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Evidence for change in current flux coupling of GLT1 at high glutamate concentrations in primary forebrain neurons and GLT1a expressing COS7 cells

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Abstract

Glutamate is the major excitatory neurotransmitter of the central nervous system and is toxic to neurons even at low concentrations. GLT1, the rodent analog of human EAAT2, is primarily responsible for glutamate clearance in the cerebrum. GLT1 was thought to be expressed exclusively in astrocytes in the mature brain. Recently, however, GLT1a was demonstrated in excitatory axon terminals where synaptic glutamate concentration rises above 1 mM during excitatory transmission. However, GLT1 function in neurons with accurate control of both intracellular and extracellular solutions mimicking synaptic concentration gradients has never been studied. Here we characterized the kinetics of coupled glutamate transporter current in whole-cell configuration and [³H]-L-glutamate uptake in cultured rat cerebral neurons across the entire range of synaptic glutamate concentrations. In both neurons and GLT1a transfected COS-7 cells, the kinetics were similar and revealed two specific components: a high affinity component with glutamate K_D value around 15 μ M and low affinity component with K_D value around 0.2 mM. The specific low affinity component was discovered due to significant deviation of the transporter current from Michaelis-Menten kinetics in the 100 – 300 μ M concentration range. Activation of the specific low affinity component led to a twofold decrease in the current/flux ratio implying a change in the transport coupling. Our data indicate that GLT1 endogenously expressed in cultured rat forebrain neurons displays high and low glutamate affinity uptake components that are different in current/flux coupling ratios. This property is intrinsic to the protein because it was also observed in GLT1a transfected COS-7 cells.

Keywords

GLT1; patch-clamp; whole cell; current/flux coupling; excitotoxicity; presynaptic; synapse

Introduction

Glutamate is the major excitatory neurotransmitter of the central nervous system, which is toxic to neurons even at low concentrations (Rosenberg & Aizenman, 1989; Rosenberg *et al.*, 1992). The only known clearance mechanism for glutamate is provided by five excitatory amino acid transporters (EAAT1-5) (Danbolt, 2001). GLT1, the rodent analog of human EAAT2, is the major glutamate transporter of the brain. GLT1 knockout mice die several weeks after birth and have only about 5% of wild-type levels of glutamate uptake

activity (Tanaka *et al.*, 1997). Inhibition of GLT1 by intrahippocampal microinjection of the antagonist WAY-855 in rat *in vivo* killed the majority of both CA1–4 pyramidal cells and dentate gyrus granule cells (Selkirk *et al.*, 2005).

GLT1a has been demonstrated by immunocytochemistry in 20–30% of excitatory axon terminals in the CA3 and CA1 fields of the rat hippocampus (Chen *et al.*, 2004). Comparing D-aspartate uptake immunocytochemistry in wildtype and GLT1 knockout mice, it has been estimated that up to 80% of excitatory terminals transport D-aspartate in a GLT1 dependent manner (Furness *et al.*, 2008). In another line of evidence, glutamate uptake in synaptosomes was found to be pharmacologically similar to GLT1 (Ferkany & Coyle, 1986; Robinson *et al.*, 1993; Koch *et al.*, 1999), and GLT1 co-purified with synaptosomes (Suchak *et al.*, 2003).

There are four variant forms of GLT1. Alternative polyadenylation at the 3'-end of GLT1 mRNA leads to two forms, GLT1a and GLT1b, with different C-termini. The GLT1b C-terminus was first found in mouse liver (Utsunomiya-Tate *et al.*, 1997). The complete GLT1b cDNA was subsequently cloned from a neuronal cDNA library (Chen *et al.*, 2002) and from the retina (Schmitt *et al.*, 2002) where it was called GLT1 *v*. GLT1b interacts with the synaptic PDZ domain protein PICK1 (Bassan *et al.*, 2008). A partial cDNA for the third carboxyl-terminal splice-variant is GLT1c that was found in retina (Rauen *et al.*, 2004). The fourth GLT1 variant is an exon 9-skipping form that is expressed primarily in populations of white matter astrocytes (Macnab & Pow, 2007).

The glutamate concentration in the synaptic cleft rises over 1 mM during excitatory synaptic neurotransmission (Clements *et al.*, 1992; Raghavachari & Lisman, 2004), while the affinity of transporter for L-glutamate in different preparations was found to range between 1 and 40 μ M (Danbolt, 2001), implying saturation of the transporters during neurotransmission. However, in the present study we found that in cultured rat forebrain neurons GLT1 appears to change its current/flux coupling at glutamate concentrations higher than 150 μ M, which would allow GLT1 to transport more glutamate molecules per transported electric charge. This change in the current/flux coupling was reproduced in GLT1a stably transfected COS-7 cells in the same glutamate concentration range. Our data suggest that in addition to high glutamate affinity uptake, GLT1 displays a specific, low affinity uptake component with different current/flux coupling ratio.

Materials and methods

Ethics

All procedures on animals were performed in conformance with Children's Hospital (CH) policy that all research involving animals is conducted under humane conditions, with appropriate regard for animal welfare. CH is a registered research facility with the United States Department of Agriculture (USDA), and is committed to comply with the Guide for the Care and use of Laboratory Animals (Department of Health and Human Services), the provisions of the Animal Welfare Act (USDA), and all applicable federal and state laws and regulations. CH has developed institutional standards for the humane care and use of animals that are maintained through published policies. An Animal Care Committee has been established to ensure compliance with all applicable federal and state regulations for the purchase, transportation, housing and research use of animals. CH has filed appropriate assurance of compliance with the Office for the Protection of Research Risks of the National Institutes of Health. Discomfort and injury to animals were limited to that which was unavoidable in the conduct of scientifically valuable research. Analgesic, anesthetic and tranquilizing drugs were used where indicated and appropriate to minimize discomfort and

pain to animals. Specifically, animals to be used as a source of tissue for tissue culture or immunoblot experiments were anesthetized by CO₂ narcosis and then decapitated.

Neuronal cultures

Neuronal cultures were prepared from embryonic day 16 Sprague Dawley rat fetuses as described previously (Wang *et al.*, 1998; Chen *et al.*, 2002). Contamination by astrocytes was determined by immunochemical labeling with anti-glial fibrillary antibody and was found to be < 0.2% of total cells. Cultured neurons were used in electrophysiological and uptake experiments between 14 to 28 days *in vitro*.

GLT1a stably transfected COS-7 cells

COS-7 cells were stably transfected with GLT1a cDNA as described previously (Chen *et al.*, 2002).

Electrophysiology

Electrophysiological experiments were performed in the whole cell, nucleated patch, and outside-out configurations with an Axopatch 200A (Axon Instruments, USA) or WPC-100 amplifier (E.S.F., Germany), pClamp 8 or pClamp 10 software (Molecular Probes, USA), and MP-225 micromanipulator (Sutter Instrument, USA). Temperature of the chamber (Cat. # TC-344B, Warner Instruments) was adjusted to 37°C. Fast solution exchange was performed with the Fast Step perfusion system SF-77B and computer controlled valves VC-6 (both from Warner Instruments). Several records obtained during subsequent applications of the same set of experimental solutions were averaged to improve signal to noise ratio (e.g., see Fig. 1A).

Artificial cerebrospinal fluid (ACSF) contained (in mM): 160 NaCl, 2.5 KCl, 2.5 CaCl₂, 1.3 MgCl₂, 10 D-glucose, 10 HEPES (pH 7.4, adjusted with N-methyl-D-glucamine). The intracellular solution contained (in mM): 1 NaCl, 110 KOH, 20 tetraethylammonium (TEA), 1 CaCl₂, 5 MgCl₂, 5 EGTA, 10 HEPES (pH 7.2 was adjusted with gluconic acid). Osmolality in all solutions was adjusted to 330 mmol/kg with mannitol. With these solutions $E_{Cl} = -70$ mV. In experiments shown in Figures 2C, intracellular potassium was adjusted to obtain $E_K = -70$ mV, by replacement with N-methyl-D-glucamine. Glutamate activated channels in experiments using cerebral neurons were blocked with 10 μ M (*RS*)-3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP), 100 μ M 5,7-dichlorokynurenic acid, 10 μ M 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzoquinoxaline-7-sulfonamide (NBQX), and 50 μ M 4-(8-methyl-9*H*-1,3-dioxolo[4,5-*h*][2,3]benzodiazepin-5-yl)-benzenamine (GYKI 52466) (Bergles & Jahr, 1998). Electrical activity in neurons was blocked with 0.3 μ M TTX. To reduce GABA associated noise we added 25 μ M bicuculline.

Uptake studies

To study L-[³H]glutamate transport independent of the complicating effects of excitotoxicity, in all experiments we included the NMDA receptor antagonist MK-801 (10 μ M), as described previously (Wang *et al.*, 1998). In experiments examining the concentration dependence of glutamate transport, in which high concentrations of glutamate were used, the noncompetitive non-NMDA receptor antagonist GYKI 52466 at 10 μ M was incorporated into the assay in addition to MK-801. In all cases cells (neurons or GLT1a stably transfected COS-7 cells) were plated in 24 well plates (Corning, Cat# 3524). Assays were performed in 24-well plates with 6 experimental conditions performed in triplicate and 6 control wells in which sodium was replaced with choline. In all experiments sodium independent uptake was small at or below 1 mM glutamate ($11 \pm 1\%$ at [glutamate]_e 1 mM, and 31-32% at 3 and 10 mM [glutamate]_e, but it was always subtracted from

appropriate average values in the presence of sodium. All experimental solutions with radioactive glutamate were distributed into an empty 24-well plate and preheated to 37°C in a water bath. First, cells were washed two times with preheated 37°C ACSF (see “solutions”) or choline-ACSF, 1 mL. Then, at appropriate times “wash” solutions were removed and appropriate experimental solutions (0.5 mL) were added to wells with cells for 6 minutes. After 6 minutes, the cells were washed two times with ice-cold choline-ACSF containing 1% bovine serum albumin to remove residual radioactive glutamate. Cells in each well were dissolved in 0.5 mL of 0.1 N NaOH, transferred into scintillation glass vials with 10 mL scintillation cocktail, and uptake was measured by the method of liquid scintillation. The absence of the time dependent saturation of the uptake at high glutamate concentrations was confirmed by the linearity of uptake time dependence at 1, 2 and 10 minutes of exposure times at 1, 10, 1000, and 10000 μM glutamate concentrations.

Immunoblot analysis

Proteins from control COS-7 cells and GLT1a stably transfected COS-7 cells were analyzed by immunoblot as described previously (Chen *et al.*, 2002). Polyclonal antibody against the C terminus of GLT1a protein based on the published sequence (amino acid 559–573 of GLT1) was generously provided by Dr. J. Rothstein (Johns Hopkins University) and has been previously characterized with respect to its specificity and localization in brain (Rothstein *et al.*, 1994; Chen *et al.*, 2004). An anti-actin antibody (Sigma, cat. # A2103, dilution 1:10,000) was used to test for equal protein loading. A secondary goat anti-rabbit IgG (H+L)-HRP conjugate (Bio-Rad, cat. # 172-1019, dilution 1:5,000) was used for detection.

Statistics and fitting of the data

Experimental data in the figures and in the text are presented as mean $\pm$ s.e.m. Non-directional Student's two-tailed *t*-test was conducted to compare the means.

For kinetic analysis and comparison of the current and uptake glutamate concentration dependences in different experiments, we normalized data to the obtained values at 300 μM glutamate in each experiment. Therefore, the normalized curves have no error bars at 300 μM glutamate (see Figs. 2 and 4).

To determine the glutamate concentration dependence of the current/flux ratio we used Hill equation. Since the measurements of current and uptake were made in separate independent experiments (with similar number of the experiments for each), we calculated the standard errors of the mean of the current/uptake ratios as $\delta_{\text{ratio}} = (\text{uptake}^2 \delta_{\text{current}}^2 + \text{current}^2 \delta_{\text{uptake}}^2)^{1/2} / (\text{uptake}^2)$, where δ corresponds to a standard error of the mean of the parameter written in the subscript. The fitting of our neuronal data gave us the following parameters of Hill equation: $\text{ratio}/\text{ratio}_{\text{max}} = 0.5 / (1 + ([\text{Glu}]_e / 167 \mu\text{M})^3) + 0.5$. In all curve fittings, we used Origin (version 6; OriginLab Corporation, Northampton, MA 01060, U.S.A.).

Results

There are two different glutamate transporter currents that can be activated by glutamate: 1st current is stoichiometrically coupled with glutamate transport, and 2nd current is stoichiometrically uncoupled from the glutamate transport current and this is a glutamate-activated anionic current. In all our experiments, we minimized the non-coupled anionic channel current by nullifying the driving force for chloride. This was accomplished by equalizing the Nernst equilibrium potential for chloride (E_{Cl}) to the holding membrane potential of -70 mV. Thus, we recorded only the coupled glutamate transporter current

(i_{Glu}) in both cultured rat cerebral neurons and transfected COS-7 cells in whole cell configuration. All glutamate-activated channels were blocked (see Materials and methods).

Glutamate transport kinetics in neurons

Application of 30 μ M glutamate to neuron elicited inward current of 0.6 ± 0.1 pA/pF ($n = 13$) in whole cell configuration ($C_{mem} = 31 \pm 3$ pF) (Fig. 1A). Most of this current was inhibited by the specific GLT1 inhibitor dihydrokainate (Pines *et al.*, 1992; Arriza *et al.*, 1994; Wang *et al.*, 1998) (DHK) at 320 μ M. Consecutive application of a set of experimental solutions evoked reproducible current responses that were averaged to improve the signal to noise ratio (Fig. 1A). The dashed line shows a slight outward drift of the extrapolated baseline that was taken into account for determination of the concentration dependence of the DHK inhibition of the current. The best fit of the DHK concentration dependence of the current gave us IC_{50} value for DHK at 30 μ M glutamate equal to 73 ± 4 μ M and $n_{Hill} = 1.5 \pm 0.1$. The DHK-independent component contributed only 7 ± 2 % to the total glutamate activated current (Fig. 1B). Therefore, we conclude that about 90% of the coupled transporter current in these cells is mediated by GLT1. The DHK-inhibited current was inwardly rectifying with zero glutamate in the patch pipette and sodium dependent (Fig. 1C-D), confirming the transporter origin of the current. A typical I-V curve is shown in the inset of Fig. 1C and the solid line is an exponential fit with a decay constant of 58 ± 2 mV. The competitive nature of DHK inhibition was also confirmed when at the same DHK concentration (300 μ M) the current was less inhibited at higher glutamate concentration (200 μ M) than at lower concentration (30 μ M) (Fig. 1D).

We next determined the glutamate concentration dependence of neuronal i_{Glu} (Fig. 2A), and derived an apparent $k_{D,Neuro,Current}$ value = 12 ± 4 μ M. Since at 30 μ M glutamate the IC_{50} value for the competitive inhibitor DHK was 73 ± 4 μ M (Fig 1B), the dissociation constant for DHK (k_i) was calculated by using the Chen-Prusoff equation (Cheng & Prusoff, 1973): $k_i = IC_{50} / (1 + [Glu] / k_{D,Neuro,Current})$. The calculated k_i value for DHK is 21 ± 5 μ M.

Lineweaver-Burke transformation of neuronal [3H]L-glutamate uptake data (insert in Fig. 2C) clearly showed high and low affinity components of the uptake with $k_{D,Neuro,Uptake1} = 9 \pm 1$ μ M (solid line) and $k_{D,Neuro,Uptake2} = 5.1 \pm 0.3$ mM (dashed line) respectively. The value of $k_{D,Neuro,Uptake1}$ is in agreement with high affinity dissociation constant calculated from the current response (Fig. 2A). Increase in a non-saturating current at glutamate concentrations higher than 1 mM (Fig. 2B) is in agreement with the low affinity component determined from the uptake data.

Curiously, increasing extracellular glutamate from 100 to 300 μ M produced a significant ($P < 0.02$) decrease of i_{Glu} (Fig. 2A). The presence of this decrease in the absence of intracellular glutamate (Fig. 2A) implies independence of this phenomenon from glutamate exchange or reverse of uptake because both require the presence of intracellular glutamate. A possible alternative explanation for this phenomenon might be accumulation of intracellular submembrane glutamate due to uptake and limited glutamate diffusion (Sylantsev *et al.*, 2008) in the submembrane space away from sites of transport. With 1 pA/pF GLT1 current density and average 31 pF cell membrane capacitance each neuron would have 3400 μ m² surface area (Gentet *et al.*, 2000) and glutamate influx rate of $1.6 \cdot 10^{-16}$ mol/sec. In the case of a spherical shape of the cells, this would translate into a rising intracellular glutamate concentration at the rate of 0.01 mM per second; in the case of a non-spherical shape, the change would be larger. Submembrane diffusion limitations, as has been shown for calcium (Kabakov & Hilgemann, 1995) and ATP (Kabakov, 1998), could further increase the glutamate concentration in the submembrane space. Thus, when intracellular glutamate concentration is low (Fig. 1A), active glutamate transport and diffusion limitations could lead to a decrease in the driving force for glutamate. If the diffusion

limitations indeed were responsible for the effect seen in Fig. 1A, at high intracellular glutamate concentration (10 mM) the relative submembrane glutamate concentration increase would be small and should not cause the reduction of the current. However, incorporation of 10 mM L-glutamate into the patch pipette (pH was adjusted with NMG, see methods) did not eliminate the effect ($P < 0.01$; $n = 4$; Fig. 2B) and therefore the effect was not due to accumulation of glutamate in the submembrane space.

The decrease of the inward current could be also explained if at high glutamate concentrations glutamate itself has a blocking effect on its own influx. Instead, the glutamate uptake continuously increased with increase in glutamate concentration in all concentration ranges (Fig. 2C). Therefore, the ratio of normalized i_{Glu} to normalized glutamate influx was also significantly decreased when glutamate concentration was increased from 100 to 300 μM (Fig. 2D). Since the coupled current and the uptake are carried only by GLT1 as transported electrical charges and glutamate molecules respectively, we conclude the decrease in the current/flux ratio is due to a decrease in the number of elementary charges transported by GLT1 per one glutamate molecule, i.e. a decrease increase in the n_e/n_{Glu} ratio.

Interestingly, fitting of the normalized concentration dependence of the current/flux ratio with the Hill equation (Fig. 2D) gave us $n_{Hill} = 3.2 \pm 0.6$, $k_{D, \text{Ratio}} = 167 \pm 23 \mu\text{M}$, $\text{Ratio}_{[Glu] = 0} / \text{Ratio}_{[Glu] = \text{infinity}} = 2.0 \pm 0.1$. Here $k_{D, \text{Ratio}}$ is the apparent dissociation constant for a second glutamate low affinity binding site. $\text{Ratio}_{[Glu] = 0} / \text{Ratio}_{[Glu] = \text{infinity}}$ is a ratio of the best fit values of the current/flux ratios at small (zero) glutamate and high (infinite) glutamate concentrations that indicates a two-fold decrease in n_e/n_{Glu} .

Glutamate transport kinetics in GLT1a transfected COS-7 cells

To test whether the low affinity glutamate uptake in neurons is GLT1-specific or not, and the variation of the current/flux ratio is an intrinsic property of GLT1 or an artifact, we evaluated the properties of GLT1 (GLT1a) stably expressed in COS-7 cells (Fig. 3A). We had partially characterized these cells previously and found that at 0.5 μM total glutamate, [^3H]-L-glutamate uptake in GLT1a transfected COS-7 was DHK-sensitive with $k_i = 53 \pm 3 \mu\text{M}$ (Chen *et al.*, 2002). Here we show that the glutamate activated coupled current in GLT1a expressing COS-7 cells was 15 times larger than in control cells even at 3 mM glutamate (Fig. 3B and C), and it was sodium dependent (Fig. 3D and E). The contribution of GLT1a non-specific [^3H]-L-glutamate uptake in COS-7 cells was negligible compared to GLT1a specific uptake in the 0 – 300 μM glutamate concentration range (Fig. 4C).

The glutamate concentration dependence of the current in GLT1a transfected cells also revealed the high affinity component and the current reduction in the 100 – 300 μM glutamate concentration range that is presumably is due to the low affinity component with different current/flux coupling. It should be noted that in individual GLT1a transfected COS-7 cells as well as in neurons the glutamate concentration corresponding to the maximal dip in the current dose dependence curve varies between 300 and 1000 μM . This might be related to limitations in the control of intracellular ion concentrations and voltage clamp in areas distant from the patch electrode and cell soma. A non-specific component clearly emerged at glutamate concentrations above 1 mM (Fig. 4A-B). The value of high affinity $k_{D, \text{GLT1a, Current}}$ was found to be $11 \pm 3 \mu\text{M}$ ($n = 8$), which is almost the same as $k_{D, \text{Neuro, Current}}$.

The glutamate concentration dependence of [^3H]-L-glutamate uptake revealed a specific high affinity component in GLT1a transfected COS-7 cells and a very low affinity, non specific component in non-transfected wild COS-7 cells (Fig. 4C). It is clear that the very low affinity component is non-specific because it was not saturated even at high glutamate

concentrations implying that $k_{D, \text{Non-specific, Uptake}} > 2 \text{ mM}$. This non-specific component makes a significant contribution to the total uptake in GLT1a transfected COS-7 cells only at glutamate concentrations higher than 1 mM, but not at 300 μM or lower (Fig. 4C). We assume that neuronal uptake also includes a significant contribution from the non-specific component only at glutamate concentrations higher than 1 mM. Thus, at least in the 0 – 300 μM glutamate concentration range the total sodium-dependent uptake can be considered as glutamate transporter specific in both GLT1a transfected COS-7 cells and neurons (see horizontal arrows in Figs. 4C and 2D).

When GLT1a-specific [^3H]-L-glutamate uptake was determined as a difference between uptakes of GLT1a stably transfected COS-7 cells and wild type COS-7 cells (solid triangles in Fig. 4C), the apparent value of $k_{D, \text{GLT1a, Uptake}}$ for “pure” GLT1a glutamate uptake was $70 \pm 6 \mu\text{M}$, that is, larger than high affinity k_D value and lower than the low affinity k_D value determined above. This discrepancy is probably related to the involvement of both high ($k_D = 11 \mu\text{M}$) and low ($k_D \approx 0.2 \text{ mM}$) affinity GLT1a-specific components.

As in neurons, GLT1a-specific [^3H]-L-glutamate uptake showed a monotonic increase throughout the 0 – 300 μM concentration range (Fig. 4C), and the current/flux ratio of GLT1a in COS-7 cells was significantly lower at 300 compared to 100 μM glutamate (Fig. 4D). Therefore, the apparent deviation from conventional GLT1 coupling observed in neurons appears to be the intrinsic property of GLT1 that does not depend on the specific neuronal environment.

Estimation of the low affinity component contribution

The low affinity glutamate transport component has $k_D = k_{D, \text{Ratio}} = 167 \mu\text{M}$, and $n_{\text{Hill}} = 3.2$ (Fig. 2D). When glutamate rises from 30 to 300 μM , the contribution of the low affinity component to the total flux would be rise from 0.4% to 85.8% of its maximal contribution in accordance with the Hill equation. The high affinity component with $k_D = 10 \mu\text{M}$ will rise from 75% to 96% of its maximal contribution over the same concentration range in accordance with Michaelis-Menten equation. Therefore, the high affinity flux component at 300 μM is $(96\%)/(75\%) = 1.28$ times larger than at 30 μM . We can calculate the low affinity component contribution at 300 μM as the difference, (low affinity component of flux at 300 μM) = (total flux at 300 μM) – (1.28 (total flux at 30 μM)). This allows us to estimate relative contribution of the low affinity component to the total uptake at 300 μM as 58% in GLT1a transfected COS-7 cells. In this case, the apparent k_D of the total simulated uptake (42% of maximal uptake with $k_D = 10 \mu\text{M}$ plus 58% of uptake with $k_D = 167 \mu\text{M}$) as 100 μM that is relatively close to experimentally found value of 70 μM (Fig. 4C). A similar calculation applied to the neuronal flux gave us the relative contribution of the low affinity component as 35%. Thus, the low affinity component of glutamate transport by GLT1 significantly contributes to the total uptake at high synaptic glutamate concentrations.

Discussion

Despite the potential importance for synaptic physiology of glutamate transport in axon terminals by the major glutamate transporter of the brain, GLT1, little is known about the properties of GLT1 expressed in primary neurons or under conditions likely to be present in or near synapses. Difficulties in the detection of the neuronal GLT1 protein arose from its highly polarized distribution into neuronal terminals, low abundance in neurons relative to astrocytes, and other technical issues (Chen *et al.*, 2004). To determine the kinetic parameters of GLT1 transporter coupled current in neurons by using the patch-clamp technique we have taken advantage of our previous findings that GLT1 is the major glutamate transporter in these cultures and that GLT1 is expressed in the cell bodies (Wang *et al.*, 1998; Chen *et al.*, 2002). Expression in the cell body reduces access resistance,

improves voltage clamp at the sites of transport and increases the signal to noise ratio. Since the non-GLT1 mediated current, which is probably EAAC1 mediated, contributed less than 7% to the total current as shown in Fig. 1B, the high affinity component of the coupled transporter current mediated by GLT1 in neurons and by GLT1a expressed in COS-7 cells characterized electrophysiologically in the present study is similar to the high affinity component previously found with [^3H]-L-glutamate uptake measurements (Wang *et al.*, 1998). However, in the present studies we observed the novel finding that the coupled GLT1 current decreased significantly in the 100 – 300 μM glutamate concentration range (Fig. 2A). This phenomenon is potentially of substantial significance for our understanding of the function of GLT1 at synaptic concentrations of glutamate, and so it is important to consider the potential artifacts that might produce such a phenomenon.

Could the i_{Glu} decrease be an artifact?

First, depression of the transporter associated current could be due to accumulation of intracellular submembrane glutamate that may significantly affect the glutamate concentration gradient and consequently decrease total transporter driving force. The effect might be potentiated if glutamate diffusion in submembrane space is limited as has been shown for ATP (Kabakov, 1998). However, we found that the current reduction was also observed even at 10 mM intracellular glutamate (Fig. 2B) when the relative increase in $[\text{Glu}]_i$ is negligible. Thus, the current reduction was not due to accumulation of glutamate in the submembrane space.

A second possible artifact would be decrease of the coupled current at high glutamate concentrations due to a self-blocking effect of glutamate on its own influx as has been shown for GABA_A receptors (Mercik *et al.*, 2002). However, the glutamate uptake increased monotonically with increase in glutamate concentration from 100 to 300 μM (Figs. 2C and 4C), and the current/flux ratio significantly decreased over the same concentration range (Fig. 2D and 4D).

A third possible artifact may occur due to poor voltage clamp in neuronal processes. If processes were inadequately clamped, then the uncoupled chloride current (Fairman *et al.*, 1995) might contribute to the measured current. However, this is unlikely because the uncoupled anionic current was found to be negligible in EAAT2, the human homolog of GLT1 (Wadiche *et al.*, 1995). Even when chloride is replaced with a more permeable anion, thiocyanate, the GLT1 current is less than 1/10th of the coupled current (Bergles *et al.*, 2002). In addition, the phenomenon was also seen in COS-7 cells (Fig. 4), which are geometrically and electrically more compact than neurons and have a more reliable spatial distribution of the voltage clamp at E_{Cl} (see methods).

The fourth potential artifact is that due to glutamate exchange or reversal of uptake. However, the decrease of i_{Glu} was observed in the absence of intracellular glutamate (Fig. 2A) that is required for both the exchange and the reversal of uptake.

Thus, we have excluded all plausible artifacts and conclude that the decrease in i_{Glu} between 100-300 μM glutamate is indeed due to a decrease in the GLT1 coupled current.

Basis for glutamate concentration dependent decrease in i_{Glu}

Once artifacts are eliminated from consideration, a decrease in the current to flux ratio must be due to a decrease of the ratio of the number of transported elementary charges to the number of transported glutamate molecules per cycle, i.e. n_e/n_{Glu} (Zerangue & Kavanaugh, 1996). This change in n_e/n_{Glu} could be due to deviation from the conventional (Zerangue & Kavanaugh, 1996; Levy *et al.*, 1998) transporter coupling to a new one with non-zero probability of transport of a second glutamate molecule per transport cycle. This would

decrease the transported charge from 2 elementary charges ($2 = 3 - 1 + 1 - 1$; for 3 Na^+ , 1 Glu^- , 1 H^+ are being cotransported and 1 K^+ countertransported) to 1 elementary charge ($1 = 3 - 2 + 1 - 1$; i.e., 3 Na^+ , 2 Glu^- , 1 H^+ ions cotransported and 1 K^+ ion countertransported).

Formally, the fitting of a twofold decrease of the current/flux ratio (Fig. 2D) supports a hypothetical model in which glutamate molecules cooperatively bind to each of three subunits (Yernool *et al.*, 2004) of GLT1 with a K_D value of $167 \mu\text{M}$ leading to transport of a second glutamate molecule per transport cycle with 50% probability. In this case, at high synaptic glutamate concentrations, on average 1.5 glutamate molecules and 1.5 elementary charges are transported per transport cycle ($1.5 = 3 - 1.5 + 1 - 1$; i.e., 3 Na^+ , 1.5 Glu^- , 1 H^+ and 1 K^+) leading to $n_e/n_{\text{Glu}} = 1.5/1.5 = 1$, instead of $n_e/n_{\text{Glu}} = 2/1 = 2$.

Interestingly, a cooperative interaction with $n_{\text{Hill}} = 3$ has been also shown for activation of the anion channel of the human neuronal glutamate transporter EAAT4 (Torres-Salazar & Fahlke, 2006). In addition, the affinity of D-aspartate for the activating the anionic current of the glutamate transporter EAAC1 after selective elimination of the coupled current by mutation (I421C) was found to be 0.18 mM (Borre *et al.*, 2002), and the EC_{50} of the GLT1 peak response to L-glutamate with a KSCN-based internal solution was found to be 0.14 mM (Bergles *et al.*, 2002). These values are similar to the K_D value determined here for the low affinity component ($167 \mu\text{M} \approx 0.17 \text{ mM}$), suggesting a possible connection between regulation of the anionic conductance and GLT1 transport stoichiometry, which might be due, for example, to conformational changes in GLT1 facilitating both transport of the second glutamate and the uncoupled anionic current.

Other transporters have coupling deviations

It should also be noted that deviation from conventional transport coupling has been reported for the bacterial lactose transporter, LacY (DeFelice, 2004), the $\text{ClC-ec1 H}^+-\text{Cl}^-$ -exchange transporter (Accardi & Miller, 2004), the dopamine transporter (Sonders *et al.*, 1997), the norepinephrine transporter (Galli *et al.*, 1998), the serotonin transporter (Petersen & DeFelice, 1999; Adams & DeFelice, 2003), and the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (Kang & Hilgemann, 2004).

The deviation from conventional transporter coupling found here for GLT1 in neurons appears to be an intrinsic property of GLT1 because the same shift of the current/flux ratio was observed in GLT1a transfected COS-7 cells. However, the exact changes in GLT1 transport stoichiometry have yet to be determined since there is a possibility that the numbers of other transported per cycle ions (Na^+ , H^+ , or K^+) are also changing.

Synaptic GLT1 is exposed to high glutamate concentrations

Although the baseline concentration of glutamate in the extracellular space has been reported to be 25 nM (Herman & Jahr, 2007) in the synaptic cleft, near vesicular release sites it has been estimated to rise to over 1 mM (Clements *et al.*, 1992; Raghavachari & Lisman, 2004). The possible physiological relevance of the low affinity component of GLT1 transport at sub-millimolar glutamate concentrations is suggested by the direct demonstration that GLT1a is expressed in excitatory presynaptic terminals (Chen *et al.*, 2004; Furness *et al.*, 2008). GLT1a mRNA has also been demonstrated in CA3 neurons of the hippocampus (Chen *et al.*, 2004; Berger *et al.*, 2005). Neuronal expression of GLT1 has been supported by subsequent observations in other laboratories (Regan *et al.*, 2007) (and Dwight Bergles, personal communication). Deviation from conventional invariant current/flux coupling in GLT1 may have a significant effect on the clearance capacity of the transporter expressed in the presynaptic membrane near vesicular release sites. Under normal physiological conditions, the ability of presynaptic GLT1 to transport a 2nd

glutamate per cycle implies the existence of a second glutamate binding site on GLT1 that would facilitate synaptic glutamate clearance via binding of the second glutamate, and subsequent transport. Our estimates show that almost half of the transport at 300 μM glutamate occurs via the low affinity component. On the other hand, in the case of energy failure and collapse of transmembrane ionic gradients (especially that of sodium), this deviation from the conventional coupling during glutamatergic transmission would lead to reduction of the total driving force for forward GLT1 transport, or even to reverse of GLT1 leading to non-vesicular release of glutamate.

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Abbreviations

DHK	dihydrokainate
EAAT	excitatory amino acid transporter
GLT1	glutamate transporter one
IC₅₀	half maximal inhibitory concentration
<i>i_{Glu}</i>	coupled glutamate transporter current
k_D	dissociation constant for agonist
k_{D,Neuro,Current}	dissociation constant for agonist determined from current dose response in neurons

$k_{D,Neuro,Uptake}$	dissociation constant for agonist determined from L-[3H]glutamate uptake dose response in neurons
$k_{D,GLT1a,Current}$	dissociation constant for agonist determined from current dose response in GLT1a transfected COS-7 cells
$k_{D,GLT1a,Uptake}$	dissociation constant for agonist determined from L-[3H]glutamate uptake dose response in GLT1a transfected COS-7 cells
k_i	dissociation constant for inhibitor

Figure 1.

Glutamate transporter current (i_{Glu}) measured in cultured cerebral neurons is produced primarily by GLT1. (A) Two consecutive current traces of a 20 pF neuron and their average are shown during applications of glutamate (solid bar) and dihydrokainate (DHK, open bars) at -70 mV. (B) Cumulative data ($n = 4$). DHK inhibited i_{Glu} with $IC_{50} = 73 \pm 4 \mu\text{M}$. (C) The glutamate activated current (30 μM , solid bar) was reduced 15 times when choline (upper dashed line) replaced the sodium (lower dashed line). DHK (300 μM , open bar) inhibits only sodium-dependent current. Inset: I-V of the DHK sensitive current. (D) Cumulative data. DHK inhibited i_{Glu} more at 30 μM compared to 200 μM glutamate ($P < 0.02$) that confirms competitive nature of DHK inhibition. Residual sodium-independent glutamate activated currents are presented on the right and are around 5% of the current in presence of sodium. Error bars in all figures represent s.e.m. of appropriate measurements.

Figure 2.

In neurons, the ratio of glutamate transporter coupled current (i_{Glu}) to L-[3 H]glutamate influx varies with glutamate concentration. (A) Glutamate concentration dependence of i_{Glu} in absence of intracellular glutamate (3 $[Glu]_e/\mu$ M 300; $n = 6$). All currents were normalized to appropriate amplitudes at 300 μ M glutamate. Dashed line is Michaelis-Menten kinetic fit. Significant decrease of i_{Glu} occurs between 100 and 300 μ M. Inset: typical record of i_{Glu} at different glutamate concentrations. (B) Concentration dependence of i_{Glu} with 10 mM glutamate in pipette (30 $[Glu]_e/\mu$ M 3000; $n = 4$). Significant decrease of i_{Glu} occurs in the same region, between 100 and 300 μ M. Note similarity in appearance of GLT1a non-specific component at $[Glu]_e > 1$ mM in Fig. 4C. (C) Concentration dependence of L-[3 H]glutamate uptake. Inset: Lineweaver-Burke plot. (D) Superimposed concentration dependences of i_{Glu} (data combined from 2A and 2B, solid circles), L-[3 H]glutamate uptake (as in 2C, open triangles), and calculated (i_{Glu}) / ([3 H]glutamate uptake) ratios (open squares). The Hill equation (solid black line) was used in fitting the ratio curve (thick gray line). * corresponds to $P < 0.02$, and ** – to $P < 0.005$.

Figure 3.

Stable transfectant COS-7 cells express functional GLT1a. (A) An anti-cGLT1a antibody recognized a band in lysate of transfected cells (+), but not in control cells (-). Representative (B) and cumulative (C) data show glutamate activated current in transfected cells that was on average 15 times larger than in control cells. (D) and (E) show sodium dependence of glutamate activated current in transfected cells is similar to the one shown in Fig. 1D.

Figure 4.

Variation of current/flux ratio is an intrinsic property of GLUT1 as confirmed by experiments with stable transfectant COS-7 cells. Concentration dependence of glutamate activated current in representative GLUT1a transfected COS-7 cell (A) and cumulative data (B) (open circles, $n = 5$). Neuronal data from 2D are shown without error bars for comparison as solid circles in (B). Current decreased significantly, when extracellular glutamate increased from 100 and 300 μ M was. (C) Concentration dependence of L-[3 H]glutamate uptake in transfectant (open triangles) and non-transfectant (open squares) cells. GLUT1a specific component of the uptake in transfectant cells (closed triangles) is the difference between uptakes in transfectant and non-transfectant cells. GLUT1a specific uptake increases significantly, when glutamate increases from 100 to 300 μ M. The top arrow shows concentration range where the total glutamate influx in transfectant cells is GLUT1 specific because the GLUT1 non-specific uptake is negligibly small. (D) Normalized current/flux ratio at 100 and 300 μ M glutamate. * corresponds to $P < 0.05$.