SUPPLEMENTAL MATERIALS AND METHODS

Biotinylation
Biotinylation was performed as previously described (Davis, K.E., D.J. Straff, E.A. Weinstein, P.G. Bannerman, D.M. Correale, J.D. Rothstein, and M.B. Robinson. 1998. J. Neurosci. 18:2475–2485) with some modifications. In brief, confluent HEK293T cells in 10-cm plates were transfected with transporter cDNA by using FuGENE 6 transfection reagent (Roche) according to the supplied protocol. The cells were washed two to three times with ice cold PBS plus 0.1 mM Ca$^{2+}$ and 1 mM Mg$^{2+}$ at 48 h after transfection. 2 ml biotinylation reagent (2 mg/ml, EZ-Link Sulfo-NHS-Biotin; Pierce Chemical Co.) was added to the plate and the cells were kept at 4°C for 30 min while gently shaking. The cells were washed with ice cold PBS plus 0.1 mM Ca$^{2+}$, 1 mM Mg$^{2+}$, and 100 mM glycine three times; then the same solution was added to the plate at 4°C and incubated for 45 min to quench any unreacted biotin reagent. The cells were lysed by adding 1 ml RIPA/lysis buffer (100 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, plus protease inhibitors) to the cell and by subsequent vortexing for 1 h at 4°C (al-

Figure S1. (A) [Na$^{+}$] dependence of the glutamate-induced current for EAAC1$^{WT}$; the glutamate concentration was 1 mM, 140 mM NaSCN and 10 mM glutamate were in the pipette solution, $V_{\text{hold}} = 0$ mV. Currents were normalized to those at 140 mM [Na$^{+}$]. The solid line represents a fit to the Hill equation with a Hill coefficient of $n = 1$ and $K_{m}(WT) = 8 \pm 1$ mM. (B) Similar experiments for D454N (open squares), E373Q (open triangles), and D439N (closed triangles) as in A, except 10 mM glutamate was used for D439N. All currents were normalized to those of wild-type currents at 140 mM [Na$^{+}$]. Data of D454N and E373Q can be fitted with a Hill equation with a Hill coefficient of $n = 1$ and $K_{m}(D454N) = 7 \pm 1$ mM, $K_{m}(E373Q) = 7 \pm 2$ mM, which are represented by solid lines. The solid line for D439N represents a fit to Eq. 4 in the Appendix with parameters $G_{TNa} = 1.2$, $G_{TNa} = 0.5$, $G_{TGS} = 1$, $K_{N1} = 110$ mM, $K_{N2} = 300$ mM, and $K_{S} = 500 \mu$M. Also see Figs. 4 and 8 and the corresponding explanation in the main text. (C) $K_{m}$ of glutamate vs. [Na$^{+}$] relationships for wild-type EAAC1 (circles) and D439N (triangles) at 0 mV.

Figure S2. Comparison of wild-type EAAC1 and D439N mutant transporter expression levels in HEK293T cell membranes. HEK293T cells transfected with wild-type or D439N mutant transporters were treated with biotinylation reagent and the different fractions (T, total; U, unbound; E, elute) were analyzed by Western blotting. The wash fractions are not shown because there was no band detected in these fractions. Lanes 1, 3, and 5 represent wild-type transporter; lanes 2, 4, and 6 are EAAC1$^{D439N}$. The bands attributed to the EAAC1 monomer and multimers are indicated on the righthand side. Although minor differences are seen in this blot in the proportion of dimer and multimer bands between wild-type EAAC1 and EAAC1$^{D439N}$, such differences were not consistently observed in other, similar Western blots. The bottom panel shows the actin controls, demonstrating only little immunoreactivity in the membrane fractions.

Figure S3. Dose response data used to determine the kinetic parameters of EAAC1$^{WT}$ and mutant transporters listed in Table I. The data were normalized to a maximum response of 1. The membrane potential was 0 mV.
though the intense vortexing resulted in generation of foam, this foam disappeared after subsequent centrifugation steps). After centrifuging at 20,000 g to sediment nucleic acid and debris for 10 min at 4°C, 100 μl of 50% slurry protein A beads (Upstate) in PBS were added to preclear the sample at RT for 1 h. The beads were spun down and the supernatant transferred to a new tube (this is the total fraction, T). After adding 300 μl of 50% slurry Avidin beads (Pierce Chemical Co.) in PBS to 300 μl precleared sample and keeping the sample at RT for 1 h, the beads were sedimented by centrifugation and the supernatant was transferred to a new tube (this is the unbound fraction, U). The beads were washed with RIPA/lysis buffer six times, 1 ml each time (this is the wash fraction, W). The protein was eluted from the beads by adding 150 μl 2X Laemmli buffer (1X Laemmli buffer contains 62.5 mM Tris, pH 6.8, 2% SDS, 20% glycerol and 5% 2-mercaptoethanol). Each fraction was adjusted to comparable volumes with Laemmli buffer and analyzed by Western blotting using EAAC1 antibody obtained from Alpha Diagnostics. The blots were visualized with enhanced chemiluminescence (Amersham Biosciences). Anti-actin antibodies (Abcam) reacted with the total protein fraction, but not with the biotinylated fraction, indicating that the biotinylated fraction did not contain detectable amounts of intracellular proteins (Fig. S2).

Figure S4. SCN⁻ has no fundamental effect on EAAC1_WT and EAAC1_D439N. (A) Glutamate-induced transport currents were recorded from an EAAC1_WT-expressing HEK cell before (left) and after (right) application of the permeable anion SCN⁻ (middle, forward mode with KCl in the pipette, \(V_{\text{hold}} = 0\) mV). Application of glutamate in the presence of SCN⁻ outside of the cell resulted in an outward current due to the inward flow of SCN⁻. (B) Na⁺-induced currents recorded before (left) and after (right) the application of 140 mM NaSCN to EAAC1_D439N show that the application of SCN⁻ does not result in long-lasting irreversible effects after SCN⁻ removal.

Figure S5. The apparent affinity of the aspartate-bound form of EAAC1_D439A for Na⁺ is high. \([\text{Na}^+]\) dependence of the aspartate-induced maximum current (at saturating [aspartate], 20 mM) for EAAC1_D439A (A) and EAAC1_D439N (B). The experiments were performed under exchange conditions, \(V_{\text{hold}} = 0\) mV (the \(K_m\) of EAAC1_D439A for aspartate is 0.97 ± 0.16 mM). The apparent affinity of the aspartate-bound form of EAAC1_D439A mutant was determined as 28 ± 1 mM from the fit of a Michaelis-Menten–like equation to the data (solid line).