Regulation of Glial Glutamate Transporters by C-terminal Domains

Ariane Leinenweber, Jan-Philipp Machtens, Birgit Begemann, and Christoph Fahlke

Excitatory amino acid transporter 2 (EAAT2) is a high affinity glutamate transporter predominantly expressed in astroglia. Human EAAT2 encompasses eight transmembrane domains and a 74-amino acid C-terminal domain that resides in the cytoplasm. We examined the role of this region by studying various C-terminal truncations and mutations using heterologous expression in mammalian cells, whole-cell patch clamp recording and confocal imaging. Removal of the complete C terminus (K498X EAAT2) results in loss of function because of intracellular retention of truncated proteins in the cytoplasm. However, a short stretch of amino acids (E500X) EAAT2 within the C terminus results in correctly processed transporters. E500X reduced glutamate transport currents by 90%. Moreover, the voltage and substrate dependence of E500X EAAT2 anion currents was significantly altered. WT and mutant EAAT2 anion channels are modified by external Na\(^+\) because of intracellular retention of truncated proteins in the cytoplasm. Whereas Na\(^+\) stimulates EAAT2 anion currents in the presence of l-glutamate, increased [Na\(^+\)] reduces such currents without glutamate. In cells internally dialyzed with Na\(^+\), WT, and truncated EAAT2 display comparable Na\(^+\) dependence. With K\(^+\) as main internal cation, E500X drastically increased the apparent dissociation constant for external Na\(^+\). The effects of E500X can be represented by a kinetic model that allows translocation of the empty transporter from the outward- to the inward-facing conformation and stabilization of the inward-facing conformation by internal K\(^+\). Our results demonstrate that the C terminus modifies the glutamate uptake cycle, possibly affecting the movements of the translocation domain of EAAT2 glutamate transporter.

Glutamate is the major excitatory neurotransmitter in the mammalian central nervous system. After release from presynaptic nerve terminals, glutamate is quickly taken up into glial and neuronal cells by glutamate transporters belonging to the excitatory amino acid transporter (EAAT)\(^2\) family (1–4). Five different mammalian EAAT isoforms have been identified. Two of those are mainly expressed in glia, EAAT1 and EAAT2, whereas EAAT3, EAAT4, and EAAT5 are considered to be neuronal transporters. All EAAT glutamate transporters function as stoichiometrically coupled co-transporters of one glutamate, three sodium ions, and one proton, while one potassium ion is counter-transported (5, 6). In addition, they are capable to function as anion channels (7). EAAT anion channel opening is coupled to conformational changes within the glutamate uptake cycle (8, 9). Different EAAT isoforms differ in the relative contribution of anion currents to the total transporter-mediated current (7, 10–12).

The glial glutamate transporter EAAT2 appears to be crucial for glutamate homeostasis. Mice that lack EAAT2 show lethal spontaneous seizures and increased susceptibility to acute cortical injury (13). In contrast, mouse models lacking other glial or neuronal glutamate transporters, such as EAAT1 (14), EAAT3 (15), or EAAT4 (16), show much milder phenotypes without pronounced neurodegeneration.

There are many neurological diseases that appear to be associated with increased levels of external glutamate, such as schizophrenia (17), Alzheimer’s disease (18), multiple sclerosis (19, 20), and amyotrophic lateral sclerosis (ALS) (21, 22). For ALS, a progressive degenerative motor neuron disease, reduced EAAT2 glutamate transport has been suggested to contribute to the disease phenotype (21). Recently, oxidative stress was shown to activate caspase-3, resulting in a cleavage of EAAT2 in the cytosolic C-terminal domain. This result suggested a functionally important role of the C terminus and linked excitotoxicity and activation of caspase-3 as converging mechanisms in the pathogenesis of ALS (24, 25).

Channels and transporters are often modulated by cytoplasmic domains. In certain ion channels, such domains are necessary for intersubunit interactions (26), or determine conformational changes of adjacent transmembrane domains (27, 28). We studied the role of the EAAT2 C-terminal domain by heterologous expression of mutant proteins, confocal imaging and electrophysiological and radiotracer flux analyses. We demonstrated that EAAT2 tolerates removal of the majority of its C terminus without functional alterations. A region close to the distal end of the last transmembrane domain modifies interaction with transport substrates and membrane insertion.

**EXPERIMENTAL PROCEDURES**

Heterologous Expression of WT and Mutant EAAT2—The coding region of monomeric YFP (mYFP) was linked to the 5’-end of the cDNA encoding human EAAT2 (kindly provided by Dr. M. Hediger, University of Bern, Switzerland) via
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a BsrGI restriction site into an open reading frame and subcloned into pRcCMV using flanking Ncol and NotI restriction sites. Truncations in EAAT2 and rEAAT4 (kindly provided by Dr. J. Rothstein, Johns Hopkins University, Baltimore, MD) and point mutations in EAAT2 were introduced using PCR-based strategies or QuikChange® site-directed mutagenesis kit (Stratagene, La Jolla, CA). All constructs were verified by restriction analysis and DNA sequencing. For each construct, two independent recombinants from the same transformation were examined and shown to exhibit indistinguishable functional properties. Transient transfection of tsA201 cells using the Ca3(OH)2 technique was performed as previously described (12).

Electrophysiology—Standard whole-cell patch clamp recordings were performed using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA). Borosilicate pipettes were pulled with resistances of 1.5–2.5 MΩ. To reduce voltage errors, we routinely compensated more than 80% of the series resistance by an analog procedure and excluded cells with more than 10 nA maximum anion currents from the analysis. Currents were filtered at 5 kHz (~3dB) and digitized with a sampling rate of 50 kHz using a Digidata AD/DA converter (Molecular Devices, Sunnyvale, CA). Cells were clamped to 0 mV for at least 5 s between test sweeps. Four combinations of internal/external solutions were used to separate distinct EAAT2 current components. To determine electrogenic glutamate transport currents, the internal solution contained (in mM) 115 KCl, 2 MgCl2, 5 EGTA, 10 HEPES, pH 7.4, and the external solution (in mM) 140 NaCl, 4 KCl, 2 CaCl2, 1 MgCl2, 5 HEPES, pH 7.4. In some of the experiments, KCl and NaCl were equimolarly substituted by the corresponding gluconate salts. To measure reverse glutamate transport, we used an internal solution containing (in mM) 115 Na-L-glutamate, 2 MgCl2, 5 EGTA, 10 HEPES, and an external solution containing (in mM) 142 K-gluconate, 2 Na-gluconate, 2 CaCl2, 1 MgCl2, 5 HEPES, pH 7.4. To measure the K+-dependence of reverse transport, external K-gluconate was substituted by Na-gluconate. EAAT anion currents were either measured with a KCl-based internal solution and an external solution in which NaCl was equimolarly substituted by NaSCN, or in symmetrical [NO3] using a pipette solution containing (in mM) 115 Na/KNO3, 2 MgCl2, 5 EGTA, 10 HEPES, pH 7.4, and a bath solution with (in mM) 140 NaNO3, 4 KCl, 2 CaCl2, 1 MgCl2, 5 HEPES, pH 7.4. To determine the Na+ dependence of EAAT anion currents, a NaNO3-based internal solution and a NaCl-based bath solution was used. External Na+ was equimolarly substituted by choline. In some of the experiments, 0.5 mM L-glutamate was added to the external solution as indicated. We compared myFP-EAAT2 and EAAT2 currents under various ionic conditions and did not observe any change of function by covalently linking myFP to the N terminus of EAAT2.

Confocal Microscopy—tsA201 cells were transiently transfected with WT or mutant mYFP EAAT2 and cultivated on glass bottom dishes (Ibidi, Munich, Germany). Confocal imaging was carried out on living cells with an Olympus IX81 inverted motorized microscope using the Fluoview FV1000 system (Olympus, Hamburg, Germany). mYFP was excited at 515 nm, and the emission was detected after filtering with a 535–565 nm bandpass filter.

Surface Biotinylation and Western Blotting—Cell surface expression of WT and mutant EAAT2 was assayed with a modification of cell surface biotinylation methods as described previously (29, 30). WT and mutant mYFP EAAT2 were expressed in tsA201 cells, grown until 70% confluence, detached with phosphate-buffered saline (PBS) (~Mg2+, ~Ca2+) and transferred into 15-ml tubes. After washing with PBS, they were incubated with 2 mg/ml biotin (sulfo-NH-SS-biotin from Pierce) in triethanolamine buffer (in mM: 2 CaCl2, 150 NaCl, 10 C6H15NO3, pH 7.5) for 1 h. The reaction was quenched by repeated washing with quenching buffer (100 mM glycine in PBS). After washing with PBS, the cells were dispensed in lysis buffer (150 mM NaCl, 1% Triton X-100 and 5 mM EDTA with 50 mM Tris, pH 7.5), and transferred after 15 min of incubation on ice to a 1.5-ml tube. The probes were centrifuged at 14,000 × g for 15 min at 4 °C, and the cell lysate was collected. Subsequently, lysates were incubated with Ultralink immobilized NeutrAvidin beads (Pierce) for 2–3 h. After washing with high salt wash buffer (0.1% Triton X-100, 500 mM NaCl, 5 mM EDTA, 50 mM Tris, pH 7.5) and salt-free wash buffer (50 mM Tris, pH 7.5), proteins on the beads were released by incubation with SDS loading buffer containing 200 mM dithiothreitol (DTT) and 4.1% SDS. The proteins were separated on 10% SDS-polyacrylamide gradient gels. Fluorescence intensities were visualized by scanning the wet SDS-PAGE gels with a fluorescence scanner and given as fraction of whole lysate fluorescence. The gels were blotted onto polyvinyldene (Bio-Rad), and actin was detected with rabbit anti-actin antibody (Sigma-Aldrich) and anti-rabbit Cy5 antibody (GE Healthcare, Freiburg, Germany). Results were used only from experiments in which no actin was detected in purified membrane fractions.

Kinetic Modeling—Simulations of human EAAT2 anion currents were performed by solving differential equations (31) on the basis of a modified rat EAAT2 model (32) with self-written programs in the MATLAB environment (The MathWorks). Several parameters of the model were adjusted to match observed sodium and glutamate dependence. All simulations were performed under the following ionic conditions (in mM): 115 K+/Na+, pH 7.4 (internal solution) and 140 Na+, 4 K+, 0 or 0.5 L-glutamate, pH 7.4 (external solution). Our kinetic model does not distinguish between different anionic conditions. Channel open probabilities were calculated as sums of fractional occupancies in channel open states. Whole-cell ion currents were calculated from simulated open probabilities using the experimentally observed instantaneous current-voltage relationship.

Data Analysis—Data were analyzed with a combination of pClamp 9 (Molecular Devices, Sunnyvale, CA) and SigmaPlot 9 (Jandel Scientific, San Rafael, CA). Relative errors for apparent dissociation constants were obtained as standard errors of fit estimates from fitting Michaelis-Menten relationships to the concentration dependence of EAAT2 anion currents (Fig. 6). For statistical evaluations, Student’s t test and paired t test with p ≤ 0.05 (*) as the level of significance were used (p ≤ 0.01, **, p ≤ 0.001, ***). Significance levels are given in com-
RESULTS

C-terminal Truncations Affect EAAT2-mediated Glutamate Transport—A recent report demonstrated that the protease caspase-3 can cleave EAAT2 within its C terminus and generate the truncation mutation S506X EAAT2 (Fig. 1A). S506X removes 68 out of 74 C-terminal amino acids of EAAT2. We engineered a plasmid encoding S506X EAAT2, expressed WT and mutant EAAT2 in tsA201 cells and measured EAAT-associated currents through whole-cell patch clamping.

EAATs are not only secondary-active glutamate transporters, but also anion channels. Because glutamate transport is highly voltage-dependent and decreases to values around zero at positive voltages (5, 6), currents at positive voltages predominantly represent anion currents. With intra- and extracellular Cl⁻-based solutions, application of glutamate increases current amplitudes only at negative voltages but not at positive voltages, indicating that EAAT2-associated anion currents are negligible under these conditions. Transport currents that are due to electrogenic glutamate transport were therefore determined by subtracting whole-cell currents before application of 0.5 mM L-glutamate from currents measured in the same cell after substrate application (Fig. 1B, inset) (5, 34, 35). These current components required internal K⁺ and were blocked by external TBOA (data not shown). Moreover, complete substitution of internal and extracellular Cl⁻ with gluconate did not change the amplitude of glutamate-evoked currents (Fig. 1B, open bar).

A comparison of transport current amplitudes calculated in this fashion revealed indistinguishable results for WT and S506X EAAT2 (Fig. 1B). We next engineered additional truncations with decreasing length of the C terminus (I504X, D502X, L501X, E500X, K498X, and H495X EAAT2) and determined glutamate transport currents (Fig. 1B). K498 is the...
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last amino acid of transmembrane domain 8 (TM8) (36), and H495 is a conserved residue within TM8. Whereas I504X, D502X, and L501X decrease glutamate-dependent currents in Cl⁻-based solutions to ~40%, E500X reduces such glutamate currents to ~12.3 ± 3.8% (n = 7) of WT currents. Whereas glutamate-dependent currents were still above background for E500X EAAT2, H495X, and K498X currents were indistinguishable from untransfected cells (Fig. 1B).

Deletions might affect the contribution of other EAAT current amplitudes to the total glutamate-evoked current amplitudes in Cl⁻-based solutions. We thus repeated experiments on E500X EAAT2 in Cl⁻–free internal and external solutions to assess the influence of EAAT2 anion currents. E500X EAAT2 currents were comparable with and without Cl⁻, indicating that the contribution of EAAT anion channels is negligible to E500X total currents (Fig. 1B, open bar).

Subcellular Distribution of Truncated EAAT2 Transporters—
Reduced macroscopic current amplitudes in cells expressing truncated transporters might be due to an impaired surface targeting or altered function of the expressed proteins. To study the subcellular distribution of truncated EAAT2 transporters, we generated fluorescent protein-tagged constructs with mYFP covalently attached to the N terminus of WT and truncated EAAT2 and examined transfected cells by confocal imaging (Fig. 1C). Cells expressing WT, as well as S506X, I504X, D502X, L501X (data not shown), and E500X EAAT2, showed almost exclusive staining of the surface membrane. In contrast, K498X (Fig. 1C) and H495X (data not shown) EAAT2 are predominantly located within the cytoplasm. We conclude, therefore, that the first two amino acids of the cytoplasmic C-terminal domain of EAAT2 are either sufficient for surface membrane insertion or necessary for retention of the proteins at the plasma membrane.

Reversed Transport Mediated by WT and E500X EAAT2—
Coupled glutamate transport by EAAT2 transporters can reverse its direction upon inversion of its driving force (5, 6). We determined reverse transport currents by dialyzing cells with 115 mM Na-glutamate and perfusing cells with varying [K⁺]. Anion currents were suppressed by substitution of internal Cl⁻ with glutamate⁻ and external Cl⁻ with gluconate⁻. Under these conditions, changing [K⁺], from 2 to 142 mM caused the occurrence of outward currents at 0 mV in cells expressing WT (Fig. 1D, upper traces) as well as E500X EAAT2 (Fig. 1D, lower traces). As expected for reverse glutamate transport currents, these currents are increased by external [K⁺] and blocked by TBOA (Fig. 1E). Moreover, application of external glutamate decreases the current amplitude (data not shown). For various [K⁺]o, reverse transport current amplitudes are identical for WT as well as for E500X EAAT2. E500X thus reduces electrogenic glutamate inward current (Fig. 1B), but not reverse glutamate transport (Fig. 1E).

C-terminal Truncations Affect EAAT2-associated Anion Currents—We next determined WT and mutant EAAT2 anion currents at positive potentials after substitution of external Cl⁻ by the more permeant SCN⁻ (8, 12) (Fig. 2A). Under these ionic conditions, currents are outwardly rectifying and reversed at negative potentials as expected for EAAT anion currents (12). Application of L-glutamate increases current amplitudes over the whole voltage range and modifies voltage-dependent activation. Whereas anion currents display only slight time-dependent increases upon positive voltage steps in the absence of glutamate, we observed pronounced voltage-dependent activation after substrate application (Fig. 2A).

Whereas S506X does not alter EAAT2 anion current amplitudes, we observed changes in the amplitude and substrate dependence of I504X, D502X, L501X, and E500X EAAT2. In the absence of glutamate, SCN⁻ currents were decreased by I504X, but unaffected by S506X, D502X, L501X and E500X (Fig. 2B). Anion currents in cells expressing H495X or K498X EAAT2 were indistinguishable from background currents (Fig. 2B). Glutamate-induced current increases are significantly decreased by L501X and E500X, but increased by I504X and D502X (Fig. 2C). We conclude that C-terminal truncations change the glutamate uptake cycle of EAAT2 resulting in reduced transport currents and modified anion currents.

Sequence Requirements for Coupled Glutamate Transport by EAAT2—The EAAT family encompasses five members with distinct primary structures and functions (2–4). Among the known mammalian EAATs, EAAT2 and EAAT4 are functionally the most diverse. EAAT4 exhibits only small glutamate transport rates (37), resulting in electrogenic transport currents that are negligible compared with EAAT4 anion currents (10, 38). We generated two deletions (supplemental Fig. S1A) in EAAT4 and tested their effects on EAAT4 anion conduction. A529X EAAT4, that is homologous to S506X EAAT2, displays functional properties virtually identical to WT (supplemental Fig. 1, B–E). Similar to the corresponding mutation in EAAT2 (Fig. 2), E523X causes a significant reduction in relative glutamate-induced current amplitudes (supplemental Fig. S1E). C-terminal truncations thus exert similar functional effects on EAAT2 and EAAT4.
We next generated a chimeric transporter that substitutes the region adjacent to TM8 from EAAT2 by the corresponding EAAT4 region (DTID(502–505)ELQE S506X EAAT2 (EAAT2chimEAAT4)), (Fig. 3A). This chimera is functionally indistinguishable from WT EAAT2 (Fig. 3).

Besides excitatory amino acid transporters, the solute carrier 1 family (39) contains two neutral amino acid transporters, ASCT1 and ASCT2, that do not mediate net flux of amino acids but merely exchange Na+/H+ and amino acids across the membrane (40). ASCT1 shares 40% sequence identity with EAATs. Substituting the EAAT2 protein segment adjacent to TM8 by the corresponding ASCT1 sequence (ELDTID(500–505)ATKKGE S506X EAAT2 (EAAT2chimASCT1)) results in dramatically reduced electrogenic glutamate transport currents (Fig. 3B). The current reduction in EAAT2chimASCT1 is partially due to a smaller number of chimeric than WT transporters. Confocal microscopy of mYFP-fused chimeric proteins expressed in tsA201 cells shows a signal located close to the surface membrane for both EAAT2chimASCT1 and E500X EAAT2 for comparison. Means ± S.E., n as indicated.

![Figure 3. Functional properties of chimeric transporters.](image)

A, alignment of C-terminal amino acid sequences for S506X EAAT2, EAAT2chimEAAT4- and EAAT2chimASCT1- mean values of glutamate transport current amplitudes at +185 mV (standard internal and external solution). B, confocal images of tsA201 cells expressing chimeric mYFP EAAT2 (scale bar = 5 µm) and relative fluorescence intensities of different cellular compartments. C, cell surface biotinylation assay of mYFP-fused WT, EAAT2chimEAAT4 or EAAT2chimASCT1. D, mean values of absolute late anion current amplitudes (F) and glutamate-induced increases of anion current amplitudes (G) at + 95 mV for WT and chimeric constructs. Dotted lines in C, F, and G represent values of S506X and E500X EAAT2 for comparison. Means ± S.E., n as indicated.
lated that the total charge of these two six amino acid stretches might be the determinant of coupled transport. To test such a scenario we engineered two point mutations at position 500, E500Q, and E500K (Fig. 4). E500Q neither reduces coupled transport (Fig. 4B), anion current amplitudes nor glutamate-induced current increases (Fig. 4, A, C, D, and E). In contrast, E500K dramatically reduces the coupled transport (Fig. 4B). The mutation additionally increases the anion current amplitude in the absence of glutamate (Fig. 4, A and C), but leaves the glutamate-dependent anion current amplitude (Fig. 4D) unaffected. We conclude, therefore, that the charge content of this region is not the main determinant of isoform-specific transporter function.

**C-terminal Truncations Alter Substrate Dependence of EAAT2 Anion Currents**—Because E500X EAAT2 mediates only small electrogenic transport currents (Fig. 1), analysis of EAAT-associated anion currents was used to determine which step within the glutamate uptake cycle is modulated by the C-terminal domain. Opening and closing of EAAT anion channel is tightly coupled to transitions in the glutamate uptake cycle (31, 32, 37, 41). Anion channel opening occurs only from certain states of the transport cycle, and each of these states is associated with distinct open probabilities of the anion channel. Changes in the EAAT2 transport cycle are thus expected to result in altered EAAT2 anion currents. We tested modulation of WT, S506X, and E500X EAAT2 anion currents by external Na⁺/H⁻ and L-glutamate, in the presence of internal Na⁺/H⁻ or K⁺/H⁻ (Fig. 5). In these experiments, we used NO₃⁻ as internal and external anion to increase EAAT2 anion currents and to permit anion current measurements over a broad voltage range.

Fig. 5, A and B give representative recordings and normalized current amplitudes for WT and truncated EAAT2 before and after application of glutamate using cells internally dialedyzed with K⁺/H⁻-based solution. Under these conditions, WT
EAAT2 anion currents in the absence of external glutamate. Application of glutamate results in an increased current amplitude and moreover in the occurrence of a small deactivating component that measures at \(-185 \text{ mV}\) about 10 ± 1% (n = 9) of the late current (Fig. 5A). Whereas S506X resulted only in minor modification of EAAT2 anion current, we observed pronounced changes of E500X EAAT2 anion channel gating in the absence as well as in the presence of glutamate (Fig. 5, A and B) and of the degree of glutamate-induced current increases (Fig. 5, C and D).

With internal K\(^+\), E500X EAAT2 anion currents activated upon hyperpolarization before and after application of glutamate (Fig. 5A). Moreover, in cells internally dialyzed with K\(^+\), application of glutamate resulted in significantly smaller relative current increases for E500X EAAT2 than for S506X and WT EAAT2 (Fig. 5, C and D).

EAAT2 anion channel gating is different in cells with internal Na\(^+\) (Fig. 5E). With internal Na\(^+\), external glutamate results in a pronounced deactivating current component at negative voltages (relative amplitude: 54 ± 1% for WT EAAT2 at \(-185 \text{ mV}\) (n = 9)). Under these conditions, WT and mutant anion currents exhibit similar time and voltage dependence in the absence of glutamate (Fig. 5, E and F). Whereas substrate-dependent current increases at negative voltages are similar between WT and mutant EAAT2 (Fig. 5, G and H), there are more pronounced glutamate-induced current increases for E500X EAAT2 than for S506X and WT EAAT2 at +95 mV.

**E500X Modifies the Na\(^+\) Dependence of EAAT2 Anion Currents**—For various EAATs, external Na\(^+\) has been shown to be necessary for anion channel activity (8, 38, 42). EAAT2 anion channels exhibit unique sodium dependence. In the absence of glutamate, EAAT2 anion current amplitudes are substantial, also in the absence of external Na\(^+\), and are not increased but rather decreased upon application of external Na\(^+\) (Fig. 6, A and C). In the presence of glutamate, late current amplitudes increase with rising external [Na\(^+\)] (Fig. 6, B and D).

With internal K\(^+\) and in the presence of glutamate, the Na\(^+\) concentration dependence of WT EAAT2 could be fit with a Michaelis-Menten relationship with a \(K_m\) of 27 ± 6 mm (Fig. 6B). Internal Na\(^+\) increases the \(K_m\) to 52 ± 11 mm (Fig. 6D). In the absence of glutamate, \(K_m\) values are smaller than in its presence, resulting in concentration dependence with a \(K_m\) of 11 ± 6 mm with internal K\(^+\) and a \(K_m\) of 15 ± 6 mm with internal Na\(^+\) (Fig. 6, B and D).

E500X changed the concentration dependence only slightly with internal Na\(^+\) (Fig. 6D) (without glutamate, \(K_m\) = 31 ± 14 mm; with glutamate, \(K_m\) = 68 ± 27 mm) (Fig. 6D). However, when K\(^+\) was used as main intracellular cation (Fig. 6B) the apparent sodium dissociation constants of EAAT2 were drastically increased by E500X. In the absence of glutamate \(K_m\) was determined to be of 109 ± 68 mm. The presence of glutamate increased \(K_m\) to 264 ± 62 mm, so that not even application of 500 mM Na\(^+\) did reach saturating concentrations.

**C-terminal Modulation of K\(^+\) Binding Suggested by Kinetic Modeling**—Coupled glutamate transport can be modeled as binding/unbinding transitions between distinct states (32, 43). EAAT-associated anion channel properties are included in these models by connecting several states of the transport cycle with open conformations of the EAAT-associated anion channel (Fig. 7). All existing models predict low anion currents in the absence of external sodium (32, 44). To account for the inhibition of anion currents by Na\(^+\) in the absence of glutamate (Fig. 6), we added additional branching channel open states to ToK and To with opening rates that are higher than for the two following Na\(^+\)-bound states. We furthermore adjusted Na\(^+\)-binding/unbinding rate constants of the outward-facing states (Fig. 7A and supplemental Table S1) to account for experimentally determined apparent Na\(^+\) dissociation constants (Fig. 6).

The so-obtained kinetic model allowed the simulation of transport rates (Fig. 7B) and of voltage- and substrate-dependent anion channel gating (Fig. 7, C, D, F, and G) as well as modulation of anion currents by external Na\(^+\) for WT EAAT2 (Fig. 7, E and H). Because of the pronounced effects of internal K\(^+\) on E500X EAAT2 (Figs. 5 and 6), we analyzed all K\(^+\)-dependent reactions in the model and tested whether their modification results in a reduced transport rate and in
the observed changes of Na\(^+\)/H\(^+\) affinity with internal K\(^+\)/H\(^+\). The reduction of electrogenic glutamate uptake could be due to lower translocation rates between TiK and ToK. However, such changes cannot account for the observed modulation of sodium affinities in E500X EAAT2 by internal K\(^+\)/H\(^+\). These effects require that internal K\(^+\)/H\(^+\) reduces the probability that E500X EAAT2 enters outward-facing states.

As the substrate binding sites are thought to undergo substantial conformational changes during translocation from the outward to the inward conformation and vice versa (45), an allosteric modulation of outward-facing Na\(^+\) binding sites by internal K\(^+\) seems unlikely. K\(^+\) binding to its putative binding site is predicted to be mutually exclusive with glutamate binding (46). It is therefore not probable that K\(^+\) ions can occupy their binding sites in outward-facing states to impair association of Na\(^+\)/H\(^+\) with E500X.

A recent cross-linking study indicated that a purified Glt\(_{ph}\) transporter can be trapped in the inward-facing conformation in substrate- and cation-free conditions and demonstrated that inward translocation was even slowed down in the presence of transport substrates (45). Because the Glt\(_{ph}\) protein used in the studies exhibits a C terminus of comparable length as E500X EAAT2, we first modified the kinetic model by allowing transitions between Ti and To in E500X EAAT2. This model correctly predicts the K\(^+\)/H\(^+\)-dependent alteration of sodium affinities in EAAT2. However, it predicts two properties that are in obvious contrast to experimental results obtained for E500X EAAT2, i.e. electrogenic glutamate transport in the absence of internal K\(^+\)/H\(^+\) and absent anion current deactivation at negative potentials.

We thus hypothesize inward movement from To to a novel inward-facing state Ti\(_*\) in mutant transporters (Fig. 7A). The additional K\(^+\) binding site in E500X (TiK\(^*\)) predicts block of

**FIGURE 7. Kinetic model of WT and E500X EAAT2.** A, state diagram of the coupled glutamate transport cycle adapted from a published rEAAT2 model (32). Gray circles refer to open channel states. Ti\(_*\) and TiK\(^*\) are only available in E500X EAAT2. B, predicted forward and reverse glutamate transport rates in the WT and E500X transporter at −90 mV (forward) or +90 mV (reverse). External or internal glutamate was set to 500 \(\mu\)M, respectively. C and F, simulated current responses of WT and E500X EAAT2 with internal K\(^+\) (C) or Na\(^+\) (F). Anion currents are calculated from simulated open probabilities as the relative distribution of the transporter in open channel states. D and G, current-voltage dependence of simulated WT (○) and E500X (□) anion currents with internal K\(^+\) (D) or Na\(^+\) (G). E and H, simulated [Na\(^+\)] dependence for WT and E500X EAAT2 with internal K\(^+\) (E) or Na\(^+\) (H). Currents are normalized to 140 mM NaCl in the presence of l-glutamate. Calculated apparent affinities: WT with internal K\(^+\): \(K_m\) Na\(^+\) = 12.0 mM without glutamate, \(K_m\) Na\(^+\) = 26.4 mM with glutamate; E500X with internal K\(^+\): \(K_m\) Na\(^+\) = 62.5 mM without glutamate, \(K_m\) Na\(^+\) = 200.0 mM with glutamate; WT with internal Na\(^+\): \(K_m\) Na\(^+\) = 14.2 mM without glutamate, \(K_m\) Na\(^+\) = 1.9 mM with glutamate.

The decrease in electrogenic glutamate uptake could be due to lower translocation rates between TiK and ToK. However, such changes cannot account for the observed modulation of sodium affinities in E500X EAAT2 by internal K\(^+\)/H\(^+\). These effects require that internal K\(^+\) reduces the probability that E500X EAAT2 enters outward-facing states.

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EAAT2 transporter function by internal K\(^+\). The introduction of this binding site reduces glutamate transport current amplitudes to experimentally observed values (Figs. 1B and 7B). In close agreement with our experimental findings (Fig. 1, C and D), the resulting kinetic model predicted that reverse glutamate transport rates are not affected (Fig. 7B and supplemental Table S1).

E500X EAAT2 furthermore differs from WT in altered gating in the presence of internal Na\(^+\) (Fig. 5E). Anion currents activate upon membrane depolarization, and the rectification of instantaneous and late current amplitudes is altered in truncated transporters (Fig. 5F). These gating alterations cannot be predicted by changes in the transport cycle since gating is unchanged at negatives voltage range and apparent Na\(^+\) affinities are almost unchanged with internal Na\(^+\). We modeled this effect by assuming voltage-dependent channel opening of the transporter in the inward-facing state TiNa3GH, which may arise from local changes in electrostatics by E500X. These modifications allow the prediction of virtually all experimentally observed features of E500X EAAT2.

**DISCUSSION**

Mammalian EAATs exhibit cytoplasmic C-terminal domains encompassing between 41 and 83 amino acids with pronounced isoform-specific differences in amino acid sequences. Functional importance of the EAAT2 C terminus was highlighted by a recent report by Boston-Howes et al. (24) that described a loss of function of EAAT2 after activation of caspase-3. The authors identified a caspase-3 cleavage site within the C terminus and demonstrated that caspase-3-mediated C terminal proteolysis results in loss of glutamate uptake. Caspase-3 cleavage did not seem to result in transporter internalization, and the authors speculated that C-terminal cleavage is most likely due to loss of function by removal of functionally important segments (24).

We generated S506X EAAT2 as well as various additional truncations of decreasing C-terminal length. In mammalian cells, S506X modified neither transport nor the anion currents (Figs. 1 and 2). The truncation left the subcellular distribution of the transporter proteins unaffected (Fig. 1C). Moreover, S506X did not cause major changes of the voltage or substrate dependence of EAAT2 anion channels (Figs. 2 and 5). At present, the reason remains unclear for the differences between our and preceding studies (24). A possible explanation is that activity of endogenous exoproteases in Xenopus oocytes results in further truncation of S506X EAAT2 and thus in decreased glutamate uptake capability.

TM8 of EAAT2 ends with Ser-497 (36). Truncations directly at the end of TM8 (K498X) or within TM8 (H495X) abolished surface insertion of EAAT2 (Fig. 1C). Only two amino acids are sufficient for normal subcellular trafficking. To identify sequence determinants responsible for these functional changes we generated two chimeric constructs substituting the protein segment between E500 and S506 with corresponding segments of EAAT4 or ASCT-1. Whereas EAAT2 and EAAT4 complete a transport cycle by countertransport of K\(^+\) and mediate electrogenic transport, the related ASCT-1 is only capable of mediating homo- and hetero-exchange. ASCT-1 is insensitive to K\(^+\) (40), although all known structural determinants of K\(^+\) exchange are conserved (46–48). EAAT2\(_{\text{chimEAAT4}}\) exhibited unaltered functional properties (Fig. 3). In contrast, electrogenic glutamate transport was reduced in EAAT2\(_{\text{chimASCT1}}\) (Fig. 3), suggesting that isoform-specific properties might be partially determined by the C terminus.

To identify the basis for the functional alterations of truncated EAAT2 we performed a detailed study of WT and E500X EAAT2 transport and anion currents. E500X causes a drastic reduction of glutamate inward current (Fig. 1B), but leaves reverse glutamate transport unaffected. E500X reduces the glutamate-dependent increase in anion current amplitude with internal K\(^+\), and augments this parameter with internal Na\(^+\) (Fig. 5). Voltage-dependent gating is modified in E500X EAAT2 when K\(^+\) is the major internal cation. Only with internal K\(^+\), but not with internal Na\(^+\), WT and E500X EAAT2 anion currents differ in the external [Na\(^+\)] dependence. Under these conditions, E500X increased the apparent $K_m$ for Na\(^+\) in the absence as well as in the presence of glutamate.

To study the effect of internal cations on EAAT2 anion currents it is necessary to separate anion currents from transport currents in the presence of internal K\(^+\). In WT EAAT2, electrogenic glutamate uptake generates currents with amplitudes that measurably contribute to total currents with Cl\(^-\) as internal and external anion (Fig. 1B). However, with NO\(_3\) as main permeant anion, total currents are ~6-fold larger (Fig. 5). A recent publication reported that NO\(_3\) reduces transport rates of glutamate transporters in salamander retina glial cells (49) suggesting that electrogenic transport current amplitudes will be even smaller in NO\(_3\) than in Cl\(^-\). Moreover, reversal potentials are closely similar in cells dialyzed with internal Na\(^+\) or K\(^+\). Lastly, all existing EAAT2 state models predict higher apparent Na\(^+\) dissociation constants for electrogenic uptake than for anion currents. A significant contribution of transport currents would result in an overestimation of $K_m$ values in WT EAAT2 and thus only decrease the difference in $K_m$ for WT and E500X EAAT2 with internal K\(^+\). Transport currents thus represent only a minor component of total currents and do not affect our conclusion about the effects of E500X on the substrate dependence of EAAT2 anion channels.

So far, EAAT anion channels have been assumed to be strictly sodium-dependent (44). For EAAT1 (50), EAAT3 (33), EAAT4 (38) EAAT-associated anion channels were shown to be stimulated by increased external [Na\(^+\)] and to exhibit very low activity in the absence of external Na\(^+\). EAAT2-associated anion channels display a unique Na\(^+\) dependence. In the case that glutamate is not present, EAAT2 anion channels exhibit maximum activity in the absence of external Na\(^+\) (Fig. 6). Moreover, EAAT2 anion channels are even active with choline\(^+\) as sole internal and external cation.\(^3\) The molecular basis and the functional impact of this unique cation dependence of the glial isoform EAAT2 remains to be determined.

To identify transitions within the uptake cycle that are modified by the C terminus we used kinetic modeling em-

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\(^3\) C. Fahlke, unpublished observation.
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ploying a state model developed by Bergles et al. (32) (Fig. 7). Minor modifications of this scheme resulted in good agreement of simulated voltage and glutamate dependence of WT EAAT2 with experimental data (Fig. 7). To account for the altered sodium dependence of E500X EAAT2 anion currents it was necessary to predict that internal K⁺ slow progression of the uptake cycle. We thus assumed translocation of the empty transporter from the outward- to a novel inward-facing conformation (Tᵢ'). We furthermore hypothesized that the transporter can bind K⁺ from this conformation. TᵢK' differs from TᵢK in its inability to complete the translocation through the membrane. Such modifications of the Bergles model accurately predict the alteration of EAAT2 forward transport, and the lack of reverse transport modification by E500X (Fig. 7). Moreover, simulation of voltage and substrate dependence of WT and E500X EAAT2 anion currents are in good agreement with experimental results (Fig. 7).

At present, high resolution structures only exist for the archetypical transporter Glt_{pr}. Two conformations have been resolved so far, one with substrate binding sites accessible to the external medium, in the outward-facing conformation (36), and the other in the inward-facing conformation (45). The two conformations differ in the position of a “translocation domain” (containing TM3, TM6, TM7, and TM8) that undergoes substantial movements during the glutamate transport process (45). In contrast, a “trimerinization domain” (containing TM1, TM2, TM4, and TM5) is largely immobile (45). Reyes et al. (45) recently demonstrated for mutant Glt_{prv} carrying two cysteine substitutions K55C/A364C that the transporter can shuttle from the outward- to the inward-facing conformation even in the absence of bound substrates. E500X exhibits a C terminus comparable with the crystallized form of Glt_{pr}, suggesting that such transitions are possible for the truncated transporter and that C-terminal domains adjacent to TM8 might interfere with movement of the empty translocation domain. This hypothesis would provide a structural explanation for the states we introduced into the kinetic model to explain our experimental results.

The effects of internal cations (Figs. 5 and 6) let us postulate that E500X EAAT2 binds internal K⁺ after shuttling in an empty state into the inward-facing conformation and that the bound K⁺ prevents the subsequent translocation through the membrane from this particular conformation. At present, little is known about the K⁺ hemicycle in WT or mutant EAAT2. Tyr-403 and Glu-404 were demonstrated to be necessary for K⁺-coupled glutamate transport in rat EAAT2 (47, 48). Grewer et al. (23) subsequently demonstrated that the homologous residue in EAAT3 (E373) does not represent a binding site for K⁺, but rather for protons. In a recent publication, Holley and Kavanaugh (46) postulated that D444 in EAAT3 (homologous to D475 in EAAT2) contributes to glutamate and K⁺ binding and that association of K⁺ is only possible after dissociation of glutamate. Glu-404 and Asp-475 are only accessible when hairpin loop 1 is open. It appears therefore unlikely that K⁺ can associate to this particular site after translocation of the empty carrier. We therefore have to postulate an additional binding site that is either not accessible or not existing in EAATs with intact C terminus. In summary, we have demonstrated that partial removal of the C terminus of EAAT2 results in changes in glutamate uptake and anion conduction. These functional alterations suggest that the impairment of certain conformational changes by the C terminus is necessary for coupled transport by EAAT2.

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