A point mutation associated with episodic ataxia 6 increases glutamate transporter anion currents

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Introduction

Episodic ataxia is a rare genetic condition characterized by paroxysmal cerebellar incoordination and additional neurological symptoms. Six forms of episodic ataxia can be distinguished based on differences in the clinical phenotype as well as on genetic information (Jen et al., 2007). Whereas episodic ataxia types 1, 2 and 5 are caused by mutations in genes encoding voltage-gated cation channels, mutations in SLC1A3, encoding a glial glutamate transporter in cerebellum, diencephalon and caudal brainstem (Banner...
The genetic basis of the disease type episodic ataxia type 6 (Jen et al., 2005) appears to be the genetic basis of the disease type episodic ataxia type 6 (Jen et al., 2005). The genetic basis of episodic ataxia type 3 and 4 has not been defined.

Episodic ataxia type 6 differs clinically from other episodic ataxia forms in long duration of attacks, epilepsy and absent myokymia, nystagmus and tinnitus. So far, it has only been reported in two families with two different disease-causing SLC1A3 missense mutations (Jen et al., 2005; de Vries et al., 2009). In the family with the more severe clinical symptoms, there was only one affected member who was heterozygous for a mutation predicting substitution of proline by arginine in the fifth transmembrane domain (TM5), P290R (Fig. 1A) (Jen et al., 2005). The effects of P290R on glutamate transport capability and on subcellular distribution have already been studied in heterologous expression systems. In this study, P290R was reported to reduce the surface density of excitatory amino acid transporter 1 (EAAT1) transporters in heterologous expression systems, and this change in subcellular distribution was suggested to be the basis of reduced cellular glutamate uptake capability and clinical episodic ataxia type 6 symptoms (Jen et al., 2005). However, the clinical phenotype of this particular patient with episodic ataxia type 6 is different from the neurological symptoms observed in animal models lacking EAAT1 (Watase et al., 1998) suggesting that P290R might not only reduce the glutamate transport, but also affect yet to be defined other functions of EAAT1. EAATs do not only mediate secondary-active glutamate transport, but also function as anion transporters 

**Materials and methods**

**Heterologous expression of wild-type and mutant excitatory amino acid transporters**

The coding region of human EAAT1 (kindly provided by Dr S. Amara, University of Pittsburgh) was sub-cloned into pcDNA3.1 using PCR-based strategies. Point mutations as well as yellow fluorescent protein (YFP)- or mCherry-fusion proteins were generated by PCR-based techniques as described (Leinenweber et al., 2011). Transient transfection of HEK293T cells using the Ca3(PO4)2 technique or Lipofectamine™ (Invitrogen) was performed as previously described (Melzer et al., 2003). Oligoclonal cell lines stably expressing wild-type or mutant human EAAT1 were obtained by selection for resistance to the antibiotic Geneticin® (G418; Boehringer Mannheim) in HEK293 cells as previously described (Melzer et al., 2005).

**Electrophysiology**

Standard whole-cell patch clamp recordings were performed using EPC10 (HEKA) or Abimek WPC-100 (Abimek) amplifiers as described (Melzer et al., 2005; Kovermann et al., 2010). Cells were clamped to 0 mV for at least 5 s between test sweeps. Standard bath solution contained (in mM): 140 NaCl/NaNO3, 4 KCl, 2 CaCl2, 1 MgCl2, 5 HEPES, 5 tetrathylammonium chloride, pH 7.4, standard pipette solution 115 KCl/KNO3, 5 EGTA, 10 HEPES, pH 7.4 (Figs 1B,C and 2A, B and D). In some experiments, external NaCl was equimolarly substituted with NaSCN (Supplementary Figs 2, 3 and 5). To determine human EAAT1 currents at an almost physiological chloride gradient, [Cl]outside was reduced to 44 mM by equimolar substitution of Cl− by gluconate− (Supplementary Fig. 2). In experiments with variable [SCN−] gradients (Supplementary Fig. 2), the bath solution contained 30/70/140 NaSCN, 110/70/0 NaGluconate, 1 MgCl2, 4 KCl, 2 CaCl2, 5 tetrathylammonium chloride, 10 HEPES, pH 7.4 and the pipette solution contained 10 NaSCN, 105 Na Gluconate, 1 MgCl2, 5 EGTA, 10 HEPES pH 7.4. Sodium dependencies were determined by equimolar substitution of external NaCl with CholineCl in the presence of 5 mM l-glutamate and a KNO3-based pipette solution. Junction potentials were corrected using the JPCalc software (Barry, 1994).

**Noise analysis**

Data were digitized at 50 kHz and filtered with a low-pass Butterworth filter with a cut-off frequency of 10 kHz. Power spectrum analysis was performed on the steady-state phase of 1 s long pulses to different potentials using Clampfit 10.0 (Molecular Devices) and fitted with a double Lorentzian function (Origin8, Microcal Inc.).
Figure 2 P290R increases human EAAT1 anion currents. (A and B) Current-voltage relationships from HEK293T cells transiently expressing wild-type (WT) or P290R human EAAT1 in the absence (A) or in the presence (B) of 0.5 mM L-glutamate \((n > 8)\). Cells were dialysed with internal KCl-based solution and perfused with NaCl-based external solutions. (C) L-glutamate concentration dependence of wild-type and P290R human EAAT1 anion currents (at \(-125 \text{ mV}\)) in stably transfected HEK293 cells at \(-125 \text{ mV}\). Solid lines represent fits to Michaelis–Menten relationship with \(K_{\text{Ms}}\) in mM of 14.8 (WT), or 9.6 \((P = 0.25, n = 4/6)\). (D) Sodium concentration dependence of wild-type and P290R human EAAT1 anion currents (at \(-125 \text{ mV}\)) in stably transfected HEK293 cells in the presence of 5 mM L-glutamate. Solid lines represent fits to Michaelis–Menten relationship with \(K_{\text{Ms}}\) in mM of 14.8 \pm 1.8 \(^{\text{WT}}\), or 23.3 \pm 3 \((P290R)\). Data are given as means \pm SEM \((P < 0.01, n = 4/6)\).

\[
S = \frac{S(O_1)}{1 + (f/fc)^2_1} + \frac{S(O_2)}{1 + (f/fc)^2_2}, \tag{1}
\]

where \(S\) and \(f\) denote the spectral density and the frequency, \(S(O)\) and \(f_c\) are the amplitude and corner frequency of the corresponding Lorentzian term.

To determine single-channel amplitudes \((i)\) and absolute open probability \((p_o)\) we employed a variation of stationary variance analysis that was developed to study ion channels with voltage-dependent unitary conductances (Torres-Salazar and Fahlke, 2007). In case of Lorentzian noise, the current variance \((\sigma^2)\)-corrected for the background variance \((\sigma^2_0)\)-depends on the unitary current amplitude \(i\), the absolute open probability \(p_o\) and the number of channels \(N\).

\[
\frac{\sigma^2 - \sigma^2_0}{I_{ss}} = i \cdot (1 - p_o) = i - \frac{I_{ss}}{N} \tag{2}
\]

In many cases, the voltage dependence of unitary current amplitudes can be estimated from isochronal current amplitudes \((I_{iso})\) determined 1 ms after the voltage step. \(I_{iso}\) depends on the unitary current amplitude \(i\), the number of channels \(N\), and the isochronal open probability \(p_{iso}\).

\[
I_{iso} = N \cdot i \cdot p_{iso}. \tag{3}
\]

If the absolute open probability does not change during the voltage step, \(p_{iso}\) is defined by the holding potentials and will be the same for all voltage steps. In this case, the isochronal current amplitude is proportional to the unitary current amplitude \((I_{iso} = const \cdot i)\) so that dividing both sides of the equation by the isochronal current amplitude results in:

\[
\frac{\sigma^2 - \sigma^2_0}{I_{iso}} = i - \frac{I_{iso}}{N} \tag{4}
\]

We plotted the variance divided by the product of isochronal and late current amplitudes \((\sigma^2/I_{iso} \cdot I_{iso})\) against the ratio of the mean late current amplitude during the last 50 ms of the voltage step by the isochronal current \((I_{iso})\) amplitude measured 1 ms after the voltage step. A linear fit provides the number of channels as slope factor \(-1/N\), and single channel current amplitudes were calculated as product of the \(y\)-axis intercept and the isochronal current amplitudes \((I_{iso} \cdot \text{const}^{-1})\). The absolute open probability was determined from \(p_o = \frac{1}{\text{const}}\).

We additionally calculated unitary current amplitudes using the slope factor rather than the \(y\)-axis intercept, using

\[
i = \frac{\sigma^2 - \sigma^2_0}{I_{iso}} + \frac{I_{iso}}{N}. \tag{5}
\]

The two methods to calculate unitary current amplitudes yielded indistinguishable results.

This analysis is only possible within a voltage range in which the isochronal current amplitude \((I_{iso})\) is proportional to the unitary current amplitude \((i)\). A deviation from this proportionality is possible in case of very fast gating steps that change the number of open channels during the voltage step. This assumption was tested using noise analysis (Hebeisen et al., 2003; Melzer et al., 2005) (Supplementary material and Supplementary Fig. 6).
Radioactive uptakes

Uptake of radio labelled L-[^3H]-glutamate into stably transfected HEK293 cells was assessed in a buffer containing (in mM) 140 NaCl, 4 KCl, 2 CaCl₂, 1 MgCl₂, 5 HEPES, pH 7.4, with 0.5 mM L-glutamate and 6.3 nM L-[^3H]-glutamate at 20°C. Uptake was terminated by washing cells with ice cold Na⁺-free solutions and solubilizing in 0.1% SDS prior to scintillation counting. Each uptake was determined in triplicate for every incubation time. Transport rates were determined from linear fits to measured values from separate experiments and given as mean ± SEM with n ≥ 3. Mean L-[^3H]-glutamate background values obtained from untransfected cells were subtracted from wild-type as well as from P290R human EAAT1 data. Since human EAAT1 glutamate transport rates are voltage-dependent (Wadiche et al., 1995), we compared resting membrane potentials of HEK293 cells stably expressing wild-type or P290R human EAAT1 in the uptake buffer containing glutamate. No significant differences between the two data sets could be observed.

Flow cytometry analysis, confocal microscopy and biochemical characterization of excitatory amino acid transporter fusion proteins

Flow cytometry experiments were performed on HEK293T cells transiently expressing wild-type or P290R human EAAT1–YFP/mCherry using the MoFlo high-speed sorter (Beckmann-Coulter) equipped with an argon-laser (488/515 nm, Coherent Inc.). Geometric means (ξ) and median values (ηmed) were determined using Summit 4.1 (Dako Cytomation) for each set of transfections, assuming a threshold fluorescence intensity of 500 relative units. Confocal imaging was carried out on living cells with a TCS SP2 AOBS scan head and an inverted Leica DM IRB. YFP was excited at 514 nm and the emission was detected after filtering with a 520-580 nm band pass filter. Human EAAT1 fusion proteins were purified from HEK293T cells as previously described (Leinenweber et al., 2011). Surface expression of human EAAT1 was quantified with a modification of cell surface biotinylation method as described previously (Leinenweber et al., 2011).

Data analysis

Data were analysed with a combination of Clampfit (Molecular Devices), PatchMaster (HEKA Electronic), SigmaPlot (Jandel Scientific), Matlab (Mathworks) and Excel (Microsoft Corp.) programs. Current-voltage relationships at various [L-glut] were constructed by plotting macroscopic current amplitudes determined 75 ms (wild-type) or 150 ms (P290R) after the voltage step versus the membrane potential. All data are given as means ± SEM. For statistic evaluation the Student’s t-test with a level of significance of P < 0.05 was used.

Results

P290R increases human excitatory amino acid transporter-1-associated currents in heterologous expression systems

We expressed wild-type and P290R human EAAT1 transiently in mammalian cells and studied human EAAT1-mediated currents through whole-cell patch clamp. Cells were internally dialysed with K⁺-based solutions to permit all physiologically occurring transitions in the glutamate uptake cycle (Watzke et al., 2001). We used either Cl⁻ or NO₃⁻ as main anion in the internal and in the external solution. NO₃⁻ increases human EAAT1 anion currents (Wadiche and Kavanaugh, 1998) and was therefore used for the biophysical characterization of human EAAT1 anion currents (Melzer et al., 2003). Experiments with the physiological anion Cl⁻ provide insights into the effects of the mutation on human EAAT1 anion currents under native conditions.

Figure 1B and C show representative whole-cell current responses to voltage steps between −185 mV and +55 mV followed by a fixed step to −125 mV from HEK293T cells expressing wild-type (Fig. 1B) or P290R human EAAT1 (Fig. 1C) with symmetrical Cl⁻ as main internal and external anion. In the absence of L-glutamate, wild-type human EAAT1 anion currents were small, and application of 0.5 mM L-glutamate resulted in a ~2-fold increase of the current amplitude (Figs 1B–C and 2A–C). P290R causes dramatic alterations of the time and voltage dependence of human EAAT1 currents (Fig. 1C). Voltage steps to negative potentials resulted in prominent current activation that was much more pronounced in the presence of L-glutamate than in its absence. At positive potentials, currents were small and time-independent, without appreciable effect of L-glutamate. Untransfected cells exhibited negligible glutamate-independent currents (without L-glutamate, −22 ± 3 pA, with L-glutamate, −17.0 ± 1.6 pA, P = 0.25, n = 8/12) under these experimental conditions.

In the absence (Fig. 2A) as well as in the presence of external glutamate (Fig. 2B) whole-cell currents (Fig. 2) were significantly larger in HEK293T cells transiently expressing P290R human EAAT1 than in cells expressing wild-type human EAAT1 (at −125 mV without L-glutamate, wild-type: −51.3 ± 11.9 pA, P290R: −162.3 ± 51.3 pA, P < 0.02, n = 8/5; with L-glutamate, wild-type: −92.7 ± 19.5 pA, P290R: −250.7 ± 83.3 pA, P < 0.05, n = 8/5). We also compared current densities of transiently transfected HEK293T cells with similar outcome [without L-glutamate: −3.1 ± 0.9 pA/pF (wild-type), −8.9 ± 2.1 pA/pF (wild-type), P < 0.02, n = 8/5; with L-glutamate: 5.0 ± 1.0 pA/pF (wild-type), P290R: 12.9 ± 3.4 pA/pF (P290R), P = 0.02, n = 8/5] (Supplementary Fig. 1). P290R human EAAT1 whole-cell currents were not only increased in symmetrical Cl⁻, but also in other tested anion distributions, i.e. with internal KCl- and external NaSCN-based solution (Supplementary Fig. 2).

P290R increases human excitatory amino acid transporter-1 anion currents

Under our experimental conditions, wild-type human EAAT1 currents consist of two current components, one generated by electrogenic glutamate uptake and another one conducted by EAAT1-associated anion channels (Wadiche et al., 1995). The increased current amplitudes for P290R in the presence as well as in the absence of glutamate and the marked alterations in the time and voltage dependence of P290R human EAAT1 currents either suggest the upregulation of human EAAT1 anion...
currents or the occurrence of novel conductances with distinct selectivities. Experiments with reduced [Cl\(^-\)]\(_{\text{ext}}\) and standard [Cl\(^-\)]\(_{\text{ext}}\) resulted in decreased inward currents and a shift of the reversal potential to negative values (Supplementary Fig. 2A and B). Moreover, in ion substitution experiments varying [SCN\(^-\)]\(_{\text{ext}}\) as sole permeant anion, we observed current reversal potentials as predicted by the Nernst relation for wild-type and P290R human EAAT1 anion currents (Supplementary Fig. 2E). Experiments with P290R human EAAT1 using varying [Na\(^+\)]\(_{\text{ext}}\) and [H\(^+\)]\(_{\text{ext}}\) demonstrated that there is no upregulation of proton (Tzingounis et al., 1998) or cation (Melzer et al., 2005) currents (Supplementary Fig. 3). We conclude that anion currents are predominant under our experimental conditions and that P290R causes a gain-of-function of human EAAT1-associated anion current amplitudes.

**P290R modifies the substrate dependence of human excitatory amino acid transporter-1-associated anion currents**

Human EAAT anion currents are glutamate-dependent with glutamate dependences arising from a tight coupling of anion channel opening and the glutamate uptake cycle in which certain states are associated with open anion channels and others with closed ones (Wadiche and Kavanaugh, 1998; Watzke et al., 2001; Bergles et al., 2002; Machtens et al., 2011b). To test whether P290R affects the substrate dependence of human EAAT1 anion currents we determined anion current amplitudes at various external glutamate concentrations (Fig. 2C) or at various sodium concentrations (Fig. 2D) in the presence of 5 mM L-glutamate. Due to the very small wild-type current amplitudes in the absence of L-glutamate, we were unable to study Na\(^+\) association to glutamate-free transporters. We determined all concentration dependences at –125 mV since this voltage is close to physiological membrane potentials but nevertheless negative enough to result in measurable current amplitudes.

Fitting these concentration dependences to Michaelis–Menten relationships provided \(K_{M\text{S}}\) for L-glutamate that were similar for wild-type (12.9 ± 1.6 μM, \(n = 4\)) and P290R (9.6 ± 1.2 μM, \(n = 6\), \(P = 0.25\)). However, we observed an increase of the relative glutamate-independent current amplitude from 33.0 ± 2% (wild-type human EAAT1) to 48.2 ± 3.4% (P290R human EAAT1) (\(P < 0.01\), \(n = 8/9\), Fig. 2C). In the presence of 5 mM L-glutamate, \(K_{M\text{S}}\) for Na\(^+\) were determined to be 14.8 ± 1.8 mM for wild-type and 23.3 ± 3 mM for P290R human EAAT1 (\(P < 0.01\), \(n = 4/6\), Fig. 2D). We repeated these experiments for various potentials between –105 and –145 mV and apparent Na\(^+\) and L-glutamate dissociation constants for wild-type as well as for mutant human EAAT1 appeared to be voltage-independent within this voltage range (Supplementary Fig. 4). We conclude that P290R modifies the substrate dependence of human EAAT1 anion currents.

P290R leaves unitary current amplitudes unaffected, but changes voltage dependences of open probabilities of human excitatory amino acid transporter-1-associated anion channels

We next used noise analysis to study unitary current amplitudes \(I\) and absolute open probabilities \(p_i\) of wild-type and P290R human EAAT1 anion channels. We first determined frequency spectra from macroscopic anion currents for wild-type and P290R human EAAT1. In both cases, power spectra from macroscopic anion currents could be well fit with a sum of two Lorentzian components with corner frequencies \((f_c)\) in accordance with the relaxation time constants of macroscopic currents (WT\(_{1/2/1/2} = 1.5/19.8\) Hz; P290R\(_{1/2/1/2}\) = 1.1/15.5 Hz) (Supplementary Fig. 5). Untransfected HEK293T cells showed lower current variances and power spectra resembling 1/f noise under the same conditions (Supplementary Fig. 5). Secondary-active transport does not contribute to the current variance, and noise analysis thus exclusively provides information about anion channels (Machtens et al., 2011a). These results demonstrate that human EAAT1 anion current-associated noise arises from the random opening and closing of individual channels and can be thus used to characterize the underlying unitary current events (Melzer et al., 2003; Torres-Salazar and Fahlke, 2007; Kovermann et al., 2010).

To determine unitary current amplitudes \(I\) and absolute open probabilities \(p_i\) by noise analysis we employed stationary noise analysis (Sigworth and Zhou, 1992; Sesti and Goldstein, 1998; García-Olivares et al., 2008; Machtens et al., 2011a). In these experiments, NO\(_3\) was used as main permeant anion on both membrane sides, a condition that increases EAAT1 anion current amplitudes (Wadiche and Kavanaugh, 1998) without affecting the time and voltage dependences of wild-type and P290R human EAAT1 currents (Figs 1 and 3). P290R human EAAT1 anion currents are inwardly rectifying, raising the question whether unitary current conductances are voltage-dependent or whether rapid gating processes modify the number of open anion channels during the voltage step. To distinguish between these two possibilities we plotted isochronal standard deviations versus the isochronal current amplitude determined at test potentials between –150 and +30 mV (Supplementary material and Supplementary Fig. 6). For such a plot, the isochronal standard deviation depends on the isochronal current amplitude \(I_{iso}\) and the isochronal open probability \(p_{iso}(V)\) (Hebeisen et al., 2003; Melzer et al., 2005).

\[
SD_{iso}(V) = \sqrt{\sigma_2^2 - \sigma_1^2} = I_{iso}(V) \left[ \frac{1}{N} \left( \frac{1}{p_{iso}(V)} - 1 \right) \right]^{1/2}
\]

(6)

For wild-type as well as for P290R human EAAT1 linear relationships between these two parameters were observed (\(R^2_{WT} = 0.997/0.992\); \(R^2_{P290R} = 0.9946/0.9433\)). In such a plot, the slope of the linear regression depends on the absolute open probability (Supplementary material), indicating that the \(p_{iso}(V)\) is constant in the voltage range. This analysis demonstrates that unitary current amplitudes are proportional to isochronal current...
amplitudes and can be expressed as factor of the isochronal current amplitude \( I_{\text{iso}} \) and a scaling factor \( i = \text{const} \cdot I_{\text{iso}} \). Since different slopes were determined at negative or at positive potentials, we had to restrict the subsequent analysis to negative voltages.

We next plotted the ratio of the stationary current variance \( \sigma_{I_{\text{ss}}}^2 \) by the product of the steady-state current amplitudes and the instantaneous current amplitudes \( \sigma_{I_{\text{ss}}}^2 \) (Fig. 3B). Fitting a straight line to experimental data provided the number \( N \) of active human EAAT1 anion channels as inverse slope factor \( -1/N \) and the scaling factor \( \text{const}^{-1} \) as y-axis intercept (Fig. 3B). These two values were then used to calculate unitary current amplitudes and absolute open probabilities from measured noise and current amplitudes (Fig. 3C and D).

P290R did not affect the mean unitary current amplitudes of human EAAT1 anion channels (at \(-150 mV\): wild-type: \(-173 \pm 38 fA\); P290R: \(-195 \pm 19 fA\), \(P = 0.7\), \(n = 6/4\)). However, we observed changes in the voltage dependence of the absolute open probability \( p_o \). At \(-150 mV\) the absolute open probability of wild-type human EAAT1 reaches a maximum at \(0.73 \pm 6.2\) (\(n = 4\)) P290R causes pronounced changes in the voltage dependence of human EAAT1 anion channel opening. Absolute open probabilities of P290R human EAAT1 reach maximum values at negative potentials (at \(-150 mV\), \(p_o = 0.96 \pm 0.02\), \(P < 0.01\), \(n = 6/4\)) and decrease upon depolarization (at \(-20 mV\), \(p_o = 0.35 \pm 0.05\)). P290R thus increases \( p_o \) at negative voltage and decreases \( p_o \) at positive voltages. We conclude that P290R do not cause major alterations of the EAAT1 anion conduction pathway, but changes the voltage dependence of anion channel opening.

**P290R decreases the number of human excitatory amino acid transporter-1 in the surface membrane**

P290R was reported to decrease the surface membrane density of EAAT1 by confocal analyses and surface biotinylation experiments. Confocal images show almost exclusive insertion of wild-type human EAAT1–YFP into surface membrane or in domains in close proximity. Experiments with cells expressing P290R human EAAT1–YFP demonstrated lower expression levels of fluorescent fusion proteins and additional staining of intracellular compartments (Fig. 4C). Quantification of the membrane insertion of P290R human EAAT1 by surface biotinylation revealed a P290R-induced reduction of surface membrane-inserted human EAAT1 to 65 \(\pm\) 10\% \((P < 0.01\), \(n = 3\)) (Fig. 4D and E). The results from flow cytometry and surface biotinylation taken together predict a P290R-mediated reduction of the surface membrane density of human EAAT1 to <50\%. P290R thus increases macroscopic anion currents despite a reduced number of mutant transporters in the surface membrane.

**P290R reduces the glutamate transport capability of human excitatory amino acid transporter**

Modification of macroscopic glutamate uptake rates by P290R has already been studied (Jen et al., 2005), but information about individual transport rates is still missing. We generated cell lines stably expressing wild-type or P290R human EAAT1 and quantified glutamate transport rates by measuring L-[\(^{3}\)H]-glutamate...
Figure 4 P290R decreases human EAAT1 transporter density in the surface membrane and glutamate uptake rates. (A) Single parameter histograms showing the fluorescence intensities of transiently transfected HEK293T cells (wild-type: wild-type, solid line, n = 4004, P290R: light grey, n = 4043). (B) Mean values of geometric means and medians for untransfected cells, wild-type, and mutant fluorescence intensities. Results obtained from single transfections are given for human EAAT1–YFP (open circles) and human EAAT1-mCherry (crossed boxes) after normalization to wild-type data. (C) Confocal images of living HEK293T cells transiently expressing wild-type or mutant human EAAT1. (D) Fluorescent scan of a 10% SDS–PAGE of cleared lysate and purified biotinylated surface proteins from HEK293T cells transiently expressing wild-type and P290R human EAAT1–YFP (arrowhead indicates 75 kDa position of mass marker). (E) Membrane surface insertion of wild-type and mutant human EAAT1 was quantified by dividing the fluorescence of surface-biotinylated EAAT1–YFP by the fluorescence of the total cleared lysate and normalized to the ratio found in the presence of wild-type human EAAT1. (F) Glutamate accumulation in HEK293 cells stably expressing wild-type or P290R human EAAT1 within the first 3 min. Solid lines give linear fits to experimental data. (G) Initial uptake velocities for cells stably expressing wild-type or P290R human EAAT1 normalized to wild-type human EAAT1. Data are given as means ± SEM (n ≥ 3). Asterisks indicate levels of significance (**P < 0.01, ***P < 0.001).
accumulation in these cells. Clonal cell lines are expected to exhibit a uniform subcellular distribution that allows calculation of the percentage of transporters in the surface membrane using biochemical approaches. Cells expressing wild-type human EAAT1 accumulate L-glutamate to levels that increase nearly linear within the first minutes after starting the reaction (Fig. 4F). During this time period, the velocity of glutamate accumulation exclusively depends on the transport rate of individual transporters and their number in the surface membrane. P290R glutamate uptake is reduced to values only slightly above background levels ($P < 0.001$, Fig. 4F and G). In the stable cell line used for glutamate uptake measurements, the number of biotinylated P290R human EAAT1 proteins averaged ~65% of the corresponding value in the wild-type cell line. Changes in the number of transporters in the surface membrane thus do not explain the negligible transport rates in clonal P290R human EAAT1 cell lines. We conclude that P290R reduces individual human EAAT1 glutamate transport rates.

**Discussion**

We evaluated the functional consequences of a SLC1A3 missense mutation that was found in a patient suffering from a severe form of episodic ataxia and that predicts the substitution of proline by arginine at position 290 (Jen et al., 2005). EAATs are secondary-active transporters and glutamate-activated anion channels, and the disease-associated mutation was found to exert distinct effects on both functions. P290R augments the amplitude of human EAAT1 anion currents about 2- to 4-fold and alters their voltage and substrate dependence (Figs 1 and 2, Supplementary Figs 1 and 2). Noise analysis revealed unaltered unitary channel conductances (Fig. 3C), and the higher macroscopic current amplitudes in cells expressing P290R human EAAT1 must thus be due to increased open probabilities of mutant human EAAT1 anion channels. Since openings and closings of EAAT anion channels are tightly coupled to transitions within the uptake cycle (Bergles et al., 2002; Tao et al., 2006; Machens et al., 2011a), altered voltage- and substrate-dependent gating of human EAAT1 anion channels indicates changes in the glutamate uptake cycle by P290R. Indeed, we observed a P290R-mediated reduction of glutamate uptake rates to values only slightly above background (Fig. 4), whereas the surface membrane density of mutant human EAAT1 was ~65% in the cell line used to quantify glutamate uptake. The distinct degree in reduction demonstrates reduced unitary glutamate transport rates for P290R human EAAT1. We conclude that P290R modifies the subcellular distribution, reduces glutamate transport, but increases anion currents by human EAAT1.

EAAT anion current amplitudes are small in homologous as well as in heterologous expression systems. To study such currents we and others normally use unphysiological anions with higher permeability. However, anion currents in cells expressing P290R human EAAT1 are sufficiently large to be also observed at symmetrical Cl$^{-}$/C0$^-$ (Figs 1, 2 and Supplementary Fig. 1) or even with a physiological internal [Cl$^-$] (Supplementary Fig. 2). These results provide direct evidence that P290R will increase human EAAT1 anion currents also under native ionic conditions in Bergmann glia cells of affected individuals.

Our finding that P290R increases anion currents in addition to reducing glutamate transport provides a possible explanation for the differences in neurological symptoms between a mouse model with disruption of EAAT1 (GLAST) and the affected patient (Jen et al., 2005). Homozygous GLAST knock-out mice only suffer in challenging coordination tasks, but do not experience major alterations in intention movements (Watase et al., 1998). Heterozygous GLAST knock-out mice are without phenotype. The mild phenotype can be explained by the existence of additional EAAT glutamate transporters in Bergmann glia, and indeed glutamate uptake capability of the cerebellum in homozygous GLAST knock-out mice is only reduced by half (Watase et al., 1998). The clinical phenotype of the patient carrying the P290R mutation is also severe, with episodes of ataxia, headache and hemiplegia as additional symptoms. Based on the results of this study, we propose that these symptoms originate from the P290R-mediated gain-of-function of anion channels. The only known patient is heterozygous for the mutation, and gain-of-function of EAAT1 anion currents also provides an explanation for the severe symptoms in the presence of one mutant and one wild-type allele. In the heterozygous patient with episodic ataxia type 6, only a fraction of EAAT1 transporters carries the disease-causing mutation, but this fraction of mutant transporters conducts significantly more anion current. Gain-of-function accounts for clinical symptoms in heterozygous patients in several inherited diseases with channel/transporter dysfunction (Weber et al., 2008; Jurkat-Rott et al., 2010).

All EAATs exhibit an anion conductance that is small in the absence of glutamate and stimulated by transport substrates (Wadiche et al., 1995). For some EAAT isoforms, electrogenic uptake currents exceed anion currents by far, while in other isoforms, anion currents represent the predominant transporter-mediated current component (Fairman et al., 1995; Wadiche et al., 1995; Watzke and Grewer, 2001; Melzer et al., 2003). The functional effects of the disease-causing mutation P290R suggest that increased anion currents might result in pathological conditions demonstrating the importance of only small anion current amplitudes of certain EAATs.

At present, we can only speculate about the consequence of the increased human EAAT1 anion currents on cerebellar function. P290R increases human EAAT1 anion currents in the absence as well in the presence of external glutamate (Figs 1 and 2) and thus will increase the resting anion conductance of Bergmann glia cells. Such cells exhibit large potassium and only negligible anion conductances (Walz, 2002), and it thus appears unlikely that changes in EAAT1 anion current amplitude could modify electrical parameters of this type of astrocytes. However, increased anion conductance might modify volume regulation of astrocytes and partially account for the observed oedema in the patient (Ernest et al., 2005; Jen et al., 2005; Habela et al., 2009). Moreover, it might affect glial [Cl$^-$]$_{int}$. The small anion conductance of normal astrocytes permits active accumulation of glial [Cl$^-$]$_{int}$ by Na$^+$-K$^+$-$2Cl^-$ co-transporters, resulting in glial [Cl$^-$]$_{int}$ between 22 and 55 mM (Bekar and Walz, 2002). Increased resting EAAT1 anion currents might permit chloride effluxes that exceed Na$^+$-K$^+$-$2Cl^-$ transport.
rates and thus reduce glial [Cl\(^-\)]\(_{int}\) resulting in multiple variations of synaptic transmission in the cerebellum.

Since high [Cl\(^-\)]\(_{int}\) in astrocytes and oligodendrocytes causes gamma-aminobutyric acid (GABA)\(_A\)-mediated excitation (Kettenmann et al., 1984; MacVicar et al., 1989; Meier et al., 2008), human EAAT1 anion channel gain-of-function might impair GABAergic astrocyte excitation. Recently, astrocytes in the mouse barrel cortex were shown to inhibit nearby neurons and thus to directly affect neuronal signalling (Benedetti et al., 2011). Such interaction is likely to take place in the cerebellum. Moreover, Bergmann glial cells express a variety of secondary-active GABA transporters that control [GABA\(^-\)]\(_{ext}\) (Beenhakker and Huguenard, 2010) by transporting one GABA\(^-\) stoichiometrically coupled to two Na\(^+\) and one Cl\(^-\). Reduced astrocytic [Cl\(^-\)]\(_{int}\) will increase the driving force for GABA uptake and decrease [GABA\(^-\)]\(_{ext}\). Gain-of-function of EAAT1 anion currents may thus attenuate GABA\(_A\)- and GABA\(_B\)-mediated synaptic transmission by stimulation of glial GABA reuptake.

Our data thus suggest that the P290R mutation impairs GABAergic synaptic activity in the cerebellum, in addition to stimulating glutamatergic transmission by reduced glutamate uptake (Jen et al., 2007). Since correct cerebellar function is based on the interplay of excitatory and inhibitory synapses, this pathomechanism fully accounts for the cerebellar symptoms of the patient carrying the P290R mutation.

Acknowledgements

The authors would like to thank Dr Susan Amara for providing an expression construct for human EAAT1, Drs Alexi Alekov, David Ewers, Martin Fischer, Jasmin Hotzy, Ariane Leinenweber, Jan-Philipp Machtens, Nicole Schneider and Gabriel Stölting for helpful discussions, and Petra Kilian, Toni Becher, Birgit Begemann and Silke Schmidt for excellent technical assistance.

Funding

These studies were supported by the Deutsche Forschungsgemeinschaft (FA301/9 to Ch. F.). The authors would also like to acknowledge the assistance of the Department of Nuclear Medicine, the Confocal Core Facility and the Cell Sorting Core Facility of the Medizinische Hochschule supported in part by Braukmann-Wittenberg-Herz-Stiftung and Deutsche Forschungsgemeinschaft.

Supplementary material

Supplementary material is available at *Brain* online.

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