,21]. Furthermore L-aspartate and DL-threo-hydroxy-aspartate exhibited opposing effects (Table I), while the astroglial transporter reacted much more sensitively with L-aspartate its synaptosomal counterpart interacted more strongly with DL-aspartate-$\beta$-hydroxamate were quite distinct for the astroglial and synaptosomal glutamate uptake. While both compounds acted as competitive inhibitors on the synaptosomal transport in the case of glial glutamate uptake.

$K_m$ as well as $K_{max}$ were affected, indicating a linear non-competitive inhibition. Since DL-aspartate-$\beta$-hydroxamate is a racemate, it is possible that each of the two stereoisomers might inhibit the transporter differently. Quisqualate, a putative inhibitor of $Cl^-$-dependent glutamate uptake [22-23], and D-glutamate in contrast had no effect on the sodium- and potassium-dependent neuronal or glial glutamate uptake system studied here. On the basis of the calculated $K_i$ values given in Table I the following rank order of inhibition capacity of the analogues for the astroglial glutamate uptake is suggested: L-aspartate ($K_i$ 0.55 $\mu$M) > L-trans-PDC ($K_i$=1.1 $\mu$M) $\geq$ DL-threo-$\beta$-hydroxy-aspartate ($K_i$: 1.4 $\mu$M) > D-aspartate ($K_i$: 2.9 $\mu$M) > DL-aspartate-$\beta$-hydroxamate ($K_i$ = 87.8 $\mu$M). For the synaptosomal glutamate transport the rank order of inhibition was determined as follows: DL-threo-$\beta$-hydroxy-aspartate ($K_i$ = 1.9 $\mu$M) $\geq$ L-trans-PDC ($K_i$ = 2.1 $\mu$M) > L-aspartate ($K_i$ = 3.4 $\mu$M) $\geq$ D-aspartate ($K_i$ = 3.6 $\mu$M) > DL-aspartate-$\beta$-hydroxamate ($K_i$ = 18.9 $\mu$M).

As a whole, our data imply that the astroglial and synaptosomal glutamate transporter from mouse cerebral cortex exhibit significant structural differences in their glutamate binding site, whilst appearing more or less identical in their ion dependence. Considering this, it is quite conceivable that the regional heterogeneity recently observed for the pharmacological features of L-glutamate transport in the brain of rat [19] could at least partly be attributed to varying proportions of the glial and neuronal glutamate transport system in different brain areas.

Comparative immunoblot analysis of mouse astroglial membrane proteins using polyclonal antibodies raised against the glial glutamate transporter of rat brain [9] showed a strongly immunoreactive band at about 75 kDa (Fig. 3A, lane 1), which is very close to the value reported for the purified rat brain transporter

[13]. This compound was highly enriched in the WGA-binding glycoprotein fraction, containing the glutamate transport activity (Fig. 3B, lane 1), whereas the non-WGA-binding proteins (flow-through fraction) of astroglial membranes, exhibiting only a weak transport activity were just faintly immunolabeled (Fig. 3B, lane 2). In the synaptosomal membrane protein fraction an immunostained band of corresponding electrophoretic mobility was revealed too (Fig. 3A, lane 2; and B, lane 3). This could be due to a glial contamination of the synaptosomal membrane preparation, but we cannot exclude the possibility of some immunological crossreactivity with a neuronal form of the glutamate transporter or the presence of a common molecular subunit. Accordingly it could be envisaged that the transport activity of the neuronal glutamate carrier in the synaptosomal membrane preparation was partly masked by those of the glial carrier. Hence it is likely that the pharmacological differences between both transporter subtypes described here on a molecular level may be even more pronounced. It is also conceivable that differences in the microenvironment of both transporters might have contributed to the divergent pharmacological behaviour. It is worth mentioning in this context that in the case of the neuronal and glial GABA-transport system two proteins with distinct pharmacological and even antigenic properties were revealed [24,25].

Double-labeling immunostaining of cultured cortical mouse astrocytes using monoclonal anti-GFAP antibodies in conjunction with the above-mentioned polyclonal anti-rat glutamate transporter antiserum revealed that virtually all of the astrocytes were intensely labelled with the anti-glutamate transporter antiserum; typically the immunofluorescence was not homogeneously distributed over the plasma membrane but exhibited an irregularly arranged patchy network of staining.

In future a detailed comparison of the two glutamate transporter molecules on the level of cDNA-sequencing will be necessary to yield more precise information as to the molecular structures underlying the divergent pharmacology of either glutamate carrier.

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