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ORIGINAL PAPER

Distribution of Glutamate Transporter GLAST in Membranes of Cultured Astrocytes in the Presence of Glutamate Transport Substrates and ATP

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Abstract Neurotransmitter L-glutamate released at central synapses is taken up and "recycled" by astrocytes using glutamate transporter molecules such as GLAST and GLT. Glutamate transport is essential for prevention of glutamate neurotoxicity, it is a key regulator of neurotransmitter metabolism and may contribute to mechanisms through which neurons and glia communicate with each other. Using immunocytochemistry and image analysis we have found that extracellular D-aspartate (a typical substrate for glutamate transport) can cause redistribution of GLAST from cytoplasm to the cell membrane. The process appears to involve phosphorylation/dephosphorylation and requires intact cytoskeleton. Glutamate transport ligands L-*trans*-pyrrolidine-2,4-dicarboxylate and DL-*threo*-3-benzyloxyaspartate but not *anti,endo*-3,4-methanopyrrolidine dicarboxylate have produced similar redistribution of GLAST. Several representative ligands for glutamate receptors whether of ionotropic or metabotropic type, were found to have no effect. In addition, extracellular ATP induced formation of GLAST clusters in the cell membranes by a process apparently mediated by P2 receptors. The present data suggest that GLAST can rapidly and specifically respond to changes in the cellular environment thus potentially helping to fine-tune the functions of astrocytes.

Keywords Astrocytes · Glutamate synapses · Glutamate transport · GLAST · GLT · Brain metabolism · Na^+, K^+-dependent ATPase · Neurotransmitter metabolism · Regulation of transporters · Phosphorylation and dephosphorylation of membrane proteins

Introduction

Acidic amino acids L-aspartate (L-asp) and L-glutamate (L-glu) act on a set of membrane-located receptors that function as ion-gated channels, changing membrane permeability to Na^+ and Ca^{2+} ("ionotropic glutamate receptors", iGluR) and depolarizing ("exciting") the neurons in the central nervous system. In addition, L-glu, but not L-asp, acts on distinct G-protein linked receptors ("metabotropic glutamate receptors", mGluR) that activate intracellular signalling pathways [1]. Thus L-glu and L-asp, can act as signalling agents in the intercellular communication and, indeed, L-glu appears to be the major neurotransmitter at the excitatory synapses in brain (reviews [2, 3]).

L-Glu is stored in presynaptic vesicles and is released from synaptic nerve endings by depolarization-coupled, Ca^{2+}-dependent mechanisms. Active Na^+/K^+-dependent transport of L-glu (GluT) provides a mechanism that regulates concentrations of L-glu at the synapse, preventing excessive stimulation of glutamate receptors that could be harmful and cause pathological neurodegeneration [4].

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L-Glutamate transport (GluT) in the brain is mediated mainly by glutamate transporters expressed in astrocytes, GLAST and GLT (also known as EAAT1 and EAAT2, respectively; reviews [5–7]). Long-term regulation of GluT, over periods of hours and days involves factors that can change overall levels of GLAST and GLT in astrocytes and are probably mediated by manipulation of protein synthesis [8]. There is, however, evidence that the efficacy of GluT can be strongly influenced by rapid shifts of transporters in and out of plasma membranes [9, 10] (for reviews see [11, 12]) and/or by their clustering in astrocytic processes [13] (for review see [5]). Such movements may occur in response to changes in the activity of glutamatergic synapses and, therefore, activity of L-glu transport could be of importance not only for the normal operation of the synaptic excitation [5] but also as an indicator of local excitatory activity that would have to be matched by local changes in the metabolism and blood flow. Indeed, it has recently been demonstrated that not only pharmacological manipulation of glutamate receptors but, in particular, interference with GluT, can elicit specific metabolic responses in brain tissue [14–17].

In the present study, we have used immunofluorescence labelling combined with deconvolution microscopy to visualize GLAST transporter distribution in cultured astrocytes. We have been investigating changes in the distribution of GLAST in response to compounds known as specific ligands for glutamate receptors or as substrates/inhibitors of glutamate transporters.

Materials and Methods

Cultured Astrocytes

Cultured astrocytes were prepared from neocortices of neonatal (aged up to three postnatal days) Sprague–Dawley rats. The tissue was freed of meninges, sliced with micro-scissors and dissociated with trypsin (0.25% in Hanks balanced salt solution). Cells were maintained in Dulbecco modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (FBS), initially in 25 cm^2 flasks (usually 2–3 brains per flask) until confluent (10–14 days), then passaged onto coverslips (13 mm in diameter) and grown for further 12–14 days. At this time, astrocytes were evenly distributed over the area of the coverslips and displayed characteristic astrocyte morphology [18].

All procedures involving animals were carried out in accordance with the guidelines of the National Health and Medical Research Council of Australia and were approved by The University of Sydney Animal Ethics Committee.

Immunocytochemistry and Image Analysis

The coverslips with astrocytes were washed in serum-free DMEM (sfDMEM) and incubated with drugs such as GluT substrates and glutamate receptor ligands, dissolved in 500 µl of sfDMEM. Incubations lasted 30 min and were carried out at 37°C in 5% CO_2/95% O_2. Drug-free sfDMEM was used as a control.

Following the exposure to drugs the coverslips were first washed in 2 ml of phosphate buffered saline (PBS, pH 7.4) for 5 min, then fixed with paraformaldehyde (2% in PBS) for 10 min and again washed with 2 ml of PBS for 5 min. Fixed cells were then "blocked" with PBS containing 1% bovine serum albumin (BSA) (PBS/BSA) for 30 min. Astrocytes were double-labelled with antibodies against glial fibrillary acidic protein (GFAP, marker for astrocytes, mouse monoclonal antibodies, SIGMA Chemical Co.) and antibodies against the glutamate transporter GLAST (polyclonal antibody raised in rabbit [19]). The immunolabelling procedures were similar to those described previously [19]. The antibodies were dissolved in PBS/BSA containing 0.05% saponin and coverslips were exposed to 200 µl of solutions containing the antibodies at dilutions of 1:4,000 (GLAST) and 1:400 (GFAP). The incubations (2 h) were carried out at room temperature in a humidified environment. The coverslips were then washed three times for 5 min with 2 ml of the PBS/BSA solution and exposed to the secondary antibodies.

All procedures involving the fluorescent labelled antibodies were performed in a dark environment. Goat-generated anti-mouse IgG conjugated to Alexa Fluor 488 (AF 488) was used to visualise GFAP-like immunoreactivity (GFAP-LIR) while goat-generated anti-rabbit IgG conjugated to Alexa Fluor 594 (AF 594) was used to label the anti-GLAST antibody GLAST-like immunoreactivity, GLAST-LIR). Both secondary antibodies were diluted in the PBS/BSA solution, incubations were for 1 h at room temperature and washed as described above for the primary antibodies; three times for 5 min with 2 ml of PBS/BSA.

After washing, the coverslips were carefully blotted to remove excess moisture, mounted upside down, using 50% solution of glycerol in PBS, on slides and secured along the edges with nail polish, to prevent drying out.

Image Analysis

Deconvolution microscope (Axioplan 2, Zeiss) was used for image acquisition. AF 488 was excited at 499 nm (emission at 520 nm, filters used: BP 480/40, 50SLP and BP 535/30), AF 594 was excited at 590 (emission at 618 nm, filters used: BP560/55, 595/LP, BP 645/75). The images were optically sectioned at 0.513 µm intervals and subjected to deconvolution using an inverse filter algorithm to remove

out-of-focus (background) signals. Sections from the mid-plane of the stacks were used for the image analyses. Each image represented a randomly selected single cell.

Images of immunolabelled cells were subjected to "thresholding" to objectively estimate the background [20] while the cells contour was determined using a spline-based "snake" model developed for this project. This model has been derived from the active contour model [21]. It uses an arbitrary closed spline cube as an initial contour rather than an ellipsoidal curve. This allows the model to define a very accurate cell contour. The membrane was defined as the area between the cell contour and a line obtained by "shrinking" the "snake" 1.7 μm inside the contour. Thus, the "thickness" of membrane was taken as constant at 1.7 μm, about 5 times the lateral resolution limit of the deconvolution microscope. This is much greater than the actual thickness of cell membranes (<10 nm [22]). Rather, this value was chosen as corresponding approximately to the size of the clusters of the fluorescence-labelled GLAST-LIR appearing in the membrane following exposure of the astrocytes to GluT substrates so as to capture most of the label typically present in, or, in the immediate vicinity of, the membrane under such experimental conditions.

Mean fluorescent density (MFD) was then determined (using Image-J software, http://rsbweb.nih.gov/ij/) in both the cytoplasm and membrane (cMFD and mMFD) and the membrane/cytoplasm ratio of fluorescent intensity (RFI = mMFD/cMFD) was used as an index of the distribution of GLAST-LIR between membrane and cytoplasm.

In order to assess the distribution of GLAST-LIR along the plasma membrane, fluorescence intensity was sampled using squares 1.7 × 1.7 μm (10 × 10 pixels) along the membrane, every 8.5 μm (50 pixels). The values were then analysed for Gaussian distribution, using D'Agostino and Pearson normality test ($P < 0.05$ as a limit) and the distribution was considered "clustered" if it was not normal (Gaussian).

In addition, in some experiments, "compactness" of the cells, defined as a square of the cell perimeter divided by the area [23, 24] was calculated and correlated by linear regression with RFI. There was no correlation ($r^2 = 0.1737$) indicating that the values of RFI were not influenced by the shape of astrocytes.

Sources of Materials

(*RS*)-α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), D-aspartic acid, DL-*threo*-3-benzyloxyaspartic acid (DL-TBOA), (*2S,3S,4R*)-carboxy-4-(1-methylethenyl)-3-pyrrolidineacetic acid (kainic acid), 8,8′-[carbonyl*bis*[imino-3,1-phenylenecarbonylimino(4-methyl-3,1-phenylene)carbonylimino]]*bis*-1.3.5-naphtalenetrisulphonate hexasodium (suramin), 6-chloro-3,4-dihydro-3-(2-norbornen-5-yl)-2H-1,2,4-benzothaidiazine-7-sulphonamide-1,1-dioxide (cyclothiazide), 9,10-deepithio-9,10-didehydroacanthofolicin (okadaic acid), (*2S,2′R,3′R*)-2-(2′,3′-dicarboxycyclopropyl)glycine (DCG IV), (*S*)-3,5-dihydroxyphenylglycine (DHPG), L-*trans*-pyrrolidine-2,4-dihydroxylic acid (L-*t*-PDC), L-*anti,endo*-3,4-methanopyrrolidinedicarboxylic acid (*a,e*-MPDC) and (*RS*)-(tertrazol-5-yl)glycine (TZG) were purchased from Tocris Cookson Ltd, Northpoint, Fourth Way, Avonmouth, Bristol, BS11 8TA, UK; 1-[6-[(3-Acetyl-2,4,6-trihydroxy-5-methylphenyl)methyl]-5,7-dihydroxy-2,2-dimethyl-2*H*-1-benzopyran-8-yl]-3-phenyl-2-propen-1-one) (rottlerin) came from Calbiochem (EMD Biosciences, Inc., 10394 Pacific Center Court, San Diego, CA 92121, USA); bovine serum albumin (BSA), antirabbit and antimouse secondary antibodies, adenosine triphosphate (ATP) and uridine triphosphate (UTP) came from Sigma-Aldrich Pty Ltd, Unit 2/14 Anella Ave, Castle Hill, NSW 2154, Australia; materials for cell culturing originated from Gibco, purchased via Invitrogen Corporation, 1600 Faraday Avenue, P.O. Box 6482 Carlsbad, California 92008, USA or BioScientific Pty. Ltd, P.O. Box 78, Gymea, NSW 2227, Australia, except for foetal bovine serum: Cosmic Calf Serum, Progen, Global Science & Technology Ltd, P.O. Box 101253 North Shore Mail Centre, Auckland, New Zealand.

Results

The presence of a typical transporter substrate D-asp caused a significant shift of GLAST towards the cell membrane (Fig. 1a, b), with RFI about double the control value (Fig. 2). The effect of D-asp was inhibited by cytochalasin D (Fig. 2), by the phosphatase 1/2A inhibitor okadaic acid (Figs. 1c, 2) and by PKC-delta inhibitor rottlerin (data not shown; cf. [25]).

Synthetic ligands for the glutamate transporter binding site L-*t*PDC [26], and DL-TBOA [27, 28] but not *a,e*-MPDC [26] significantly changed the distribution of GLAST-LIR in a manner similar to that observed in the presence of D-asp (Figs. 1d–f, 2). In contrast, none of the ligands for iGluR or mGluR had any effect either on RFI (Fig. 2) or on the distribution of GLAST-LIR within the membrane (data not shown): (tetrazol-5-yl)-glycine (TZG, neurotoxic agonist on NMDA receptors [29, 30]), kainic acid, AMPA (whether alone or in the presence of cyclothiazide to slow down the receptor desensitization [31]), dihydroxyphenylglycine (DHPG, specific activator of metabotropic GluR of

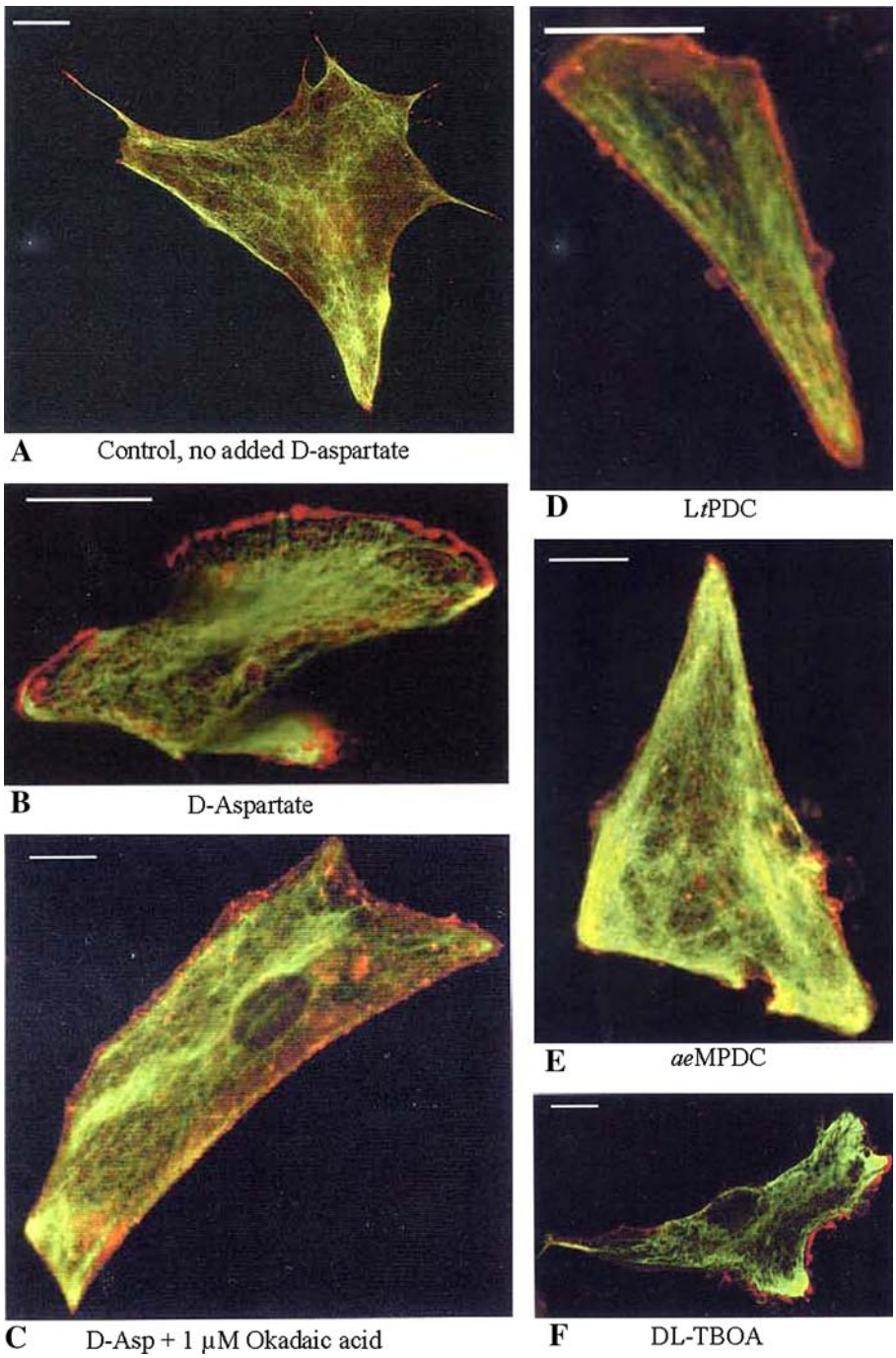


Fig. 1 Subcellular distribution of GLAST in cultured astrocytes in the presence of glutamate transport substrates. *Green* fluorophore AF 488 marks glial fibrillary acidic protein-like immunoreactivity (GFAP-LIR) and identifies the cells as astrocytes. *Red* fluorophore AF 594 marks the distribution of GLAST-LIR. Except for the controls, cells were incubated for 30 min in the presence of 500 µm concentrations of test compounds: D-aspartate (D-asp), L-*t*PDC (L-*trans*-pyrrolidine-2,4-dicarboxylate), *ae*-MPDC (*anti,endo*-metanopyrrolidine-3,4-dicarboxylate) and DL-TBOA (DL-*threo*-3-benzyloxyaspartate). Okadaic acid (**c**) is a broad-spectrum phosphatase inhibitor. In several preparations, some cells were selected at random and the distribution of GLAST-LIR was evaluated in more detail (Fig. 2). Scale bar = 20 µm. (For interpretation of the references to color in this figure legend, the reader is referred to the online version of this article)

group I [32]) and DCG IV (specific activator of metabotropic GluR of group II [1, 33]).

Cells exposed to ATP displayed a characteristic distribution of GLAST-LIR along the membrane (Fig. 3a). The effect was inhibited by suramin (Fig. 3b) and it could not be reproduced by UTP (Fig. 3c, d). In addition to the increased frequency of clusters the presence of ATP resulted in a net redistribution of GLAST in the direction of cell membrane but the effect was much smaller than that observed with D-aspartate (Fig. 3c).

Discussion

The present data demonstrate that GluT substrates, in particular D-asp can cause significant changes on the distribution of GLAST-LIR in astrocytes cultured from brains of neonatal rats. The process appears to be dependent on the intact cytoskeleton as evidenced by the inhibitory effect of cytochalasin D and might involve phosphorylation as indicated by the effect of 1/2A phosphatase inhibitor okadaic acid. Okadaic acid has been shown to have no effect

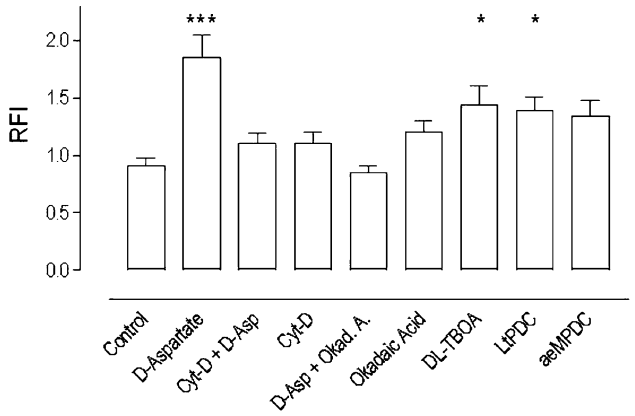


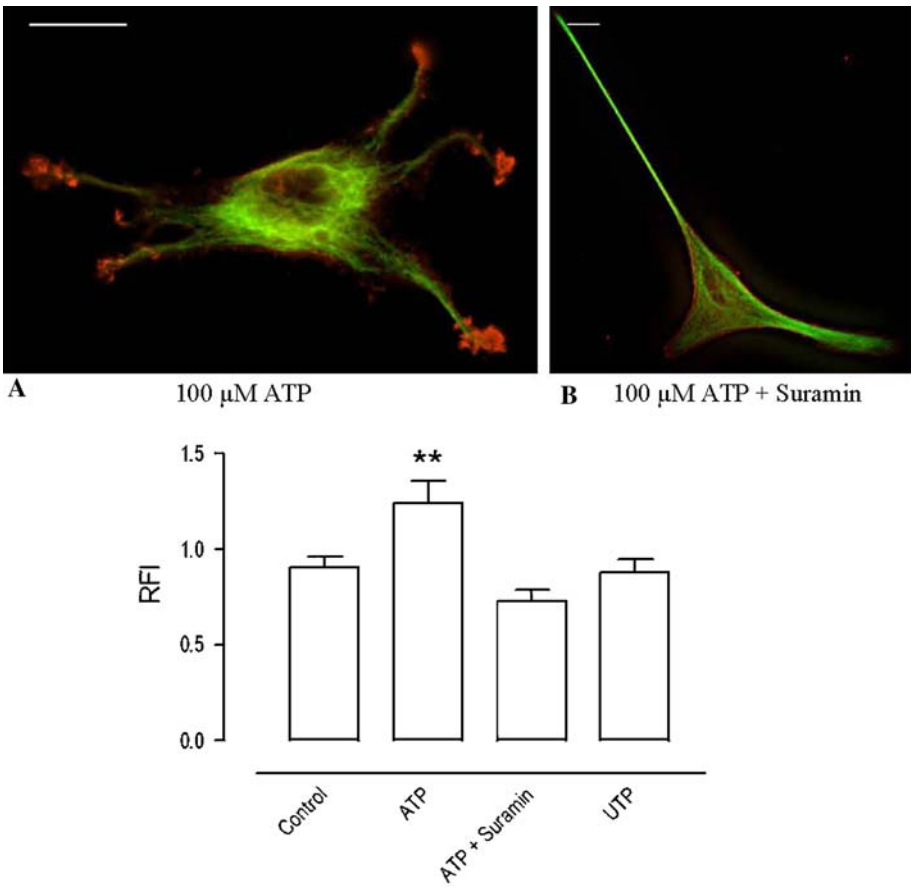
Fig. 2 Effect of drugs and glutamate transport substrates/inhibitors on the subcellular distribution of GLAST-LIR. Cells were selected at random from two or three experiments and the distribution of the *red* dye (AF 594, GLAST) was estimated as described in "Methods and Materials". Data are presented as mean ± SEM and the asterisks mark the statistical significance of the difference from controls (incubation without D-aspartate and with no drug): * $P < 0.05$; *** $P < 0.001$ by ANOVA (Newman–Keul's test, GraphPad Prism). The numbers of cells (n) included in the analysis were 20 for controls and 9, 10 or 11 in all other cases. RFI stands for "ratio of fluorescence intensities", see "Methods and Materials" for further details

on GluT in glioma C6 cells where the principal glutamate transporter is EAAC1 [34]. In the present studies okadaic acid did inhibit the effect of D-asp on the distribution of GLAST and this is consistent with phosphorylation/dephosphorylation of GLAST being involved in the mechanism of the rapid regulation of GluT. This is in accordance with the findings obtained using glial plasmalemmal vesicles [35] or astrocytes where GLAST is known to be the principal glutamate transporter [8, 36]. The present observations are also consistent with the effect of the PKC-delta inhibitor rottlerin [37]. PKC and PKC-delta in particular might play a role in the regulation of GluT [11, 38], however, rottlerin has recently been reported to act on the subcellular distribution of GLAST via a PKC-independent mechanism [10] and the mechanism by which it inhibits the effect of D-asp on GLAST redistribution may not be exclusively dependent on phosphorylation by protein kinases [25].

Available data seem to suggest no great difference between the affinities of D-aspartate and L-*t*PDC on GLAST [26] whereas *ae*-MPDC seems to be a rather poorly transportable inhibitor of GluT in synaptosomes [39]. More recent experiments in cultured cells which, on other evidence, appear to express GLAST as the main glutamate transporter indicate little if any difference in the potencies of the presently used compounds as substrates or inhibitors of GluT. The inhibitory potencies (expressed as IC_{50} or K_i values, μM) are, in human fibroblasts [40]: D-asp 17.6, L-*t*PDC 36.8; in human astrocytoma line (U373 [41]): D-asp 29.8, L-*t*PDC 17.6, *ae*-MPDC 30.1; in primary murine astrocytes [42]: DL-TBOA 40.6, L-*t*PDC 74.1, *ae*-MPDC 87.5; in EAAT1 transfected COS-1 cells [28]: DL-TBOA 33.0. In a fluorescent assay, using EAAT1 transfected HEK293 cells [43], the values were reported as K_m for transportable substrates D-aspartate (25 μm) and L-*t*PDC (20 μm) and K_i for DL-TBOA (2.9 μm). In particular, the observed ability of the GluT ligands to cause redistribution of GLAST does not appear to be related to whether they are substrates or inhibitors of GluT. Data obtained in GLAST-expressing primary cultures of astrocytes (similar to the presently used experimental model), indicated that L-*t*PDC was transported as a substrate while DL-TBOA and *ae*-MPDC were transported only poorly or not at all [42]. Thus there seems to be no simple correlation between the potencies of the substrates/inhibitors of GluT on GLAST redistribution (Fig. 2) and their actions on GluT as reported in the literature, particularly when these are likely to be mediated by GLAST (EAAT1; for additional discussion of the affinities of the abovementioned substrates/inhibitors of GluT see [12, 18, 26, 44]). The best correlation with the present data seems to be offered by a set of IC_{50} values obtained in [^{3}H]L-aspartate binding studies using fresh/frozen sections of rat brain (summarized in [45]): D-aspartate ($IC_{50} \sim 0.3$–1.4 μm) is about 10 times more potent than L-*t*PDC (less potent than D-aspartate in the present study, Fig. 2) and about 100 times more potent than *ae*-MPDC (no significant effect, Fig. 2). Interestingly, the distribution of [^{3}H]L-aspartate binding in rat brain closely resembles that of GLAST: it is significantly greater in the cerebellar cortex than in the forebrain regions [45, 46]. As the binding to GLAST in frozen tissue is not likely to involve any active substrate transport, the most parsimonious conclusion from the observed correlation would seem to be that high affinity for the substrate binding site on GLAST is more important in the mechanism of the substrate-assisted redistribution of GLAST in astrocytes than the ability of the substrates to be actually transported.

It is remarkable that none of the glutamate receptors tested produced any effect. Glutamate receptors have been shown to exist on astrocytes [47, 48] but the present results suggest that none are likely to play a prominent part in the distribution of GLAST. The lack of effect of receptor ligands also implies that the changes in the distribution of GLAST are not influenced by variations of intracellular pH; kainic acid that causes decrease in cytoplasmic pH similar to that observed after the application of D-asp [49], had no effect.

ATP had only a small influence on the actual distribution of GLAST between cytoplasm and cell membrane. The presence of ATP, however, resulted in the appearance of distinct clusters of GLAST-LIR that were found mainly in the astrocytic processes. Several findings seem to



C Distribution of GLAST in the presence of ATP

	CD	GD	Diff. from control
Control	5	15	---
ATP	10	2	P = 0.003
ATP + Suramin	4	6	n.s.
UTP	6	4	n.s

D Frequency of "clustered" distributions (CD) v. "non-clustered" (normal, Gaussian, GD) distributions analysed by Fisher exact test

Fig. 3 Effect of ATP on the distribution of GLAST-LIR in the membranes of cultured astrocytes. Typical distribution of GLAST-LIR following a 30-min incubation in the presence of 100 µm ATP is shown in the *panel a* (meaning of fluorophores cf. Fig. 1). The P2 antagonist suramin was used at 100 µm concentration (**b**). The shift of GLAST-LIR to the cell membrane appeared to be less pronounced than after the incubation with D-aspartate (**c**; cf. Fig. 2). UTP was used at 100 µm. Details of the analysis in *panel c* are same as in Fig. 2, for the numbers of cells analysed, see *panel d*. The magnitude and statistical significance of changes in the distribution of ATP towards the "clustered" pattern was investigated by Fisher exact test (**d**). Scale bar = 20 µm

suggest that it is the distribution of glutamate transporters along the membrane that is decisive for the role of GluT in the regulation of glutamate in the vicinity of glutamatergic synapses [5]. Such perisynaptic and/or clustered distributions have been described mainly for GLT [13, 50] (for reviews and additional references see [5, 12]). It has been suggested that the degree of utilization of GluT (particularly that portion mediated by GLAST) could act as a sensor of glutamatergic excitation, translate, via a coupled Na^+, K^+-dependent ATPase, the variations in the synaptic activity into changes in the astrocytic metabolism and eventually regulate glucose uptake from blood [51–53]; see also [54–56]). However, as indicated by the sensitivity to inhibition by suramin, the ATP effect on GLAST distribution seems to be mediated by P2 receptors. The Na^+, K^+-dependent ATPase inhibitor oubain (100 µM) had little or no effect on the ATP-linked redistribution of GLAST-LIR (results not shown). Additional experiments have, however, indicated, that ouabain at that concentration was not a strong inhibitor of Na^+, K^+-dependent ATPase in membranes prepared from the cultured astrocytes (KTD Nguyen, unpublished) implying that the expression of Na^+, K^+-ATPase protein subunits conferring inhibitor sensitivity on the enzyme is, in presently used astrocytic cultures, different from that in other types of cultured astrocytes [51]. Release of glutamate by astrocytes has been reported

to be linked to P2X7 receptors [57]. The release may have been mediated by a glutamate transporter such as GLAST that could be, in situ, subject to a feedback regulation by extracellular ATP which is also released, as a signalling compound, by astrocytes [58]. The GLAST shifts in the membrane would thus contribute to mechanisms involved in the communications among astrocytes [59] or to a feedback mechanism in a process by which astrocytes communicate with neurones [60].

One limitation of the present study is the low resolution of the immunolabelled cell images, even when using deconvolution microscopy. The "artificial" membrane thickness (1.7 μm) is about two orders of magnitude greater than the actual thickness of the plasma membrane. Thus we may have included, as "membrane-bound", also GLAST molecules not yet incorporated into the membrane but merely transferred to the immediate vicinity of it (so close that they are fixed to it during the exposure to paraformaldehyde).

In summary, using fluorescence immunocytochemistry and a computer-assisted image analysis of deconvolution microscopy we have demonstrated that the glutamate transporter GLAST expressed in astrocytes cultured from neonatal rat brain can be redistributed from the cytoplasm to the cell membrane (or its immediate vicinity) by substrates of glutamate transport. The process appears to be phosphorylation/dephosphorylation dependent and requires intact cytoskeleton. In addition, ATP, acting in a suramin-sensitive (P2 recepotor-like) manner, was found to induce formation of distinct clusters in the astrocytic membranes. The present data indicate that glutamate transport in astrocytes can rapidly respond to the changes in cell environment possibly as a part of mechanisms fine-tuning the functions of the astrocytic metabolic compartment in brain tissue.

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