

Regulation of the Glial Na^+ -Dependent Glutamate Transporters by Cyclic AMP Analogs and Neurons

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Received September 11, 1997; Accepted November 14, 1997

This paper is available online at <http://www.molpharm.org>

ABSTRACT

Sodium-dependent transport into astrocytes is critical for maintaining the extracellular concentrations of glutamate below toxic levels in the central nervous system. In this study, the expression of the glial glutamate transporters GLT-1 and GLAST was studied in primary cultures derived from cortical tissue. In primary astrocytes, GLAST protein levels were approximately one half of those observed in cortical tissue, but GLT-1 protein was present at very low levels compared with cortical tissue. Maintenance of these astrocytes in medium supplemented with dibutyryl-cAMP (dbcAMP) caused a dramatic change in cell morphology, increased GLT-1 and GLAST mRNA levels ≈ 5 -fold, increased GLAST protein ≈ 2 -fold, and increased GLT-1 protein ≥ 8 -20-fold. These increases in protein expression were accompanied by 2-fold increases in the V_{\max} and K_m values for Na^+ -dependent L -[^3H]glutamate transport activity. Although GLT-1 is sensitive to inhibition by dihydrokainate in heterologous expression systems, no dihydrokainate sensitivity was observed in astrocyte cultures that expressed GLT-1. Biotinylation with a membrane-impermeant reagent, separation of the biotinylated/cell surface proteins, and subsequent Western blotting demonstrated that both GLT-1 and GLAST were present at the cell surface. Coculturing of astrocytes with neurons also induced expression of GLT-1, which colocalized with the glial specific marker, glial fibrillary

acidic protein. Neurons induced a small increase in GLAST protein. Several studies were performed to examine the mechanism by which neurons regulate expression of the glial transporters. Three different protein kinase A (PKA) antagonists did not block the effect of neurons on glial expression of GLT-1 protein, but the addition of dbcAMP to mixed cultures of neurons and astrocytes did not cause GLT-1 protein to increase further. This suggests that neurons do not regulate GLT-1 by activation of PKA but that neurons and dbcAMP regulate GLT-1 protein through convergent pathways. As was observed with GLT-1, the increases in GLAST protein observed in cocultures were not blocked by PKA antagonists, but unlike GLT-1, the addition of dbcAMP to mixed cultures of neurons and astrocytes caused GLAST protein to increase ≈ 2 -fold. Neurons separated from astrocytes with a semipermeable membrane increased GLT-1 protein, indicating that the effect of neurons was mediated by a diffusible molecule. Treatment of cocultures with high concentrations of either *N*-methyl-D-aspartate or glutamate killed the neurons, caused GLT-1 protein to decrease, and caused GLAST protein to increase. These studies suggest that GLT-1 and GLAST protein are regulated independently in astrocyte cultures and that a diffusible molecule secreted by neurons induces expression of GLT-1 in astrocytes.

Glutamate, the predominant excitatory neurotransmitter in the mammalian central nervous system, has been implicated as a neurotoxic agent in neurodegenerative diseases and in central nervous system insults such as ischemia and epilepsy (for a review, see Choi, 1992).

Extracellular glutamate levels are regulated primarily by Na^+ -dependent transport of glutamate into glia and neurons (for reviews, see Danbolt, 1994; Robinson and Dowd, 1997). It is thought that glutamate transport is crucial for preventing the accumulation of neurotoxic levels of extracellular glutamate. Pharmacological studies in synaptosomes and astrocytes provide evidence for the existence of multiple subtypes of Na^+ -dependent glutamate transporters (for a review, see Robinson and Dowd, 1997). Molecular

This work was supported by Grants NS29868 and HD26979 (M.B.R.), NS33958 (J.D.R.), and NS36465 (M.B.R., J.D.R.).

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ABBREVIATIONS: dbcAMP, dibutyryl-cAMP; 8-Br-cAMP, 8-bromo-cAMP; rpcAMP, adenosine-3',5'-cyclic monophosphothioate triethylammonium salt; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TBS, Tris-buffered solution; TGT, 5% normal goat serum/0.1% Triton-X 100; DAPI, 4',6-diamidino-2-phenylindole dihydrochloride hydrate; NMDA, *N*-methyl-D-aspartate; PKA, protein kinase A; GFAP, glial fibrillary acidic protein; TTX, tetrodotoxin.

cloning led to the isolation of cDNAs for three glutamate transporter subtypes in nonhuman systems: GLAST (Storck *et al.*, 1992), GLT-1 (Pines *et al.*, 1992), and EAAC1 (Kanai and Hediger, 1992). Human homologs of these three transporters also have been cloned (EAAT1, EAAT2, EAAT3), as well as two additional transporters, EAAT4 and EAAT5 (Arriza *et al.*, 1994, 1997; Fairman *et al.*, 1995). The cloning of these transporters and subsequent development of subtype-specific antibodies have allowed for localization of the transporter subtypes *in vivo*. Immunocytochemical studies indicate that EAAC1 and EAAT4 are expressed in neurons, whereas GLT-1 and GLAST are present in glia (Rothstein *et al.*, 1994; Lehre *et al.*, 1995; Furuta *et al.*, 1997b). Selective reduction of individual transporter subtypes using antisense oligonucleotides has provided evidence that the astroglial transporters GLAST and GLT-1 may be of primary importance in maintaining low extracellular concentrations of glutamate, thereby protecting neurons against excitotoxicity *in vivo* (Rothstein *et al.*, 1996). Selective genetic knock-out of GLT-1 in mice provides further evidence for the importance of this particular transporter (Tanaka *et al.*, 1997).

Despite the importance of glutamate transport for normal brain physiology, little is known about its regulation (for a review, see Gegelashvili and Schousboe, 1997). Recent data from several groups suggest the occurrence of both transcriptional and post-transcriptional regulation of individual transporter subtypes. For instance, in the suprachiasmatic nuclei, EAAC1 mRNA levels vary with circadian rhythm (Cagampang *et al.*, 1996), and in a renal epithelial cell line, EAAC1 mRNA levels decrease in response to amino acid deprivation (Plakidou-Dymock and McGivan, 1993). Lowered levels of GLT-1 mRNA are observed of postischemic rat hippocampus, suggesting a possible mechanism for the decreased clearance of glutamate in ischemia models (Torp *et al.*, 1995). Moreover, immunoreactivity of GLT-1 and GLAST decrease after disruption of the corticostriatal glutamatergic pathway (Ginsberg *et al.*, 1995), indicating that neurons may participate in the regulation of glutamate transporter expression. These observations provide evidence that glutamate transport *in vivo* is regulated by several pathways; the underlying mechanisms, however, remain largely unexplored.

Recent observations describing the developmental regulation of glutamate transporters in the maturing rat brain suggest a close connection between adult patterns of glutamate transporter expression and synapse formation/astrocyte development. GLAST and GLT-1 protein and mRNA levels are reported to increase with maturation, whereas EAAC1 protein levels peak in neonatal brains, around postnatal day 16 (P16), and decrease to adult levels by P26 (Sutherland *et al.*, 1996; Furuta *et al.*, 1997b). This suggests that GLT-1 expression is a correlate of maturation of the central nervous system.

The goal of the current study was to examine the expression and regulation of the astrocytic glutamate transporters using primary cell cultures derived from cortical tissue. In this study, we demonstrate that GLT-1 protein levels are increased dramatically through treatment of astrocytes with dbcAMP or coculturing of astrocytes with neurons. Similar but lesser effects on GLAST expression also were observed. Evidence is presented to indicate that the effect of neurons on

astrocytic expression of GLT-1 is mediated by a diffusible molecule and is reversible. Some of this work was presented first in abstract form by two groups simultaneously (Stein *et al.*, 1997; Vondrasek *et al.*, 1997).

Experimental Procedures

Materials. FBS was obtained from Hyclone (Logan, UT). All other cell culture reagents were from GIBCO BRL (Gaithersburg, MD). Anti-GFAP antibody, poly-D-lysine, anti-actin antibody, dbcAMP, 8-Br-cAMP, and propranolol were obtained from Sigma Chemical (St. Louis, MO). L-[³H]Glutamate was obtained from DuPont-New England Nuclear (Boston, MA). Donkey anti-rabbit horseradish peroxidase IgG, rainbow molecular mass markers, [α -³²P]dCTP, Hybond N⁺, and enhanced chemiluminescence kits (ECL kits) were purchased from Amersham (Arlington Heights, IL). Immobilon P membrane was from Millipore (Bedford, MA). Dihydrokainate was purchased from Genosys (The Woodlands, TX). Adenosine-3',5'-cyclic monophosphothioate, rpcAMP (Rp isomer), H89, KT 5720, forskolin, and TTX were purchased from Calbiochem (La Jolla, CA). Isoproterenol and MK801 were purchased from RBI (Natick, MA). (+)- α -Methyl-4-carboxyphenylglycine was from Tocris Cookson (St. Louis, MO). N-Glycosidase F was from Boehringer-Mannheim Biochemicals (Indianapolis, IN). EZ-Link Sulfo-succinimidobiotin (biotin) and Immunopure Immobilized Monomeric Avidin beads (avidin) were purchased from Pierce Chemical (Rockford, IL). Vectashield and goat serum were purchased from Vector Labs (Burlingame, CA). Anti-mouse IgG fluorescein and anti-rabbit IgG rhodamine were obtained from Jackson ImmunoResearch (West Grove, PA). Medium-molecular-mass neurofilament antibody was a gift from Dr. V. Lee (University of Pennsylvania, Philadelphia, PA). GLT-1 and EAAC1 cDNAs in pBluescript SK⁻ were generous gifts from Drs. B. Kanner (Hebrew University, Jerusalem, Israel), and M. A. Hediger (Harvard University, Cambridge, MA), respectively. GLAST cDNA was generated by reverse transcription-polymerase chain reaction using specific primers and cloned into pBluescript SK⁻.

Cell culture. Cortical astrocyte cultures were prepared from the cortices of neonatal rats (1–3 days old) as described previously (Garlin *et al.*, 1995) and grown in DMEM supplemented with 10% heat-inactivated FBS, 10% Ham's F-12, and 0.24% penicillin/streptomycin (10,000 units/ml penicillin/10,000 μ g/ml streptomycin). Cells were plated at a uniform density of 2.5×10^5 cells/ml (3×10^4 cells/cm²) onto sterile polystyrene dishes (either 10-cm, 12-well, or 6-well dishes). The cultures were maintained in a 5% CO₂ incubator at 37° and fed with a complete medium exchange twice a week until used. Cells reached confluency after 10–14 days. At 14 days *in vitro*, >95% of the cells in these cultures were astrocytes based on expression of cell-specific immunohistochemical markers (Garlin *et al.*, 1995). These cultures were fed with medium containing cAMP analogs and harvested with untreated sister cultures.

Neuron/astrocyte mixed cultures were prepared from embryonic day 17–19 rat cortices using a procedure similar to that described above with a few exceptions: cells were plated onto poly-D-lysine (50 μ g/ml)-coated plastic dishes and maintained in a 7% CO₂ incubator at 37°. The cultures were fed with a one third medium exchange once a week. After the first week in culture, cells were fed with medium containing 5% FBS. After 7–10 days, neurons in these cultures sit on top of a confluent monolayer of astrocytes and represent <50% of the cells.

In some experiments, astrocytes were prepared as described above and plated onto six-well plates (no. 3046; Falcon Plastics, Oxnard, CA) and maintained for 10–12 days. Neuronal cultures were prepared by plating cultures into inserts and then maintained in DMEM with 5% FBS. After 3 days *in vitro*, cytosine arabinoside (20 μ M) was added to these inserts. Twenty-four hours later, this medium was removed, and the inserts were lowered into the six-well plates that contained astrocytes. These cells were maintained in the astrocyte-

conditioned medium, which was supplemented with 25% fresh DMEM. These cells were maintained as described for the neuron/astrocyte mixed cultures for 14 days.

Neuron-enriched cultures (astrocyte-poor cultures) were prepared as described for the cocultures of neurons and astrocytes. After 3 days *in vitro*, cytosine arabinoside (20 μ M) was added to these cultures. Twenty-four hours later, this medium was changed to Neurobasal medium (GIBCO BRL, Gaithersburg, MD) containing 30% conditioned medium (from cocultures of neurons and astrocytes). At day 7, cells were fed with a 25% medium exchange. These cultures are >80% neurons as determined by visual inspection. At day 12, cells were harvested by scraping in buffer (20 mM HEPES, pH 7.5, 2 mM MgSO_4 , and 1 mM EDTA) supplemented with protease inhibitors. This homogenate was centrifuged at 1000 rpm for 5 min to remove nuclei and cell bodies. The supernatant then was centrifuged at 12,500 rpm for 20 min. This pellet was resuspended in DMEM. Membranes from one 10-cm dish of neurons were added to the medium of one 10-cm dish of astrocytes.

Measurement of L-[^3H]glutamate transport. The sodium-dependent transport of L-[^3H]glutamate into primary cortical astrocytes was measured at 24–27 days *in vitro* in control cultures and in sister cultures treated with 0.25 mM dbcAMP for 10 days. Triplicate transport assays were performed as described previously (Garlin *et al.*, 1995). Na^+ -dependent transport was calculated as the difference between the radioactivity accumulated by cells incubated with sodium buffer and those incubated with choline buffer and was examined at each concentration of L-glutamate.

Western analyses. Cells were washed twice with cold PBS, plates were scraped, and cells were suspended in PBS containing 0.5 mM EDTA. Cell suspensions were centrifuged at 12,000 rpm in an Eppendorf microcentrifuge for 5 min. The pellet was resuspended in buffer containing 20 mM HEPES, pH 7.5, 2 mM MgCl_2 , and 1 mM EDTA. After sonication, an aliquot was removed for protein analysis (Lowry *et al.*, 1951). Protease inhibitors (100 μ M phenylmethylsulfonyl fluoride, 10 μ g/ml leupeptin, 1.1 μ g/ml aprotinin, 1 mM EDTA) were added to the remaining suspension, which then was diluted 1:2 in sample buffer (2% SDS, 10% β -mercaptoethanol, 5% glycerol, 0.005% bromophenol blue, and 50 mM Tris-Cl, pH 7.0), boiled for 5 min, and frozen at -20° . Crude synaptosomes (P2) were prepared from cortex or cerebellum as described previously and frozen at -20° (Robinson *et al.*, 1991). At the time of electrophoresis, cell suspensions were boiled again for 5 min, and synaptosomal membranes were diluted in sample buffer and boiled before loading onto gels. Except where noted, equal amounts of protein were loaded onto each lane. Protein samples and rainbow molecular mass markers were separated by electrophoresis on SDS/10% polyacrylamide gels and transferred onto polyvinylidene fluoride membranes (Immobilon P). These immunoblots were visualized using enhanced chemiluminescence (Rothstein *et al.*, 1994). In most experiments, blots were probed with both an anti-actin antibody (diluted 1:1,000) and an anti-transporter antibody; either GLT-1 (diluted 1:10,000), GLAST (diluted 1:5,000), EAAC1 (diluted 1:75), or EAAT4 (diluted 1:200) (Rothstein *et al.*, 1994; Furuta *et al.*, 1997a, 1997b).

The density of immunoreactive bands was quantified using Image software (National Institutes of Health, Bethesda, MD). In most experiments, several immunoreactive bands were observed, but the predominant band usually had an apparent molecular mass consistent with the monomer. The apparent molecular masses of the additional bands were consistent with multimers (see Fig. 1 for examples). A recent report suggests that these bands are homomultimers that are not dissociated with boiling in SDS containing β -mercaptoethanol (Haugeto *et al.*, 1996). In the current study, the lower- and higher-molecular-mass bands were quantified and reported. The data also were calculated and compared using just the low-molecular-mass monomer species, and the results were the same.

Biotinylation of cell surface proteins. All steps were performed at 4° . Astrocyte-enriched cultures were rinsed twice with PBS, pH 7.35, supplemented with 1 mM MgCl_2 and 0.1 mM CaCl_2

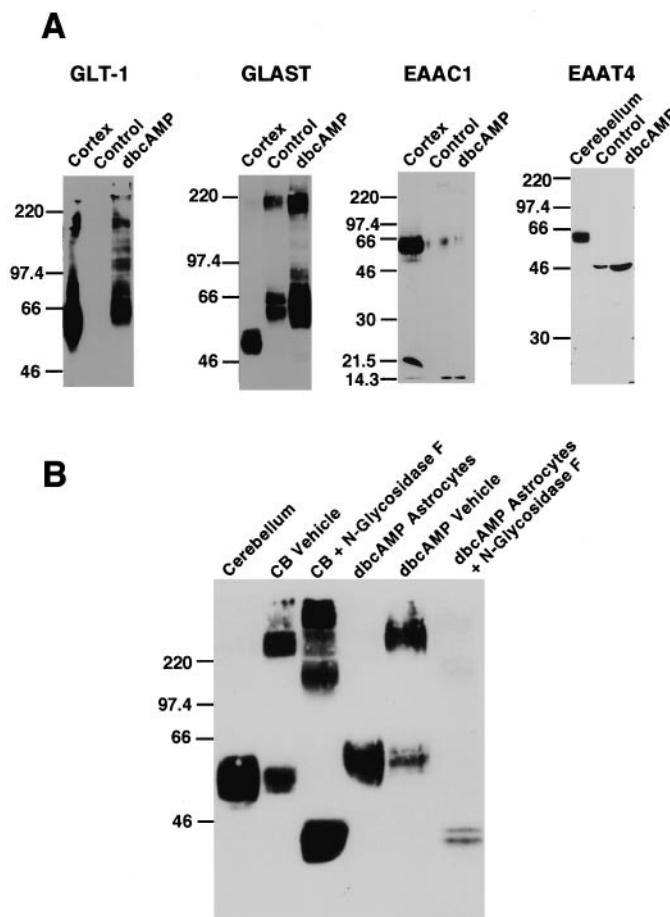


Fig. 1. A, Western blot analysis of glutamate transporter expression in brain and astrocyte cultures. Cortical membrane homogenates, control astrocyte cultures, and astrocyte cultures treated with dbcAMP (0.25 mM) for 10 days were immunoblotted with anti-GLT-1 (1:10,000), anti-GLAST (1:5,000), anti-EAAC1 (1:75), and anti-EAAT4 (1:200). For GLT-1, EAAC1, and EAAT4, the antibodies were raised against unique peptides from the carboxyl-terminal regions of the respective transporters. For GLAST, the antibody was raised against a peptide unique to the amino terminus of the protein. Except for the cortical protein with the GLT-1 blot, 50 μ g of protein was loaded in each lane. For GLT-1 blots, 5 μ g of cortical protein was loaded. Each of these Western blots were reproduced at least four times. B, Western blot analysis of GLAST immunoreactivity in cerebellum and astrocyte cultures before and after deglycosylation. Cerebellar tissue (CB) or dbcAMP-treated astrocytes was incubated in HME buffer with *N*-glycosidase F (10 units/50 μ g of protein), 0.01% SDS, and 0.01% 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate for 1 hr at 37° . Control tissue (not incubated) and tissue incubated without *N*-glycosidase F (vehicle) were included for comparison. Note the increase in larger molecular mass aggregates with incubation. In each lane, 50 μ g of protein was loaded. This Western blot was reproduced three times.

(PBS Ca/Mg). After a 20-min incubation with biotin dissolved in PBS Ca/Mg (1 mg/ml), the cells were rinsed twice in quenching solution (PBS Ca/Mg with 0.75 g/100 ml of glycine) and then incubated for an additional 20 min in this same buffer. After two rinses in PBS Ca/Mg buffer, cells were lysed with 1 ml of RIPA buffer (100 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS, pH 7.4)/10-cm dish. After scraping, this homogenate was centrifuged at 12,400 rpm for 15 min. An aliquot of this supernatant was boiled in sample buffer. Another aliquot (250 μ l) was incubated with 150 μ l of avidin beads for 2 hr and then centrifuged at 12,400 rpm for 15 min. An aliquot of supernatant from this centrifugation step (referred to as supernatant in Fig. 6) was boiled in sample buffer. The pellet (avidin beads) was washed four times in RIPA buffer, boiled in sample buffer (500 μ l), and then

centrifuged. The supernatant from this step (referred to as biotinylated fraction in Fig. 6) and the supernatant from the other steps were frozen until they were immunoblotted.

Northern analyses. Total RNA was extracted from primary astrocyte cultures and adult rat brain tissue (cortex, hippocampus, and cerebellum) were used to obtain high signals for all three transporters according to the single-step guanidinium thiocyanate-phenol-chloroform procedure as described previously (Ausubel et al., 1995). RNA samples were separated with a 1% agarose/6.6% formaldehyde gel in 1×3 -(*N*-morpholino)propanesulfonic acid buffer. RNA was transferred to a Hybond N⁺ positively charged nylon membrane and immobilized by baking at 80° for 2 hr. Membranes were prehybridized for 2–3 hr at 65° and hybridized for 16–20 hr with the specific cDNA probe at 65° as described by Church and Gilbert (1984). Washes were performed at 65° in $2-0.1 \times$ SSPE (NaCl, sodium phosphate buffer). Membranes were exposed to Kodak X-OMAT film for 12–36 hr. Radioactivity was quantified with a PhosphorImager SI (Molecular Dynamics, Sunnyvale, CA) using the ImageQuant analysis program. Data were expressed as a ratio of transporter-specific mRNA to cyclophilin mRNA.

The *NotI* fragment of GLT-1 cDNA clone (1.4 kb), *PstI/HincII* fragment of GLAST cDNA clone (0.9 kb), and *BamHI/HindIII* fragment of rat cyclophilin cDNA (0.7 kb) were used as specific probes for the corresponding mRNAs. cDNA probes were radiolabeled with [α -³²P]dCTP by nick translation.

Immunocytochemistry. Primary astrocyte-enriched and astrocyte/neuron cocultures were prepared as described. The cultures were plated onto sterile glass coverslips coated with poly-D-lysine. Cultures were rinsed briefly in PBS and then fixed in 4% paraformaldehyde in PBS for 10 min at room temperature. The cells were rinsed in TBS containing 50 mM Tris and 150 mM NaCl, pH 7.4, and then blocked in the same buffer supplemented with TGT for 30 min at room temperature. Cultures were incubated overnight at 4° in an antibody cocktail containing the monoclonal GFAP antibody (diluted 1:100) and the respective transporter antibody (diluted 1:100 for GLT-1, 1:50 for GLAST, 1:10 for EAAC1) in TGT. The cells were rinsed in TBS containing 0.1% Triton-X 100 and then incubated for 2 hr at room temperature in a cocktail containing anti-mouse IgG-fluorescein and anti-rabbit IgG-rhodamine conjugates diluted 1:200 in TGT. To identify cell nuclei, cultures were rinsed and then incubated in DAPI diluted 1:500 in PBS for 10 min at room temperature. To dehydrate the tissue, coverslips were immersed in 97% ethanol for 2 min and air-dried. The coverslips were mounted in Vectashield and sealed. Control incubations leaving out the primary or secondary antibody were performed for each antibody. Photomicrographs were taken with an Axiophot microscope (Zeiss Instruments, Thornburg, NY).

Results

Expression of glutamate transporters after treatment with cAMP analogs. The expression patterns of individual glutamate transporters GLT-1, GLAST, EAAC1, and EAAT4 in primary astrocyte-enriched cortical cultures at 24 days *in vitro* were compared with those observed in brain tissue by Western blot analysis (Fig. 1A). Cultured astrocytes expressed little EAAC1 protein (apparent molecular mass, 65 ± 3 kDa; mean \pm standard error of three observations) compared with the levels observed in cortical tissue which is consistent with the neuronal localization of EAAC1 *in vivo* (Rothstein et al., 1994). These astrocyte cultures expressed low levels of an EAAT4-immunoreactive band that had an apparent molecular mass of 47 ± 3 kDa (three observations), which was lower than the apparent molecular mass of EAAT4 in cerebellar homogenates (61 ± 0.4 kDa, three observations). Although mature astrocytes *in*

vivo express the GLT-1 transporter (Rothstein et al., 1994; Lehre et al., 1995), GLT-1 immunoreactivity was present at very low levels in cultured astrocytes (24 days *in vitro*) compared with the levels observed in cortical tissue. The levels of GLAST immunoreactivity detected in astrocyte-enriched cultures were comparable to those observed in cortical tissue, consistent with its glial localization *in vivo*. The immunoreactive band for GLAST in these experiments migrated with a different mobility in astrocytes than in cortical tissue, with apparent molecular masses of 61.4 ± 0.2 and 56.8 ± 0.6 kDa, respectively (three observations). Two approaches were used to determine whether this band represents GLAST. First, the same experiment was repeated using an antibody derived against a carboxyl-terminal peptide of GLAST with the same result (data not shown, three observations). Second, membranes from cerebellum or from dbcAMP-treated astrocytes were treated with *N*-glycosidase F to remove *N*-linked carbohydrate residues; after treatment, both proteins migrated to the same apparent molecular masses of 45.1 ± 0.1 kDa (three observations; Fig. 1B).

Factors were examined that might induce the expression of GLT-1 in cultured astrocytes. The initial hypothesis that glutamatergic neurons might regulate the expression of glutamate transporters on astrocytes led us to investigate the effects of glutamate (1 mM, added at 24-hr intervals) and the metabotropic glutamate receptor agonist (1*S*,3*R*)-1-amino-1,3-cyclopentanedicarboxylic acid (0.5 mM). Because cAMP also is known to alter the expression of many astrocytic genes and induce morphological changes similar to those occurring during maturation, we examined the effects of the cAMP analog dbcAMP. Of these treatments, only dbcAMP induced *de novo* synthesis of GLT-1 protein (data not shown, two observations). As has been observed previously (Pollenz and McCarthy, 1986), dbcAMP induced a morphological change in primary astrocytes from flat polygonal cells to process-bearing stellate cells. To explore further these effects, astrocyte cultures were treated with dbcAMP (0.25 mM) beginning at 14 days *in vitro* for a period of 10 days. This caused a dramatic increase in GLT-1 immunoreactivity (apparent molecular mass, 66 ± 4 kDa; three observations), a modest increase in GLAST immunoreactivity, a decrease in EAAC1 immunoreactivity, and a modest increase in the EAAT4 immunoreactivity (Fig. 1).

To determine whether these effects of dbcAMP are related to the nonspecific effects of butyrate that have been observed with this analog of cAMP (Yusta et al., 1988), astrocyte cultures were incubated with other cAMP analogs or with activators of cAMP synthesis. The effects of 8-Br-cAMP, forskolin, and the β -adrenergic receptor agonist isoproterenol were compared with those of dbcAMP. Although isoproterenol did not increase GLT-1 or GLAST protein levels, both forskolin and 8-Br-cAMP caused increases in both GLT-1 and GLAST (Table 1).

Time course for changes in GLT-1 and GLAST mRNA and protein. The kinetics of the changes in protein and mRNA for GLT-1 and GLAST were examined after allowing the astrocytes to grow to confluence (14–16 days *in vitro*). In untreated/control cultures, there was no significant change in GLT-1 mRNA levels during the subsequent 14 days in cultures (Fig. 2, A and C), and GLT-1 protein remained at very low levels (Fig. 2, B and D). dbcAMP caused significant increases in GLT-1 mRNA at days 7, 10, and 14 with maxi-

TABLE 1

Effects of cAMP analogs and activators on GLT-1 and GLAST protein expression in astrocytes

Astrocytes were prepared as described in the text and maintained in medium supplemented with drugs starting at day 14. After 10 days of treatment, the levels of GLT-1 or GLAST immunoreactivity were determined by immunoblotting. Values represent the mean \pm standard error of at least three independent observations.

Treatment	Immunoreactivity (relative to control)	
	GLT-1	GLAST
Control	1.0	1.0
dbcAMP (0.25 mM)	8.2 \pm 2.9	2.3 \pm 0.5
Forskolin (10 μ M)	2.2 \pm 0.2	1.4 \pm 0.2
8-Bromo-cAMP (0.25 mM)	7.0 \pm 0.7	1.7 \pm 0.1
Isoproterenol (2 μ M)	0.84 \pm 0.24	0.92 \pm 0.44

mal increases of 4–6-fold and increased GLT-1 protein \approx 20-fold above that observed in control cultures. In one experiment, GLT-1 mRNA levels increased >2 -fold within a few hours, but in five other experiments, GLT-1 mRNA levels did not increase (2-fold above control) with 8-hr treatment.

GLAST mRNA levels initially increased to a maximum of 5-fold above control after 3 and 7 days of treatment (Fig. 3, A and C). GLAST mRNA levels fell to \approx 2-fold increases above that observed under control conditions after 10–14 days of treatment. In dbcAMP-treated cultures, the levels of GLAST protein were increased \approx 2-fold above that of control (Fig. 3, B and D). Although the levels of GLAST immunoreactivity in dbcAMP-treated astrocytes approached those observed in cortical membranes (Fig. 3D), the levels of GLT-1 in dbcAMP-treated astrocytes were \approx 10% of those observed in brain homogenates. Astrocytes were treated with dbcAMP in the presence and absence of actinomycin (10 ng/ml) for 3 days. This duration of exposure to actinomycin was toxic to the cells; therefore, it was not possible to determine whether these changes in mRNA levels were due to increased message stability or increased transcription using this pharmacological approach.

Immunohistochemical localization of GLT-1 and GLAST in dbcAMP-treated astrocytes. As visualized by light microscopy, dbcAMP treatment caused a dramatic change in morphology from a flat polygonal shape to a stellate shape with elaborate processes. Immunohistochemistry with anti-GFAP antibodies demonstrates this change. The GFAP immunoreactivity changed from a diffuse morphology around the nucleus to a process bearing elaborate morphology with condensed GFAP-positive fibrils (Fig. 4). This change in morphology was associated with increased expression of GLT-1 protein in the process-bearing cells (Fig. 4). The GLT-1 immunoreactivity colocalized with GFAP immunoreactivity. A similar pattern of staining was observed with GLAST immunoreactivity.

Effect of dbcAMP on L-[3 H]glutamate transport activity. Na $^+$ -dependent glutamate transport activity was examined to determine whether the increase in GLT-1 and GLAST protein was accompanied by an alteration in transport activity (Fig. 5A). The treatment of cortical astrocytes with dbcAMP (0.25 mM) for 10 days increased the V_{\max} value of Na $^+$ -dependent L-[3 H]glutamate transport from 14.8 ± 2.4 nmol/mg of protein/min in control astrocytes to 31.2 ± 2.3 nmol/mg of protein/min in treated astrocytes ($p < 0.01$, by Student's unpaired t test). Treatment with dbcAMP also increased the K_m value from 78 ± 16 μ M in control cultures to

164 ± 19 μ M in dbcAMP-treated cultures ($p < 0.05$, by Student's unpaired t test).

Expression of GLT-1 in heterologous systems results in Na $^+$ -dependent glutamate transport that is inhibited by dihydrokainate, with an IC_{50} value of <100 μ M, whereas GLAST expression results in transport that is dihydrokainate insensitive (Arriza *et al.*, 1994). In the current study, Na $^+$ -dependent L-[3 H]glutamate transport (measured at 0.5 μ M glutamate) was insensitive to dihydrokainate in both dbcAMP-treated and control astrocyte cultures (Fig. 5B). It is possible that GLT-1 has a lower affinity for glutamate than GLAST; this could result in a smaller contribution of GLT-1 to transport activity at low concentrations of glutamate. Therefore, the sensitivity of glutamate transport to dihydrokainate was examined at both 60 and 300 μ M glutamate. There was no evidence of dihydrokainate sensitivity at concentrations of dihydrokainate up to 3 mM (at least two independent observations). Because it seemed possible that the observed result could be an artifact of significant dihydrokainate impurity, the potency of the stock solutions used in these experiments was examined in a synaptosomal transport system. Consistent with previous data (Robinson *et al.*, 1991), glutamate transport in synaptosomes was sensitive to the same dihydrokainate stocks. Human homologs of GLAST and EAAC1 are more sensitive to inhibition by L-serine-O-sulfate than is GLT-1 (Arriza *et al.*, 1994). In one experiment, the sensitivity of transport to inhibition by L-serine-O-sulfate was examined at 10, 100, and 1000 μ M in dbcAMP-treated and control cultures. At each concentration, the difference in inhibition in the two types of cultures was $<5\%$. One possible explanation for the lack of increased dihydrokainate sensitivity and the lack of decreased sensitivity to L-serine-O-sulfate was that GLT-1 was not being trafficked to the cell surface. To address this possibility, we used a membrane-impermeant biotinylation reagent that covalently modifies cell surface proteins (Qian *et al.*, 1997). After separation of biotinylated proteins using avidin immobilized to beads, the proteins in each fraction were analyzed by Western blot analysis (Fig. 6, A and B). The amount of each protein in the biotinylated fraction was expressed as a percentage of the sum of the immunoreactivity in biotinylated and nonbiotinylated fractions. Approximately 60% of the GLAST protein was on the cell surface in both control ($60 \pm 14\%$, mean \pm standard error of four independent observations) and dbcAMP-treated ($68 \pm 6\%$, mean \pm standard error of four independent observations) cultures, and \approx 60% of the GLT-1 protein was on the cell surface in dbcAMP treated cultures ($56 \pm 12\%$, mean \pm standard error of six independent observations).

Effects of coculturing astrocytes with neurons on GLT-1 expression. Neurons are known to induce differentiation of astrocytes in culture (Hatten, 1985), and there is evidence from *in vivo* studies that neurons may have a role in regulating expression of the glial transporters (see introduction). To determine whether GLT-1 and GLAST immunoreactivities were elevated in mixed cultures, protein levels were examined in neuron/astrocyte cocultures that were harvested at different times after plating. Unlike astrocyte-enriched cultures, which expressed very low levels of GLT-1 protein after 21 days *in vitro*, cocultures of neurons and astrocytes expressed clearly detectable levels of GLT-1 protein (Fig. 7A). In these cocultures, the levels of both GLT-1

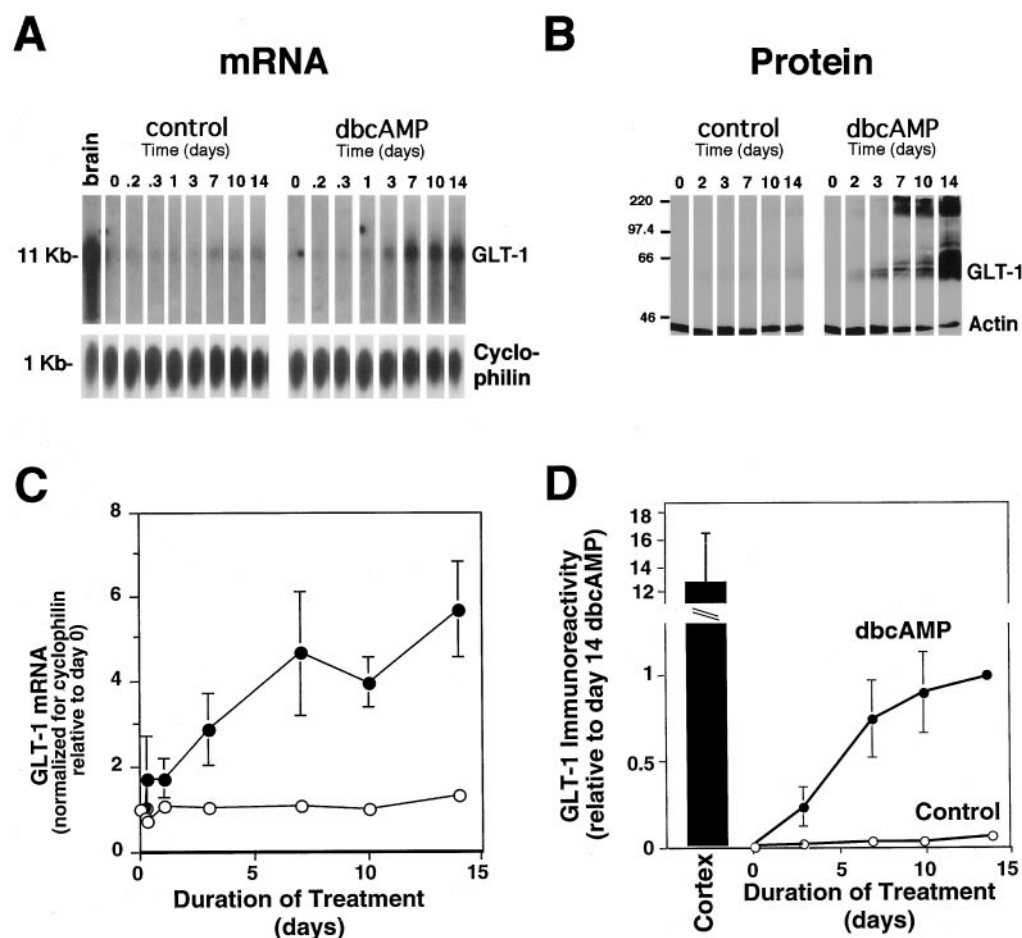


Fig. 2. Time course of changes in GLT-1 mRNA (A and C) and protein (B and D) in astrocyte-enriched cultures treated with dbcAMP (0.25 mM) for 10 days. Astrocyte-enriched cultures were grown until confluent (14–16 days *in vitro* referred to as day 0) and then treated with dbcAMP for different periods of time. Equal amounts of total RNA (25 μ g) or protein (50 μ g) were loaded in each lane except for cortical protein (5 μ g). The level of GLT-1 mRNA was normalized to cyclophilin mRNA and is expressed relative to the amount observed in astrocytes at day 0. The level of GLT-1 protein was expressed relative to that observed with 14 days of dbcAMP treatment because there were such low levels of GLT-1 protein in control astrocytes. C, Data are the mean \pm standard error of at least four observations (there were six observations at 8 hr) and were compared by analysis of variance. D, Data are the mean \pm standard error of four observations and were compared by analysis of variance. There was no significant change in GLT-1 mRNA or protein in control cultures. GLT-1 mRNA was increased significantly with 3 ($p < 0.05$), 7 ($p < 0.01$), 10 ($p < 0.01$), or 14 ($p < 0.01$) days of dbcAMP treatment. GLT-1 protein was increased significantly with 7, 10, or 14 days of dbcAMP treatment ($p < 0.001$).

and GLAST protein increased with the age of the cultures (Fig. 7, A and B). The levels of GLAST were slightly higher than those observed in cortical homogenates, whereas the levels of GLT-1 were $\approx 10\%$ of those observed in cortex.

Immunohistochemical localization of transporters in cocultures of neurons and astrocytes. To examine the localization of GLT-1, GLAST, and EAAC1 protein in mixed cultures, double-label immunohistochemistry was performed with antibodies against each of these transporters, GFAP, and neurofilament. GLT-1 and GLAST immunoreactivity was expressed at higher levels in differentiated stellate-shaped cells with elaborate processes than in the less differentiated polygonal-shaped cells (Fig. 8). Wherever GLT-1 or GLAST immunoreactivity was observed, it colocalized with GFAP. In contrast to either GLT-1 or GLAST, EAAC1 immunoreactivity was localized to a morphologically distinct population of cells and did not colocalize with GFAP-positive cells. In these same studies, nuclear staining with DAPI suggested that the increased GLT-1 or GLAST staining occurred selectively in astrocytes near neurons. To address these expression patterns more directly, double-label immunohistochemistry also was performed with neurofilament and GLT-1. This staining revealed that GLT-1 expression was much greater near clusters of neurofilament-positive cells (figure not shown but was made available to the reviewers).

Effects of dbcAMP and inhibitors on expression of GLAST and GLT-1 in cocultures of neurons and astro-

cytes. Because both dbcAMP and neurons caused an increase in GLT-1 and GLAST protein, we sought to determine whether activation of PKA might be involved in the effects of neurons on transporter expression. We also sought to determine whether blocking Na^+ channels or β , NMDA, or metabotropic glutamate receptors would eliminate the effect of neurons. In initial studies, cocultures of neurons and astrocytes were treated with the different inhibitors at the time of plating, and many of these inhibitors killed the neurons. Therefore, we treated the cultures with these inhibitors at 7 days, a time when the expression of GLT-1 and GLAST was still increasing steadily (see Fig. 7). These cultures were harvested at 14 days, and GLT-1 and GLAST immunoreactivity was quantified by Western blot analysis (Table 2). Neither MK801 nor TTX, two compounds that would be expected to significantly reduce neuronal excitability and synaptic transmission, had any significant effects on the levels of GLT-1 and GLAST protein. In the addition, neither *levo*-propanolol nor (+)- α -methyl-4-carboxyphenylglycine, antagonists of β and metabotropic glutamate receptors, had significant effects on the expression of GLT-1 or GLAST. Finally, three different PKA antagonists (rpcAMP, KT5720, H89) showed no significant effect on the levels of GLT-1 or GLAST compared with the levels observed in untreated cultures. As a positive control, KT5720 and H89 were tested in astrocytes treated with dbcAMP. GLT-1 expression was examined in astrocyte cultures treated with dbcAMP in the absence or presence of the antagonist for 7 days. KT5720 blocked the

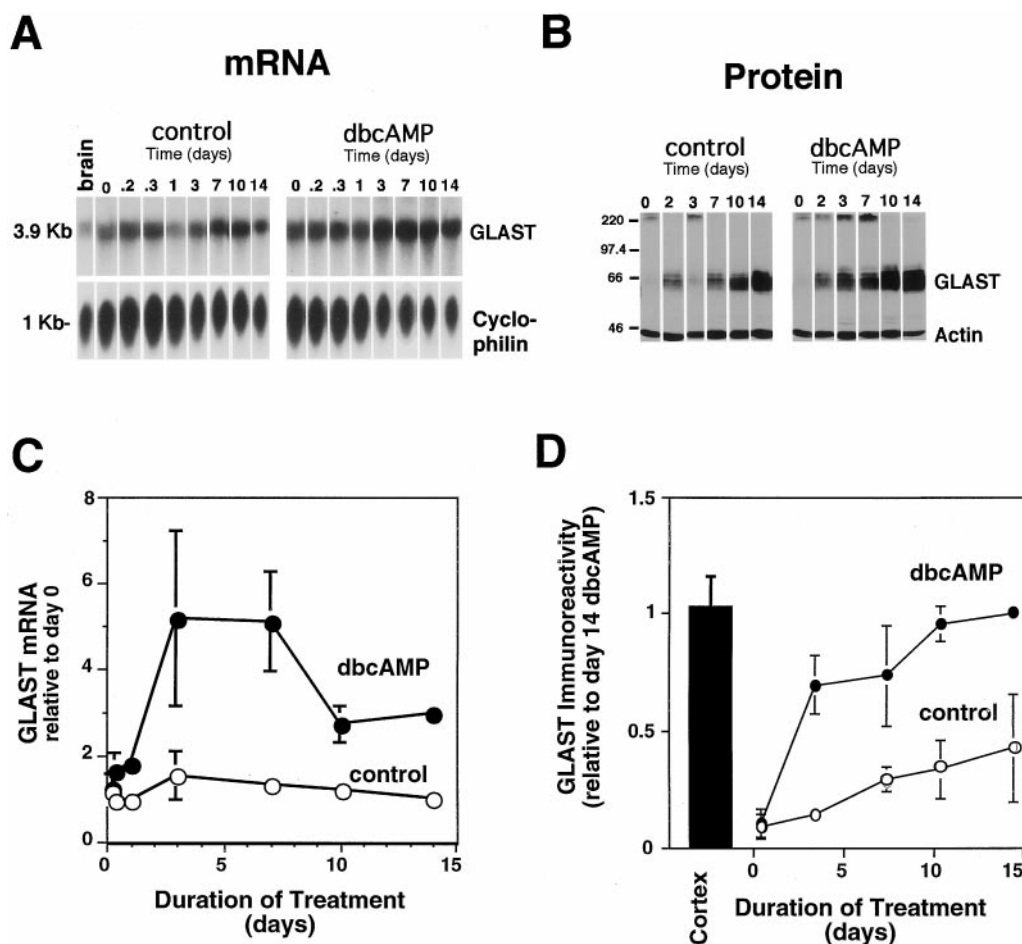


Fig. 3. Time course of changes in GLAST mRNA (A and C) and protein (B and D) in astrocyte-enriched cultures treated with dbcAMP (0.25 mM) for 10 days. Astrocyte enriched cultures were grown until confluent (14–16 days *in vitro* referred to as day 0) and then treated with dbcAMP for different periods of time. Equal amounts of total RNA (25 μ g) or protein (50 μ g) were loaded in each lane. The level of GLAST mRNA was normalized to cyclophilin mRNA and is expressed relative to the amount observed in astrocytes at day 0. The level of GLAST protein was expressed relative to that observed with 14 days of dbcAMP treatment. C and D, Data are the mean \pm standard error of at least three observations and were compared by analysis of variance. There was no significant change in GLAST mRNA or protein in control cultures. GLAST mRNA was increased significantly with 3 ($p < 0.01$) or 7 ($p < 0.01$) days of dbcAMP treatment. GLAST protein was increased significantly ($p < 0.05$) with 3, 7, 10, or 14 days of dbcAMP treatment.

effects of dbcAMP of GLT-1 expression by $>80\%$ (two observations), and H89 blocked the effects by 45% (two observations).

To determine whether the effects of dbcAMP and neurons were additive, cocultures were treated with dbcAMP for 7 days starting at day 14, a time when the levels of transporter protein were significantly elevated above those observed in freshly dissociated cultures. dbcAMP (0.25 mM) caused GLAST protein to increase to levels 2.4 ± 0.5 -fold above those observed in control cocultures (mean \pm standard error from three observations; $p = 0.06$). dbcAMP had no effect on the levels of GLT-1 protein in cocultures of neurons and astrocytes (1.1 ± 0.3 -fold above that observed in control cultures; mean \pm standard error from three observations).

Effects of neuron homogenates and separation of neurons and astrocytes by a semipermeable membrane. Two strategies were used to determine whether neurons induce expression via a contact-mediated event or secretion of a diffusible molecule. Neuron-enriched cultures were prepared and maintained for 14 days. Crude membranes were prepared from these cultures and placed on astrocytes that had been maintained for 14 days. No increases in GLT-1 or GLAST immunoreactivity were observed after 7 days (Fig. 9A). We were unable to use the medium from these cultures because it yielded unhealthy cultures with vacuolization of the astrocytes, presumably because of the lack of serum.

To determine whether a secreted molecule or molecules

from neurons can cause an increase in GLAST and GLT-1 expression in astrocyte cultures, astrocytes were plated onto and maintained in six-well plates for 10–12 days *in vitro*. Neuron-enriched cultures were plated and maintained on inserts with a semipermeable membrane. The neuron-containing inserts or empty inserts (control) were placed over the astrocytes and maintained in culture. After 14 days, the astrocytes were harvested and analyzed for GLT-1 or GLAST immunoreactivity. As was observed under control conditions (Fig. 2D), low levels of GLT-1 were observed in astrocytes under the empty inserts (Fig. 9B). The presence of neurons caused a 4-fold increase in GLT-1 protein, whereas there was no significant effect of neurons on GLAST immunoreactivity (Fig. 9, B and C).

Effect of killing neurons on GLT-1 and GLAST protein. To determine whether the effects of neurons on GLT-1 and GLAST expression are reversible, cocultures of neurons and astrocytes were maintained for 14 days and then treated with NMDA or glutamate to kill the neurons. Seven days later, the levels of GLT-1 and GLAST protein were quantified by Western blot analysis (Fig. 10). Compared with the levels observed in cocultures at day 14 (D14), glutamate or NMDA significantly reduced the levels of GLT-1 protein after 7 days, whereas the levels of GLT-1 increased slightly in untreated control cultures (D21). Both of these treatments killed all of the neurons in these cultures. In contrast to the effects on GLT-1 protein, the levels of GLAST increased dramatically with either NMDA or glutamate treatment.

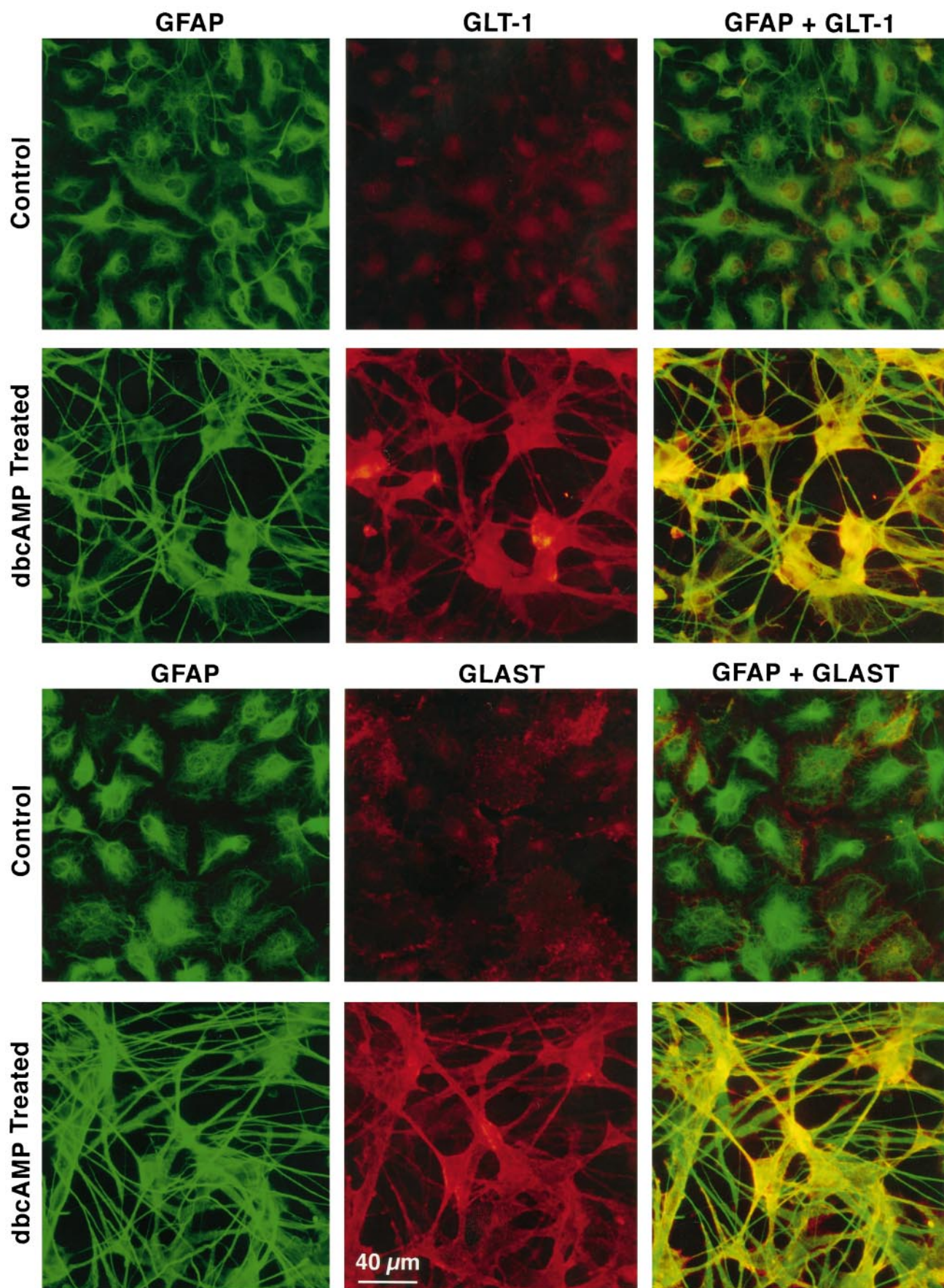


Fig. 4

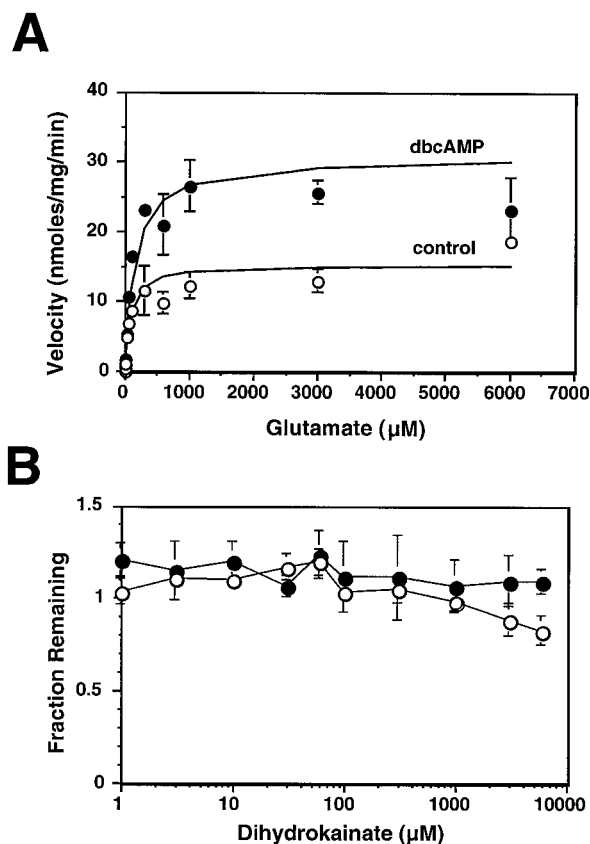


Fig. 5. A, Na^+ -dependent L-[^3H]glutamate transport in primary astrocyte-enriched cultures. The concentration dependence of Na^+ -dependent glutamate transport was measured in control (\circ) and dbcAMP-treated (\bullet) astrocyte cultures. Astrocyte cultures were treated with dbcAMP (0.25 mM) for 10 days. These data were fit by linear regression analysis of an Eadie-Hofstee transformation. For control astrocytes, the average K_m value was $78 \pm 16 \mu\text{M}$, and the average V_{\max} value was 14.8 ± 2.4 nmol/mg of protein/min. For dbcAMP-treated astrocytes, the average K_m value was $164 \pm 19 \mu\text{M}$, and the average V_{\max} value was 31.2 ± 2.3 nmol/mg of protein/min. Both the K_m and V_{\max} values were significantly different by Student's unpaired t test. Data are the mean \pm standard error of three independent observations. B, Sensitivity of glutamate transport to inhibition by dihydrokainate. Na^+ -dependent L-[^3H]glutamate (0.5 μM) transport was measured in the absence and presence of increasing concentrations of dihydrokainate. In parallel assays, transport was measured in untreated astrocytes (\circ) and in astrocytes treated with dbcAMP (0.25 mM) for 10 days (\bullet). Data are expressed as a fraction of the transport activity observed in the absence of dihydrokainate and are the mean \pm standard error of three independent observations.

Discussion

In the current study, the expression of subtypes of Na^+ -dependent glutamate transporters was examined in primary cultures derived from rat cortical tissue, with either cortical or cerebellar brain tissue as a control. Expression of transporters was examined in cocultures of neurons and astrocytes and in astrocyte-enriched cultures. cAMP analogs and neurons induced expression of GLT-1. Several experiments were performed to localize and define the mechanism of this regulation.

Although EAAC1 expression is thought to be restricted to neurons in the central nervous system, this transporter orig-

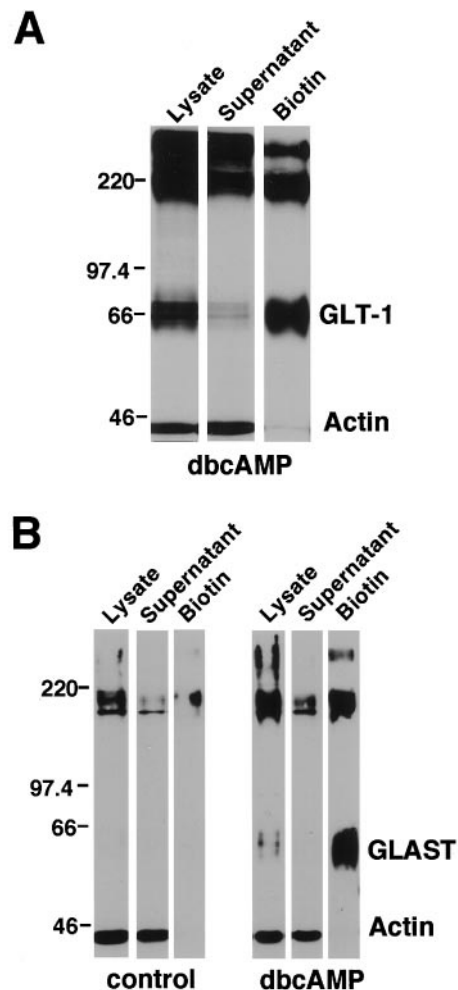


Fig. 6. Biotinylation of the GLT-1 and GLAST proteins in astrocyte cultures. Astrocytes were prepared and maintained for 14 days *in vitro* and then treated with dbcAMP (0.25 mM) for 10 days (dbcAMP). Control cultures were maintained for 24 days *in vitro*. After biotinylation and lysis (lysate), biotinylated proteins were separated with avidin beads. The supernatant from this batch extraction (supernatant) and the bound biotinylated fraction (biotin) were immunoblotted with anti-transporter antibody and with anti-actin antibody. Cell surface expression of GLT-1 (A) was only examined in the dbcAMP-treated cultures because there were such low levels of protein in the control cultures. Cell surface expression of GLAST (B) was examined in both control and dbcAMP-treated cultures. Quantitation of these studies indicate that $\approx 60\%$ of the transporters were on the cell surface (see Results for average values).

inally was cloned from an intestinal cDNA library and is expressed in several peripheral tissues (Kanai and Hediger, 1992; Rothstein *et al.*, 1994). In astrocyte-enriched cultures, EAAC1 was expressed at low levels relative to brain, and treatment of these astrocytes with dbcAMP caused a modest decrease in its expression. In cocultures of neurons and astrocytes, the expression pattern of EAAC1 was clearly distinct from that of the other transporters and from that of the astrocytic protein GFAP.

The other neuronal transporter, EAAT4, was detected as a single immunoreactive band in cerebellar homogenates with a molecular mass comparable to that observed previously

Fig. 4. Double-label immunohistochemistry of GFAP and GLT-1 or GFAP and GLAST. Localization of GFAP and GLT-1 or GLAST was examined in control astrocytes (24 days *in vitro*) and in astrocytes treated with dbcAMP (0.25 mM) for 10 days. The magnification is the same in each field (scale bar, 40 μm). None of the appropriate staining was observed with omission of primary antibody. These studies were reproduced in three independent experiments.

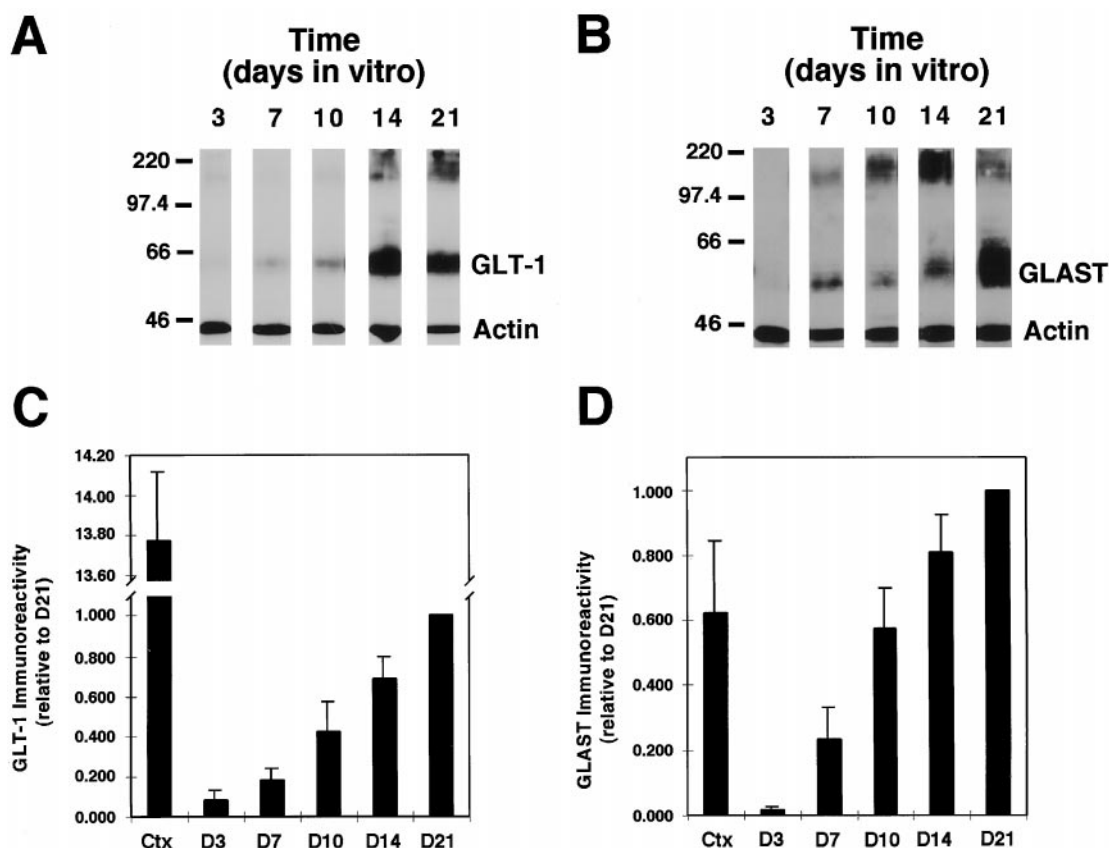


Fig. 7. Changes in GLT-1 (A and C) and GLAST (B and D) immunoreactivity in cocultures of neurons and astrocytes. Equal amounts of protein (50 μ g) were loaded in each lane. Cortical membrane homogenate (5 μ g for GLT-1 and 50 μ g for GLAST) also was included (not shown in A and B). Data were expressed relative to the immunoreactivity observed in these cocultures after 21 days. C and D, Data are the mean \pm standard error of at least five independent observations. Each time point is significantly ($p < 0.05$ by analysis of variance) different from the others with the exception of adjacent time points. Ctx, cortical membrane homogenates.

(Furuta *et al.*, 1997a). Although a single immunoreactive band also was observed in these astrocyte-enriched cultures, the apparent molecular mass was ≈ 20 kDa smaller than that observed in cerebellar homogenates. This immunoreactive band was not characterized further but is identical in size to the deglycosylated band of EAAT4 observed in cerebellar tissue (Furuta *et al.*, 1997a), suggesting that these astrocytes are unable to post-translationally process this protein.

Using antibodies derived against peptides from either the carboxyl- or amino-terminal portion of GLAST, multiple immunoreactive bands were observed; multiple immunoreactive bands also were observed for GLT-1. These higher molecular mass immunoreactive bands recently were characterized using brain tissue and cells transfected with individual cDNAs. They were attributed to oxidation of sulfhydryl groups and irreversible cross-linking to form large molecular mass aggregates (Haugeto *et al.*, 1996). The chemical nature of this cross-linking has not been defined, but the quantal nature of the band size and results of immunoprecipitation studies suggest that these are homomultimers (Haugeto *et al.*, 1996). In the current study, these high-molecular-mass bands generally were not observed in freshly prepared and boiled brain tissue but instead were observed in most cell culture homogenates. Because the length of time required to prepare these specimens is comparable, these multimers may be formed in the cell cultures before harvesting. This suggestion is supported by our observation that the

inclusion of dithiothreitol (5 mM), which prevents the formation of these multimers in brain tissue (Haugeto *et al.*, 1996), did not prevent the formation of these aggregates in specimens prepared from cell cultures.

The apparent molecular mass of the smallest GLAST immunoreactive band in cortical tissue was smaller than that observed in cell cultures by ≈ 5 kDa and was comparable in size to that observed in cerebellar homogenates. Interestingly, the size of the cortical band was ≈ 4 kDa smaller than the 60-kDa mass predicted based on the cDNA sequence (Storck *et al.*, 1992). This apparent molecular mass has been observed by others but not discussed (Storck *et al.*, 1992; Haugeto *et al.*, 1996). Four different antibodies derived against different peptide sequences recognize a band of the same size: the amino- and carboxyl-terminal antibodies used in the current study and two antibodies prepared by different groups (Haugeto *et al.*, 1996; Wahle and Stoffel, 1996). This band is observed in oocytes injected with the GLAST cRNA but not in water-injected oocytes (Storck *et al.*, 1992). In the current study, treatment of either cerebellar or astrocyte proteins with *N*-glycosidase F reduced the intensity of the GLAST band at ≈ 60 kDa and resulted in bands of ≈ 45 kDa. A band with an apparent molecular mass slightly larger than that observed in the current study has been observed after treatment with *N*-glycosidase F of oocytes injected with GLAST cRNA (Wahle and Stoffel, 1996) and in the developing nervous system (Furuta *et al.*, 1997b). The control incu-

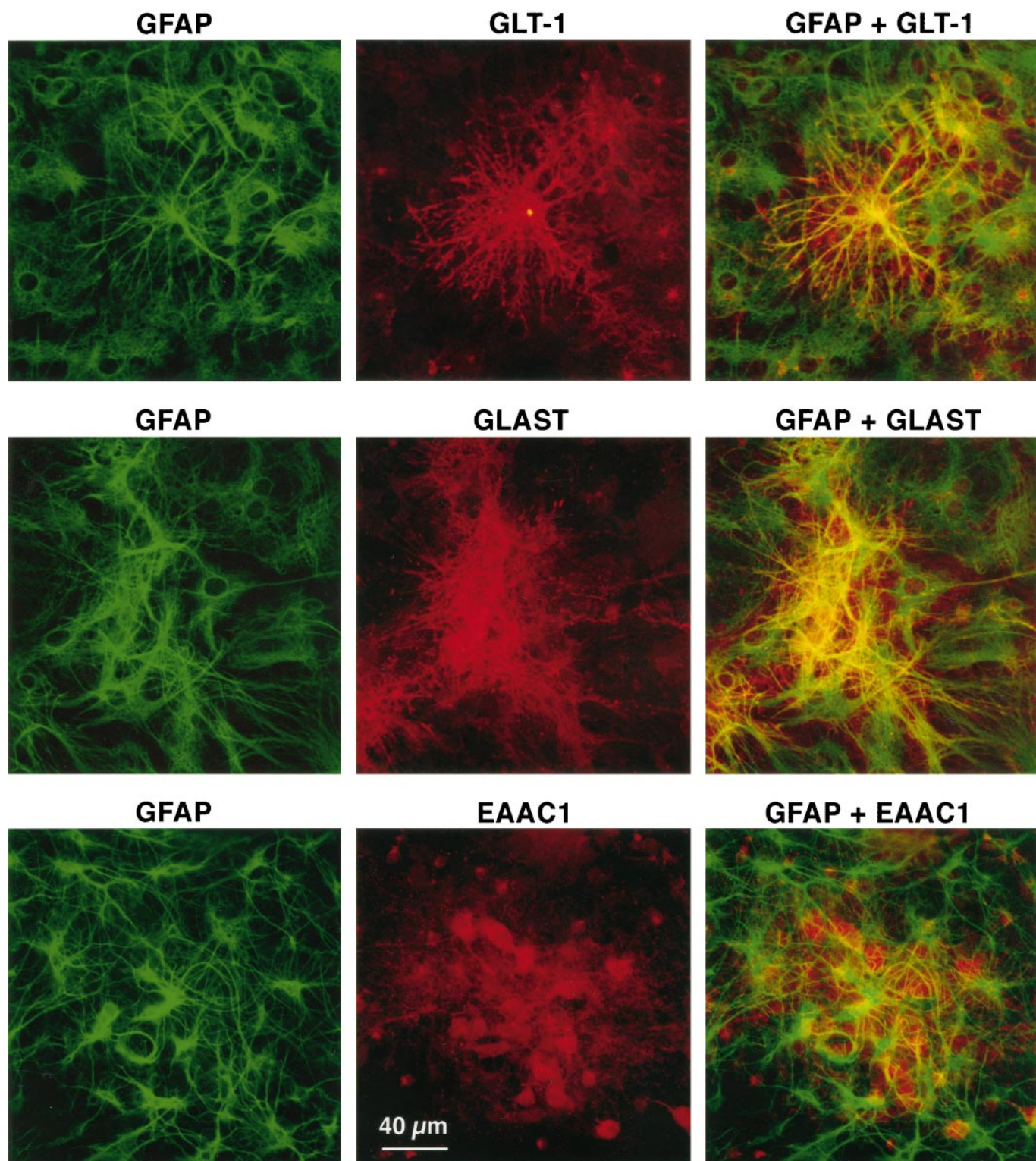


Fig. 8. Double-label immunohistochemistry in cocultures of neurons and astrocytes. Cocultures were prepared and maintained *in vitro* for 21 days. For EAAC1, the field chosen represents an area with a high density of neurons and highly differentiated astrocytes. The magnification is the same in each field (scale bar, 40 μ m). These different patterns of immunoreactivity and the observation that no immunoreactivity was observed when the appropriate primary antibody was omitted provide evidence for the specificity of these immunohistochemical studies. These studies have been reproduced at least three times.

bation (vehicle) did not result in the appearance of smaller immunoreactive bands (Fig. 1), suggesting that the small size of this protein relative to that predicted from the cDNA sequence cannot be attributed to proteolysis unless deglyco-

sylation increases the rate of proteolysis. Because four different groups have predicted the same size for GLAST through the isolation of cDNA sequences (for original references, see Robinson and Dowd, 1997) and reverse transcrip-

TABLE 2

Effects of PKA antagonists, receptor antagonists, and TTX on expression of GLT-1 and GLAST in cocultures of neurons and astrocytes

Cocultures of neurons and astrocytes were prepared as described in the text. After 7 days, compounds were added to the medium to provide the final concentration indicated in parentheses. After an additional 7 days, cells were harvested, and the levels of GLT-1 and GLAST immunoreactivity were determined by immunoblotting. Values represent the mean \pm standard error. The number of observations is provided in parentheses. There was no significant effect for any of the treatments as determined by analysis of variance.

Treatment	Immunoreactivity (relative to control)	
	GLT-1	GLAST
Control	1.0	1.0
KT5720 (5 μ M)	0.83 \pm 0.03	1.35 \pm 0.22 (3)
H89 (2 μ M)	0.71 \pm 0.12	0.93 \pm 0.18 (5)
rpcAMP (0.5 mM)	1.02 \pm 0.08	0.84 \pm 0.01 (2)
Propranolol (10 μ M)	0.79 \pm 0.16	0.79 \pm 0.21 (5)
(+)-MCPG (0.2 mM)	0.92 \pm 0.22	1.12 \pm 0.11 (6)
MK801 (1 μ M)	0.83 \pm 0.16	0.80 \pm 0.07 (6)
TTX (1 μ M)	0.92 \pm 0.19	0.86 \pm 0.19 (5)

tion-polymerase chain reaction does not reveal transcripts of different lengths in the developing nervous system (Furuta *et al.*, 1997b), this smaller molecular mass is not likely to be due to alternative splicing of this transporter. Together, these data provide strong evidence that this protein represents GLAST but that either GLAST migrates aberrantly in SDS gels, GLAST is cleaved post-translationally to remove a middle portion of the protein, or deglycosylation increases the susceptibility of the protein to proteolysis.

The most surprising observation in these control astrocyte-enriched cultures was that the expression of the glial transporter GLT-1 was very low, because it is generally accepted that GLT-1 is expressed at high levels by astrocytes *in vivo* (see Rothstein *et al.*, 1994; Lehre *et al.*, 1995; for a review, see Robinson and Dowd, 1997). Several strategies were used to induce expression of GLT-1 protein. Of the strategies studied, dbcAMP and coculturing with neurons induced expression of GLT-1 immunoreactivity. The apparent molecular mass of GLT-1 in astrocytes was comparable to that observed in cortical tissue and similar to that reported previously (Pines *et al.*, 1992; Rothstein *et al.*, 1994; Haugeto *et al.*, 1996). dbcAMP also increased the levels of GLAST protein. These effects of dbcAMP also were mimicked by 8-Br-cAMP and forskolin, providing evidence that the effects of dbcAMP were not related to previously described nonspecific effects of butyrate (Yusta *et al.*, 1988) but are due to activation of a cAMP-dependent process. The observations that the PKA antagonists attenuated the effects of dbcAMP provide evidence that these effects are caused by PKA activation. At this time, it is unclear why forskolin and isoproterenol were less effective than the cAMP analogs; this could be related to degradation of these molecules with chronic incubation or desensitization.

In many systems, cAMP and its analogs rapidly regulate transcription of various proteins through phosphorylation of a cAMP-responsive element binding protein (for review, see Montminy, 1997). In the systems examined to date, the increases in mRNA occur within hours. Although mRNA levels for both GLT-1 and GLAST are increased in response to dbcAMP, the increases in both mRNAs were delayed somewhat. This slow increase suggests that transcription of these mRNAs is not regulated directly through a cAMP-responsive element but rather that either transcription is regulated

indirectly through dbcAMP-induced expression of other transcription factors or that dbcAMP increases the stability of these mRNAs. Our initial attempts at determining whether the effects of dbcAMP were related to increased transcription or increased mRNA stability failed due to actinomycin toxicity. Although both mRNA and protein levels changed in the same direction, the increase in protein did not always correlate with the increase in mRNA levels. For example, GLAST mRNA increased 4–6-fold, but the increase in protein expression was 2-fold. This lack of correlation between mRNA and protein may be related to differential stabilization of the mRNAs or assembly and degradation rates of the proteins. These aspects of glutamate transporter regulation have not been examined.

In analyzing the functional effects of GLAST and GLT-1 protein induction, we found that dbcAMP treatment for 10 days caused a 2-fold increase in V_{\max} for Na^+ -dependent glutamate transport. This change in V_{\max} was lower than that predicted based on the 2-fold increase in GLAST protein observed and the increase in GLT-1 protein observed. The K_m value for transport also was elevated after treatment. This increase in K_m value is consistent with induction of GLT-1, which has a higher K_m value for glutamate than for GLAST in heterologous expression systems (Arriza *et al.*, 1994). It also is possible that the higher K_m value is an artifact of the increased capacity observed in these cultures. If the transport capacity is sufficiently large, it theoretically is possible that glutamate is present at a lower concentration near the transporter than in the bulk medium. Under these conditions, the apparent K_m value for transport, calculated based on the concentration of glutamate added to the bulk medium, would be higher than the true K_m value (for a discussion, see Garthwaite, 1985). Because GLT-1 expression in heterologous systems generally results in dihydrokainate sensitivity (Pines *et al.*, 1992; Arriza *et al.*, 1994), whereas GLAST expression generally results in dihydrokainate-insensitive Na^+ -dependent glutamate transport (Arriza *et al.*, 1994; Klöckner *et al.*, 1994), we expected that increased expression of GLT-1 would be accompanied by an increase in dihydrokainate sensitivity. Because no increases in dihydrokainate sensitivity were observed, cell surface proteins were biotinylated with a membrane-impermeant reagent to determine whether GLT-1 was being retained in a subcellular compartment. The biotinylated fractions were isolated with avidin beads, and both the biotinylated and nonbiotinylated fractions were analyzed by Western blot. Consistent with its intracellular localization, actin immunoreactivity was found only in the nonbiotinylated fraction. GLT-1 and GLAST immunoreactivity was present in both fractions, suggesting that both transporters are being trafficked to the cell surface. There are two possible explanations for these data: either the level of GLT-1 expression relative to GLAST is not sufficient to contribute to transport activity or GLT-1 is not active in these preparations. It is possible that GLT-1 requires either post-translational processing or coassembly with an interacting protein for activity. Although the biotinylation studies suggest that GLT-1 is accessible in these cultures, it also is possible that GLT-1 is buried by overlapping membranes and that rapid clearance of glutamate by GLAST limits access of glutamate to GLT-1 (Garthwaite, 1985). Although we originally considered studying the dihydrokainate sensitivity of transport in mixed cultures of neurons and astrocytes, this

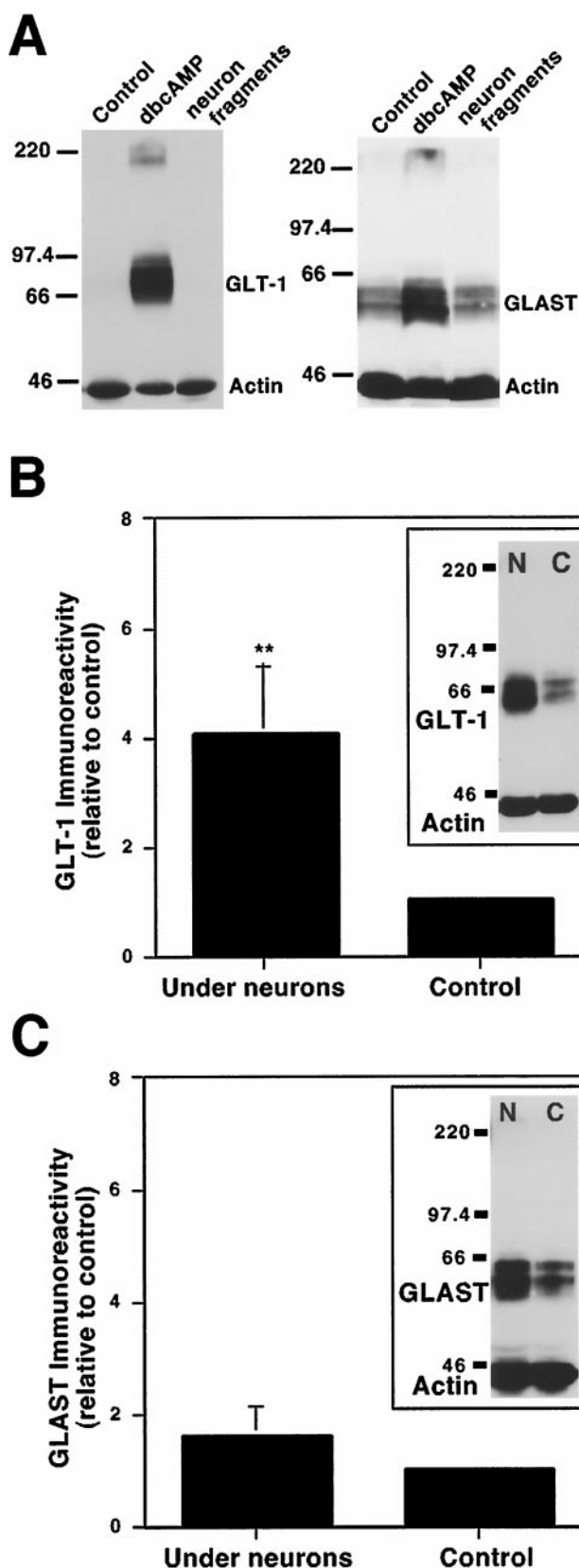


Fig. 9. Effects of neuron homogenates (A) and of separation of neurons and astrocytes by a semipermeable membrane (B and C) on the expression of GLT-1 (A and B) and GLAST (A and C) in astrocytes. After maintenance for 14 days *in vitro*, astrocytes were treated with neuronal membranes prepared from neuron-enriched cultures. Seven days later, these cultures were harvested and immunoblotted for GLT-1 immunore-

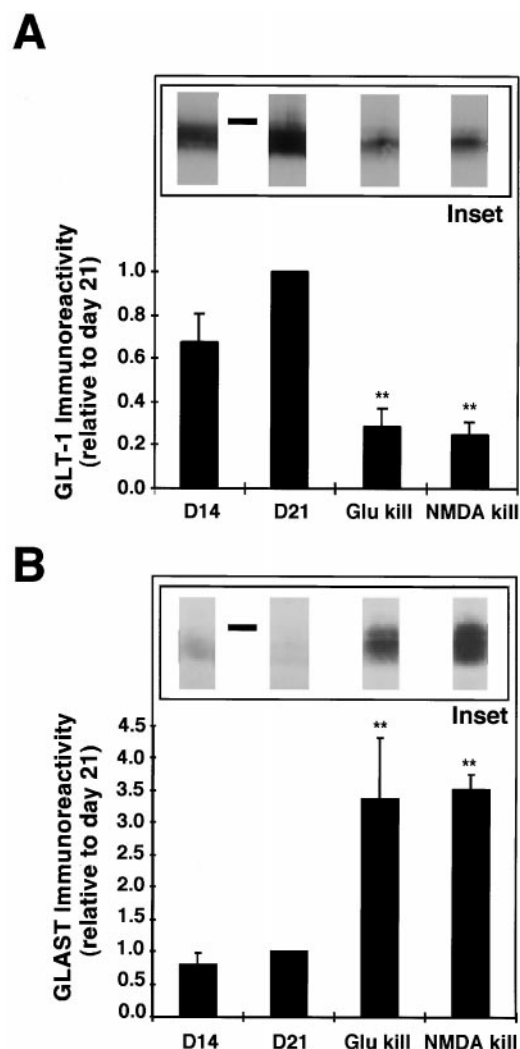


Fig. 10. Effects of treatment of cocultures of neurons and astrocytes with NMDA (1 mM) or L-glutamate (10 mM) on GLT-1 (A) and GLAST (B) protein. Cocultures were maintained for 14 days *in vitro*, and then either L-glutamate (*Glu*) or NMDA was added to the media. Twenty-four hours later, all neurons in these cultures were dead. Seven days after killing the neurons, cultures were harvested for immunoblotting. In each experiment, protein was harvested from control cultures at 14 days (D14) and 21 days *in vitro* (D21). *Insets*, representative immunoblots. *Horizontal bar*, migration of the 66-kDa molecular mass marker. Data are the mean \pm standard error of three or four determinations and are expressed relative to the amount of immunoreactivity observed at day 21.

experiment would be uninformative because recent data suggest that neurons in culture express a dihydrokainate-sensitive transport process (Wang *et al.*, 1996).

We demonstrated that coculturing astrocytes with neurons also has a dramatic effect on GLT-1 expression in astrocytes. Double-label immunohistochemistry using anti-GFAP antibodies and antitransporter antibodies was used to determine whether astrocytes were expressing GLT-1. In these cul-

activity. After maintenance for 4 days *in vitro*, neuron-enriched cultures (neurons and *N*) or empty inserts (control and *C*) were placed over astrocytes that had been maintained *in vitro* for 10 days. After an additional 14 days in culture, the cells were harvested and probed with antitransporter and anti-actin antibodies; 50 μ g of protein was loaded per lane. Immunoreactivity was calculated relative to control, yielding a significant difference with GLT-1 but not with GLAST ($p < 0.05$). Data are the mean \pm standard error of four independent observations.

tures, the neuronal transporter EAAC1 was expressed in a different population of cells than GFAP. These studies provide strong evidence that neurons are inducing expression of GLT-1 in astrocytes and that the appearance of GLT-1 is not due to expression in neurons.

Several experiments were performed to examine the mechanism behind the neuron-induced increases in astrocytic GLT-1 and GLAST protein levels. The experiments in which neurons were separated from astrocytes by a semipermeable membrane indicate that neurons secrete a diffusible molecule that induces expression of GLT-1 in astrocytes. Because neuronal membrane proteins had no effect on GLT-1 expression, we attribute the effect of neurons in cocultures to a diffusible molecule with no contribution by a contact-mediated event. We cannot rule out, however, the possibility that astrocyte/neuron interactions induce expression of the additional neuronal molecules that contribute to the regulation of GLT-1 in cocultures. In the immunohistochemical analyses of GLT-1 and GFAP in cocultures, GLT-1 seemed to be expressed at detectable levels in only a subpopulation of astrocytes that were near neurons, based on DAPI staining of cell nuclei. The expression of neurofilament and GLT-1 also was examined in these cocultures, and it was found that expression of GLT-1 always was in astrocytes near neuronal cell bodies. Together, these experiments suggest that neurons secrete a molecule to increase expression of GLT-1 but that this molecule is cleared by or binds to astrocytes, limiting the effectiveness of the molecule to the local environment.

A pharmacological approach was used to begin to define the type of molecule and the signaling that might mediate the effect of neurons on glial expression of GLT-1 and GLAST. Two systems widely known to increase cAMP production in glial cells are metabotropic glutamate and β -adrenergic receptors; chronic blockade of either of these receptor systems had no significant effect on the increase of GLT-1 or GLAST expression caused by neurons. Although the concentrations of the blockers used are effective in acute experiments, without measurement of the degradation of these compounds in these chronic experiments, the lack of an effect of these compounds cannot be taken as proof that these systems do not mediate the effects of neurons. However, the lack of an effect of glutamate receptor antagonists is consistent with the observation that glutamate had no effect on glial expression of GLT-1. Of note, recent studies suggest that glutamate may regulate expression of GLAST through activation of the ionotropic glutamate receptors (Gegelashvili *et al.*, 1996). We chose to examine the effect of chronic blockade of Na^+ channels (with TTX) or NMDA receptors (with MK-801) with the goal of determining whether neuronal excitability is required for the neuronal effect on GLT-1 expression. These compounds had no significant effect on the increase in transporter expression caused by neurons.

Because the increases in transporter expression caused by both dbcAMP and neurons correlated with a dramatic change in morphology of the astrocyte, it seemed possible that the effects are mediated by a common signaling pathway. To address this possibility, the effects of PKA antagonists and dbcAMP on GLT-1 and GLAST expression were examined in cocultures. Three different PKA antagonists had no significant effect on the increase in transporter expression caused by neurons, but the effects of neurons and dbcAMP on GLT-1 expression were not additive. These observations suggest

that the effect of neurons is not mediated by activation of PKA but that neurons and dbcAMP activate signaling pathways that converge to increase steady state levels of GLT-1 protein. In contrast to the effects on GLT-1, the effects of dbcAMP and neurons on GLAST expression were additive, suggesting that the regulations of GLT-1 and GLAST differ in this system.

Several early *in vivo* studies demonstrated that lesioning of glutamatergic projections results in decreased expression of glutamate transport in the target area (for a review, see Robinson and Dowd, 1997). The simplest interpretation of this result is that glutamate transporters are present on the presynaptic nerve terminal. Although not widely discussed, the alternative explanation is that the loss of the nerve terminal results in decreased expression of glutamate transport in the surrounding astrocytes. In the current study, high concentrations of glutamate or NMDA caused a decrease in GLT-1 expression in cocultures of neurons and astrocytes, and as expected, these treatments killed all of the neurons in these cultures. Because there is little evidence for expression of functional NMDA receptors on astrocytes, we interpret these data as indicating that neurons not only induce protein expression of GLT-1 in astrocytes but also are required to maintain expression of GLT-1. Killing the neurons increased GLAST expression. Recent studies have shown that lesions that destroy identified excitatory (cortical) inputs to the striatum are accompanied by decreases in GLT-1 and GLAST protein levels (Ginsberg *et al.*, 1995). This demonstrates that the neuronal dependence of glial glutamate transporter expression occurs both *in vivo* and *in vitro*.

In summary, we present evidence that GLT-1 and GLAST expression in astrocytes is regulated by cAMP analogs and neurons; a similar report of these observations was accepted while the current study was first under review (Swanson *et al.*, 1997). The time courses for these changes and the mechanisms involved in this regulation were examined. The increases in both GLAST and GLT-1 were relatively slow processes requiring several days. We demonstrated that GLT-1 and GLAST were present at the cell surface in dbcAMP-treated astrocytes but found little evidence that GLT-1 was functional in this system. We demonstrated that neurons release a diffusible molecule that regulates GLT-1 expression. Although PKA antagonists did not block the effects of neurons, the effects of neurons and dbcAMP were not additive, suggesting that dbcAMP and neurons regulate GLT-1 expression by converging signaling pathways. Finally, we demonstrated that killing neurons *in vitro* causes loss of GLT-1 expression. Given the demonstrated importance of GLT-1 for excitatory amino acid physiology and pathology (Tanaka *et al.*, 1997), this loss of GLT-1 expression may contribute to neurodegenerative processes such as amyotrophic lateral sclerosis and Alzheimer's disease. In these neurodegenerative diseases, a decrease in GLT-1 protein and/or a decrease in glutamate transport has been reported (for original citations, see Gegelashvili and Schousboe, 1997). If the factor responsible for regulating GLT-1 expression is not specific for glutamatergic neurons, a loss of GLT-1 expression in neurodegenerative diseases of nonglutamatergic neurons may result in increased vulnerability of the remaining neurons to an excitotoxic insult.

Acknowledgments

We thank Dana Correale, Naomi Fukuyama, Andrew Goldfine, and Andy Shulman for their technical assistance; Dr. Lisa Dowd Pedicone for initial discussions and generation of GLAST cDNA; and Drs. David Lynch, Brian Baskai, Jeffrey Golden, Louis Littman, Karen Davis, and David Pleasure for their helpful discussions and critical reviews of this manuscript. We also thank Dr. Virginia Lee for the mouse monoclonal antibody to medium molecular mass neurofilament.

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