Mutating a Conserved Proline Residue within the Trimerization Domain Modifies Na\(^+\) Binding to Excitatory Amino Acid Transporters and Associated Conformational Changes*

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**Background:** The mutation P290R in the excitatory amino acid transporter 1 (EAAT1) causes episodic ataxia type 6.

**Results:** Voltage clamp fluorometry demonstrates changes in the time course of EAAT3 fluorescence signals by the homologous P259R mutation.

**Conclusion:** P259R alters EAAT3 transport functions by decelerating conformational changes associated with sodium binding.

**Significance:** Studying naturally occurring mutations promises identification of unexpected determinants of transporter functions.

Excitatory amino acid transporters (EAATs) are crucial for glutamate homeostasis in the mammalian central nervous system. They are not only secondary active glutamate transporters but also function as anion channels. A naturally occurring mutation, which was identified in a patient with episodic ataxia type 6 and that predicts the substitution of a highly conserved proline at position 290 by arginine (P290R), was recently shown to reduce glutamate uptake and to increase anion conduction by hEAAT1. Here we used voltage clamp fluorometry to define how the homologous P259R mutation modifies the functional properties of EAAT3. P259R inverts the voltage dependence, changes the sodium dependence, and alters the time dependence of EAAT3 fluorescence signals. Kinetic analysis of fluorescence signals indicate that P259R decelerates a conformational change associated with sodium binding to the glutamate-free mutant transporters. This alteration in the glutamate uptake cycle accounts for the experimentally observed changes in glutamate transport and anion conduction by P259R hEAAT3.

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2 The abbreviation used is: EAAT, excitatory amino acid transporter.

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**EXPERIMENTAL PROCEDURES**

Expression of WT and Mutant hEAAT Transporters in Xenopus laevis Oocytes and Mammalian Cells—To permit site-directed fluorescence labeling of WT and P259R hEAAT3, we chose M205C for fluorophore attachment (see Fig. 1A) and...
additionally mutated the endogenous Cys-158 to serine to prevent possible modifications of this endogenous cysteine by fluorophores. The P259R mutation was inserted into the vector pTLN2 using PCR-based strategies. For comparison of glutamate uptake by P259R hEAAT3 and P290R hEAAT1, the coding region of WT (kindly provided by Dr. S. Amara, National Institute of Mental Health) or P290R hEAAT1 (6) was subcloned into pTLN2 using PCR-based strategies.

Capped cRNA was synthesized from MluI-/NheI-linearized pTLN2-hEAAT3/hEAAT1 through use of MESSAGE machine kits (Ambion, Austin, TX). Collagenase-treated, defolliculated stage IV–V oocytes were microinjected with 10 ng of RNA (Nanoliter 2000, World Precision Instruments, Sarasota, FL) and incubated at 18 °C in ND96 (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 5 mM Hepes, pH 7.6, supplemented with 2.5 mM sodium pyruvate and 50 μg/ml gentamicin sulfate). Experiments were performed 3–7 days after injection.

For heterologous expression in mammalian cells, P259R was introduced into pcDNA3.1-hEAAT3 (10) using the QuikChange mutagenesis kit (11). Two independent recombinants from the same transformation were examined and shown to exhibit indistinguishable functional properties. WT or mutant hEAAT3 and hEAAT1 transporters were expressed in HEK293T cells using the Ca₃(PO₄)₂ technique as described (6, 12).

**Voltage Clamp Fluorometry**—Voltage clamp fluorometry on oocytes expressing M205C hEAAT3 or M205C-P259R hEAAT3 was performed as described (9). Oocytes were voltage-clamped using a CA-1B amplifier (Dagan Corp., Minneapolis, MN) under the control of an ITC-18 computer interface in combination with Patchmaster (HEKA Elektronik, Lambrecht, Germany). For fluorescence experiments, oocytes were labeled with 2–3 h with 10 μM fluorescent maleimide probe, Alexa Fluor 546 (Invitrogen). Incubation times were optimized for each construct as described (9). A short arc mercury lamp (HBO 103W/2, Osram, München, Germany) combined with a Uniblitz shutter (VS25S2ZMOR1–24 shutter, VCM-D1 shutter driver, Uniblitz, Vincent Associates, Rochester, NY) was used as light source. Fluorescence was monitored under voltage clamp by a photodiode (PIN020-A, AMS Technologies AG, Martinsried, Germany) and a rhodamine filter cube (HQ535/565LP, HQ610/75m, Chroma Technology Corp., Bel lows Falls, VT) attached to an inverted fluorescence microscope (IX71, Olympus, Hamburg, Germany). For fluorescence signals, oocytes were clamped as described (9) to 0 mV at 0.150 mV and 0 mV, respectively. Data are given as the means ± S.E. For statistical evaluations, Student’s paired t test was used with p ≤ 0.05 (*), p ≤ 0.01 (**), or p ≤ 0.001 (***). Apparent dissociation constants and Hill coefficients were calculated by fitting the data with Equation 1.

\[
\frac{F}{F_{0.150 \text{ mV}}} = F_0 + a \times \left( \frac{[\text{Na}^+]_{\text{H}}}{{K_D}^{\text{H}} + [\text{Na}^+]_{\text{H}}} \right)
\]

where \(F\) is the fluorescence emission intensity at a certain voltage, \(F_{0.150 \text{ mV}}\) at +150 mV and \(F_0\) at 0 mV, \(a\) is the slope, \([\text{Na}^+]_{\text{H}}\) is the sodium concentration, \(n_H\) is the Hill coefficient, and \(K_D\) is the apparent dissociation constant.

**Data Analysis**—Electrophysiological and fluorescence data were analyzed with a combination of Patchmaster (HEKA Elektronik, Lambrecht, Germany), pClamp10 (Molecular Devices), MATLAB (The MathWorks, Natick, MA), and SigmaPlot (Jandel Scientific, San Rafael, CA). Apparent dissociation constants and Hill coefficients were calculated by fitting the data with Equation 1.

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**Simulation of P259R hEAAT3 Fluorescence Signals and Transport Properties**—Voltage and substrate dependences of EAAT fluorescence signals as well as glutamate uptake and anion currents were simulated using a kinetic scheme recently established for WT hEAAT3 based on voltage- and substrate-dependent fluorescence signals, as well as on uptake currents and glutamate-sensitive relative current amplitudes (9). This model was adapted to the P259R mutant by modifying sodium-binding rates (reactions 1 and 2 and 12 and 13) using a genetic algorithm as described (9). Parameters were simultaneously optimized against fluorescence signals at different \([\text{Na}^+]\) and experimentally determined relative glutamate uptake and relative glutamate-induced current amplitudes at −100 mV. Identical anion channel open probabilities were chosen for WT and P259R hEAAT3 (9). The robustness of the fit was tested by repeating the stochastic fitting procedure with similar fitting results. Electrical distances (δ) were adjusted manually to account for the time and voltage dependence of P259R.
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hEAAT3 anion currents. In all cases, a detailed balance was preserved. For simulation and fitting, we assumed intracellular concentrations of \([\text{Na}^+] = 10 \text{ mM}, [\text{K}^+] = 70 \text{ mM}, [\text{glucose}] = 12 \text{ mM}, \text{and a pH 7.3 in oocytes (14); extracellular concentrations were set to experimental conditions.}

RESULTS

P259R Reduces hEAAT3 Glutamate Uptake and Increases Associated Anion Currents—The naturally occurring point mutation P290R was recently reported to change glutamate uptake as well as anion conduction by hEAAT1 (6). We used heterologous expression in *Xenopus* oocytes to quantify the effect of the homologous P295R mutation on hEAAT3 glutamate uptake (9, 10) and expression in mammalian cells to describe changes in hEAAT3 anion current amplitudes and kinetics (6).

Under chloride-free conditions, EAAT anion currents as well as endogenous anion currents are absent, and glutamate uptake currents can be determined as differences in current amplitudes before and after glutamate application (Fig. 1C) (9, 10). We compared glutamate uptake currents in oocytes expressing WT, M205C, or M205C-P259R hEAAT3. Whereas M205C does not affect hEAAT3 function (9), P259R results in a significant reduction of hEAAT3 uptake (WT, \(I_{\text{uptake}} = -1.17 \pm 0.14 \mu\text{A}; \text{M205C}, I_{\text{uptake}} = -1.07 \pm 0.13 \mu\text{A}; \text{M205C-P259R}, I_{\text{uptake}} = -0.18 \pm 0.04 \mu\text{A}; n = 13; P_{\text{WT,M205C}} = 0.858; P_{\text{WT,M205C-P259R}} = 0.001).

Openings and closings of EAAT anion channels are tightly coupled to transitions within the uptake cycle (3, 15, 16), and analysis of time and voltage dependences of anion currents thus provide additional insights into P259R-mediated changes of the glutamate uptake cycle. EAAT anion currents are often small under physiological anion composition; however, the use of a hytropic anion such as SCN\(^-\) or NO\(_3\) greatly increases their amplitudes. Expression in mammalian cells and the use of symmetrical NO\(_3\) permits direct measurements of EAAT anion currents through whole-cell patch clamp measurements (Fig. 1E) (11, 17). In these experiments, cells were internally diazyl with high [K\(^+\)] to permit all physiologically occurring transitions in the glutamate uptake cycle. In the absence as well as in the presence of glutamate, cells expressing P259R hEAAT3 display inwardly rectifying anion currents that are significantly larger than currents of WT hEAAT3 in the negative voltage range (at \(-125 \text{ mV}, \text{WT hEAAT3}_{\text{(-glutamate)}} = -0.11 \pm 0.001 \text{nA}, \text{WT hEAAT3}_{\text{(+glutamate)}} = -0.37 \pm 0.04 \text{nA} (n = 4); \text{P259R hEAAT3}_{\text{(-glutamate)}} = -0.27 \pm 0.04 \text{nA}, \text{P259R hEAAT3}_{\text{(+glutamate)}} = -1.23 \pm 0.19 \text{nA} (n = 13)). P259R did not only modify hEAAT3 anion current amplitudes but also the voltage and time dependence of these currents (Fig. 1E and G). Upon hyperpolarizing voltage steps, P259R hEAAT3 currents display time-dependent increases that are more pronounced in the presence of glutamate than in its absence (Fig. 1E, right column). Under similar experimental conditions, WT hEAAT3 anion current amplitudes are comparable at positive and at negative voltages with only minor time-dependent relaxation (Fig. 1E, left column). The effects of P259R on hEAAT3 glutamate uptake and anion currents closely resemble those of P290R on hEAAT1 (Fig. 1, D, F, and H).

P259R Modifies Fluorescence Signals of M205C hEAAT3—Fig. 2A shows representative fluorescence recordings of M205C or M205C-P259R hEAAT3 in absence (top) or in the presence of glutamate (bottom). Oocytes were held at 0 mV, and voltage steps between \(-150 \text{ mV and } +150 \text{ mV were applied in 50 mV intervals. M205C hEAAT3 displays pronounced voltage- and substrate-dependent fluorescence signals (Fig. 2A, left column). Application of glutamate decreases fluorescence intensity of M205C hEAAT3 over the whole tested voltage-range and furthermore changes the voltage dependence of the fluorescence signal (Fig. 2B, left column) (9). P259R inverts the voltage dependence and modifies the substrate dependence of these fluorescence signals (Fig. 2, A and B, right column). M205C-P259R hEAAT3 fluorescence intensities increase upon membrane hyperpolarization, and positive membrane potentials lead to slight decreases. Glutamate decreases fluorescence intensities at negative voltages and leaves them unaffected at positive potentials (Fig. 2B, right column).

P259R also modifies the time course of M205C hEAAT3 fluorescence signals. Hyperpolarizing voltage steps cause fast reductions of M205C hEAAT3 fluorescence that can be fit with monoeponential functions resulting in time constants of \(~3 \text{ ms (Fig. 2C, left column). Within the tested voltage range, M205ChEAAT3 time constants change only little. For M205C-P259R hEAAT3, time constants of fluorescence increase upon hyperpolarization increase from 170 to 370 ms at voltage steps between \(-150 \text{ and } -50 \text{ mV (Fig. 2C, right column).}

P259R alters the sodium dependence of M205C hEAAT3 fluorescence signal—In the absence of glutamate, M205C hEAAT3 fluorescence changes report on sodium-binding (9), and the pronounced effects of P259R on these signals suggest that the mutation modifies these particular substrate association steps within the EAAT transport cycle. Fig. 3A gives the voltage dependence of averaged fluorescence intensities of M205C hEAAT3 at various external [Na\(^+\)]. Reduction of external sodium results in larger fluorescence amplitudes at negative voltages, whereas values at positive potentials were unaffected. Complete substitution of Na\(^+\) by choline nearly abolishes the voltage dependence of fluorescence signals. We next fitted the sodium dependences of fluorescence amplitudes at certain membrane potentials with Hill equations (Equation 1) (Fig. 3A, inset) to obtain apparent dissociation constants (\(K_D\)) and Hill coefficients (\(n_H\) for sodium binding and subsequent protein movements. M205C hEAAT3 exhibits apparent dissociation constants in the range of 11–14 mM (Table 1). P259R increases apparent \(K_D\) values >5-fold (Table 1) so that even doubling of external [Na\(^+\)] to 197 mM was insufficient to reach saturation for M205C-P259R hEAAT3 (Fig. 3A, right column). For M205C as well as for M205C-P259R hEAAT3, apparent \(K_D\) values decrease slightly with hyperpolarizing voltages. For WT as well as for P259R hEAAT3, Hill coefficients are >1 (Table 1), in agreement with the notion that two Na\(^+\) bind prior to glutamate (18–21).

Changes in external [Na\(^+\)] modify the time course of M205C as well as of M205C-P259R hEAAT3 fluorescence changes upon voltage steps (Fig. 3B). Fluorescence increases upon subsequent voltage steps back to the holding potential are due to sodium dissociation and related conformational changes and...
can be also described with a single time constant that changes with [Na\(^+\)]. We determined apparent rate constants of fluorescence change as inverse time constants (Fig. 3C). For M205C as well as for M205C-P259R hEAAT3, we observed a saturating Na\(^+/\)H\(^+\) dependence for these apparent rates. This deviation from a linear Na\(^+/\)H\(^+\) dependence indicates that the fluorescence signal does not result from one step association of Na\(^+\) to the transporter (21). The saturation behavior rather suggests that there is an additional reaction limiting the overall rate at high Na\(^+\) once all sodium binding sites are saturated. This rate-limiting reaction is clearly voltage-dependent (Fig. 3C). P259R causes a significant deceleration of this rate-limiting reaction.

**FIGURE 1.** P259R impairs hEAAT3 glutamate uptake and causes gain of function of hEAAT3 anion channels. A, localization of M205C and P259R in the transmembrane topology model of EAAT3. B, alignment of the amino acid sequence of different EAAT proteins and non-mammalian orthologs. C and D, averaged glutamate uptake currents at -100 mV of oocytes expressing WT hEAAT3, M205C-, or M205C-P259R hEAAT3 (C) as well as of oocytes expressing WT or P290R hEAAT1 (D). Uptake currents were calculated as difference of current amplitudes with (+glu) and without glutamate (w/o glu) measured in a chloride-free solution. Data are given as the means ± S.E.; n ≥ 13. E and F, representative current recordings from HEK293T cells expressing WT (left column) or P259R (right column) hEAAT3 (E) or WT or P290R hEAAT1 (F) with standard KNO\(_3\)-based internal solution and a NaNO\(_3\)-based external solution before (top) and after application of 1 mM glutamate (bottom). G and H, voltage dependence of mean current amplitudes from cells expressing WT or P290R hEAAT1 (G) or WT or P290R hEAAT1 (H) in the presence as well as in the absence of glutamate. Data are given as the means ± S.E.; n ≥ 4.
The data presented so far indicate that P259R changes Na$^+$ association and processes linked to Na$^+$ binding to the glutamate-unliganded transporter. To test whether P259R also affects binding of a third Na$^+$ to the glutamate-bound transporter (18, 19, 22), we used voltage clamp recordings from oocytes expressing WT, M205C, or M205C-P259R hEAAT3. We could not study this partial reaction using fluorescence signals because glutamate resulted in significant reductions of these signals (Fig. 2). We therefore studied the [Na$^+$] dependence of glutamate uptake currents at −100 mV in the presence of saturating glutamate concentrations. Under these conditions WT, M205C, and M205C-P259R hEAAT3 display sodium dependences with similar apparent dissociation constants and Hill coefficients that agree well with published data (Fig. 4) (23). The results support the notion that P259R leaves Na$^+$ association rates to glutamate-bound transporters unaltered.
We here used voltage clamp fluorometry to study how a point mutation resulting in substitution of a conserved proline in the trimerization domain by arginine (P259R) affects the function of the human glutamate transporter hEAAT3. We observed a significant deceleration of P259R hEAAT3 fluorescence signals in the absence of glutamate and changes in the glutamate effects on these signals (Figs. 2–3). Kinetic analysis of fluorescence signals under glutamate-free conditions at various external \([Na^+]/[H^+]\) (Fig. 3) revealed a non-linear relationship between the apparent rate constants of fluorescence changes and external \([Na^+]/[H^+]\) (Fig. 3C). P259R modified the \([Na^+]/[H^+]\) dependence of these apparent rates and the maximum values at saturating \([Na^+]/[H^+]\) (Fig. 3C). Such behavior demonstrates that P259R does not exclusively alters \([Na^+]/[H^+]\) binding but also modifies a conformational change preceding or following \([Na^+]/[H^+]\) association. A possible candidate for such a conformational change might be the opening of HP2 that is triggered by association of \([Na^+]/[H^+]\) to the empty transporter (19).

**TABLE 1**

| Voltage (mV) | M205C | M205C-P259R |
|-------------|-------|-------------|
|             | \(K_D\) | \(n_H\)     | \(K_D\) | \(n_H\)     |
| −150        | 11.7 ± 4 | 1.9 ± 0.9 | 50.7 ± 7.7 | 1.5 ± 0.2 |
| −100        | 13.4 ± 4.6 | 2 ± 1.3 | 63.7 ± 2.4 | 1.3 ± 0.04 |

**DISCUSSION**

We here used voltage clamp fluorometry to study how a point mutation resulting in substitution of a conserved proline in the trimerization domain by arginine (P259R) affects the function of...
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![Graphs showing sodium dependence of WT, M205C, and M205C-P259R hEAAT3 at saturating glutamate.](image)

EAAT glutamate transport and anion channel function is usually described by a kinetic scheme that is based on the transport cycle (3, 8, 24), with open anion channels represented by additional branching open channel states (11, 25). The transport cycle proceeds via consecutive association/dissociation steps of the transport substrates, interrupted by two major conformational changes during forward and backward isomerization from outward- to inward-facing states. Additional conformational changes have been described (21, 26); however, these transitions appear to be very fast and are therefore usually lumped together with substrate association steps. Deceleration of such a conformational change permits observation of one of the kinetics of Na\(^+\) association and this conformational change. At present, we can therefore not accurately describe the effects of the P259R mutation on the glutamate uptake cycle using kinetic modeling.

However, kinetic simulations permit resolving the question whether the observed alterations in Na\(^+\) association are sufficient to predict the dramatic changes in P259R hEAAT3 anion channel function (Fig. 1). We used a kinetic scheme recently developed to describe WT hEAAT3 fluorescence and transport properties (9) (Fig. 5 and Table 2) and tested whether modification of the rate constants describing sodium binding and associated conformational changes results in similar alterations of glutamate uptake and anion currents as observed experimentally.

We optimized forward and backward reaction rates for sodium association to the outward-facing transporter (reactions 1 and 2, Fig. 5) and the corresponding reactions in the inward-facing conformation (reaction 12 and 13, Fig. 5) against experimentally observed fluorescence data at various external [Na\(^+\)], relative current amplitudes at −100 mV, and relative glutamate uptake currents at −100 mV using a genetic fitting algorithm (9, 16). Because the fitted rate constants do not only describe Na\(^+\) association/dissociation but also additional slower processes (Fig. 3), we permitted fit parameters that are significantly lower than values expected for substrate association events (Table 2).

Without modifying the voltage dependences of these rates, the model predicted time-independent current responses that differed from the characteristic slow activation of P259R hEAAT3 anion currents upon hyperpolarizing voltage steps (Fig. 1E). Because the conformational changes associated with Na\(^+\) binding appear to be voltage-dependent (Fig. 3C), we also modified the voltage dependence of rates 1, 2, 12, and 13 (Table 2). These modifications resulted in a kinetic model that nicely predicts fluorescence responses, transport properties, and anion currents by mutant hEAAT3 (Fig. 5). Changes in Na\(^+\) binding and associated conformational changes are thus sufficient for predicting experimental data on P259R hEAAT3 glutamate transport and anion currents.

At present, no structural information is available for mutant transporters, and we can thus only speculate about structural correlates of the observed changes in substrate association to P259R hEAAT3. Proline often acts as structural disruptor of \(\alpha\)-helices and \(\beta\)-sheets, and the conserved Pro-259 at the hinge of transmembrane domain 5 might represent the structural determinant of the kinked shape of transmembrane domain 5. P259R might abolish the transmembrane domain 5 kink and cause an overall structural change of the trimerization domain of mutant transporters. A resulting change in the orientation of the transport domain toward the trimerization domain might impair certain conformational changes within the transport domain and thus explain the inverted voltage dependence of conformational changes (Figs. 2 and 3).

EAAT1 and EAAT3 share basic mechanisms of glutamate transport and exhibit similar anion conduction properties (2, 14, 27). However, despite the strict conservation of proline 259 in the whole transporter family, we cannot exclude the possibility that P259R acts differently on hEAAT3 than P290R on hEAAT1. However, the functional consequences of the homologous mutations P259R and P290R are remarkably similar. Both reduce glutamate uptake currents and increase the amplitudes of EAAT anion currents in the absence as well as in the...
presence of glutamate (Fig. 1). Moreover, we observed comparable modifications of the time and voltage dependence of P259R hEAAT3 and P290R hEAAT1 anion currents (Fig. 1). Our results strongly suggest that P290R modify hEAAT1 function also by affecting conformational changes associated with Na+/H+ binding.

In a preceding study on P290R hEAAT1 (6), we observed dramatically increased hEAAT1 anion currents but decreased surface membrane expression levels (5, 6). Noise analysis measurements revealed identical unitary current amplitudes for WT and P290R hEAAT1 anion channels, indicating that the ~4-fold increase of hEAAT1 anion currents by P290R must be due to a pronounced increase in the absolute open probability (6). However, experimentally determined absolute open probabilities are >0.5 for WT hEAAT1 (6). These seemingly incom-

![Kinetic model and simulated fluorescence signals and currents for M205C and M205C-P259R hEAAT3.](image-url)

** FIGURE 5.** Kinetic model and simulated fluorescence signals and currents for M205C and M205C-P259R hEAAT3. A, 15-state model with branching anion channel states (gray circles), grouped into four fluorescence states (dotted boxes), corresponding to four conformations shown as protomers. B, voltage dependence of measured (circles) or simulated fluorescence amplitudes (solid lines) without (gray) or with glutamate (black). C, simulated fluorescence amplitudes at different [Na+/H+] (197, 98.5, 49.25, 24.625, 10, and 0 mM). D and E, comparison of the simulated (gray) and measured (black) glutamate uptake (D) and anion currents in the absence of glutamate (E) at -100 mV, normalized to the current amplitudes of M205C. F and G, simulated time dependences of fluorescence changes (F) and current amplitudes (G) upon the indicated voltage steps for M205C or M205C-P259R hEAAT3 either in the absence (w/o glu) or in the presence of 1 mM glutamate (+ glu).
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TABLE 2
Rate constants of the WT and P259R hEAAT3 uptake cycle used in the simulations in Fig. 5

| Reaction | Forward | Backward | Changed parameters for P259R |
|----------|---------|----------|-------------------------------|
| 1        | 1.00 \times 10^{-9} \text{M}^{-1} s^{-1} | 2.50 \times 10^{-9} \text{M}^{-1} s^{-1} | 0.20 | 0.50 | 80 \text{M}^{-1} s^{-1} | 51.73 s^{-1} |
| 2        | 1.00 \times 10^{-9} \text{M}^{-1} s^{-1} | 2.50 \times 10^{-9} \text{M}^{-1} s^{-1} | 0.20 | -0.10 | 55 \text{M}^{-1} s^{-1} | 78.61 s^{-1} |
| 3        | 6.00 \times 10^{-11} \text{M}^{-1} s^{-1} | 3.00 \times 10^{-10} \text{M}^{-1} s^{-1} | -0.40 |
| 4        | 6.00 \times 10^{-11} \text{M}^{-1} s^{-1} | 7.00 \times 10^{-10} \text{M}^{-1} s^{-1} | 0.40 |
| 5        | 6.00 \times 10^{-11} \text{M}^{-1} s^{-1} | 7.00 \times 10^{-10} \text{M}^{-1} s^{-1} | 0.45 |
| 6        | 6.00 \times 10^{-11} \text{M}^{-1} s^{-1} | 3.00 \times 10^{-10} \text{M}^{-1} s^{-1} | 0.55 |
| 7        | 1.00 \times 10^{-10} \text{M}^{-1} s^{-1} | 1.00 \times 10^{-10} \text{M}^{-1} s^{-1} | 0.45 |
| 8        | 5.50 \times 10^{-10} s^{-1} | 5.00 \times 10^{-10} \text{M}^{-1} s^{-1} | 0.20 |
| 9        | 8.00 \times 10^{-10} s^{-1} | 4.00 \times 10^{-10} \text{M}^{-1} s^{-1} | 7.57 \times 10^{-4} s^{-1} |
| 10       | 6.00 \times 10^{-10} s^{-1} | 9.00 \times 10^{-10} \text{M}^{-1} s^{-1} | 4.64 \times 10^{-10} \text{M}^{-1} s^{-1} |
| 11       | 3.00 \times 10^{-10} s^{-1} | 4.00 \times 10^{-10} \text{M}^{-1} s^{-1} | 2.20 s^{-1} |
| 12       | 5.00 \times 10^{-10} s^{-1} | 2.00 \times 10^{-10} \text{M}^{-1} s^{-1} | 2.39 \times 10^{-10} \text{M}^{-1} s^{-1} |
| 13       | 4.00 \times 10^{-10} s^{-1} | 1.00 \times 10^{-10} \text{M}^{-1} s^{-1} | 0.20 |
| 14       | 1.00 \times 10^{-10} s^{-1} | 1.00 \times 10^{-10} \text{M}^{-1} s^{-1} | 7.8 M^{-1} s^{-1} |
| 15       | 50 s^{-1} | 5.00 s^{-1} | 0.40 |
| 16       | 8.00 \times 10^{-02} s^{-1} | 1.00 \times 10^{-02} \text{M}^{-1} s^{-1} | 0.40 |
| 17       | 0.80 s^{-1} | 1.00 \times 10^{-10} \text{M}^{-1} s^{-1} | 0.40 |

Patible data indicate that absolute open probabilities must have been incorrectly determined by noise analysis. Our novel data on the mechanistic basis of P259R induced transporter dysfunction provide a likely explanation for this overestimation of WT open probabilities. Very fast transitions between individual functional states of ion channels result in pseudo-equilibrium conditions between different branches of the kinetic scheme so that only channels that are in certain functional states are counted by noise analysis (28). Noise analysis therefore underestimates the number of open channels and overestimates absolute open probabilities. An example for such underestimation was recently shown to be the basis for the glutamate transport rates and thus the glutamate transport rate. These findings, together with earlier work on EAAT4 (7) and EAAT5 (29), illustrate how tightly high anion channel open probabilities and large anion current amplitudes are linked to slow glutamate transport.

Pro-259/Pro-290 are conserved in the whole family, and variation in this amino acid therefore does not account for isoform-specific differences in EAAT function. However, the functional properties of P259R/P290R hEAAT3/hEAAT1 closely resemble those of a prototypic low capacity transporter EAAT5. EAAT5 exhibits very small Na\textsuperscript{+} association rates, and slow Na\textsuperscript{+} association was recently shown to be the basis for the slow activation time course and low glutamate transport rates (29). Our results demonstrate that the high capacity glutamate transporters EAAT1 or EAAT3 can be converted into low capacity glutamate transporters functionally resembling EAAT5 by a single point mutation and that such a change can cause cell dysfunction and disease. These findings beautifully illustrate the importance of the evolutionary optimization of EAAT isoforms with distinct functional properties.

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