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High Affinity Glutamate Transport in Rat Cortical Neurons in Culture

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ABSTRACT

We assayed glutamate transport activity in cultures of rat cortical neurons containing <0.2% astrocytes. Using [^{3}H]L-glutamate as the tracer, sodium-dependent high affinity glutamate transport was demonstrated [K_m = 17.2 ± 2.4 μM; V_{max} = 3.3 ± 0.32 nmol/mg of protein/min (n = 5)]. Dihydrokainate (1 mM) inhibited uptake of radioactivity by 88 ± 3% and had a K_i value of 65 ± 7 μM. L-α-Aminoadipate (1 mM) inhibited uptake by only 25 ± 4%. L-*trans*-2,4-Pyrrolidine dicarboxylate, L-serine-*O*-sulfate, and kainate potently inhibited transport activity with K_i values of 5.1 ± 0.3, 56 ± 6, and 103 ± 9 μM, respectively (n = 3). Voltage-clamp studies of GLT1-expressing oocytes showed that, as in cortical neurons, glutamate transport was not inhibited by L-α-aminoadipate. Dihydrokainate was a potent inhibitor (K_i = 8 ± 1 μM), and L-serine-*O*-sulfate produced a GLT1-mediated current with a K_m value of 312 ± 33 μM. Immunoblot analysis showed that neuronal cultures express excitatory amino acid carrier 1 (EAAC1), shown previously to be relatively insensitive to dihydrokainate, plus a trace amount of GLT1, but no GLAST. These studies establish that a major component of the glutamate transport activity of cortical neurons is dihydrokainate sensitive and distinct from the previously recognized neuronal transporter excitatory amino acid carrier 1.

The physiology of neurotransmitters depends on clearance mechanisms to maintain low concentrations of transmitter in the extracellular space. Excitatory neurotransmission presents a special case because the excitatory neurotransmitters glutamate and aspartate are actually potent neurotoxins, requiring efficient clearance mechanisms to be present not only for the normal function of synapses but also for the survival of neurons. It has been shown that glutamate uptake provides remarkable protection against glutamate neurotoxicity *in vivo* (Mangano and Schwarcz, 1983) as well as *in vitro* (Rosenberg and Aizenman, 1989; Rosenberg *et al.*, 1992; Robinson *et al.*, 1993a). It would be desirable to be able to understand at a molecular level how the various glutamate transporters participate in normal excitatory neurotransmission as well as in protecting neurons against excitotoxicity, but to do this, it is necessary to know all the transporters that might be involved.

Pharmacological evidence suggests that glutamate uptake in the central nervous system is not a uniform process (Ferkany and Coyle, 1986; Robinson *et al.*, 1991). Specifically, glutamate uptake into cortical and striatal synaptosomes was potently inhibited by DHK but not by L-α-AA, the inverse pattern of that observed with cerebellar synaptosomes. The suggestion that glutamate transport is heterogeneous in the brain was proved by the cloning of different glutamate transporters, designated GLT1 (Pines *et al.*, 1992), GLAST (Storck *et al.*, 1992), EAAC1 (Kanai and Hediger, 1992), and EAAT4 (Fairman *et al.*, 1995). In addition, recently a fifth glutamate transporter was cloned, EAAT5, with expression predominantly in the retina but little in the brain (Arriza *et al.*, 1997). Immunocytochemical studies have shown that GLT1 and GLAST are expressed primarily in astrocytes (Rothstein *et al.*, 1994). EAAC1 normally is a neuronal transporter with a somatodendritic localization; it is not present in excitatory presynaptic terminals (Rothstein *et al.*, 1994). Thus, available information suggests that if excitatory presynaptic terminals possess one or more glutamate transporters, which is likely (Gundersen *et al.*, 1993), these transporters may not have been identified.

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ABBREVIATIONS: DHK, dihydrokainate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SOS, L-serine-*O*-sulfate; L-α-AA, L-α-aminoadipate; NMDA, *N*-methyl-D-aspartate; PDC, L-*trans*-2,4-pyrrolidine dicarboxylate; GFAP, glial fibrillary acidic protein; BOAA, b-*N*-oxalyl-L-a,b-diaminopropionate; AMG, α-methyl-DL-glutamate; KA, kainate; SDS, sodium dodecyl sulfate; GLAST, glutamate/aspartate transporter; EAAC, excitatory amino acid carrier; EAAT, excitatory amino acid transporter.

Glutamate transport in cortical synaptosomes is inhibited potently by DHK and SOS but not by L-α-AA (Robinson *et al.*, 1993b). Paradoxically, the neuronally expressed EAAC1 (Kanai and Hediger, 1992; Rothstein *et al.*, 1994) is relatively insensitive to DHK (Arriza *et al.*, 1994; Dowd *et al.*, 1996). In contrast, the glial transporter GLT1 is inhibited potently by DHK (Pines *et al.*, 1992) but is not thought to be expressed significantly in cortical neurons (Rothstein *et al.*, 1994). Because cortical synaptosomes are contaminated with glial membrane vesicles (Robinson *et al.*, 1993b), the question remains whether the DHK-sensitive glutamate transport in cortical synaptosomes is associated with contaminating glia or is a bonafide component of uptake into neurons.

In this study, we took advantage of selective culturing techniques to produce nearly pure neuronal cultures (<0.2% astrocytes). We found that glutamate transport in these cultures exhibited a pharmacological profile similar to that of cortical synaptosomes, suggesting that the dominant transport activity in cortical synaptosomes is of neuronal origin and that cortical neurons therefore possess a novel glutamate transport activity. An abstract has appeared reporting these results in a preliminary form (Wang *et al.*, 1996).

Materials and Methods

Cultures. Neuronal cultures were prepared from embryonic day 16 Sprague-Dawley rat fetuses using methods similar to those described previously (Rosenberg, 1991). Cultures were initially plated onto poly-L-lysine-coated 24-well plastic plates (Costar, Cambridge, MA) using an 80:10:10 (v/v) mixture of Dulbecco's modified Eagle's medium (11960–010; GIBCO, Grand Island, NY), Ham's F-12 (N-4888; Sigma Chemical, St. Louis, MO), heat-inactivated iron supplemented calf serum (A2151; Hyclone Laboratories, Logan, UT), containing 2 mM glutamine, 25 mM HEPES, 24 units/ml penicillin, and 24 μg/ml streptomycin in a 5% CO_2 (balance air) incubator at 36°. Cell proliferation was inhibited by exposure to 5 μM cytosine arabinoside at 24 hr *in vitro* for 72 hr. On the fourth day of culture, the medium was completely removed and replaced with 90% minimal essential medium, 10% NuSerum IV (Collaborative Research, Bedford, MA), 2 mM glutamine, 5 mM HEPES, containing 10 μg/ml superoxide dismutase (Boehringer-Mannheim Biochemicals, Indianapolis, IN), 1 μg/ml catalase (CV-40; Sigma), 11 mM total glucose, and 9.3 mM total sodium bicarbonate, plus 2% B27 supplement (17504–036; GIBCO). Medium was not changed subsequently. To prevent evaporation of water, culture dishes were kept on 60-mm "wet dishes" containing a filter paper circle that was always kept wet. The percentage of astrocytes in these cultures was determined by counting cell nuclei, labeled by bisbenzamide, yielding total cells, and GFAP-positive cells (astrocytes) across the longitudinal and horizontal diameters of the coverslip with a total of approximately 25 microscopic fields counted/coverslip using cultures at 3 to 4 weeks. Only 3 ± 1 GFAP-positive cells were found of the total of 2013 ± 144 cells counted in three experiments, with each run in triplicate. Thus, these cultures contained <0.2% astrocytes and will subsequently be called "neuronal cultures." Neurons were identified by morphology, which has been shown previously by staining with tetanus toxin and electrophysiology to be a reliable guide to neuronal identification in a similar culture system (Rosenberg, 1991). In addition, we made positive identification of neurons using a cocktail of antineurofilament monoclonal antibodies. In three separate experiments, we found that 92.8 ± 2.4% (mean ± standard error) of total cells were positively labeled by anti-neurofilament antibody cocktail (SMI 311; Sternberger Monoclonals, Baltimore, MD). Using this technique, some neurons cannot be identified positively because they are in an aggregate or are weakly labeled. These cells are likely to be neurons on the basis of their size, shape, and nuclear morphology. Therefore, it is our impression that use of antineurofilament antibodies results in an underestimate of the number of neurons that are present.

Uptake studies. Cortical neurons in culture are vulnerable to excitotoxicity mediated by NMDA and non-NMDA receptors. To study glutamate transport independent of the complicating effects of excitotoxicity, in all experiments we included the NMDA receptor antagonist MK-801 (10 μM) (Wong *et al.*, 1986). Non-NMDA receptor activation also may produce reversible and irreversible forms of neuronal injury but typically require longer exposure to agonists to become manifest (Koh and Choi, 1988). In experiments examining the concentration dependence of glutamate transport, in which high concentrations of glutamate were used, the noncompetitive non-NMDA receptor antagonist GYKI 52466 (Le Peillet *et al.*, 1992) at 10 μM was incorporated into the assay in addition to MK-801. Previous studies have shown that glutamate uptake in cultured astrocytes is linear for ≤5 min, and we found this to be true in neuronal cultures as well from 0.5 μM to 500 μM L-glutamate. In all experiments, therefore, we chose a 5-min exposure to ensure that initial uptake rates were being measured. At 5 min, at 0.5 μM L-glutamate, 84 ± 3% (three experiments) of the original radioactivity was still present in the medium. We found that the sodium independent uptake at 5 min in 0.5 μM L-glutamate was <1% of the total.

For the investigation of glutamate transport into cultures derived from cerebral cortex, we followed the procedures of Garlin *et al.* (1995). To isolate sodium-dependent transport, cultures were exposed to tritiated substrate in the presence (sodium buffer) or absence (choline buffer) of sodium, and the radioactivity associated with the cultures in the absence of sodium was subtracted from that associated with the cultures in the presence of sodium. Cultures grown onto 24-well plates were washed twice (1 ml/well) with sodium or choline buffer (at 37°) as appropriate. Cultures then were exposed to the test conditions at 37° in the air for 5 min in the presence of 0.5 μM [^{3}H]L-glutamate (final specific activity, 80 nCi/nmol) (TRK445; Amersham, Arlington Heights, IL; specific activity, 46 Ci/mmol) plus 10 μM MK-801 and 10 μM GYKI 52466 (0.5 ml/well) with gentle shaking. The assay was stopped by the addition of choline buffer containing 1% bovine serum albumin (measured temperature, 1–2°C) and washing for a total of three times. After removal of the third wash, the cultures were solubilized by the addition of 0.5 ml/well 10% sodium dodecyl sulfate, which was transferred into liquid scintillation vials, followed by a single 0.5-ml wash with distilled water, after which radioactivity was assayed by liquid scintillation counting.

The physiological saline used was composed of 140 mM NaCl or choline chloride, 2.5 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM K_2HPO_4, 10 mM glucose, 5 mM Tris base, and 10 mM HEPES, pH 7.4 (osmolality, 300 mOsmol).

Electrophysiology. Capped RNA was transcribed from linearized plasmid containing the coding region of rat GLT1 (Pines *et al.*, 1992) (clone 81; a gift of Dr. B. Kanner) using T7 polymerase (Boehringer-Mannheim). RNA (50 ng) was injected into stage V oocytes, and experiments were performed 2–6 days later.

Transporter-mediated currents were recorded using a two-microelectrode voltage-clamp circuit (Arriza *et al.*, 1994). Oocytes were superfused continually with Ringer's solution, pH 7.5, containing 96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, and 5 mM HEPES. The concentration-responses of the current (I) induced by L-glutamate and analogs were fitted by least-squares to the equation $I = I_{max}([S]/[S] + K_m)$, where [S] is the concentration of amino acid applied, and K_m and I_{max} values represent the mean ± standard error from fits to individual oocytes. The concentration dependence of the transport antagonist DHK was determined from the inhibition of the current induced by 10 or 100 μM L-glutamate at −60 mV by coapplication of DHK at concentrations of 1–100 μM according to the equation: Inhibition (%) = 100 [[DHK]/([DHK] + IC_{50})]. IC_{50} values were used to estimate the K_i value of DHK according to the Cheng-Prusoff equation: $K_i = IC_{50}/(1 + [\text{L-glutamate}]/K_{m\text{Glu}})$.

Data analysis. Experiments were conducted under conditions of initial velocity, and concentrations of excitatory amino acids did not change significantly during the incubation. Data from experiments determining K_m values for excitatory amino acid uptake were plotted by nonlinear regression using Prism software (GraphPAD Software, San Diego, CA). This analysis demonstrated that these data obtained in the concentration range from 50 nM to 500 μM (30 times the K_D value) were best fitted by a single-site Michaelis-Menten model.

Inhibition constants were calculated from IC_{50} values using the equation described by Cheng and Prusoff. In this equation, the K_m value used was the K_m value for transport, and the concentration of substrate was 0.5 μM.

Statistical comparisons were accomplished by analysis of variance with the *post hoc* Tukey-Kramer multiple comparisons test using the InStat2 program (GraphPAD). When descriptive statistics are provided in the text or tables summarizing transport data (K_m, K_i, V_{max} values) from multiple experiments, the mean ± standard error value is given. Otherwise, the mean ± standard deviation value is used.

Immunoblot analysis. Peptide-specific antibodies raised against the glutamate transporters EAAC1, GLAST, and GLT1 were kindly provided by Dr. Jeffrey Rothstein (The Johns Hopkins University, Baltimore, MD). A previously published protocol for immunoblot analysis of glutamate transporters was followed with some modifications (Rothstein *et al.*, 1994). Briefly, cells were washed once in buffer containing 5 mM $MgCl_2$, 5 mM EGTA, 50 mM KCl, 0.1 mM dithiothreitol, 17 μg/ml leupeptin, and 10 μg/ml phenylmethylsulfonyl fluoride in 20 mM Tris·HCl, pH 7.4, and then scraped off the culture plate, spun down in an Eppendorf microcentrifuge at 15,000 rpm for 4 min at 4°, and homogenized with a Teflon homogenizer using Eppendorf tubes in the same buffer. The homogenate was centrifuged again in an Eppendorf microcentrifuge at 15,000 rpm for 10 min at 4°. The pellet (membrane fraction) was dissolved in 50–100 μl of 1% SDS and stored at −80°. The protein content of the pellet was determined according to a modified Lowry method (Markwell *et al.*, 1981). Aliquots of membrane proteins (10 μg) were subject to SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes (DuPont-New England Nuclear Research Products, Boston, MA) by electroblotting (200 mA, overnight). Blots were blocked for 1 hr in blocking buffer containing 1% nonfat dry milk and 0.1% Tween 20 in TBS (consisting of 50 mM Tris, 200 mM NaCl, pH 7.4) at room temperature and incubated for 1 hr with anti-GLT1, anti-GLAST, or anti-EAAC1 antibodies diluted to 0.01, 0.08, or 0.3 μg/ml, respectively, in blocking buffer. Blots were then washed with blocking buffer five times for 5 min, incubated for 1 hr with horseradish peroxidase-conjugated donkey anti-rabbit IgG (Amersham; 1:2500 in blocking buffer), and washed with blocking buffer five times for 5 min. The immunoreactive proteins were visualized with enhanced chemiluminescence (DuPont-New England Nuclear Research Products).

To confirm that protein loading was the same in all lanes, gels were stained for protein after transfer with a silver method (Daiichi Silver Stain SE140001; Integrated Separation Systems, Natick, MA). In addition, immunoblots were stained for protein after enhanced chemiluminescence detection with colloidal gold (170–6517; BioRad, Hercules, CA).

Immunocytochemistry. Neuronal cultures grown on 12-mm glass coverslips were rinsed with Hanks' balanced salt solution; fixed in 4% paraformaldehyde (P-6148; Sigma) in phosphate-buffered saline, pH 7.4, for 10 min at room temperature; and washed three times with TBS. Following washes cells were preincubated for 30 min at room temperature with TBS containing 5% normal goat serum and 0.1% Triton X-100. Cells were incubated overnight at 4° with antibodies against EAAC1, GLAST, and GLT1 diluted in the preincubation solution (anti-EAAC1, 0.1 μg/ml; anti-GLAST, 0.4 μg/ml; anti-GLT1, 0.7 μg/ml). After four washes with TBS plus 0.1% Triton X-100, cells were incubated for 30 min at room temperature with tetramethylrhodamine isothiocyanate-conjugated secondary antibody, also in preincubation solution (1:200; goat anti-rabbit IgG; Sigma). Cells were washed four more times with TBS plus 0.1% Triton X-100 and mounted with Fluoromount-G (Southern Biotechnology Associates, Birmingham, AL). Controls were performed without the primary antibodies and showed no staining. The cells were examined and photographed using fluorescence and phase contrast microscopy. For most experiments, we used antibodies raised against the carboxyl termini of EAAC1 (cEAAC1) and GLT1 (cGLT1) and the amino terminus of GLAST (nGLAST) to detect the expression of EAAC1, GLAST, and GLT1 in our cultures. Antibodies raised against the amino termini of EAAC1 (nEAAC1) and GLT1 (nGLT1) and the carboxyl terminus of GLAST (cGLAST) were found to result in staining in cortical cultures similar to that of cEAAC1, cGLT1, and nGLAST, except nGLT1 also stained cytoplasmic fibrous networks in astrocytes.

Cell-marking studies were done using a polyclonal anti-GFAP (1:400; Sigma) to label astrocytes or a cocktail of monoclonal anti-panneurofilament antibodies (SMI 311, 1:200; Sternberger Monoclonals).

Results

Kinetics of glutamate transport in neuronal cultures. Uptake of [^{3}H]L-glutamate into neuronal cultures was measured at increasing concentrations of total glutamate, with the amount of radioactivity maintained at a constant level (Fig. 1A), and was found to approach saturation at 500

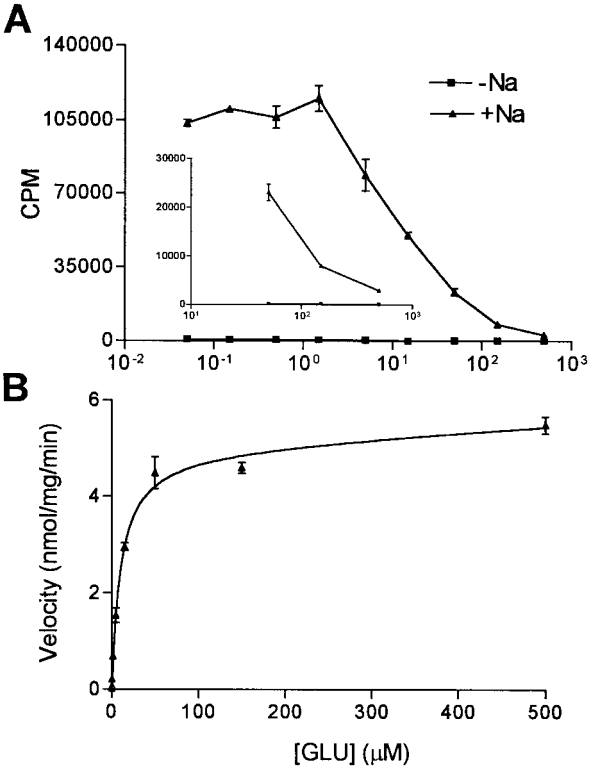

Fig. 1. Concentration dependence of high affinity sodium-dependent [^{3}H]L-glutamate transport in cortical neuronal cultures. Neuronal cultures were exposed to selected concentrations of [^{3}H]L-glutamate ranging from 50 nM to 500 μM for 5 min in the presence or absence of sodium, after which they were washed and solubilized in SDS, and radioactivity associated with the cells was assayed by liquid scintillation. A, Effect of increasing L-glutamate concentration on uptake of radioactivity by neuronal cultures. ■, Data obtained in the absence of sodium; ▲, data obtained in the presence of sodium. B, Effect of increasing L-glutamate concentration on total uptake of L-glutamate. Data were fit to a curve by nonlinear regression analysis, indicating best fit by a one-site model. In this experiment, the high affinity site had a K_m value of 11.2 μM and a V_{max} value of 5.0 nmol/mg of protein/min.

μM (Fig. 1B). Nonlinear regression analysis showed that total glutamate uptake (Fig. 1B) could best be described as a single process. In five experiments, kinetic parameters were determined. The K_m value for glutamate transport was 17.2 ± 2.4 μM, and the V_{max} value was 3.3 ± 0.3 nmol/mg of protein/min (mean ± standard error)

Potency of inhibitors of transport. To characterize pharmacologically glutamate transport in the neuronal cultures and compare this activity with that reported by others for synaptosomes and glial cultures and in systems expressing cloned transporters, several inhibitors of glutamate transport were tested; these included DHK and L-α-AA, a pair of inhibitors that together distinguish glutamate transport by cortical synaptosomes, cerebellar synaptosomes, and GLT1. In addition, several other inhibitors were tested that are potent inhibitors of some or all of the known cloned transporters, including PDC (Bridges *et al.*, 1991), KA, SOS, and others that have been shown to display a ≥10-fold difference in potency against cortical versus cerebellar transport (BOAA and AMG) (Robinson *et al.*, 1993b). Of these inhibitors, only DHK, PDC, KA, and SOS produced >90% inhibition at 1 mM. These were studied further in complete concentration-dependent experiments (Fig. 2 and Table 1).

In all experiments with inhibitors, a glutamate concentration of 0.5 μM was used. DHK was found to be a potent inhibitor, with a mean K_i value in five experiments of 65 ± 7 μM. A representative experiment with an IC_{50} value of 45 μM is shown in Fig. 2A. PDC was tested in three experiments and found to have a mean K_i value of 5.1 ± 0.3 μM. A representative experiment with an IC_{50} of 5.7 μM is shown in Fig. 2B. KA has been shown to be a potent inhibitor of EAAT2 (Arriza *et al.*, 1994). In addition, it is a potent inhibitor of glutamate uptake into both cerebellar and cortical synaptosomes (Garlin *et al.*, 1995). In these preparations, KA inhibition revealed two components, a KA-sensitive component, accounting for 83% and 71% of transport in the cerebellar and cortical synaptosomes, respectively, and a KA-insensitive component. KA was shown to have a mean K_i value in three experiments of 103 ± 9 μM. A representative experiment is shown in Fig. 2C, in which an IC_{50} value of 95 μM was found. SOS, which has been shown to be a more potent inhibitor of EAAT1 and EAAT3 than of EAAT2 (Arriza *et al.*, 1994), was found to be a potent inhibitor with a mean K_i value in neuronal cultures of 56 ± 6 μM. A representative experiment is shown in Fig. 2D, in which an IC_{50} value of 49 μM was found. The K_i values obtained in neuronal cultures are summarized in Table 1.

Other inhibitors that were tested include L-α-AA, BOAA, and AMG, all at a concentration of 1 mM. L-α-AA inhibited uptake by only 25 ± 4% (four experiments). BOAA and AMG did not significantly inhibit transport at a concentration of 1 mM.

The sensitivity of glutamate transport to DHK is seen in only one of the known cloned glutamate transporters, GLT1. Thus, we were especially interested in other similarities and differences between the observed neuronal transport activity and GLT1. Because we found that neuronal transport was not inhibited significantly by L-α-AA, it was important to know the sensitivity of GLT1 to this compound. This point is unclear from the literature; Pines *et al.* (1992) reported that GLT1 expressed in HeLa cells was sensitive to L-α-AA (71% inhibition at 20 μM), whereas Arriza *et al.* (1994) reported

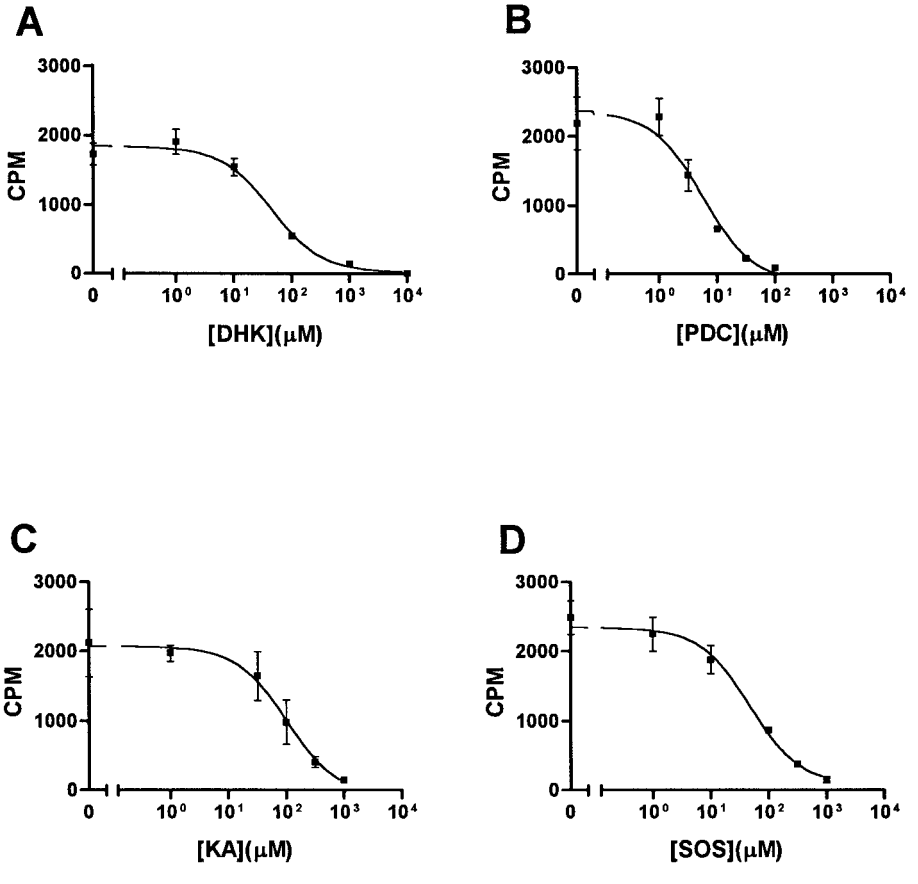

Fig. 2. Effect of transport inhibitors on [^{3}H]L-glutamate uptake in cortical neuronal cultures. Neuronal cultures were exposed to inhibitors plus 0.5 μM [^{3}H]L-glutamate in the presence or absence of sodium for 5 min, after which they were washed and solubilized, and radioactivity associated with the cells was assayed by liquid scintillation. In each case, results are representative of at least three experiments (see Table 1). The IC_{50} values obtained in each experiment shown are given. A, Effect of DHK on [^{3}H]L-glutamate uptake. The IC_{50} value in this experiment was 45 μM. B, Effect of PDC on [^{3}H]L-glutamate uptake. The IC_{50} value in this experiment was 5.7 μM. C, Effect of KA on [^{3}H]L-glutamate uptake. The IC_{50} value in this experiment was 95 μM. D, Effect of SOS on [^{3}H]L-glutamate uptake. The IC_{50} value in this experiment was 49 μM.

TABLE 1

K_i or K_m values (micromolar) for DHK, L-α-AA, PDC, KA, and SOS interaction with glutamate transport systems

	DHK	L-α-AA	PDC	KA	SOS
Neuronal cultures (rat; this study)	65 ± 7 (n = 5)	>1 mM	5 ± 0.3 (n = 3)	103 ± 9 (n = 3)	56 ± 6 (n = 3)
Cortical synaptosomes (rat)[a]	110	720	2.1	72 (71%; IC_{50})	2.6 (26%; IC_{50})
				3,500 (21%; IC_{50})	55 (74%; IC_{50})
GLT1 (rat; this study)	8 ± 1 (n = 3)	>1 mM			312 ± 33 (n = 3)
GLT[b,e] (rat)	3	8	0.7		
EAAC[f] (rat)	1120	>1 mM	14	>1 mM	
EAAT1[c,e] (human)	>3 mM	>1 mM	79	>3 mM	107
EAAT2[c,e] (human)	23	>1 mM	8	59	1157
EAAT3[c,e] (human)	>3 mM	>1 mM	61	>3 mM	150
EAAT4[d,e] (human)		170	2.6	>5 mM	

[a] Garlin et al., (1995); [b] Pines et al., (1992); [c] Arriza et al., (1994); [d] Fairman et al., (1995); [e] Robinson and Dowd (1996); [f] Dowd et al., (1996).

that the human homolog of GLT1, EAAT2, was insensitive to L-α-AA at 1 mM. Therefore, we tested the sensitivity of GLT1 to L-α-AA as well as other inhibitors in an oocyte expression system (Fig. 3 and Table 1).

Effects of DHK, SOS, and L-α-AA on GLT1 expressed in oocytes. Both SOS and L-glutamate induced voltage-dependent currents in oocytes expressing GLT1 (Fig. 3, A and B). At −60 mV, the K_m value for the current induced by SOS was 312 ± 33 μM, and the maximum current was 78 ± 10% of the maximum current induced by L-glutamate (three experiments; Fig. 3C). The K_m value for L-glutamate was 21 ± 3 μM (three experiments).

A concentration of 300 μM L-α-AA induced a current in oocytes expressing GLT1 that was <2% of the current induced by the same concentration of L-glutamate, suggesting that it is not transported with high affinity (Fig. 3, A and B). To examine whether L-α-AA might act as a nontransported inhibitor, it was coapplied with L-glutamate to cells expressing GLT1 that were voltage-clamped at −60 mV. Superfusion of 1 mM L-α-AA with 10 μM L-glutamate caused an 11 ± 2% reduction of the current seen in the absence of L-α-AA (three experiments). These data are consistent with a low affinity interaction with GLT1, similar to results with the corresponding human subtype EAAT2 (K_i> = 1 mM) (Arriza *et al.*, 1994).

Similar to its actions on currents mediated by the human transporter EAAT2 (Arriza *et al.*, 1994), DHK inhibited currents mediated by GLT1 without inducing a current (Fig. 3D). The IC_{50} value of DHK for inhibition of the GLT1 current induced by 10 μM glutamate was 12 ± 1 μM (three experiments). Assuming a competitive interaction with L-glutamate, this indicates that the K_i value for DHK binding to GLT1 is 8 ± 1 μM (see Materials and Methods). The K_i and K_m values obtained for glutamate transport inhibitors interacting with GLT1 expressed in oocytes are summarized in Table 1.

Immunoblot analysis of glutamate transporters in neuronal cultures. We determined the expression of glutamate transporters in neuronal cultures by using antibodies raised against known glutamate transporters. The expression of glutamate transporters was compared in neuronal cultures and in conventional mixed cultures of neurons and astrocytes. The mixed cultures were prepared according to a published procedure from the same cell suspension as was

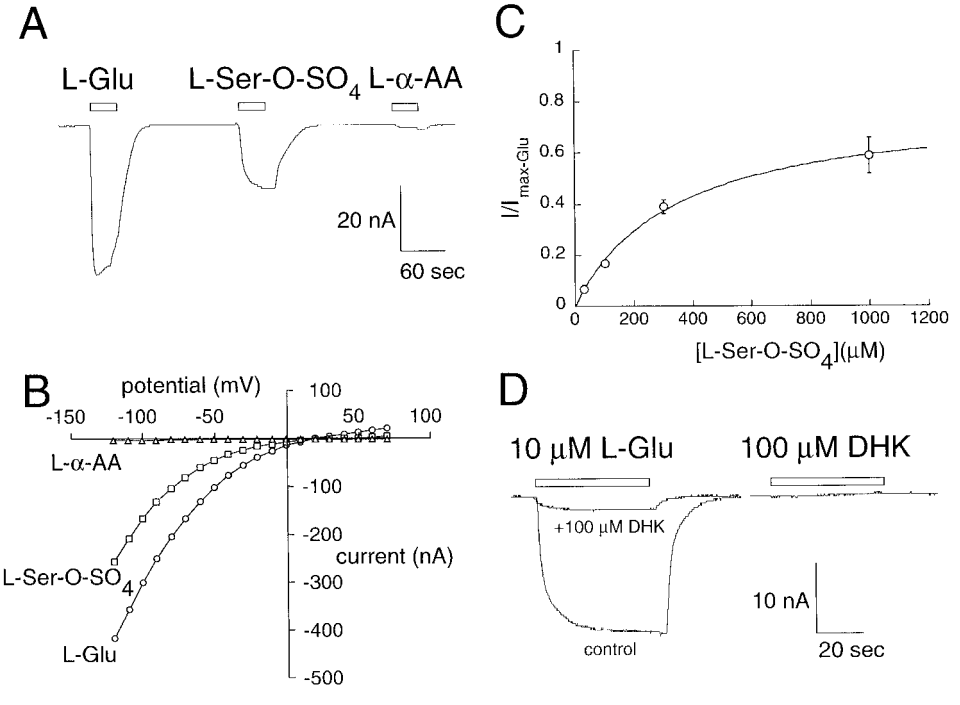

Fig. 3. Electrophysiological analysis of SOS, L-α-AA, and DHK actions on GLT1. A, Superfusion of L-glutamate, SOS, and L-α-AA (all at 300 μM) onto a representative oocyte expressing GLT1 for the duration (*bars above trace*). No currents were seen in uninjected control oocytes (data not shown). Membrane potential was −60 mV. B, Voltage dependence of currents induced by application of the same compounds. Steady state currents follow a 500-msec pulse to the indicated potential. Similar results were seen in three cells. C, Concentration dependence of currents induced by SOS. Currents were normalized to the maximum current (at −60 mV) induced by L-glutamate in the same cells (K_m = 312 ± 33 μM, relative I_{max} = 0.78 ± 0.10; three experiments). D, Coapplication of 100 μM DHK and glutamate. DHK inhibited the current induced by 10 μM L-glutamate (*left*), whereas application of DHK alone did not induce a current in the same oocyte.

used to prepare the neuronal cultures (Rosenberg, 1991). Mixed cultures contain ~95% astrocytes and ~5% neurons (Rosenberg, 1991) and were used as a positive control and basis for comparison in preference to astrocyte cultures because the latter express little GLT1 when cultured without neurons (Swanson *et al.*, 1997). With an equal loading of membrane proteins extracted from neuronal cultures and astrocyte-rich cultures (10 μg/lane), it was found that EAAC1 was expressed strongly in neuronal cultures and less so in mixed cultures of neurons and astrocytes, which is consistent with EAAC1 localization primarily in neurons (Fig. 4, *EAAC1*). The glial transporter GLAST was not detected in neuronal cultures but was strongly expressed in astrocyte-rich cultures (Fig. 4, *GLAST*). A doublet of immunoreactivity appearing in diffuse bands sometimes was observed. Previous work has shown that GLAST immunoreactivity appears in a wide band, as is the case with GLT1 and EAAC1, which is consistent with significant and heterogeneous glycosylation of the proteins (Rothstein *et al.*, 1994). Finally, the glial transporter GLT1 was present in only trace amounts in neuronal cultures compared with astrocyte-rich cultures (Fig. 4, *GLT1*). The apparent molecular masses of EAAC1, GLAST, and GLT1 relative to markers were similar to the values of ~69, ~65, and ~73 kDa, respectively, found previously (Rothstein *et al.*, 1994).

Immunocytochemistry. In neuronal cultures (Fig. 5A), anti-GLT1 stained the rare astrocytes that are present in such cultures (Fig. 5B). Fig 5, A and B, shows phase contrast and fluorescence microscopic views, respectively, of the same field. Neuronal labeling was very weak and diffuse, as can be seen in neurons surrounding the central glial cell in Fig. 5B (*small arrows*). Rarely, intracellular labeling of neuronal cell bodies was seen, as in Fig. 5B near the top of the photograph (*arrowhead*). Anti-GLAST also labeled astrocytes in neuronal cultures, but it did not label neurons (Fig. 5C). Anti-EAAC1 strongly labeled neurons in neuronal cultures. The antibody labeled both neuronal cell bodies and neuronal processes with a punctate appearance (Fig. 5D). No cells with a glial morphology in these cultures were labeled by anti-EAAC1.

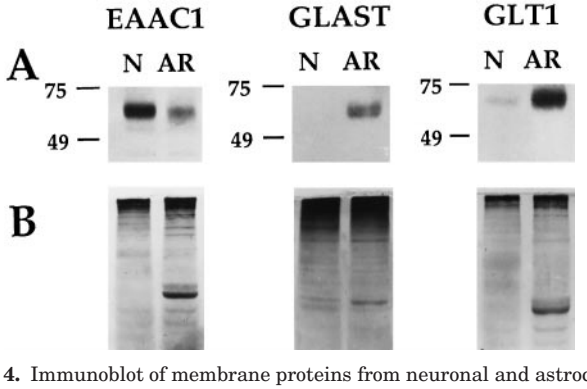

Fig. 4. Immunoblot of membrane proteins from neuronal and astrocyte-rich cultures using antibodies against glutamate transporters. A, Aliquots of membrane proteins (10 μg/lane) were obtained from neuronal (*N*) cultures. For comparison and as a positive control, membrane proteins (10 μg/lane) from astrocyte-rich (*AR*) cultures were run in the same experiment for each antibody tested. Proteins were subjected to SDS-polyacrylamide gel electrophoresis and immunoblotted with antibodies raised against the indicated glutamate transporter subtypes: the carboxyl-terminal peptide of EAAC1, the carboxyl-terminal peptide of GLT1, and the amino-terminal peptide of GLAST. B, Silver staining of the gels used to prepare the immunoblots shown in *A*, after transfer, showing the equivalence of loading of protein in each "neuron/astrocyte-rich" pair.

Discussion

The data presented in this report characterize the pharmacology of glutamate transport into cortical neurons in culture. Several glutamate transport inhibitors have proved to be useful in distinguishing regional differences in glutamate transport and between different cloned glutamate transporters. These inhibitors have been used here to test for similarities and differences between glutamate transport by cortical neurons and by the cloned transporters and synaptosomal preparations. The most important observations are that glutamate transport into cortical neurons can be demonstrated and that this transport is inhibited by DHK, with a K_i value of 65 μM. This sensitivity to DHK sets glutamate transport in cortical neurons apart from all the other cloned transporters except for GLT1; therefore, we sought to clarify the pharmacology of GLT1 to better understand the molecular basis of glutamate transport in cortical neurons. We found in voltage-clamp studies using oocytes expressing GLT1 that, in agreement with Arriza *et al.* (1994) in their work with EAAT2, the human homolog, L-α-AA was ineffective as an inhibitor against GLT1 (Table 1). In addition, we determined a K_m value for SOS-induced current mediated by GLT1 of 312 ± 33 μM. As expected, DHK produced no current of its own and inhibited the glutamate-induced current with a K_D value of 8 ± 1 μM. Interestingly, the potency of DHK against GLT1-mediated glutamate transport is significantly greater than its potency against glutamate transport into cortical neurons (8 versus 65 μM), and the potency of SOS is significantly less (312 versus 56 μM). These differences pharmacologically distinguish the behavior of cloned GLT1 from that of the glutamate transport activity in cortical neurons.

Although GLT1 is expressed in the neuronal cultures, it is expressed weakly compared with mixed cultures of neurons and astrocytes (Fig. 4). This is consistent with previous observations using immunogold immunocytochemistry showing ~10% expression of GLT1 on synaptic membranes compared with adjacent astrocyte membranes (Chaudhry *et al.*, 1995). Studies using *in situ* hybridization have demonstrated the presence of GLT1 mRNA in neurons (Torp *et al.*, 1995; Schmitt *et al.*, 1996); therefore, our observation of weak expression of GLT1 in neurons is not novel. The fact that GLT1 is expressed at all in neurons leaves open the possibility that it contributes in a significant way to the transport observed.

Another possibility is that glutamate transport into cortical neurons is mediated by EAAC1, which is abundantly expressed in these cultures, as revealed here by immunoblot and immunocytochemical studies. However, thorough examination of the pharmacology of EAAC1 in oocytes (Dowd *et al.*, 1996) suggests that the neuronal transporter EAAC1 may contribute little to the net transport activity of either cortical synaptosomes or cortical neurons in culture because transport in both preparations is inhibited at a significantly lower concentration (K_i = 110 and 65 μM, respectively) of DHK than EAAC1 expressed in *Xenopus laevis* oocytes (K_i = 1120 μM) (Dowd *et al.*, 1996). The human homolog of EAAC1, EAAT3, is even less sensitive, if at all, to DHK (Table 1). Thus, the behavior of neither of the cloned glutamate transporters shown to be expressed in neurons (EAAC1 and GLT1) matches the pharmacology of glutamate uptake into these cells. On the other hand, the pharmacological signature we found for glutamate transport into cortical neurons is mim-

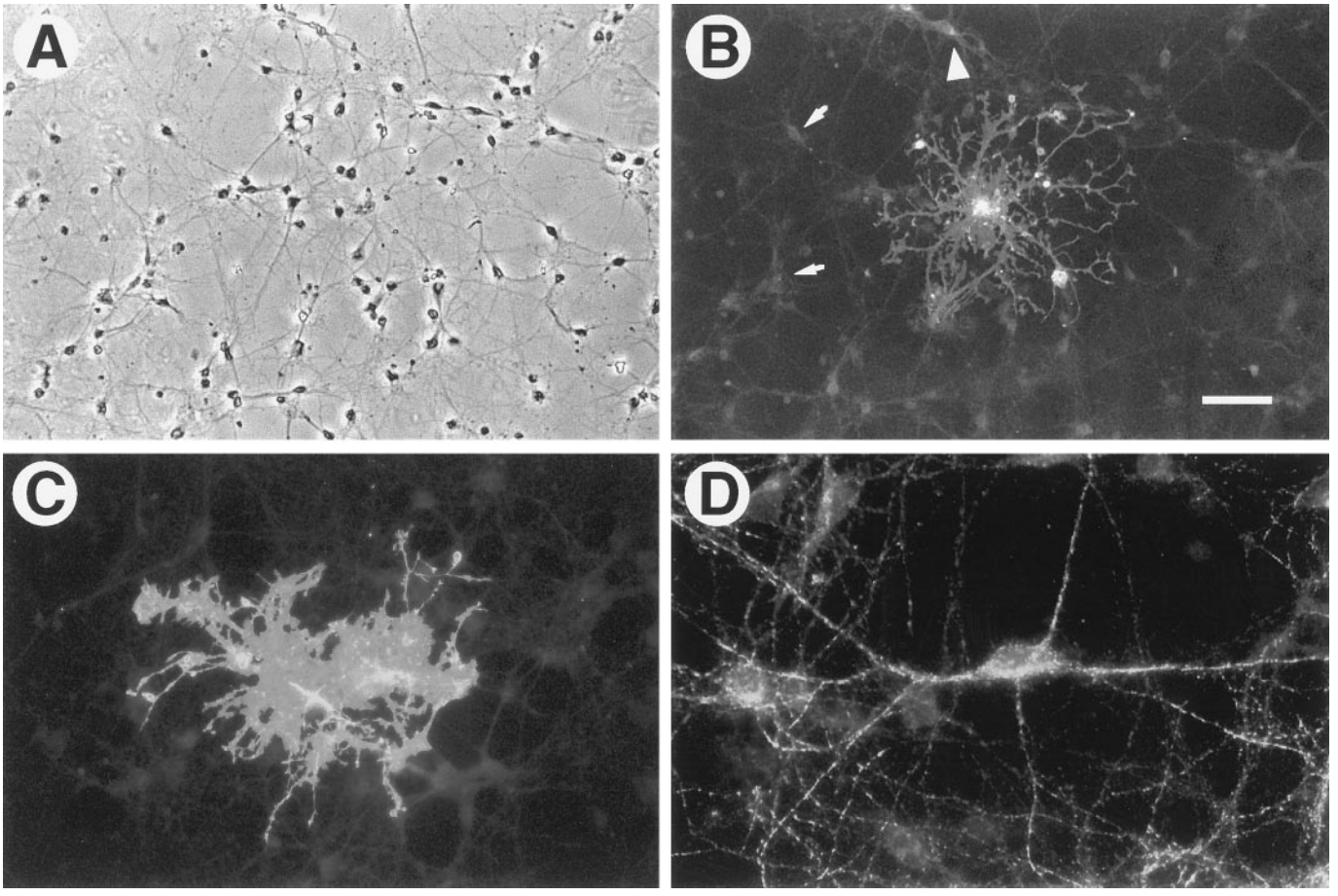

Fig. 5. Immunocytochemistry of glutamate transporters in cortical neuronal cultures. A, Phase contrast photograph of cortical neuronal cultures. B. Neuronal culture stained with antibody against carboxyl-terminal peptide of GLT1. A rare astrocyte was labeled strongly by GLT1; one such cell (*center*) has many surrounding neurons. Neuronal labeling in most cases was very weak and diffuse (*arrows*) compared with the labeling in astrocytes, although apparent intracellular labeling was observed occasionally in some neurons (*arrowhead*). C, Neuronal culture stained with antibody against amino-terminal peptide of GLAST. Rarely, an astrocyte was found that was stained using this antibody. The staining in neurons was at background levels. No obvious intracellular labeling of neuronal cell bodies was seen. D, Neuronal culture stained with antibody against carboxyl-terminal peptide of EAAC1. Anti-EAAC1 stained both neuronal cell bodies and neuronal processes in a punctate pattern. Astrocytes in these cultures were not stained by EAAC1. *Scale bar*, 40 μm in A and B, 20 μm in C, and 13 μm in D.

icked quite closely by cortical synaptosomes in being inhibited by DHK, SOS, KA, and PDC but not L-α-AA (Robinson *et al.*, 1993b) (Table 1).

We determined a K_m value for glutamate transport into cortical neurons of 17.2 μM. This compares with a value of 1–5 μM obtained for cortical synaptosomes (Robinson *et al.*, 1993b). In contrast, we found that glutamate transport into astrocytes in culture had a K_m value of 76 $\pm$ 17 μM (five experiments) (Chung HJ, Schnuer J, Rosenberg PA, unpublished observations), which is similar to the value of 91 μM obtained by others (Garlin *et al.*, 1995). K_m values for L-glutamate transport reported for the cloned transporters are 20, 18, 28, and 3.3 μM for EAAT1 through EAAT4, respectively (Arriza *et al.*, 1994; Fairman *et al.*, 1995). Because of the similarity in K_m values for the different transporters, there is doubtful whether it would be possible to detect through kinetic analysis participation by two different transporters in the neuronal cultures, such as EAAC1 and GLT1.

We determined the V_{max} value for glutamate transport in cortical neurons in culture to be 3.3 nmol/mg of protein/min. This is comparable to the V_{max} values observed in other preparations derived from the central nervous system that actively transport glutamate. Thus, a V_{max} value of 2.7 nmol/mg of protein/min was obtained for cortical synaptosomes (Robinson *et al.*, 1991), and 7.5 nmol/mg of protein/min was obtained for glial cultures (Garlin *et al.*, 1995). One therefore would expect a comparable density of transport sites in the cortical neurons as in these other preparations, assuming an equivalence of transport activity per transporter molecule in the neurons and other preparations. The protein that is immunoreactive with the peptide-specific anti-GLT1 antibody that we used is present on the basis of immunoblot analysis in small amounts in neuronal cultures relative to the expression of GLT1 in mixed cultures of astrocytes and neurons and barely detectable by immunochemistry in the neurons. Therefore, it is difficult to accept that this protein could itself account for the glutamate transport activity observed in cortical neurons. However, a variant form of GLT1 that was not very immunoreactive with the anti-GLT1 antibody used (e.g., due to amino acid variation in the target carboxyl-terminal peptide sequence) is not excluded.

The fact that glutamate transport in neuronal cultures closely resembles that in cortical synaptosomes establishes for the first time that the DHK-sensitive glutamate transport in cortical synaptosomes is associated with neurons. What is

this neuronal DHK-sensitive glutamate transporter? A number of lines of evidence suggest the possibility of an important role for GLT1 or a variant form of GLT1, as follows: (1) GLT1 is the only transporter known to be sensitive to DHK; (2) GLT1 protein (Chaudhry *et al.*, 1995), as well as mRNA (Torp *et al.*, 1995), has been shown to be expressed in neurons; (3) the results of our studies using peptide-specific antibodies are consistent with expression of GLT1 protein in neurons; (4) immunoprecipitation using a polyclonal antibody against whole GLT1 protein nearly completely eliminated glutamate transport activity from vesicles reconstituted from brain membranes, according to preliminary reports (Danbolt *et al.*, 1992; Torp *et al.*, 1995); 5) GLT1 has been shown to form homomultimers (Haugeto *et al.*, 1996), suggesting the possibility of heteromultimer formation with other transporters; and (6) cortical synaptosomes from a GLT1 knockout mouse significantly reduced glutamate transport compared with synaptosomes derived from control animals (Tanaka *et al.*, 1997).

A variant form of GLT1 may be the consequence of alternative splicing, alternative promoter usage, or RNA editing. In addition, a variant form of GLT1 with altered function and immunoreactivity might be produced by post-translational modification. It is known that glutamate transporters are glycosylated, and differences in glycosylation might account for differences in function; other types of post-translational modification are also possible, such as phosphorylation. Other possible explanations for the unusual pharmacology of glutamate uptake in cortical neurons and synaptosomes that would be consistent with an important role for the transporters shown to be present are as follows: (1) a regulatory protein or other substance may be present that alters the pharmacology of one or more known transporters that are localized there, and (2) native transporters may be heteromeric and have a different pharmacology than cloned transporters that are expressed individually. Finally, a novel glutamate transporter may be present to account for the unusual pharmacology, just as the discovery of EAAT4 accounted for the unique transport activity of cerebellar synaptosomes (Fairman *et al.*, 1995). Discrimination among these possibilities will require further work. The neuronal cultures described here should prove useful in pursuit of this issue.

It is conceivable that the transport activity we found in cortical neurons may be present at excitatory terminals at which glutamate uptake has been demonstrated (Gundersen *et al.*, 1993) but no known transporters are localized (Rothstein *et al.*, 1994). It is not known how glutamate is returned to the presynaptic terminal after synaptic release. Strong evidence exists for a glutamine/glutamate cycle (Westergaard *et al.*, 1995). In this scheme, glutamate released from the presynaptic terminal is taken up by astrocytes, by which it is converted to glutamine. The glutamine then is released, by an undefined process; taken up by neurons; and deaminated by glutaminase to glutamate. An alternate possibility is that glutamate is metabolized in astrocytes to α-ketoglutarate, which then is transferred to neurons, by which it is metabolized to glutamate (Shank and Campbell, 1984). The simplest mechanism would be reuptake of transmitter by the presynaptic terminal itself (Gundersen *et al.*, 1993).

Characterization of the molecular basis of neuronal/synaptosomal glutamate transport will be of use in understanding pathological mechanisms. It is well established that glutamate transport protects neurons *in vitro* and *in vivo* against the toxicity of exogenous glutamate. It is likely that glutamate transport also protects neurons against the toxicity of endogenous glutamate. Interestingly, a recent report suggests that DHK-sensitive transport is important in this capacity because application of DHK *in vivo* caused significant neurotoxicity (Massieu *et al.*, 1995), suggesting that DHK-sensitive transport activity is required for protection of neurons against the toxicity of endogenous glutamate. This hypothesis is supported further by our recent observations that 20–24-hr exposure of neuronal cultures to DHK is toxic and the neurotoxicity of DHK seems to be entirely due to its ability to block glutamate transport in these cultures (Blitzblau *et al.*, 1996).

Studies by Rothstein *et al.* (1992), who used synaptosomal preparations derived from the brain and spinal cord of patients dying from amyotrophic lateral sclerosis, showed that this disease is associated with a loss of glutamate uptake activity in cortical synaptosomes. It is not known whether this phenomenon is a cause or an effect of the neuronal loss in this disease, or, even if it is an effect, whether it might contribute ultimately to the neurodegeneration by rendering neurons more vulnerable to glutamate toxicity. In any case, to better understand the molecular pathogenesis of this disease, it will be important to know specifically which transporters mediate synaptosomal glutamate uptake.

Glutamate transport seems to be important in conferring protection against glutamate toxicity, but it also has been suggested to be an important source of the abnormal concentrations of glutamate that accumulate in the setting of energy failure (Attwell *et al.*, 1993). Although the driving force for glutamate transport is still inwardly directed in moderately ischemic conditions (Zerangue and Kavanaugh, 1996), the presynaptic transporter on excitatory terminals is located in an especially important site from this perspective; putative glutamatergic neurons contain intraterminal glutamate concentrations of 12–27 mM (Storm-Mathisen *et al.*, 1992), with significantly lower concentrations found in astrocytes (0.3–5 mM) (Bramhan *et al.*, 1990). Because of the higher glutamate concentrations in neurons, it might be predicted that slowing or reversal of neuronal transporters would preferentially contribute to the pathological rise of extracellular glutamate, especially in the face of a profound disruption in ion gradients. Elucidation of the molecular basis of the transport activity found in cortical neurons and synaptosomes is thus of great interest because it may play an important role in the normal and abnormal physiology of the presynaptic terminal.

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