Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical Pharmacology 67 (2004) 2115–2127
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Pharmacological characterization of human excitatory amino acid transporters EAAT1, EAAT2 and EAAT3 in a fluorescence-based membrane potential assay

Anders A. Jensen*, Hans Bräuner-Osborne

Department of Medicinal Chemistry, The Danish University of Pharmaceutical Sciences, Universitetsparken 2, DK-2100 Copenhagen, Denmark

Received 1 December 2003; accepted 9 February 2004

Abstract

We have expressed the human excitatory amino acid transporters EAAT1, EAAT2 and EAAT3 stably in HEK293 cells and characterized the transporters pharmacologically in a conventional [^{3}H]-D-aspartate uptake assay and in a fluorescence-based membrane potential assay, the FLIPR® Membrane Potential (FMP) assay. The K_m and K_i values obtained for 12 standard EAAT ligands at EAAT1, EAAT2 and EAAT3 in the FMP assay correlated well with the K_i values obtained in the [^{3}H]-D-aspartate assay (r^2 values of 0.92, 0.92, and 0.95, respectively). Furthermore, the pharmacological characteristics of the cell lines in the FMP assay were in good agreement with previous findings in electrophysiology studies of the transporters. The FMP assay was capable of distinguishing between substrates and non-substrate inhibitors and to discriminate between ''full'' and ''partial'' substrates at the transporters. Taking advantage of the prolific nature of the FMP assay, interactions of the EAATs with substrates and inhibitors were studied in some detail.

This is the first report of a high throughput screening assay for EAATs. We propose that the assay will be of great use in future studies of the transporters. Although conventional electrophysiology set-ups might be superior in terms of studying sophisticated kinetic aspects of the uptake process, the FMP assay enables the collection of considerable amounts of highly reproducible data with relatively little labor. Furthermore, considering that the number of EAAT ligands presently available is limited, and that almost all of these are characterized by low potency and a low degree of subtype selectivity, future screening of compound libraries at the EAAT-cell lines in the FMP assay could help identify structurally and pharmacologically novel ligands for the transporters.

Keywords: Excitatory amino acid transporter; EAAT1; EAAT2; EAAT3; Glutamate; HEK293; FLIPR® Membrane Potential assay; High throughput screening (HTS)

1. Introduction

L-Glutamate (L-Glu) is the major excitatory neurotransmitter in the mammalian CNS, where it mediates a wide range of physiological functions important for cognition, memory formation and learning [1,2]. However, at higher extracellular concentrations L-Glu can also act as a neurotoxin, and excessive glutamatergic signaling appears to be implicated in numerous neurological and psychiatric disorders [1–3]. L-Glu is synthesized and stored in specialized glutamatergic neurons, and upon neuronal excitement the neurotransmitter is released into the synaptic cleft, where it exerts its effects through two distinct receptor classes: the ionotropic glutamate receptors (iGluRs) and the metabotropic glutamate receptors (mGluRs) [3–5]. The termination of glutamatergic transmission is achieved by desensitization and/or internalization of postsynaptic iGluRs and mGluRs, by inhibition of the synaptic release of L-Glu through activation of presynaptic GluRs, and by the reuptake of L-Glu from the synaptic cleft mediated by a

Abbreviations: ALS, amyotrophic lateral sclerosis; Asp, aspartate; *cis*-ACBD, *cis*-1-aminocyclobutane-1,3-dicarboxylate; DHK, dihydrokainate; DMEM, Dulbecco's Modified Eagle Medium; EAAT, excitatory amino acid transporter; FMP, FLIPR® Membrane Potential; FU, fluorescent unit; Glu, glutamate; HEK, Human Embryonic Kidney; HTS, high throughput screening; iGluR, ionotropic glutamate receptor; KA, kainate; mGluR, metabotropic glutamate receptor; (±)-T3MG, (±)-*threo*-3-methylglutamate; DL-TBOA, DL-*threo*-β-benzyloxyaspartate; (*S*)-TDPA, (*S*)-2-amino-3-(3-hydroxy-1,2,5-thiadiazol-4-yl)propionic acid; L-THA, L-(−)-*threo*-β-hydroxyaspartate; L-*trans*-2,4-PDC, L-*trans*-pyrrolidine-2,4-dicarboxylate

* Corresponding author. Tel.: +45-3530-6491; fax: +45-3530-6040.
E-mail address: aaj@dfuni.dk (A.A. Jensen).

0006-2952/$ – see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2004.02.013

family of sodium-dependent high-affinity glutamate/aspartate transporters, the excitatory amino acid transporters (EAATs) [6,7].

The EAATs are polypeptides of 500–600 amino acid residues, which exhibit up to 60% amino acid sequence identity with each other [6,7]. To date five human EAAT subtypes have been cloned: EAAT1 (termed GLAST-1 in rat), EAAT2 (GLT-1 in rat), EAAT3 (EAAC1 in rat), EAAT4 and EAAT5 [6,7]. The mRNAs and proteins of the five EAAT subtypes are highly differentially expressed in the CNS and in the periphery. EAAT2 is expressed abundantly throughout the CNS, EAAT1 is also widely expressed with greatest levels in cerebellum, EAAT3 is widely distributed in both brain and in peripheral tissues, EAAT4 is predominantly found on GABAergic Purkinje cells in cerebellum, and EAAT5 is found exclusively in retina. At the cellular level, EAAT1 and EAAT2 are predominantly located to glia cells, whereas EAAT3 and EAAT4 are neuronal transporters [7].

Extracellular L-Glu concentrations in the synapse increase from nanomolar to millimolar concentrations during the period of synaptic release, whereas the intracellular concentrations of the neurotransmitter are stable in the millimolar ranges [8]. The EAATs use the cellular Na^+/K^+ gradient as driving force for the translocation of L-Glu against its concentration gradient and couple the transport of one substrate molecule with the cotransport of three sodium ions and one proton and the countertransport of a potassium ion [9–11]. Furthermore, an uncoupled chloride flux is associated with the transport, in particular in the case of EAAT4 and EAAT5 [6,7,9]. These two transporters are also characterized by low Glu transport capacities, and thus EAAT1, EAAT2 and, to a lesser extent, EAAT3 are believed to be the predominant mediators of synaptic L-Glu uptake.

The EAATs play a key role in the maintenance of L-Glu concentration below neurotoxic levels [12,13]. Consequently, failure or inadequacy of L-Glu reuptake by the EAATs have been proposed to be implicated in a wide range of acute neurotoxic states like stroke and ischemia and in chronic neurodegenerative disorders such as Alzheimer's disease, amyotrophic lateral sclerosis (ALS) and epilepsy [7,12–14]. Furthermore, reversal of glutamate transport contributes significantly to states of acute cerebral ischemia and epilepsy, especially under conditions when energy supply is reduced [14,15]. Another indication of EAAT involvement in the pathogenesis of these disorders is the substantial loss of EAAT2 observed in both ALS and Alzheimer's disease [16]. The perspectives in EAATs as therapeutic targets have been explored in numerous animal studies. A few examples of the findings of these are the lethal spontaneous seizures and increased susceptibility to neurodegeneration displayed by EAAT2 knock out mice and the elevated extracellular L-Glu levels and varying degrees of neurotoxicity produced by antisense oligonucleotide inhibition of EAAT1, EAAT2 and EAAT3 expression in rats [17,18].

Despite the obvious perspectives in EAATs as therapeutic targets, the medicinal chemistry efforts in the field have been surprisingly small compared with those in the iGluR and mGluR fields [3,14]. The vast majority of the ligands presently available for the transporters are amino acid-derived structures characterized by low potency and poor EAAT subtype selectivity, and many of the ligands also possess activities at mGluRs and/or iGluRs [14]. The search for structurally and pharmacologically novel EAAT ligands would be greatly facilitated by the availability of high throughput screening (HTS) assays for the transporters. In the present study, we have characterized the pharmacological properties of human EAAT1, EAAT2 and EAAT3 stably expressed in Human Embryonic Kidney 293 (HEK293) cells in a conventional [^{3}H]-D-aspartate uptake assay and in the fluorescence-based FLIPR® Membrane Potential (FMP) assay.

2. Materials and methods

2.1. Materials

Culture media, serum, antibiotics, G-418 sulfate and buffers for cell culture were obtained from Invitrogen (Groningen, The Netherlands). The cDNAs encoding the human EAAT1, EAAT2 and EAAT3 were a kind gift from Dr. Susan G. Amara (Howard Hughes Medical Institute, Portland, OR). L-Glu, D-glutamate (D-Glu), L-aspartate (L-Asp) and D-aspartate (D-Asp) were obtained from Sigma (St. Louis, MO), whereas DL-*threo*-β-benzyloxyaspartate (DL-TBOA), L-(−)-*threo*-β-hydroxyaspartate (L-THA), L-*trans*-pyrrolidine-2,4-dicarboxylate (L-*trans*-2,4-PDC), *cis*-1-aminocyclobutane-1,3-dicarboxylate (*cis*-ACBD), (±)-*threo*-3-methylglutamate [(±)-T3MG], kainate (KA), and dihydrokainate (DHK) were purchased from Tocris Cookson (Bristol, UK). (*S*)-2-Amino-3-(3-hydroxy-1,2,5-thiadiazol-4-yl)propionic acid [(*S*)-TDPA] was synthesized at the department [19]. The chemical structures of the compounds used in this study are depicted in Fig. 1.

2.2. Molecular biology

The cDNAs for EAAT1, EAAT2 and EAAT3 were subcloned from their original Bluescript vectors into pCDNA3.1 as previously described [20].

2.3. Cell culture and stable transfection of HEK293 cells

The HEK293 cells were maintained at 37 °C in a humidified 5% CO_2 incubator in culture medium (Dulbecco's Modified Eagle Medium (DMEM) supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) and 10%

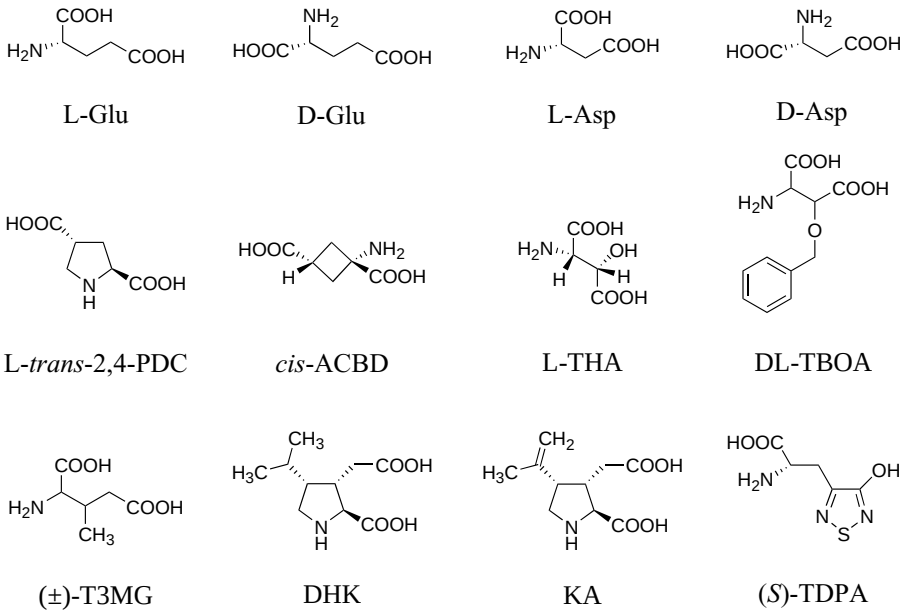

Fig. 1. Chemical structures of the compounds characterized pharmacologically at the EAATs.

fetal bovine serum). For the stable expression of human EAAT1, EAAT2 and EAAT3, the cells were transfected with EAAT1–pCDNA3.1, EAAT2–pCDNA3.1 or EAAT3–pCDNA3.1 using Polyfect as a DNA carrier according to the protocol of the manufacturer (Qiagen, Hilden, Germany) and maintained for 2–3 weeks in selection medium containing 3 mg/ml G-418. Antibiotic-resistant colonies were isolated and maintained in culture medium supplemented with 1 mg/ml G-418 and screened individually in the FMP assay using 1 mM L-Glu as substrate concentration.

2.4. [^{3}H]-D-Asp and [^{3}H]-L-Glu uptake assays

The EAAT1-, EAAT2- and EAAT3-HEK293 cell lines were characterized pharmacologically in a [^{3}H]-D-Asp assay essentially as previously described [20]. Briefly, cells were split into poly-D-lysine-coated white 96-well plates (Perkin-Elmer, Boston, MA) in DMEM supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), 10% fetal bovine serum and 1 mg/ml G-418. 16–24 h later the medium was aspirated, and cells were washed three times with 100 μl assay buffer (Hanks Buffered Saline Solution supplemented with 1 mM $CaCl_2$ and 1 mM $MgCl_2$). Then 50 μl assay buffer supplemented with radioligand and various concentrations of different ligands was added to each well, and the plate was incubated at 37 °C for 15 min. The wells were then washed with 3 × 100 μl ice-cold assay buffer, and 150 μl Microscint^{TM}20 scintillation fluid (Perkin-Elmer) was added to each well. The plate was shaken for 1 h and counted in a TopCounter (Perkin-Elmer). The experiments were performed in duplicate three to four times for each compound. In the saturation experiments, [^{3}H]-D-Asp and [^{3}H]-L-Glu concentrations up to 300 nM were used and in order to measure transport at higher concentrations the radioligands were diluted with the corresponding cold ligand. Non-specific transport was determined using 1 mM L-Glu (in the [^{3}H]-D-Asp assay) or 1 mM D-Asp (in the [^{3}H]-L-Glu assay). In the competition transport experiments, 30 nM [^{3}H]-D-Asp was used as tracer concentration.

2.5. The FLIPR® Membrane Potential (FMP) assay

The compounds were characterized functionally at the EAAT1-, EAAT2- and EAAT3-HEK293 cell lines in the FMP assay (Molecular Devices, Crawley, UK). The lipophilic, anionic, bis-oxonol dye in this kit is excited at 530 nm, and the distribution of the dye across the plasma membrane is dependent on the membrane potential of the cell. Hence, depolarization of the cells will cause more dye to enter the cells, where it will bind to intracellular proteins and lipids and cause an increase in the fluorescence signal. Conversely, the dye exits the cells upon hyperpolarization giving rise to a decrease in the fluorescence signal.

Cells were split into poly-D-lysine-coated black clear bottom 96-well plates (BD Biosciences, Bedford, MA) in DMEM supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), 10% fetal bovine serum and 1 mg/ml G-418. 16–24 h later the medium was aspirated, and the cells were washed with 100 μl Krebs buffer (140 mM NaCl/4.7 mM KCl/2.5 mM $CaCl_2$/1.2 mM $MgCl_2$/11 mM HEPES/10 mM D-glucose, pH 7.4). Then 50 μl Krebs buffer was added to each well (in the experiments with non-substrate inhibitors, the inhibitors were added to this buffer). Fifty microliters Krebs buffer supplemented with FMP assay dye was then added to each well, and the plate was incubated at 37 °C for 30 min. The plate was

assayed at room temperature in a NOVOstar^{TM} plate reader (BMG Labtechnologies, Offenburg, Germany) measuring emission at 560 nm caused by excitation at 530 nm before and up to 1 min after addition of 25 μl substrate solution (the substrate was dissolved in Krebs buffer). The experiments were performed in duplicate at least three times for each compound. The gain of the NOVOstar^{TM} was typically set at a level, where the basal fluorescence was 15,000–20,000 fluorescence units (FU). For the characterization of the non-substrate inhibitors 30 μM L-Glu was used as substrate concentration. The concentration–response curves were constructed based on the maximal responses obtained for eight different concentrations of each of the compounds.

2.6. Data analysis

Data from the [^{3}H]-D-Asp and [^{3}H]-L-Glu saturation experiments were fitted to a single-site ligand binding model, and the Michaelis transport constant (K_m) and the maximal transport rate (V_{max}) values were determined by non-linear regression. Inhibition data were fitted to the Michaelis–Menten equation and the IC_{50} values were determined. K_i values were then calculated from the ''functional equivalent'' of the Cheng–Prusoff equation [21]. Data for the substrates in the FMP assay was fitted to the simple mass equation: $R = R_{basal} + [R_{max}/(1 + (EC_{50}/[S])^n)]$, where $[S]$ is the concentration of substrate, n is the Hill coefficient and R is the response. Curves were generated by non-weighted least-squares fits using the program KaleidaGraph 3.6 (Synergy Software, Reading, PA).

3. Results

3.1. Identification of stable cell lines

We isolated clones of the EAAT1-, EAAT2- and EAAT3-HEK293 cell lines and screened them individually for response to 1 mM L-Glu in the FMP assay. We were able to identify several functional clones for EAAT1, EAAT2 and EAAT3 within the first 20–30 screened clones of each of the three transporters. Although the functional response of the EAAT1 clone selected for further characterization was significantly lower than those observed for the EAAT2- and EAAT3-clones, it was of an amplitude significant enough to use for functional characterization.

3.2. Pharmacological characterization of the EAAT-cell lines in [^{3}H]-D-Asp and [^{3}H]-L-Glu assays

The pharmacological characteristics of the EAAT1-, EAAT2- and EAAT3-HEK293 cell lines in conventional [^{3}H]-D-Asp and [^{3}H]-L-Glu uptake assays are given in Fig. 2 and Table 1. In agreement with previous studies of EAATs

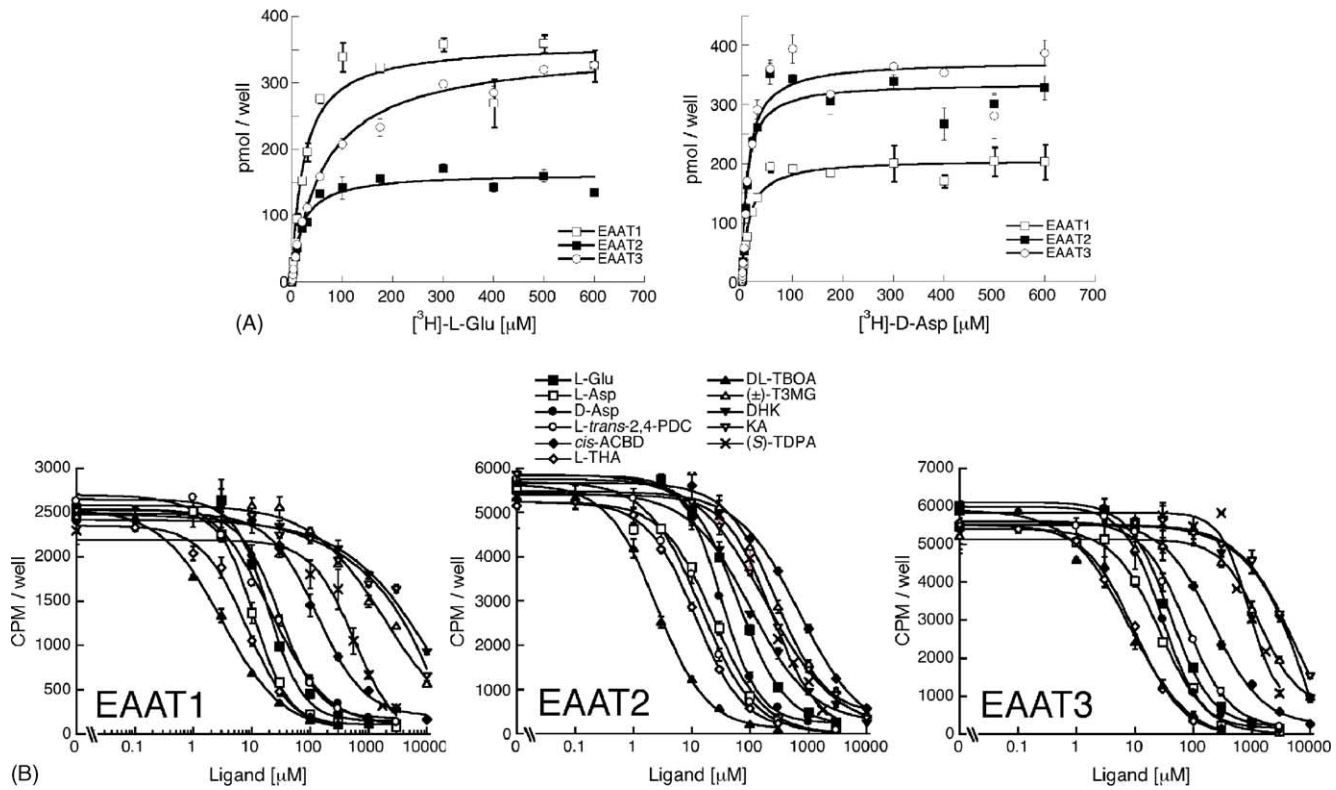

Fig. 2. Pharmacological characterization of the EAAT1-, EAAT2- and EAAT3-HEK293 cell lines in [^{3}H]-D-Asp and [^{3}H]-L-Glu uptake assays. The uptake assays were performed as described in Section 2. (A) Saturation transport of [^{3}H]-D-Asp and [^{3}H]-L-Glu. (B) Competition for transport of 30 nM [^{3}H]-D-Asp by 11 standard ligands at the EAAT1, EAAT2 and EAAT3 cell lines.

Table 1
Pharmacological characteristics of human EAAT1, EAAT2 and EAAT3 stably expressed in HEK293 cell lines in [^{3}H]-L-Glu and [^{3}H]-D-Asp uptake assays

Compound	EAAT1 K_m ± S.E.M.	EAAT2 K_m ± S.E.M.	EAAT3 K_m ± S.E.M.
[^{3}H]-L-Glu	22 ± 3	25 ± 3	42 ± 13
[^{3}H]-D-Asp	20 ± 3	31 ± 7	14 ± 5
	K_i (pK_i ± S.E.M.)	K_i (pK_i ± S.E.M.)	K_i (pK_i ± S.E.M.)
L-Glu	21 (4.7 ± 0.06)	53 (4.3 ± 0.09)	39 (4.4 ± 0.10)
D-Glu	>1000 (<3)	>1000 (<3)	>1000 (<3)
L-Asp	12 (4.9 ± 0.04)	29 (4.5 ± 0.05)	38 (4.4 ± 0.07)
D-Asp	25 (4.6 ± 0.06)	38 (4.4 ± 0.07)	34 (4.5 ± 0.09)
L-*trans*-2,4-PDC	20 (4.7 ± 0.10)	20 (4.7 ± 0.05)	109 (4.0 ± 0.11)
cis-ACBD	170 (3.8 ± 0.06)	680 (3.2 ± 0.04)	230 (3.6 ± 0.08)
L-THA	11 (4.9 ± 0.08)	19 (4.7 ± 0.08)	14 (4.9 ± 0.11)
DL-TBOA	2.9 (5.5 ± 0.09)	2.2 (5.7 ± 0.07)	9.3 (5.0 ± 0.06)
(±)-T3MG	2500 (2.6 ± 0.10)	240 (3.6 ± 0.04)	1900 (2.7 ± 0.13)
DHK	>3000 (<2.5)	89 (4.1 ± 0.05)	>3000 (<2.5)
KA	>3000 (<2.5)	200 (3.7 ± 0.10)	>3000 (<2.5)
(*S*)-TDPA	630 (3.2 ± 0.08)	190 (3.7 ± 0.07)	1010 (3.1 ± 0.09)

The assays were performed as described in Section 2. The K_i values for the 12 standard ligands were determined using [^{3}H]-D-Asp as radioligand. The K_m and K_i values (with pK_i ± S.E.M. values in parenthesis) are given in μM.

expressed in mammalian cell lines or *Xenopus* oocytes, non-transfected wild type HEK293 cells displayed uptake of both radioligands (data not shown) [20,22–25]. However, the endogenous uptake in the non-transfected wild type HEK293 cells was significantly smaller than the EAAT-mediated uptake. For example, in experiments using a similar number of non-transfected HEK293 cells and EAAT1-HEK293 cells the endogenous [^{3}H]-D-Asp uptake in HEK293 cells constituted 5–10% of the uptake in EAAT1-HEK293 at low radioligand concentrations (10–100 nM) and 2–5% at saturating concentrations. In the EAAT2- and EAAT3-HEK293 cell lines the transporter-mediated [^{3}H]-D-Asp uptake constituted an even greater component of the total uptake. The observation that EAAT2-selective ligands like DHK and KA suppressed the [^{3}H]-D-Asp uptake in the EAAT2-cell line with considerable higher potencies than in the EAAT1- and EAAT3-HEK293 cell lines also supported that the [^{3}H]-D-Asp uptake measured in the assay was indeed EAAT-mediated (Fig. 2B). The endogenous [^{3}H]-D-Asp uptake in the non-transfected HEK293 cells was not subtracted from the EAAT-mediated uptake, since this did not alter the potency of the EAAT ligands significantly.

[^{3}H]-D-Asp and [^{3}H]-L-Glu displayed very similar K_m values at the three EAATs (Fig. 2A and Table 1). This was in agreement with a previous study of EAAT1, EAAT2 and EAAT3 transiently expressed in COS-7 cells, although the K_m values in that study were two- to threefold higher than the ones in this study [24]. We did not count the number of cells used in the saturation experiments and thus could not estimate the V_{max} values of the cell lines. The pharmacological profiles and characteristics of 12 standard EAAT ligands in the [^{3}H]-D-Asp uptake assay are depicted in Fig. 2B and Table 1.

3.3. Pharmacological characterization of the EAAT-cell lines in the FMP assay

Exposure of the EAAT1-, EAAT2- and EAAT3-HEK293 cell lines to substrates in the FMP assay gave rise to solid concentration-dependent increases in fluorescent intensity (exemplified by L-Glu at EAAT2 in Fig. 3A). The increase in the fluorescence intensity in this assay reflects cell depolarization, which is in agreement with the co-transport of three Na^+ ions and one proton and the counter-transport of one K^+ ion per substrate molecule [7]. The responses could be suppressed in a concentration-dependent manner by non-substrate inhibitors (exemplified by DL-TBOA at EAAT2 in Fig. 3B).

Prior to the characterization of the EAAT-cell lines in the FMP assay, multiple assay parameters were validated. It was established that the substrate-induced responses in the EAAT-cell lines were independent of the incubation time prior to substrate application (data not shown). This meant that the fluorescence in the first well (incubation time 30 min) and the last well (incubation time 2 h) in the 96-well plate were recorded under comparable conditions. Furthermore, no significant differences were observed between FMP assays run at room temperature and at 37 °C or between assays using substrate solution volumes of 25, 33 and 50 μl (data not shown). Given that the majority of published pharmacological studies of EAATs have been performed at room temperature, we chose that temperature for further studies in order to facilitate comparison of data.

The Na^+ concentration in the assay buffer had a significant impact on the magnitude of the substrate-induced response through the transporters (exemplified with EAAT2 in Fig. 4). When $[Na^+]_{ex}$ was reduced from 140 mM (in the original Krebs buffer) to 70 mM the

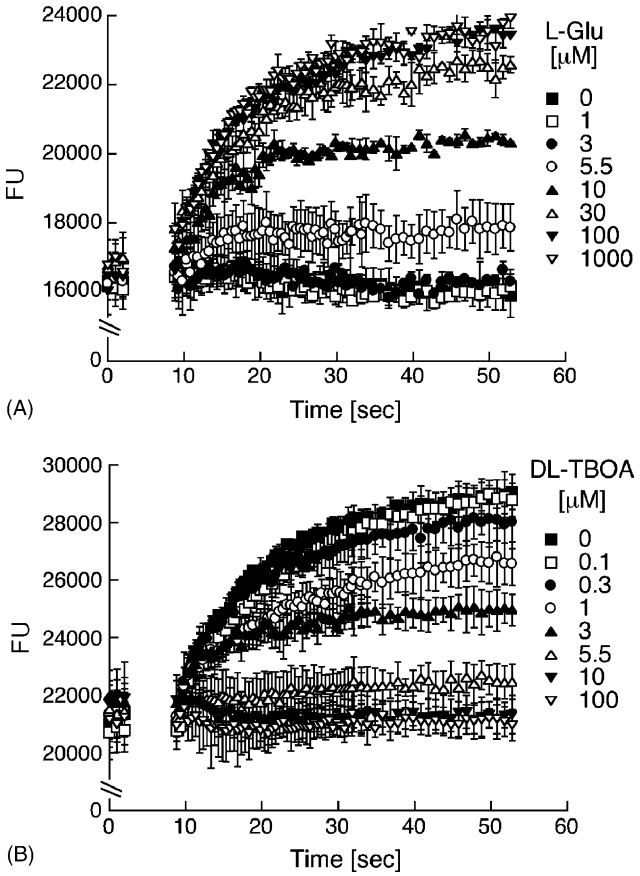

Fig. 3. EAAT2 transport in the FMP assay. Time–response curves for the L-Glu-induced response in the EAAT2-HEK293 cell line (A) and the inhibition of it exerted by DL-TBOA (B). Measurement of fluorescence was performed immediately before (seconds 0–3) and after (seconds 8.8–52) addition of substrate to the wells and mixture of the final solution. In the inhibitor experiments, 30 μM L-Glu was used as substrate concentration. The experimental procedures used are described in Section 2.

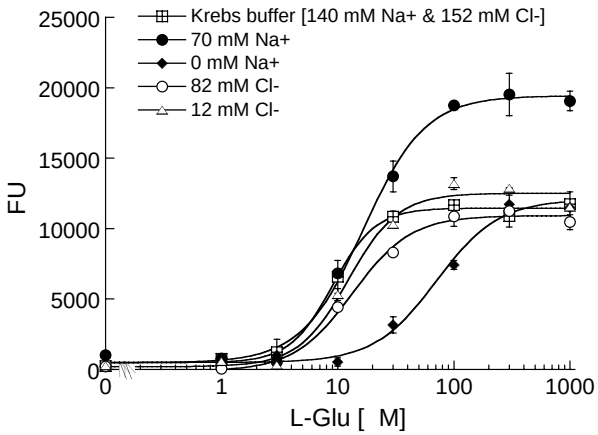

Fig. 4. Importance of Na^+ and Cl^- for EAAT2 transport of L-Glu in the FMP assay. In the Na^+ experiments (closed circle, closed diamond) the NaCl in the Krebs buffer was substituted with choline chloride, and in the Cl^- experiments (open circle, open triangle) with sodium D-gluconate. The compositions of the respective buffers were: Krebs buffer (140 mM NaCl/4.7 mM KCl/2.5 mM $CaCl_2$/1.2 mM $MgCl_2$/11 mM HEPES/10 mM D-glucose, pH 7.4), "70 mM Na^+" (closed circle) (70 mM NaCl/70 mM choline chloride/4.7 mM KCl/2.5 mM $CaCl_2$/1.2 mM $MgCl_2$/11 mM HEPES/10 mM D-glucose, pH 7.4), "0 Na^+" (closed diamond) (140 mM choline chloride/4.7 mM KCl/2.5 mM $CaCl_2$/1.2 mM $MgCl_2$/11 mM HEPES/10 mM D-Glucose, pH 7.4), "82 mM Cl^-" (open circle) (70 mM NaCl/70mM sodium D-gluconate/4.7 mM KCl/2.5 mM $CaCl_2$/1.2 mM $MgCl_2$/11 mM HEPES/10 mM D-glucose, pH 7.4) and "12 mM Cl^-" (open triangle) (140 mM sodium D-gluconate/4.7 mM KCl/2.5 mM $CaCl_2$/1.2 mM $MgCl_2$/11 mM HEPES/10 mM D-glucose, pH 7.4). The basal fluorescence levels in this experiment were 15,000–17,000 FU.

maximal response of EAAT2 was significantly increased, whereas the potency of L-Glu did not change significantly. Although the potency of L-Glu was significantly reduced in a Na^+-free buffer, the maximal response of the transporter to the substrate was comparable to that in normal Krebs buffer (Fig. 4). In contrast to importance of the Na^+ concentration, the concentration of Cl^- in the buffer could be reduced from 152 mM (in the original Krebs buffer) to 12 mM without any measurable effects on the EAAT2 signal (Fig. 4).

The findings from the experiments with the Cl^- concentration in the assay buffer is in agreement with findings that the contributions of chloride conductance to the total currents of EAAT1, EAAT2 and EAAT3 are negligible [9]. In contrast, a reduction of the sodium concentration in the assay buffer should theoretically have resulted in a reduced signal from the transporter. Furthermore, the significant response from the EAAT2-HEK cell line in the Na^+-free buffer contrasts the findings from similar experiments with EAATs expressed in oocytes, where no responses to L-Glu is observed when using a Na^+-free buffer [24]. One explanation for this discrepancy could be that the FMP assay is a more "dirty" system than a conventional electrophysiological set-up with the possibility for trace concentrations of Na^+ left in the assay. Furthermore, since we do not know the ingredients of the FMP assay dye, we cannot exclude the possibility that the dye contains trace concentrations of Na^+. Finally, the 30-min incubation of the cells in Na^+-free and 70 mM Na^+ buffers prior to L-Glu application could also be speculated to have interfered with ion gradients across the cell membrane. As the enhanced EAAT responses in the 70 mM Na^+ buffer were somewhat puzzling, and the 140 mM NaCl in the regular Krebs buffer was closer to physiological conditions, we decided to use this regular buffer for the pharmacological characterization of the EAATs.

The pharmacological profiles of 12 standard ligands were determined at the EAAT-HEK293 cell lines using the FMP assay in this manner. L-Glu, L-Asp, D-Asp, L-*trans*-2,4-PDC, L-THA and *cis*-ACBD were found to be substrates at all three EAAT subtypes, albeit with different maximal responses (R_{max}) (Fig. 5 and Table 2). The Hill slopes of all the substrates were greater than unity, typically between 1.3 and 2.0 (data not shown). Exposure of non-transfected wild type HEK293 cells to all six substrates in 3 mM concentrations did not elicit significant responses (data not shown). Analogously, application of L-Glu, D-Asp and L-Asp have been reported not to give rise

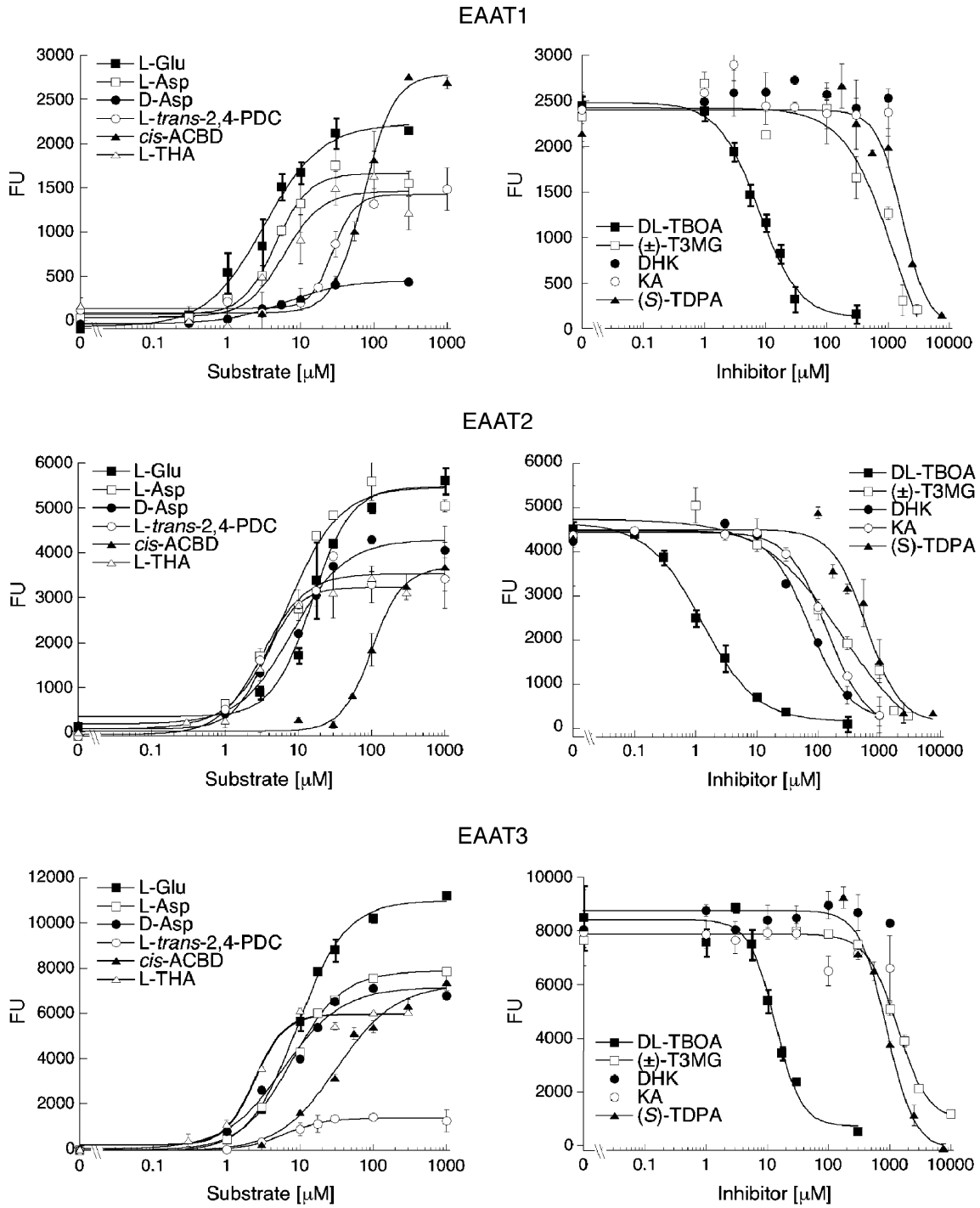

Fig. 5. Pharmacological characterization of the EAAT1-, EAAT2- and EAAT3-HEK293 cell lines in the FMP assay. Concentration–response curves for substrates and non-substrate inhibitors at EAAT1, EAAT2 and EAAT3. The basal levels of fluorescence intensity for the three cell lines were 15,000–20,000 FU. The experiments were performed as described in Section 2.

to any significant currents in wild type *Xenopus* oocytes [25]. Exposure of the EAAT-HEK293 cell lines to DL-TBOA, (±)-T3MG, DHK, KA or (*S*)-TDPA in 3 mM concentrations did not give rise to significant responses (data not shown). Instead these ligands inhibited the L-Glu-induced response in concentration-dependent manner (Fig. 5 and Table 2). Finally, D-Glu was found to be virtually inactive at all three transporters both as a substrate and as a non-substrate inhibitor at concentrations up to 1 mM (Table 2).

The K_m and K_i values obtained for substrates and inhibitors in the FMP assay were typically one- to three-fold, one- to fivefold and two- to sevenfold lower than the corresponding K_i values in the [^{3}H]-D-Asp uptake assay at EAAT1, EAAT2 and EAAT3, respectively (Tables 1 and 2). However, L-Asp, D-Asp, L-*trans*-2,4-PDC and *cis*-

Table 2
Pharmacological characteristics of human EAAT1, EAAT2 and EAAT3 stably expressed in HEK293 cell lines in the FMP assay

Compounds	EAAT1		EAAT2		EAAT3	
	K_m ($pK_m \pm$ S.E.M.)	$R_{max} \pm$ S.E.M.	K_m ($pK_m \pm$ S.E.M.)	$R_{max} \pm$ S.E.M.	K_m ($pK_m \pm$ S.E.M.)	$R_{max} \pm$ S.E.M.
Substrates						
L-Glu	6.8 (5.2 ± 0.05)	100	11 (4.9 ± 0.11)	100	6.6 (5.2 ± 0.05)	100
D-Glu	>1000 (<3)[a]	–	>1000 (<3)[a]	–	>1000 (<3)[a]	–
L-Asp	6.1 (5.2 ± 0.11)	78 ± 7	5.5 (5.3 ± 0.07)	93 ± 11	3.2 (5.5 ± 0.03)	63 ± 9
D-Asp	8.9 (5.1 ± 0.14)	21 ± 5	6.9 (5.2 ± 0.07)	87 ± 8	3.3 (5.5 ± 0.04)	52 ± 4
L-*trans*-2,4-PDC	19 (4.7 ± 0.08)	55 ± 5	7.7 (5.1 ± 0.12)	62 ± 5	11 (5.0 ± 0.07)	12 ± 6
cis-ACBD	52 (4.3 ± 0.09)	112 ± 11	170 (3.8 ± 0.13)	74 ± 8	27 (4.6 ± 0.11)	78 ± 9
L-THA	3.6 (5.4 ± 0.11)	61 ± 8	3.8 (5.4 ± 0.08)	57 ± 6	3.2 (5.5 ± 0.11)	54 ± 4
	K_i ($pK_i \pm$ S.E.M.)		K_i ($pK_i \pm$ S.E.M.)		K_i ($pK_i \pm$ S.E.M.)	
Non-substrate inhibitors						
DL-TBOA	2.8 (5.6 ± 0.10)		0.59 (6.2 ± 0.15)		1.8 (5.7 ± 0.05)	
(±)-T3MG	190 (3.7 ± 0.04)		62 (4.2 ± 0.06)		270 (3.6 ± 0.05)	
DHK	>3000 (<2.5)		31 (4.5 ± 0.09)		>3000 (<2.5)	
KA	>3000 (<2.5)		60 (4.2 ± 0.12)		>3000 (<2.5)	
(*S*)-TDPA	240 (3.6 ± 0.02)		170 (3.8 ± 0.12)		150 (3.8 ± 0.12)	

The assays were performed as described in Section 2. The K_m and K_i values are given in μM (with $pK_m \pm$ S.E.M. or $pK_i \pm$ S.E.M. values in parenthesis), and the R_{max} values for the substrates are given as % of R_{max} of L-Glu.
[a] 1 mM D-Glu gave rise to weak responses in all three cell lines.

ACBD were up to 10-fold more potent at EAAT3 in the FMP assay than in the uptake assay, and (±)-T3MG was 13-fold more potent as an EAAT1 inhibitor in the FMP assay. The K_m values for L-Glu, L-Asp, D-Asp, L-THA, L-*trans*-2,4-PDC at the three transporters in the FMP assay were generally one- to fourfold lower than those obtained by Amara and coworkers at EAATs expressed in *Xenopus* oocytes [24]. However, L-THA was eight- to ninefold more potent on EAAT1 and EAAT3, and D-Asp displayed a 14-fold higher potency at EAAT3 in the FMP assay [24]. The K_i values for the inhibitors at EAAT2 were threefold higher (DHK, KA and (±)-T3MG) or threefold lower (DL-TBOA) in the FMP assay than in electrophysiology studies using oocytes [24,26,27].

The prolific nature of the FMP assay enabled us to study the interactions of the EAAT with its substrates and inhibitors in some detail. The inhibition of the L-Glu uptake by EAAT2 mediated by DL-TBOA are depicted in Fig. 6A. The inhibitor displayed increasing IC_{50} values in presence of increasing concentrations of substrate, whereas the calculated K_i values of the compound in the presence of different L-Glu concentrations were not significantly different. Furthermore, increasing concentrations of DL-TBOA shifted the concentration–response curves for L-Glu towards the right without reducing the maximal responses of the substrate (Fig. 6A).

In the FMP assay, the substrates D-Asp, L-THA and L-*trans*-2,4-PDC displayed significantly smaller maximal responses at the three EAATs compared to those of L-Glu (Fig. 5). We used this diversity to explore the different interactions of "full" and "partial" substrates with the EAATs. Concentration–response relationships for the three partial substrates at the transporters when co-applied with two different concentrations of the full substrate, L-Glu, are depicted in Fig. 6B. The partial substrate enhanced the responses elicited by 3 μM L-Glu (EC_{20}–EC_{30}) to the level of its own maximal responses at all three EAATs. Conversely, at sufficiently high concentrations the partial substrate suppressed the response caused by 100 μM L-Glu (EC_{100}) down towards its own R_{max} value and in some cases even past it (Fig. 6B).

4. Discussion

Plasma membrane bound neurotransmitter transporters have been divided into two families: the Na^+-dependent glutamate/aspartate transporters and the Na^+/Cl^--dependent neurotransmitter transporters, which includes transporters for the monoamines norepinephrine, serotonin and dopamine, and amino acids like GABA and glycine [28]. In this study, we have taken advantage of the electrogenic transport process of the EAATs and implemented a fluorescence-based pharmacological assay for the transporters. In recent years, fluorescence-based assays have been used extensively in studies of other membrane-bound proteins like G-protein coupled receptors and voltage- and ligand-gated ion channels [29–34]. Whereas scintillation proximity assays have been reported for various transporter systems [35–37], it is to our knowledge the first time a fluorescence-based HTS assay has been reported for any neurotransmitter transporter.

The pharmacological profiles of the EAAT1-, EAAT2- and EAAT3-HEK293 cell lines in the FMP assay correlated well with those obtained in the [^{3}H]-D-Asp uptake assay (Fig. 7). The potencies of the 12 ligands were in general higher in the FMP assay than in the [^{3}H]-D-Asp uptake assay. Analogously, substrate potencies at the three

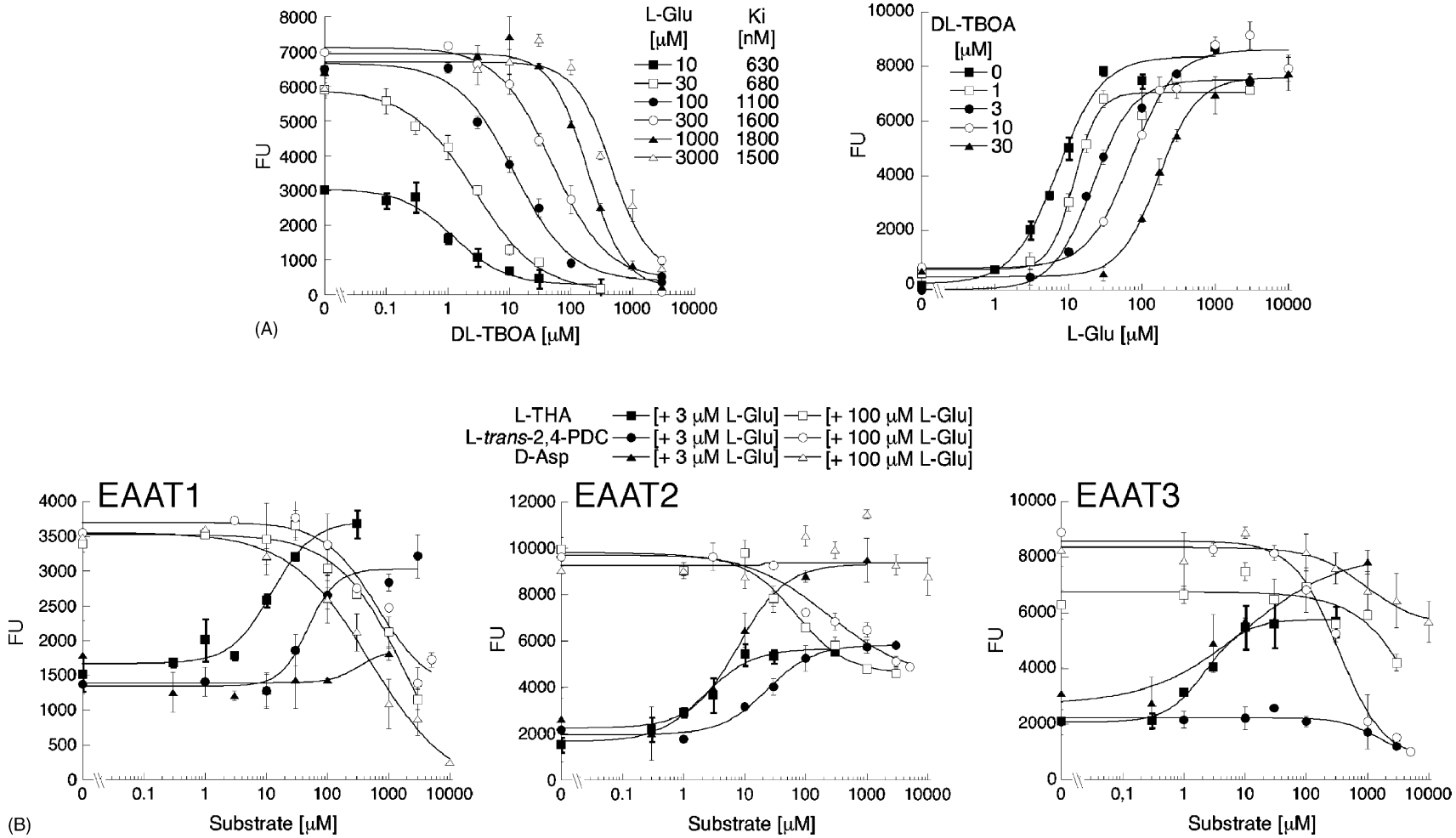

Fig. 6. Ligand–EAAT interactions. (A) Inhibition of EAAT2 uptake by DL-TBOA. Concentration–inhibition curves for DL-TBOA at the EAAT2-HEK293 cell line in the presence of various concentrations of L-Glu (right panel) and concentration–response curves for the substrate in the presence of different concentrations of DL-TBOA (Schild analysis, left panel). (B) Competition of full and partial substrates for EAAT transport. Concentration–response curves for partial agonists D-Asp, L-THA and L-*trans*-2,4-PDC in the presence of 3 μM (EC_{20}–EC_{30}) and 100 μM (EC_{100}) concentrations of L-Glu.

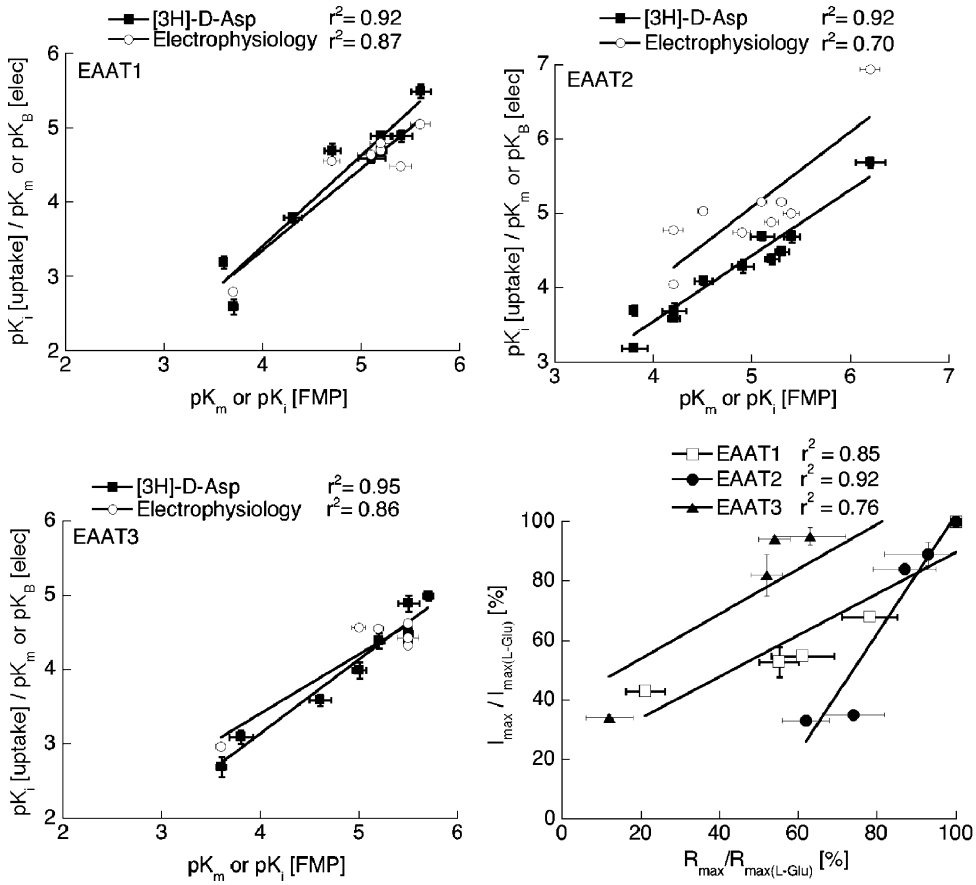

Fig. 7. The FMP assay compared to other assays. Correlations between the pK_m and pK_i values from the FMP assay and the corresponding pK_i values from the [^{3}H]-D-Asp uptake assay and pK_m and pK_B values from electrophysiological studies of the EAATs in *Xenopus* oocytes [24,27,43]. Bottom panel, right: correlations between the R_{max} values of substrates from the FMP assay and the I_{max} values from an electrophysiological study of the EAATs in *Xenopus* oocytes [24].

transporters obtained in conventional electrophysiology assays have been reported to be higher than those obtained in [^{3}H]-D-Asp and [^{3}H]-L-Glu uptake assays [24]. L-Glu, L-Asp and D-Asp were equipotent at the three EAAT subtypes, whereas D-Glu was inactive in concentrations up to 1 mM. In agreement with previous studies L-*trans*-2,4-PDC and LTHA exhibited similar K_m values as the endogenous substrates, whereas *cis*-ACBD was 5- to 30-fold weaker as a substrate at the three transporters (Table 2) [22,24,38–41]. Also in agreement with previous studies, DHK and KA were highly selective EAAT2-inhibitors, (±)-T3MG was equipotent with KA at EAAT2, and DL-TBOA turned out to be the most potent of the five inhibitors characterized (Table 2) [22,23,26,27,42,43]. The major discrepancy between the FMP assay and other assays was the degree of EAAT2-selectivity observed for the inhibitors DL-TBOA, (±)-T3MG and (*S*)-TDPA (Table 2). DL-TBOA has been reported to be equipotent at EAAT2 and EAAT3, whereas it has displayed 7- and 77-fold lower potencies at EAAT1 in [^{14}C]glutamate uptake and electrophysiological experiments, respectively [23,27]. (±)-T3MG has displayed 20- to 50-fold and 12-fold higher potencies at EAAT2 than at EAAT1 and EAAT3, respectively [26,42,43], and we have previously reported (*S*)-TDPA to be at least 10-fold more potent at EAAT2 than at EAAT1 and EAAT3 [20]. In the FMP assay, DL-TBOA, (±)-T3MG and (*S*)-TDPA displayed K_i^{EAAT1}/K_i^{EAAT2} ratios of 4.7, 3.1 and 1.4, respectively, and the corresponding K_i^{EAAT1}/K_i^{EAAT2} ratios in the [^{3}H]-D-Asp assay were 1.3, 10.5 and 3.3 (Table 1). Thus, we were able to reproduce the EAAT2 selectivity of (±)-T3MG in the uptake assay, whereas the lower degree of EAAT2 selectivity for (*S*)-TDPA and EAAT2/3-selectivity for DL-TBOA appeared to be an assay-independent observation. Since the K_i values for DL-TBOA and (±)-T3MG at EAAT2 in the FMP assay were similar to K_B or K_i values from previous electrophysiology and uptake studies, it appeared to be higher potencies of the compounds at EAAT1 that caused the reduced degree of subtype selectivity [23,26,27,43]. Interestingly, however, the prototypic EAAT2-selective inhibitors DHK and KA did not exhibit lower degrees of EAAT2-selectivity in the FMP assay (Table 2).

An important feature of the FMP assay is its ability to distinguish the actions of substrates and non-substrate inhibitors at the transporters. In conventional uptake assays, both substrates and inhibitors will reduce the uptake of the radioligand (Fig. 2). Thus, substrates and inhibitors have typically been identified in electrophysiological recordings

of EAATs expressed in oocytes or in "hetero-exchange" assays measuring the cellular exchange of radioligand (preloaded onto the cells) with the test compounds [24,41]. Considering the labor-intensive nature of these assays and the health-related concerns about working with isotopes, the FMP assay seems to be superior to both these assays in this respect. In agreement with previous studies L-Glu, L-Asp, D-Asp, *cis*-ACBD, L-*trans*-2,4-PDC and L-THA were determined to be substrates at all three EAATs in the FMP assay, whereas DL-TBOA, (±)-T3MG, DHK, KA and (*S*)-TDPA were non-substrate inhibitors (Fig. 5 and Table 2) [24,26,27,39–41,43]. A certain degree of caution is in order when interpreting the varying degrees of maximal responses exhibited by the different substrates in a membrane potential assay, since the interactions of the compounds with the transporters could alter the ion flux ratios for a number of reasons. However, the R_{max} values determined for the substrates in the FMP assay were in reasonable good agreement with those determined in a electrophysiology study of EAATs expressed in *Xenopus* oocytes, the major discrepancy being that the maximal responses of partial substrates L-Asp, D-Asp and L-THA at EAAT3 in the FMP assay were considerably smaller than in the electrophysiology study (Fig. 7) [24]. Hence, the assay appears to be capable of discriminating between full and partial substrates at the transporters.

The prolific nature and the high reproducibility of the FMP assay make it ideal for complex studies of ligand interactions and competition at the EAATs. As it was to be expected, DL-TBOA displayed a pharmacological profile at EAAT2 characteristic for an inhibitor competing with the substrate for the binding site (Fig. 6A). The four other inhibitors in this study [(±)-T3MG, DHK, KA and (*S*)-TDPA] also inhibited L-Glu transport through EAAT2 in a competitively manner, and DL-TBOA and (±)-T3MG were also determined to be competitive inhibitors of EAAT1 and EAAT3 transport (data not shown). Furthermore, the stimulatory and inhibitory effects on the L-Glu-induced responses in the EAAT-cell lines exerted by the partial substrates D-Asp, L-*trans*-2,4-PDC and L-THA correlated nicely with their respective R_{max} values (Fig. 6B). Although EC_{100} responses of L-Glu in some cases were suppressed to levels below the R_{max} values of the partial substrates, in general the observations in these experiments were in excellent agreement with the analogous concept of "partial agonism" in classical receptor theory [44]. All in all, the FMP assay seems to be an excellent assay for detailed studies of ligand–EAAT interactions. On the other hand, considering the 5.8-s period between measurements prior to and following substrate application (Fig. 3A), it may not be the assay of choice for sophisticated studies of kinetics aspects of the transport process.

As mentioned above, almost all of the EAAT ligands presently available are amino acids or amino acid-derived compounds acting as substrates or non-substrate inhibitors through the L-Glu recognition site of the transporters [14]. In addition to the low potencies and low subtype selectivities displayed by most of these compounds, the amino acid structure also presents several drug delivery problems, for example in terms of blood–brain barrier penetration. Furthermore, although inhibition of EAAT activity in a non-excitotoxic manner has been proposed as a way of intervening in the glutamatergic hypofunction associated with some cognitive disorders and in certain forms of schizophrenia, the problem in most disorders involving glutamatergic neurotransmission is an excessive signaling of the neurotransmitter [1–3]. Hence, while selective inhibitors of the reverse L-Glu transport observed in certain states of ischemia and epilepsy could be of therapeutic interest [7,15], administration of substrates or inhibitors competing L-Glu for binding to the EAAT would not be useful in most of these disorders. Since these ligands would lead to a decreased synaptic reuptake of L-Glu resulting in increased extracellular levels of the neurotransmitter, they could in fact be more harmful than beneficial. Instead, the ideal drug targeted at EAATs in states of hyperglutamatergic transmission would be a positive allosteric modulator, i.e. a compound facilitating L-Glu uptake through the EAAT by binding to an allosteric site at the transporter. EAAT function has been demonstrated to be modulated by allosteric ligands as diverse as Zn^{2+} (EAAT1 and EAAT4), arachidonic acid (EAAT2), niflumic acid (EAAT4) and phorbol esters (GLAST-1) (reviewed in [44]). However, most of the allosteric modulators identified are inhibitors, and they are unlikely candidates for drug development, if nothing else because of their low potencies [45]. The ability to screen libraries of structurally diverse compounds at EAATs in a HTS assay could facilitate the identification of novel ligand structures acting as orthosteric or allosteric ligands at the EAATs. Considering the ability of the FMP assay to quantify the maximal responses of the six substrates in this study, the assay should be able to identify compounds increasing the potency and/or the maximal response of L-Glu at the EAATs. The assay could also be useful in the search for novel compound structures to be used as leads in the development of EAAT inhibitors characterized by higher degrees of subtype selectivities and better drug delivery properties.

In conclusion, stable cell lines expressing human EAAT1, EAAT2 and EAAT3 have been constructed and a fluorescence-based HTS assay for characterization of the transporters has been implemented. In the FMP assay, the cell lines exhibited pharmacological characteristics in good agreement with the profiles obtained in a classical [^{3}H]-D-Asp uptake assay and with the properties reported from previous electrophysiological studies. The prolific nature and reproducibility of the FMP assay will be of great use in future studies of EAAT–ligand interactions, and the ability to screen compound libraries at the transporters could facilitate the discovery of EAAT ligands with novel structural and pharmacological properties.

Acknowledgments

The authors wish to thank Dr. Susan Amara for the human EAAT cDNAs. This work was supported by the Augustinus Foundation, the Director Ib Henriksen Foundation, the Danish Medical Research Council and the Lundbeck Foundation.

References

[1] Wheal HV, Thomson AM. Excitatory amino acids and synaptic transmission. London; 1995.
[2] Meldrum BS. The glutamate synapse as a therapeutical target: perspectives for the future. Prog Brain Res 1998;116:441–58.
[3] Bräuner-Osborne H, Egebjerg J, Nielsen EØ, Madsen U, Krogsgaard-Larsen P. Ligands for glutamate receptors: design and therapeutic prospects. J Med Chem 2000;43:2609–45.
[4] Jensen AA. Molecular pharmacology of the metabotropic glutamate receptors. In: Schousboe A, Bräuner-Osborne H, editors. Molecular neuropharmacology. Strategies and methods. Totowa (NJ, USA): Humana Press, 2004. p. 47–82.
[5] Dingledine R, Borges K, Bowie D, Traynelis SF. The glutamate receptor ion channels. Pharmacol Rev 1999;51:7–61.
[6] Slotboom DJ, Konings WN, Lolkema JS. Structural features of the glutamate transporter family. Microbiol Mol Biol Rev 1999;63: 293–307.
[7] Seal RP, Amara SG. Excitatory amino acid transporters: a family in flux. Annu Rev Pharmacol Toxicol 1999;39:431–56.
[8] Clements JD. Transmitter timecourse in the synaptic cleft: its role in central synaptic function. Trends Neurosci 1996;19:163–71.
[9] Wadiche JI, Amara SG, Kavanaugh MP. Ion fluxes associated with excitatory amino acid transport. Neuron 1995;15:721–8.
[10] Levy LM, Warr O, Attwell D. Stoichiometry of the glial glutamate transporter GLT-1 expressed inducibly in a Chinese hamster ovary cell line selected for low endogenous Na^+-dependent glutamate uptake. J Neurosci 1998;18:9620–8.
[11] Zerangue N, Kavanaugh MP. Flux coupling in a neuronal glutamate transporter. Nature 1996;383:634–7.
[12] Danbolt NC. Glutamate uptake. Prog Neurobiol 2001;65:1–105.
[13] O'Shea RD. Roles and regulation of glutamate transporters in the central nervous system. Clin Exp Pharmacol Physiol 2002;29: 1018–23.
[14] Campiani G, Fattorusso C, De Angelis M, Catalanotti B, Butini S, Fattorusso R, et al. Neuronal high-affinity sodium-dependent glutamate transporters (EAATs): targets for the development of novel therapeutics against neurodegenerative diseases. Curr Pharm Des 2003;9:599–625.
[15] Rossi DJ, Oshima T, Attwell D. Glutamate release in severe brain ischaemia is mainly by reversed uptake. Nature 2000;403:316–21.
[16] Rothstein JD, Van Kammen M, Levey AI, Martin LJ, Kuncl RW. Selective loss of glial glutamate transporter GLT-1 in amyotrophic lateral sclerosis. Ann Neurol 1995;38:73–84.
[17] Tanaka K, Watase K, Manabe T, Yamada K, Watanabe M, Takahashi K, et al. Epilepsy and exacerbation of brain injury in mice lacking the glutamate transporter GLT-1. Science 1997;276:1699–702.
[18] Rothstein JD, Dykes-Hoberg M, Pardo CA, Bristol LA, Jin L, Kuncl RW, et al. Knockout of glutamate transporters reveals a major role for astroglial transport in excitotoxicity and clearance of glutamate. Neuron 1996;16:675–86.
[19] Johansen TN, Janin YL, Nielsen B, Frydenvang K, Bräuner-Osborne H, Stensbøl TB, et al. 2-Amino-3-(3-hydroxy-1,2,5-thiadiazol-4-yl)propionic acid: resolution, absolute stereochemistry and enantiopharmacology at glutamate receptors. Bioorg Med Chem 2002;10:2259–66.
[20] Bräuner-Osborne H, Hermit MB, Nielsen B, Krogsgaard-Larsen P, Johansen TN. A new structural class of subtype-selective inhibitor of cloned excitatory amino acid transporter, EAAT2. Eur J Pharmacol 2000;606:41–4.
[21] Cheng Y, Prusoff WH. Relationship between the inhibition constant (K_1) and the concentration of inhibitor which causes 50 per cent inhibition (I_{50}) of an enzymatic reaction. Biochem Pharmacol 1973;22:3099–108.
[22] Dunlop J, Lou Z, Zhang Y, McIlvain HB. Inducible expression of the human excitatory amino acid transporter 2 subtype of L-glutamate transporter. Br J Pharmacol 1999;128:1485–90.
[23] Shimamoto K, Shigeri Y, Yasuda-Kamatani Y, Lebrun B, Yumoto N, Nakajima T. Syntheses of optically pure β-hydroxyaspartate derivatives as glutamate transporter blockers. Bioorg Med Chem Lett 2000;10:2407–10.
[24] Arriza JL, Fairman WA, Wadiche JI, Murdoch GH, Kavanaugh MP, Amara SG. Functional comparison of three glutamate transporter subtypes cloned from human motor cortex. J Neurosci 1994;14:5559–68.
[25] Kanai Y, Hediger MA. Primary structure and functional characterization of a high-affinity glutamate transporter. Nature 1992;360:467–71.
[26] Vandenberg RJ, Mitrovic AD, Chebib M, Balcar VJ, Johnston GAR. Contrasting modes of action of methylglutamate derivatives on the excitatory amino acid transporters EAAT1 and EAAT2. Mol Pharmacol 1997;51:809–15.
[27] Shimamoto K, Lebrun B, Yasuda-Kamatani Y, Sakaitani M, Shigeri Y, Yumoto N, et al. DL-*threo*-β-Benzyloxyaspartate, a potent blocker of excitatory amino acid transporters. Mol Pharmacol 1998;53:195–201.
[28] Nelson N. The family of Na^+/Cl^- neurotransmitter transporters. J Neurochem 1998;71:1785–803.
[29] Heding A, Elling CE, Schwartz TW. Novel method for the study of receptor Ca^{2+} signalling exemplified by the NK1 receptor. J Recept Signal Transduct Res 2002;22:241–52.
[30] Whiteaker KL, Gopalakrishnan SM, Groebe D, Shieh CC, Warrior U, Burns DJ, et al. Validation of FLIPR membrane potential dye for high throughput screening of potassium channel modulators. J Biomol Screen 2001;6:305–12.
[31] Fitch RW, Xiao Y, Kellar KJ, Daly JW. Membrane potential fluorescence: a rapid and highly sensitive assay for nicotinic receptor channel function. Proc Natl Acad Sci USA 2003;100:4909–14.
[32] Jensen AA, Mikkelsen I, Frølund B, Bräuner-Osborne H, Falch E, Krogsgaard-Larsen P. Carbamoylcholine homologs: novel and potent agonists at neuronal nicotinic acetylcholine receptors. Mol Pharmacol 2003;64:865–75.
[33] Adkins CE, Pillai GV, Kerby J, Bonnert TP, Haldon C, McKernan RM, et al. α4β3δ $GABA_A$ receptors characterized by fluorescence resonance energy transfer-derived measurements of membrane potential. J Biol Chem 2001;276:38934–9.
[34] Jensen AA, Kristiansen U. Functional characterisation of the human α1 glycine receptor in a fluorescence-based membrane-potential assay. Biochem Pharmacol (in press).
[35] Hallen S, Fryklund J, Sjostrom JE, Bonge H. Cytostar-T scintillating microplate assay for measurement of sodium-dependent bile acid uptake in transfected HEK-293 cells. Anal Biochem 2000;282: 94–101.
[36] Williams JB, Mallorga PJ, Lemaire W, Williams DL, Na S, Patel S, et al. Development of a scintillation proximity assay for analysis of Na^+/Cl^--dependent neurotransmitter transporter activity. Anal Biochem 2003;321:31–7.
[37] Graves R, Davies R, Brophy G, O'Beirne G, Cook N. Noninvasive, real-time method for the examination of thymidine uptake events—application of the method to V-79 cell synchrony studies. Anal Biochem 1997;248:251–7.
[38] Esslinger CS, Koch HP, Kavanaugh MP, Philips DP, Chamberlin AR, Thompson CM, et al. Structural determinants of substrates and inhibitors: probing glutamate transporters with 2,4-methanopyrrolidine-2,4-dicarboxylate. Bioorg Med Chem Lett 1998;8:3101–6.

[39] Koch HP, Kavanaugh MP, Esslinger CS, Zerangue N, Humphrey JM, Amara SG, et al. Differentiation of substrate and nonsubstrate inhibitors of the high-affinity, sodium-dependent glutamate transporters. Mol Pharmacol 1999;56:1095–104.

[40] Lebrun B, Sakaitani M, Shimamoto K, Yasuda-Kamatani Y, Nakajima T. New β-hydroxyaspartate derivatives are competitive blockers for the bovine glutamate/aspartate transporter. J Biol Chem 1997;272: 20336–9.

[41] Chamberlin AR, Koch HP, Bridges RJ. Design and synthesis of conformationally constrained inhibitors of high-affinity, sodium-dependent glutamate transporters. In: Amara SG, editor. Methods enzymol, vol. 296. San Diego: Academic Press; 1998. p. 175–89.

[42] Mitrovic AD, Amara SG, Johnston GA, Vandenberg RJ. Identification of functional domains of the human glutamate transporters EAAT1 and EAAT2. J Biol Chem 1998;273:14698–706.

[43] Eliasof S, McIlvain HB, Petroski RE, Foster AC, Dunlop J. Pharmacological characterization of *threo*-3-methylglutamic acid with excitatory amino acid transporters in native and recombinant systems. J Neurochem 2001;77:550–7.

[44] Kenakin T. Pharmacological analysis of drug-receptor interaction. Philadelphia, NY: Lippencott-Raven; 1997.

[45] Vandenberg RJ, Ryan RM. Allosteric modulation of glutamate transporters. In: Schousboe A, Bräuner-Osborne H, editors. Molecular neuropharmacology. Strategies and methods. Totowa (NJ, USA): Humana Press, 2004. p. 161–74.