Exon-skipping Splice Variants of Excitatory Amino Acid Transporter-2 (EAAT2) Form Heteromeric Complexes with Full-length EAAT2*

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The glial transporter excitatory amino acid transporter-2 (EAAT2) is the main mediator of glutamate clearance in brain. The wild-type transporter (EAAT2wt) forms trimeric membrane complexes in which each protomer functions autonomously. Several EAAT2 variants are found in control and Alzheimer-diseased human brains; their expression increases with pathological severity. These variants might alter EAAT2wt-mediated transport by abrogating membrane trafficking, or by changing the configuration or functionality of the assembled transporter complex. HEK293 cells were transfected with EAAT2wt; EAAT2b, a C-terminal variant; or either of two exon-skipping variants: alone or in combination. Surface biotinylation studies showed that only the exon-7 deletion variant was not trafficked to the membrane when transfected alone, and that all variants could reach the membrane when co-transfected with EAAT2wt. Fluorescence resonance energy transfer (FRET) studies showed that co-transfected EAAT2wt and EAAT2 splice variants were expressed in close proximity. Glutamate transporter function was measured using a whole cell patch clamp technique, or by changes in membrane potential indexed by a voltage-sensitive fluorescent dye (FMP assay): the two methods gave comparable results. Cells transfected with EAAT2wt or EAAT2b showed glutamate-dependent membrane potential changes consistent with functional expression. Cells transfected with EAAT2 exon-skipping variants alone gave no response to glutamate. Co-transfection of EAAT2wt (or EAAT2b) and splice variants in various ratios significantly raised glutamate EC50 and decreased Hill coefficients. We conclude that exon-skipping variants form heteromeric complexes with EAAT2wt or EAAT2b that traffic to the membrane but show reduced glutamate-dependent activity. This could allow glutamate to accumulate extracellularly and promote excitotoxicity.

Excitatory amino acid transporters (EAATs)6 are high-affinity, sodium-dependent glutamate carriers from solute carrier family 1 (SLC1). Five EAATs have been cloned from animal and human tissue. EAAT1 (SLC1A3; GLAST in rodents) and EAAT2 (SLC1A2; GLT1) are primarily expressed in astroglia (1). Overall, EAAT2 is responsible for most of the glutamate transport in the adult brain (1, 2).

The amino acid sequence of an aspartate transporter from Pyrococcus horikoshii (GltPp) is 36% homologous to EAAT2; many residues implicated in glutamate binding and transport are highly conserved (up to 90%) between all EAATs (3, 4). The crystal structure of GltPp is a trimer in which each protomer comprises eight α-helical transmembrane domains and two helical hairpins (5). It has been proposed that transmembrane domains, TM3, -6, -7, and -8, together with the two helical hairpins, HP1 and HP2, are essential for substrate translocation from the extracellular side into the cell. The first transmembrane segments, TM1, -2, -4, and -5, form the trimerization domain and provide stability to counterbalance the movements of the transport domains (3, 6, 7).

In addition to EAAT2wt, several post-transcriptionally regulated forms have been identified. Alternative splicing of 5’ untranslated regions produces four different potential N termini (8, 9). Additionally, two 3’ splice variants with alternative C termini are known (9, 10). One of these, GLT1b, was first described in liver and brain from mice. When expressed in Xenopus laevis oocytes it showed similar glutamate concentration dependence profiles to GLT-1 (EC50 32 μM). EAAT2b is only expressed in brain astrocytes, not in neurons, although some neuronal expression occurs in retina (11). Holmseth et al. (12) found both GLT1 and GLT1b mainly in astroglia and did not detect GLT1b in nerve terminals. They showed that overall GLT1b expression was much lower than GLT1 expression in the hippocampus.

Besides diverse C and N termini, exon-skipping splice variants of EAAT2 have been described in brain tissue samples (Table 1): exon 7 deletion (EAAT2Δ7; Ref. 13), exon 8 deletion (EAAT2Δ8; Ref. 14), exon 9 deletion (EAAT2Δ9; Ref. 15), and

6 The abbreviations used are: EAAT, excitatory amino acid transporter; CFP, cerulean fluorescent protein; YFP, yellow fluorescent protein; FMP, fluorescence membrane potential; ER, endoplasmic reticulum; TM, transmembrane.
EAAT2δ7 lacks part of the inter-protomer binding site and parts of the glutamate translocation site. EAAT2δ9 lacks parts of the glutamate translocation site. Expression of EAAT2δ7 or EAAT2δ9 (alone) in X. laevis oocytes or HEK293 cells generates protein that is expressed at the cell surface, but in both cases the proteins were non-functional (16). A splice variant of EAAT1, EAAT1δ9, has also been described. It lacks the region homologous to that missing in EAAT2δ9; expression of EAAT1δ9 alone in HEK293 cells generates protein that is also unable to transport glutamate. HEK293 cells co-transfected with equal amounts of EAAT1wt and EAAT1δ9 showed 44% lower glutamate uptake than cells transfected with EAAT1wt alone (17).

Because there are many EAAT2 splice variants, and a tri-meric complex appears to be required for the transporter to be functional, the question may be raised whether variants form homomeric or heteromeric structures. Fluorescence resonance energy transfer (FRET) experiments have shown that N- and C-terminal variants of GLUT1 can associate with each other, in both homomers and heteromers, but will not associate with EAAT1 (18, 19). It is not known whether EAAT2δ7 or EAAT2δ9 are able to form complexes. Here we show that EAAT2 exon splice variants can form heteromeric complexes when co-transfected with either EAAT2wt or EAAT2b and that glutamate-dependent voltage changes are contingent on the ratio of splice variant to EAAT2wt or EAAT2b.

### Experimental Procedures

**Plasmid Construction**—Plasmids encoding EAAT2 and each variant were inserted into pcDNA 3.1 mammalian expression vectors (Invitrogen). EAAT2wt and the various splice variants were tagged with cerulean fluorescent protein (CFP) or yellow fluorescent protein (YFP) at either the carboxyl or amino terminus. For the carboxyl-terminal tags, the stop codons of EAAT2wt-pcDNA3.1 and each EAAT2variant-pcDNA3.1 cDNA were removed by site-directed mutagenesis (QuickChange Site-directed Mutagenesis Kit, Stratagene; Agilent Technologies, Santa Clara, CA). XbaI restriction enzyme sites were introduced by mutagenesis at the 5’ or 3’ termini of both CFP and YFP cDNA. The EAAT2wt-pcDNA3.1 and EAAT2variant-pcDNA3.1 cDNAs were linearized with XbaI (Promega) and ligated with either CFP or YFP cDNA using a Fast-LinkTM DNA Ligation Kit (Epicenter Biotechnologies, Madison, WI). For amino-terminal tags, KpnI restriction enzyme sites were introduced at the 5’ or 3’ termini of CFP and YFP and ligated with KpnI-linearized EAAT2wt-pcDNA3.1 or EAAT2variant-pcDNA3.1 cDNA using a Fast-Link Kit (Epicenter).

**Transfection of HEK293 Cells**—For Western blot analysis and imaging, 5 × 10⁵ cells/well were aliquoted in 6-well plates. After 24 h they were transfected with 0.25–5 μg of plasmid encoding EAAT2wt or EAAT2 splice variants using a calcium phosphate precipitation protocol (20). For electrophysiology, 1 × 10⁶ cells were plated on coverslips in single well dishes and co-transfected with 0.25–5 μg of plasmid encoding EAAT2wt or EAAT2 variants and 0.25 μg of eGFP-IRES (Stratagene). 12 h after transfection cells were washed twice with PBS and maintained in 2 ml of DMEM, 10% Serum Supreme (Thermo Fisher Scientific), and used for electrophysiology studies 24 h after transfection.

For FRET experiments, HEK293 cells were plated on 24-well glass-bottom culture plates coated with poly-d-lysine (MatTek, Ashland, MA) at 5 × 10⁵ cells/well. Cells were transfected at a 1:2 ratio of cDNA encoding EAAT2 tagged with CFP (0.1 μg of plasmid DNA) and EAAT2 tagged with YFP (0.2 μg of plasmid DNA). Control experiments were conducted on cells transfected with 0.1 μg of CFP-tagged DNA and 0.2 μg of pcDNA3.1 vector DNA. A total of 0.3 μg of DNA was transfected with Fugene HD (Promega) according to the manufacturer’s instructions in serum-free DMEM (Invitrogen). Medium was replaced with DMEM containing 10% fetal bovine serum at 48 h. The cells were allowed to grow for a further 24–48 h before imaging. Prior to imaging DMEM was aspirated and cells were washed twice with Leibovitz’s L-15 medium (Invitrogen).

**Total Cell Protein Isolation**—Transfected cells were washed twice with PBS, scraped off into 1 ml of lysis buffer (1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM β-glycerophosphate, 1 mM Na₂VO₄, 2.5 mM Na₃P₂O₇, 7 mM urea, 2 mM thiourea, and Complete Mini Protease Inhibitors (Roche Diagnostics) in 20 mM Tris-HCl, pH 8.0), incubated for 15 min on ice, and dispersed with an ultrasonic pulse for 20 s. The homogenate was centrifuged for 10 min at 12,000 × g at 4 °C to remove cellular debris. Protein concentration in the supernatants was determined by the Bradford (21) method. Samples were stored at −80 °C until use.

**Cell Surface Protein Isolation**—Cell surface expression was performed with the Pierce Cell Surface protein isolation kit (Thermo Fisher) according to the manufacturer’s protocol. In brief, transfected HEK293 cells were grown in T75 flasks to 80% confluence, rinsed once with ice-cold PBS, then incubated with Sulfo-NHS-SS-Biotin for 1 h at 4 °C on a rocking platform. The biotinylation reaction was quenched to stop the labeling. Cells were washed twice with TBS and lysed on ice by ultrasonic homogenization pulses for 3 × 10 s. Cellular debris was deposited by centrifugation at 10,000 × g for 2 min at 4 °C. Labeled proteins were bound to an immobilized NeutrAvidin Gel. Proteins were eluted in SDS sample buffer (5% [v/v] glycerol, 1.7% [w/v] SDS, 2.5% [w/v] β-mercaptoethanol, 0.2% [w/v] bromophenol blue in 58 mM Tris, pH 8.5).

**Total Tissue Protein Preparation**—For Western blotting, total protein samples of human cortex were prepared as follows. Pieces were excised from frozen tissue blocks on a freezing stage, thawed in 0.32 M sucrose solution held at 37 °C, immediately transferred to a tared beaker of ice-cold 0.32 M sucrose, and weighted. Each tissue piece was transferred to 10 volumes of ice-cold water and dispersed using a motor-driven

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**Functional Expression of EAAT2 Splice Variants**

| EAAT2 splice variant characteristics | Theoretical molecular mass | Length | Missing aa (cf. wt) |
|-------------------------------------|----------------------------|--------|-------------------|
|                                     | kDa | aa                  |       |
| EAAT2wt                             | 62.1 | 574                 |
| EAAT2b                              | 61.0 | 563                 |
| EAAT2Δ9                             | 53.6 | 496                 | 289–363 |
| EAAT2δ9                             | 57.7 | 529                 | 429–474 |

Transfected with equal amounts of EAAT1wt and EAAT1/H9004/H9001/H9001/H9019, and that other regions of EAAT2 including mutations 9 are able to form complexes. Here we show that EAAT2δ7, has also been described. It lacks the region homologous to that missing in EAAT2δ9; expression of EAAT1 alone in HEK293 cells generates protein that is also unable to transport glutamate. HEK293 cells co-transfected with equal amounts of EAAT1wt and EAAT1δ9 showed 44% lower glutamate uptake than cells transfected with EAAT1wt alone (17).

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Teflon-glass homogenizer. The homogenate was centrifuged at 1,000 × g for 5 min at 4 °C. Protein concentrations were measured using the Lowry (22) method. Aliquots (1 ml) were stored at −80 °C.

**Western Blotting**—Proteins were separated by SDS-PAGE on 9% acrylamide gels and transferred to Immobilon-FL membranes (Millipore, Billerica, MA). Membranes were blocked with either 1% BLOTTO/PBST or 0.5% fish skin gelatin/PBST for 1 h, then incubated overnight at 4 °C with primary antibodies at concentrations as designated below. EAAT2-specific antibody B493 kindly provided by Dr. N. C. Danbolt was raised against residues 493–508 of EAAT2wt. Rabbit anti-p44/42 MAPK (Erk1/2) antibody (Cell Signaling Technology, Danvers, MA) was used at a dilution of 1:1000. Monoclonal anti-GFP antibody (Sigma) was used at a concentration of 1 μg/ml. Secondary Alexa Fluor® 680 goat anti-rabbit antibody (Invitrogen) was used at a concentration of 0.1 μg/ml. Membranes were scanned using the LI-COR Odyssey® scanner with Odyssey software 3.0 (LI-COR Biosciences, Lincoln, NE).

**FRET Imaging**—This was performed using a Nikon Confocal Microscopy system (C1) equipped with a high-speed Lifetime Imaging Module consisting of a pulsed 440-nm laser and a high-speed gated detection system (Nikon Instruments, Melville, NY). To ensure that only the CFP signal is detected, a 470 ± 20-nm bandpass emission filter is placed in the detection pathway. Experiments were conducted at 37 °C on a heated stage. All images were taken using a ×60 water objective (Numerical aperture = 1.4). Images were collected at 256 × 256 pixel resolution and regions of interest at the cell surface were chosen that had photon counts greater than 500 photons/pixel for Fluorescence lifetime imaging microscopy analysis. Analysis was conducted using the Nikon C1 speed Lifetime Imaging Module software.

**Imaging**—Transfected cells were transferred to transparent 384-well plates at 4 × 10^4 cells/well. After 16–24 h the medium was aspirated and cells were washed with 25 μl of Ringer solution (140 mM NaCl, 5 mM KCl, 2 mM CaCl_2_, 1 mM MgCl_2_, 10 mM HEPES, 10 mM glucose, pH 7.4). A mixture of 10 μl of Ringer solution and 15 μl of blue fluorescence membrane potential (FMP) assay dye (Molecular Devices, Sunnyvale, CA) was added to each well and the plate incubated for 1 h at room temperature. Plates were placed onto a motorized stage (Prior ProScan II, Prior Scientific Instruments, Cambridge, UK) of an Olympus IX51 inverted microscope and cells were imaged using a ×10 objective (UPlanFln, NA 0.30, Olympus, Tokyo, Japan). Fluorescence was collected by a CCD camera (coolSNAP monochrome cf/OL, Olympus). Liquid handling was performed with an LC PAL autosampler (CTC Analytics, Zwingen, Switzerland) using a 100-ml syringe. A suite of labView 7.1 and 8.2.1 (National Instruments, Dublin, Ireland) software routines written in the laboratory were used for hardware control and image acquisition. Automated image analysis was performed with a modified version of DetecTiff® image analysis software (23).

Two pictures were acquired per well, the first in 25 μl of FMP assay solution and the second 24 s after the addition of 25 μl of substrate in Ringer solution. For concentration-response assessment, each substrate was assayed in triplicate. Each experiment was repeated independently at least three times on different days.

**Data Analysis**—Results were expressed as mean ± S.E. of three or more independent experiments. Glutamate concentration-response relationships were fitted with following equation:

\[
F = F_{basal} + \frac{F_{max}}{(1 + EC_{50}/S)}^{Hill\,Slope}
\]

(Eq. 1)

where \(F\) is the fluorescence corresponding to substrate concentration \(S\), \(F_{basal}\) is the initial (control) fluorescence value, \(F_{max}\) is the maximum fluorescence, and \(EC_{50}\) is the substrate concentration that elicits a half-maximal response. A similar equation was used to calculate the IC_{50} for inhibition. For electrophysiology, fluorescence \((F)\) was substituted by current \((I)\) in the formula above. All curves were fitted by non-linear regression using Prism version 5.0b for Macintosh (GraphPad Software, San Diego, CA). Statistical significance was determined by paired Student’s \(t\) tests, or by analysis of variance with appropriate post hoc tests where indicated.

**RESULTS**

**Expression of EAAT2 Splice Variants**—Western blot analysis was carried out using a pan-EAAT2 primary antibody on HEK293 cells expressing EAAT2wt and EAAT2 splice variants. It may be seen (Fig. 1) that EAAT2wt, EAAT2b, and each splice variant were translated into protein. Several immunoreactive bands were detected for EAAT2wt and EAAT2b, including a band at ~75 kDa and bands at higher molecular mass of ~100 and ~150 kDa. The latter bands correspond to homodimers and homotrimers. Samples from human cerebral cortex and EAAT2wt not only showed a 75-kDa band but also a band at ~55 kDa that may represent intracellular unglycosylated EAAT2wt. EAAT2Δ7 and EAAT2Δ9 showed a major band at ~50–58 kDa, but only EAAT2Δ9 showed bands at high molecular mass, which suggests that EAAT2Δ9 is able to form homotrimeric but that EAAT2Δ7 is unable to do so.

Expression analysis of EAAT2wt and splice variants at the surface of HEK293 cells was performed. Transfected cells were surface biotinylated and proteins were isolated by column purification. Samples were immunoblotted and proteins were detected with the same antibody used for total protein visualization. EAAT2wt, EAAT2b, and EAAT2Δ9 were all found to be expressed both intracellularly and at the cell surface (Fig.
Functional Expression of EAAT2 Splice Variants

FIGURE 1. Immunoblot of electrophoretically separated proteins. Membranes were incubated with a primary antibody directed against EAAT2wt amino acids 493–508 (0.1 μg/ml) or anti-GFP antibody (1:2000). Each sample contained 15 μg of total protein extract from transfected HEK293 cells (0.5 μg DNA/5×10⁶ cells) or 10 μg of protein from human brain tissue. Bands were detected using Alexa Fluor 680 goat anti-rabbit antibody (0.1 μg/ml) on the LI-COR Odyssey system. A, EAAT2 splice variants displayed immunoreactive bands in accordance with their theoretical Mₚ. Total protein extracts (T) showed expression of EAAT2wt, EAAT2b (black arrowheads), EAAT2Δ7, and EAAT2Δ9 (black arrows); high Mₚ bands represent multimeric structures (empty arrowheads). Note that high Mₚ bands were not observed for EAAT2Δ7. Cell-surface (S) and intracellular (I) expression analysis of EAAT2wt and EAAT2 splice variants was carried out. EAAT2wt, EAAT2b, and EAAT2Δ9 were detected at the cell surface (S). EAAT2Δ7 was only expressed on the cell surface when co-expressed with 1:10 of EAAT2wt (0.05 μg of DNA/5×10⁶ cells). Lane 1, M, marker; lane 2, EAAT2wt; lane 3, EAAT2b; lane 4, EAAT2Δ9; lane 5, EAAT2Δ7; lane 6, EAAT2wt:EAAT2Δ7 (1:10); lane 7, untransfected cells; lane 8, human cortex; lane 9, M, marker; lane 10, EAAT2wt:GFP (1:10); T, total protein preparation; S, cell-surface expression; I, intracellular expression. B, protein samples were labeled with anti-ERK1/2 antibody. Samples from cell-surface expression did not show any ERK1/2 expression, whereas samples form intracellular expression and total protein extracts showed ERK1/2 labeling. C, co-transfection of EAAT2wt with EAAT2Δ7 or Δ9 at increasing ratios (1:2, 1:5, and 1:10). Lane 1, M, marker; lane 2, EAAT2wt:EAAT2Δ7, 1:2; lane 3, EAAT2wt:EAAT2Δ7, 1:5; lane 4, EAAT2wt:EAAT2Δ7, 1:10; lane 5, EAAT2wt:EAAT2Δ9, 1:2; lane 6, EAAT2wt:EAAT2Δ9, 1:5; lane 7, EAAT2wt:EAAT2Δ9, 1:10.

In contrast, EAAT2Δ7 protein was not expressed at the cell surface when it was transfected alone. Co-expression of EAAT2Δ7 with EAAT2wt enabled the complex to be transported to the cell surface. To check on cytoplasmic protein contamination of the membrane fraction, HEK293 cells were co-transfected with GFP and EAAT2wt and cell-surface protein complexes. CFP and YFP cDNAs were subcloned at the 5’ and 3’ ends of EAAT2wt and the above mentioned splice variant cDNAs to generate C or N termini-tagged transporters. We measured the fluorescence lifetime of the donor fluorophore, CFP, in the presence and absence of the acceptor fluorophore, YFP, as an indicator of FRET. Fluorescence lifetime can only be reduced when the two fluorophores are in close proximity (24).

Baseline CFP lifetime values were determined from cells transfected with EAAT2wt or EAAT2 variants tagged with CFP only. Differences from baseline were calculated for cells co-transfected with CFP-tagged and YFP-tagged protomers. As a positive control, cells were transfected with a tandem YFP-CFP construct, which generated a maximal decrease in CFP lifetime (Fig. 2A). A negative control comprised CFP-tagged EAAT2wt co-transfected with YFP-tagged glycine transporter GLYT1. These transporters do not associate at the cell surface (25, 26). The CFP lifetime of tagged EAAT2wt was not altered significantly by the presence of the YFP-tagged GLYT1 (Fig. 2A).

Fluorescence signals from EAAT2wt or EAAT2b were only detected at the cell membrane. For EAAT2α7 and EAAT2Δ9 some intracellular fluorescence was seen (Fig. 2C). However, FRET measurements were only taken from regions located at the surface of cells.

In cells co-transfected with YFP-EAAT2wt and CFP-EAAT2wt there was a marked and significant reduction in CFP fluorescence lifetime that did not differ significantly from the tandem CFP-YFP positive control (Fig. 2A). The reduction did not differ significantly irrespective of whether both the fluorescent protein tags were located at either the C or N terminus of EAAT2wt, or if one tag was at the C terminus and other at the N terminus (Fig. 2A). YFP-EAAT2b co-expressed with CFP-EAAT2b gave a similar reduction in CFP fluorescence lifetime (Fig. 2A). Tests were not done on co-expressed EAAT2b constructs with fluorophore proteins at opposite ends (Fig. 2A). When EAAT2wt, tagged either with CFP or YFP, was co-expressed with EAAT2b carrying the alternate tag, CFP fluorescence lifetime did not differ significantly from the positive control (Fig. 2A). The reduction in CFP fluorescence lifetime was equally strong whether the tags were at the same or opposite termini of the amino acid sequence of either transporter (Fig. 2A). These observations confirm that EAAT2wt and EAAT2b can form both homomeric and heteromeric complexes.

Co-expression of tagged EAAT2wt with either EAAT2Δ7 or EAAT2Δ9 tagged with the complementary fluorophore protein significantly decreased CFP fluorescence lifetimes, which suggests that these transporter variant combinations can associate to form heteromeric complexes (Fig. 2A). Tags at the same terminus (N or C) of EAAT2wt and EAAT2Δ9 gave CFP fluorescence lifetime values that did not differ significantly from the positive control. With tags at opposite ter-
mini of EAAT2wt and EAAT2Δ9, the reductions in CFP lifetime were significantly less than those with the positive control, although still significantly different from baseline and from the negative control (Fig. 2A). That is, the location of the tags influenced the efficiency of FRET for EAAT2wt/EAAT2Δ9 combinations. Co-transfections of EAAT2wt and EAAT2Δ7 gave the smallest fluorescence lifetime changes.

Tags at the same ends of the protein (C terminus) generated changes in CFP fluorescence lifetimes that were not significantly different from baseline and significantly less than the positive control, while those at opposite ends (one N- and one C-terminal tag) showed significant increases over baseline. The statistical probabilities are Bonferroni-corrected for multiple testing.

**Figure 2.** A, formation of EAAT2wt homomers and heteromers with EAAT2 splice variants was evaluated with FRET. Decrease in CFP lifetime, when positioned in close proximity to YFP, was measured and normalized to basal CFP lifetime levels in the absence of YFP (ΔCFP lifetime). Basal CFP lifetime is an average of all EAAT2s variants tagged with CFP in the absence of YFP. As a positive control for increased CFP lifetime a tandem CFP-YFP construct was expressed in HEK293 cells, giving maximal CFP lifetime increases (C, upper left). As a negative control, EAAT2wt was co-expressed with glycine transporter 1 (GlyT1). EAAT2wt homomers, EAAT2wt:EAAT2b, EAAT2wt:EAAT2Δ7, and EAAT2wt:EAAT2Δ9 heteromers are divided in two sets. First, the combinations where the tags were on the same (S) end of each EAAT2 variant (either both N-terminal or both C-terminal) were combined. Second, combinations where the tags were at opposite (O) ends, one C- and one N-terminal tag, were pooled. *, p < 0.01 cf basal CFP lifetime; †, p < 0.01 cf. basal CFP lifetime and positive control; ‡, p < 0.01, cf. positive control (analysis of variance with Bonferroni-corrected pairwise comparisons). B, representations of EAAT2 in 3 (upper panel) and 2 (lower panel) dimensions showing the positions of exons 7 (black) and 9 (red). The three-dimensional representation of EAAT2 is based on the structure of GltPh, with the regions corresponding to exons 7 and 9 of EAAT2 highlighted. Also shown are the positions of the proposed transmembrane (TM) and hairpin (HP) segments. Structures are based on Ref. 5. Representative cells from which CFP fluorescence lifetime measures were made. Cells expressing the following combinations are presented: CFP + YFP (positive control; upper left), CFP-EAAT2wt + YFP-EAAT2Δ9 (upper right), and CFP-EAAT2wt + YFP-EAAT2Δ7 (lower right). In all cases except CFP-YFP, areas of interest for lifetime measures were taken at the cell edges.
positive control (Fig. 2A). However, when the tags were at the opposite ends of these proteins (CFP on EAAT2wt C-terminal and YFP on EAAT2Δ7 N-terminal), the change in CFP fluorescence lifetime values were significantly different from baseline (Fig. 2A).

**Functional Characterization of EAAT2 Splice Variants**—We characterized the functional properties of EAAT2 co-expressed with various EAAT2 splice variants using a FMP dye and also by electrophysiological analysis.

**Establishment of FMP Assay**—
The FMP dye has been used previously with expressed EAATs, glycine receptors, and voltage-gated K⁺ channels (27–29). Glutamate transport is coupled to the transport of three Na⁺ ions and one proton and the countertransport of one K⁺ ion. This results in the transfer of two positive charges into the cell with each transport cycle, causing depolarization that may be monitored as an increase in fluorescence. Jensen et al. (28) showed that EAAT2wt-transfected HEK293 cells gave highly similar Km values in FMP assays, D-[3H]aspartate transport assays, and electrophysiology studies. Glutamate applied to HEK293 cells transiently transfected with EAAT2wt generated concentration-dependent increases in fluorescence, reflecting depolarization of the cells (Fig. 3). The responses were fitted to a non-linear Hill equation over a range of time points (Fig. 3). The fluorescence reached steady state at ~24 s and the EC₅₀ (33.4 ± 1.3 μM) and Hill slope values (1.1 ± 0.1) for glutamate-induced depolarization at this time point conformed well to values obtained with other assays (30–32). All measurements for further experiments were performed at 24 s. Increasing the amount of EAAT2wt cDNA used in the transfection did not significantly alter EC₅₀, Hill slope, or Fₘₐₓ values (Table 2). Similarly, increasing the amount of EAAT2wt cDNA, but with the total amount of cDNA adjusted to 5 μg with empty pcDNA 3.1 plasmid, did not significantly alter transport characteristics (data not shown). Toki et al. (33) suggest that HEK293 cells express endogenous EAAT3 and report a Km value of 75 μM. However, we observed no glutamate-dependent change in fluorescence in untransfected HEK293 cells over a range of substrate concentrations from 1 μM to 10 mM, consistent with the report from Sullivan et al. (11).

**Functional Properties of EAAT2 Variants in the FMP Assay**—Application of glutamate to untransfected HEK293 cells and cells solely expressing either of the exon-skipping splice variants (EAAT2Δ7 or EAAT2Δ9) generated no fluorescence changes (Fig. 4, A and B), which suggests that EAAT2 exon-
Functional Expression of EAAT2 Splice Variants

A

$\frac{F}{F_{\text{max}}}$

\[ \text{[L-Glutamate] } \mu M \]

- EAAT2<sub>wt</sub>
- 1:5 EAAT2<sub>wt</sub> : EAAT2Δ7
- 1:10 EAAT2<sub>wt</sub> : EAAT2Δ7
+ EAAT2Δ7

D

$\frac{F}{F_{\text{max}}}$

\[ \text{[L-Glutamate] } \mu M \]

- EAAT2b
- 1:5 EAAT2b : EAAT2Δ7
- 1:10 EAAT2b : EAAT2Δ7

B

$\frac{F}{F_{\text{max}}}$

\[ \text{[L-Glutamate] } \mu M \]

- EAAT2<sub>wt</sub>
- 1:5 EAAT2<sub>wt</sub> : EAAT2Δ9
- 1:10 EAAT2<sub>wt</sub> : EAAT2Δ9
- 1:20 EAAT2<sub>wt</sub> : EAAT2Δ9
+ EAAT2Δ9

E

$\frac{F}{F_{\text{max}}}$

\[ \text{[L-Glutamate] } \mu M \]

- EAAT2b
- 1:5 EAAT2b : EAAT2Δ9
- 1:10 EAAT2b : EAAT2Δ9

C

mean $F$ change

- EAAT2<sub>wt</sub>
- EAAT2Δ7: 1.5
- EAAT2Δ7: 1.10
- EAAT2Δ9: 1.5
- EAAT2Δ9: 1.10
- EAAT2Δ9: 1.20

F

mean $F$ change

- EAAT2<sub>wt</sub>
- EAAT2b: 1.5
- EAAT2b: 1.10
- EAAT2b: 1.5
- EAAT2b: 1.10
- EAAT2b: 1.20
skipping variants cannot form functional transporters when expressed alone. Glutamate-induced fluorescence changes in cells expressing EAAT2wt or EAAT2b (Fig. 4, A and D) were saturable over the substrate concentration range of 1 μM to 10 mM. Mean $EC_{50}$ and Hill slope values are presented in Table 3 and are in very good agreement with previous data (28).

The EAAT2-selective inhibitor dihydrokainate inhibited the glutamate-dependent changes in fluorescence for cells expressing EAAT2wt and EAAT2b. Averaged concentration-inhibition curves are shown for EAAT2wt and EAAT2b (Figs. 4, A and D, inset) with best-fit parameters summarized in Table 3. Glutamate-induced fluorescence changes were measured in cells co-transfected with EAAT2wt and EAAT2 exon-skipping splice variants. Glutamate-dependent increases in fluorescence over the concentration range of 1 μM to 10 μM were attenuated in all co-transfected cells compared with cells transfected with EAAT2wt alone (Fig. 4C). The ratio of splice variant to EAAT2wt was critical for fluorescence changes. Co-transfection of EAAT2wt with EAAT2Δ7 in a 1:5 ratio increased the $EC_{50}$ for glutamate by 8-fold (Fig. 4A and Table 3). With a 1:10 ratio, the $EC_{50}$ was increased by 11-fold (Fig. 4A and Table 3). These changes in glutamate $EC_{50}$ may be due to the formation of heteromeric complexes by EAAT2wt and EAAT2Δ7. Supporting this idea is the marked reduction in Hill slope (0.4 ± 0.1) for the 1:10 EAAT2wt:EAAT2Δ7 co-transfection, compared with Hill slopes (1.3 ± 0.2) seen with EAAT2wt alone (Table 3). This suggests that EAAT2Δ7 was incorporated into a complex with EAAT2wt and influenced the transporter complex negatively.

EAAT2Δ9 co-transfections with EAAT2wt also reduced glutamate-dependent fluorescence changes compared with EAAT2wt alone. When co-expressed with EAAT2wt in ratios of 1:5 EAAT2wt:EAAT2Δ9, glutamate-mediated fluorescence plots did not show increased $EC_{50}$ or altered Hill slopes. However, when the EAAT2wt:EAAT2Δ9 ratio was increased to 1:10 and 1:20, there were significant 4- and 12-fold increases in $EC_{50}$ estimates (Fig. 4B and Table 3). A ratio of 1:10 did not change the Hill slope, but it was reduced markedly at a 1:20 ratio (Table 3). EAAT2Δ9 might only be incorporated into a heteromeric transporter complex at higher expression levels.

EAAT2b has similar transport characteristics to EAAT2wt (11). EAAT2b and EAAT2wt can also form heteromeric complexes (see above and Ref. 18). EAAT2Δ7 and EAAT2Δ9 were co-transfected in various ratios with EAAT2b. Rightward shifts in the glutamate concentration-response curves were observed that were similar to EAAT2wt co-transfections. At a ratio of 1:10 EAAT2b:EAAT2Δ7, the $EC_{50}$ was 7-fold higher than that for EAAT2b transfected alone (Fig. 4D and Table 3). For the EAAT2b:EAAT2Δ9 co-transfections the $EC_{50}$ values for glutamate-dependent fluorescence responses were 3-fold higher at a ratio of 1:10 EAAT2b:EAAT2Δ9, and 5-fold higher for the 1:20 ratio (Fig. 4E and Table 3). Hill slopes did not change markedly in EAAT2b:EAAT2Δ7 co-transfected cells, and only moderate reductions were observed with EAAT2b:EAAT2Δ9 co-transfections (Table 3). Cells co-transfected with EAAT2b and splice variants also showed significantly lower fluorescence changes than cells solely transfected

### TABLE 3

Functional and pharmacological characteristics in the FMP assay of EAAT2wt and EAAT2b, and co-transfections of different ratios of EAAT2wt (or EAAT2b) and EAAT2 exon-skipping splice variants, expressed in HEK293 cell lines.

| Ratio       | $EC_{50}^a$ $\mu M$ | Hill slope | $IC_{50}^b$ $\mu M$ | Hill slope |
|-------------|----------------------|------------|----------------------|------------|
| EAAT2wt     | 23.5 ± 1.2           | 1.3 ± 0.2  | 286.1 ± 1.1          | 1.4 ± 0.2  |
| EAAT2wt:EAAT2Δ7 | 188.2 ± 31.9  | 0.5 ± 0.2  | 679.6 ± 4.7          | 0.5 ± 0.2  |
| EAAT2wt:EAAT2Δ9 | 259.6 ± 34.7    | 0.4 ± 0.1  | 15460 ± 9.3         | 0.5 ± 0.2  |
| EAAT2wt     | 27.1 ± 0.7           | 1.4 ± 0.1  | ND$^c$               | ND$^c$     |
| EAAT2b     | 108.6 ± 19.5         | 1.1 ± 0.4  | 629.6 ± 1.9          | 0.9 ± 0.3  |
| EAAT2Δ7    | 275.5 ± 37.7         | 0.4 ± 0.1  | 10840 ± 8.8         | 0.4 ± 0.2  |
| EAAT2Δ9    | 32.9 ± 2.0           | 1.0 ± 0.1  | 1046 ± 1.2          | 1.3 ± 0.2  |
| EAAT2b:EAAT2Δ7 | 70.3 ± 13.9   | 0.7 ± 0.2  | 686.8 ± 4.4         | 0.6 ± 0.3  |
| EAAT2b:EAAT2Δ9 | 237.6 ± 48.2  | 0.9 ± 0.3  | 11830 ± 48.0       | 0.5 ± 0.3  |

$^a EC_{50}$ values represent the average of at least three independent experiments performed in triplicate; values are mean ± S.E. Non-linear regression was carried out as described under “Results.” The inhibitor DHK was tested at 300 μM glutamate concentration.

$^b$ Indicates significance relative to EAAT2wt/EAAT2b by unpaired Student’s t test ($p < 0.01$).

$^c$ ND, not determined.
EAAT2wt also forms heteromultimers with EAAT2b (see curves are presented in Table 4. They were measured in the presence of intracellular SCN−. Exemplar recordings of the anion current requires Na+/H+/K+ -coupled glutamate transport and is a good measure of transporter function (34, 35). Exemplar recordings of the responses of HEK293 cells transfected with EAAT1, EAAT2wt, and EAAT2b to increasing glutamate concentrations are shown in Fig. 5, A, C, and E. The averaged concentration-response curves normalized to the saturating glutamate current magnitude are also shown (Fig. 5, B, D, and F). Mean parameters of best fit to the concentration-response curves are presented in Table 4.

EAAT2wt and EAAT2b can each form homomultimers, and EAAT2wt also forms heteromultimers with EAAT2b (see above). However, EAAT1 cannot form heteromultimers with EAAT2 (25). We used EAAT1 as a control to see if co-transfection with EAAT2 exon-skipping splice variants (EAAT2Δ7 and EAAT2Δ9) altered glutamate-induced current changes. Non-linear regressions of glutamate concentration-dependent currents for EAAT1 gave the EC50 and Hill slope values shown in Table 4. Co-transfections of EAAT1 with either EAAT2Δ7 or EAAT2Δ9 at ratios of 1:10 or 1:20 gave curves and fitted parameters that did not differ significantly from those with EAAT1 alone (Fig. 5, A and B, and Table 4).

Electrophysiological recordings were performed with cells co-transfected with EAAT2wt and EAAT2Δ7 or EAAT2Δ9 at a 1:10 ratio. The concentration-response curves fitted for the EAAT2wt:EAAT2Δ7 combination displayed a significant 15-fold higher EC50 and a markedly lower Hill slope (Table 4).

The data suggest there was an interaction between EAAT2wt and EAAT2Δ7. EAAT2wt:EAAT2Δ9 co-transfections showed a less than 2-fold increase in EC50, which was not significant, and an unchanged Hill slope (Figs. 5, C and D, and Table 4). These data correspond well with the EC50 and Hill coefficient values obtained with the FMP assays described above. Electrophysiological recordings from EAAT2Δ9 co-transfected with EAAT2wt at 20:1 did not give reproducible data because the currents were too small.

Similar experiments were performed with EAAT2b instead of EAAT2wt (Fig. 5, E and F). Cells co-transfected with EAAT2Δ7 and EAAT2b at a 1:10 ratio gave only a 3-fold higher EC50 than those transfected with EAAT2b alone (Table 4). That is, co-transfection of EAAT2b with EAAT2Δ7 increased the EC50 less than co-transfections of EAAT2Δ7 with EAAT2wt at the same ratio. Comparable results were seen for EAAT2Δ9 co-transfections with EAAT2b. Values for EC50 increased 1.6-fold, which are similar to values for EAAT2Δ9 co-transfected with EAAT2wt. Hill slopes decreased to the same degree seen in FMP assays (Table 4). Glutamate-dependent maximal currents from EAAT2Δ7 or EAAT2Δ9 co-expressed with EAAT2wt at a ratio of 1:10 were significantly lower than EAAT2wt currents. EAAT2Δ7 or EAAT2Δ9 co-expression with EAAT2b or EAAT1 did not change maximal currents significantly, cf. EAAT2b or EAAT1 expressed alone (Fig. 5G). In summary, co-transfections of EAAT2wt or EAAT2b with EAAT2 exon-skipping splice variants reduced glutamate-induced currents in a manner that depended on the concentration ratios of the transfectants.

**DISCUSSION**

In this study we show that EAAT2wt can form heteromeric complexes with variants EAAT2b, EAAT2Δ7, and EAAT2Δ9 that alter glutamate-induced fluorescence and current changes of the complex.

Transcripts of EAAT2wt and its splice variants EAAT2b, EAAT2Δ7, and EAAT2Δ9 are all expressed in human brain tissue (16). Exon-skipping splice variants (Δ7 and Δ9) show higher mRNA transcript expression in Alzheimer-diseased brain with increasing pathological severity. EAAT2wt and EAAT2b protein expression has been confirmed (1), but immunohistochemistry for EAAT2Δ9 showed only diffuse neural labeling in Alzheimer-diseased tissue (36), and there is no report as yet that the EAAT2Δ7 protein is expressed in human brain.

Western blot analysis here showed that all EAAT2 variants were translated into protein when expressed in HEK293 cells. EAAT2Δ9, but not EAAT2Δ7, showed the high M4 dimer and trimer bands that are characteristic of EAATs (1), which suggests that only EAAT2Δ7 can form trimers when expressed alone. Localization of EAAT2 trimers at the cell surface is

**TABLE 4**

| Ratio | EC50μM | Hill slope | n |
|-------|--------|------------|---|
| EAAT1 | 3.0 ± 0.1 | 1.0 ± 0.1 | 3 |
| EAAT1:EAAT2Δ7 | 1:1 | 9.3 ± 1.1 | 1.0 ± 0.1 | 3 |
| EAAT1:EAAT2Δ9 | 1:1 | 12.5 ± 1.4 | 1.0 ± 0.4 | 3 |
| EAAT2wt:EAAT2Δ7 | 1:1 | 23.4 ± 1.5 | 1.2 ± 0.3 | 6 |
| EAAT2wt:EAAT2Δ9 | 1:1 | 355.4 ± 1.6 | 0.4 ± 0.1 | 5 |
| EAAT2b:EAAT2Δ7 | 1:1 | 429.9 ± 1.2 | 0.9 ± 0.2 | 4 |
| EAAT2b:EAAT2Δ9 | 1:1 | 315.2 ± 2.6 | 1.1 ± 0.2 | 5 |
| EAAT2Δ7:EAAT2Δ9 | 1:1 | 96.9 ± 1.6 | 1.0 ± 0.4 | 4 |

* EC50 values represent the average of at least 3–6 separate experiments; values are mean ± S.E. Non-linear regression was carried out as described under “Results.”
* Significantly different from EAAT2wt/EAAT2b (p < 0.05, unpaired Student’s t test).

**FIGURE 5.** Whole cell patch clamp data from HEK293 cells transfected with EAAT1, EAAT2wt, and EAAT2 splice variants. A, C, and E, representative current responses for the effect of glutamate. All traces in each row were recorded from cells expressing the transporter complex as indicated on the left. B, D, and F, averaged glutamate-dependent concentration response curves from cells expressing EAAT2wt, EAAT2b, EAAT1 alone, or co-expressed with EAAT2Δ7 or EAAT2Δ9 at a ratio of 1 in 10. All response curves were generated by normalizing the corresponding currents to the maximum current, recorded in the same cell. The data plotted represent the mean ± S.E. (n = 3–5). G, mean maximal current (I) recorded from at least 3 different cells transfected with EAAT1, EAAT2wt, EAAT2b, and EAAT2 exon-skip variant, normalized to maximal EAAT1, EAAT2wt, or EAAT2b current at 300 μM glutamate. *, significantly different from EAAT2wt by unpaired Student’s t test (p < 0.05).
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essential for glutamate transport. All variants except EAAT2Δ7 were transported to the cell surface when expressed alone in HEK293 cells. This is in contrast to Lin et al. (15), who found that EAAT2Δ9-GFP fusion protein expression was restricted to the cytoplasm of COS7 cells. Low expression levels could be a reason for this, because Western blotting of untagged EAAT2Δ9 failed to detect protein. Lin et al. (15) showed that co-expression of EAAT2wt-GFP and EAAT2Δ9 did not affect EAAT2wt localization. However, co-expression of an EAAT2 partial intron-7 retention variant with EAAT2wt suggested that the variant interfered with normal EAAT2wt protein assembly and trafficking. It is possible that the 1:1 ratio of EAAT2Δ9 to EAAT2wt used by Lin et al. (15) was too low to allow EAAT2Δ9 to be expressed at the cell surface and affect EAAT2wt expression. We show that co-expression of EAAT2wt with EAAT2Δ7 at a 1:10 transfection ratio enabled EAAT2Δ7 to be trafficked to the cell surface.

EAAT2wt and EAAT2b transport glutamate when expressed alone. It has been suggested that EAAT2wt and EAAT2b expression patterns are different (37, 38). The last three amino acids of EAAT2b (TC1) comprise a PDZ domain binding motif. EAAT2b can interact with scaffolding proteins PSD-95 and PICK 1, which are involved in cell-surface trafficking (39, 40). Although PSD-95 and PICK-1 are not endogenously expressed in HEK293 cells, other PDZ-binding proteins may interact with the PDZ motif of EAAT2b. Because we saw no major differences in glutamate-dependent fluorescence and current changes between EAAT2wt or EAAT2b co-transfections with EAAT2 exon-skipping splice variants, the PDZ-interacting motif of EAAT2b might not enhance the transport characteristics of heteromeric complexes.

Co-expression of the EAAT2 exon-skipping splice variants (EAAT2Δ7 or EAAT2Δ9) with EAAT2wt or EAAT2b was necessary for them to form active transporter complexes. The ratio of splice variant to EAAT2wt was pivotal for glutamate-dependent activation. Lin et al. (15) showed that co-expression of EAAT2Δ9 with EAAT2wt at a 1:1 ratio did not interfere with glutamate transport. Those findings are in close agreement with the data presented here: EAAT2Δ9:EAAT2wt at ratios up to 5:1 did not alter EC_{50} or Hill slope. However, higher splice variant to EAAT2wt ratios reduced glutamate transport significantly, and there was a decrease in Hill slope as the ratio increased above 5:1. This suggests there may be negative cooperativity between the glutamate binding sites in complexes that include splice variants, which could be due to the loss of a binding/transport site in the trimeric complex. Together with the FRET data, this is further evidence that the splice variants were incorporated into multimeric transporter complexes at the cell surface.

FRET was used to show that EAAT2wt not only forms heteromeric complexes with EAAT2b, as has been described before (18), but also with EAAT2Δ7 and EAATΔ9. For EAAT2wt:EAAT2Δ7 or EAAT2wt:EAATΔ9 heteromers, FRET was dependent on the position of the fluorescent tags. This was not the case for EAAT2wt:EAAT2b heteromers. It is possible that the splicing out of exons 7 or 9 changed the protein structure of EAAT2Δ7 or EAATΔ9 such that their N or C termini moved away from each other, resulting in changed FRET signals. Even so, the changes in CFP lifetime for cells co-transfected with EAAT2wt and EAAT2 exon-skipping splice variants provide clear evidence that EAAT2wt can form heteromeric complexes with either EAAT2Δ7 or EAAT2Δ9.

Nevertheless, it is possible that heteromeric complexes between exon-skipping splice variants and EAAT2wt are less stable than EAAT2wt homomers, which could explain the differences in the FRET signals. Differing mechanisms could modify the stability of the protein complex. For instance, the heteromultimer might be degraded rapidly, or be prevented from trafficking from the endoplasmic reticulum (ER) to the plasma membrane, or may not be inserted correctly into the plasma membrane.

Alignment of the EAAT2Δ9 and Glt_{ph} sequences suggests that EAAT2Δ9 lacks the residues necessary for glutamate and Na^+ binding, and for glutamate translocation (5). EAAT2Δ7 lacks transmembrane domain 6 and the first helical hairpin HP1. Parts of the region responsible for trimerization, transmembrane domain 5, are missing. This could be a possible reason for reduced stability of heteromeric trimers.

Mutation studies in cultured mammalian cells have revealed that an extracellular leucine-based motif is necessary for multimer trafficking from the ER to the membrane (41). GLT1 with a leucine to alanine mutation in the HP2b-TM8 region is retained in the ER and has a dominant-negative effect on GLT1 expression (42). When expressed in mammalian cells, certain ratios of EAAT2wt to EAAT2 mutant protein complexes are able to exit the ER, suggesting that the ratio of wild-type to mutant affects either the degradation of the complex or its trafficking to the membrane. EAAT2Δ7 retains the forward tracking signal responsible for protein trafficking from the ER to the plasma membrane. EAAT2Δ9 lacks this motif.

In conclusion, the EAAT2 exon-skipping splice variants EAAT2Δ7 and EAAT2Δ9 seem to alter glutamate transport capability. They can be incorporated into multimeric transporter complexes together with either EAAT2wt or EAAT2b. Although glutamate transporters form a trimeric complex, the three protomers function as independent proteins (3, 5, 43–46) (but see Ref. 47 for a dissenting view). However, our results suggest that non-functional exon-skipping splice variants can be expressed as proteins and incorporated into membrane-resident complexes, where they interact with their fully functional protomer neighbors. This is shown by significant increases in glutamate EC_{50} as well as by altered cooperativity, which imply that variant protomers distort EAAT2wt protomers in the transporter complex. This might lead to less efficient glutamate transport properties of the complex.

Future work should aim to elucidate the mechanism by which the transport process of heteromeric complexes is altered, and the process by which heteromeric complexes form and are trafficked to the membrane. It will also be of interest to understand how the formation of heteromeric complexes in glia and neurons affects the efficiency of glutamate clearance from synapses and the extent to which this process may influence the pathogenesis of disease states such as Alzheimer disease.
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