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# Characterization of Novel L-*threo*-β-Benzyloxyaspartate Derivatives, Potent Blockers of the Glutamate Transporters

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## ABSTRACT

Nontransportable blockers of the glutamate transporters are important tools for investigating mechanisms of synaptic transmission. DL-*threo*-β-Benzyloxyaspartate (DL-TBOA) is a potent blocker of all subtypes of the excitatory amino acid transporters (EAATs). We characterized novel L-TBOA analogs possessing a substituent on their respective benzene rings. The analogs significantly inhibited labeled glutamate uptake, the most potent of which was (2*S*,3*S*)-3-{3-[4-(trifluoromethyl)benzoylamino]benzyloxy}aspartate (TFB-TBOA). In an uptake assay using cells transiently expressing EAATs, the $IC_{50}$ values of TFB-TBOA for EAAT1, EAAT2, and EAAT3 were 22, 17, and 300 nM, respectively. TFB-TBOA was significantly more potent at inhibiting EAAT1 and EAAT2 compared with L-TBOA ($IC_{50}$ values for EAAT1–3 were 33, 6.2, and 15 μM, respectively). Electrophysiological analyses revealed that TBOA analogs block the transport-associated currents in all five EAAT subtypes and also block leak currents in EAAT5. The rank order of the analogs for potencies at inhibiting substrate-induced currents was identical to that observed in the uptake assay. However, the kinetics of TFB-TBOA differed from the kinetics of L-TBOA, probably because of the strong binding affinity. Notably, TFB-TBOA did not affect other representative neurotransmitter transporters or receptors, including ionotropic and metabotropic glutamate receptors, indicating that it is highly selective for EAATs. Moreover, intracerebroventricular administration of the TBOA analogs induced severe convulsive behaviors in mice, probably because of the accumulation of glutamate. Taken together, these findings indicate that novel TBOA analogs, especially TFB-TBOA, should serve as useful tools for elucidating the physiological roles of the glutamate transporters.

L-Glutamate is the major excitatory neurotransmitter in the mammalian central nervous system. Termination of glutamate receptor activation and maintenance of low extracellular glutamate concentrations are mainly achieved by glutamate transporters located in nerve endings and surrounding glia cells (Seal and Amara, 1999; Danbolt, 2001; Amara and Fontana, 2002; Campiani et al., 2003). Among the five cloned subtypes of excitatory amino acid transporters (EAAT1–5), the two glial transporters EAAT1 (GLAST) and EAAT2 (GLT-1) and the neuronal transporter EAAT3 (EAAC1) are primarily involved in synaptic glutamate homeostasis (Kanai and Hediger, 1992; Pines et al., 1992; Storck et al., 1992; Arriza et al., 1994, 1997; Fairman et al., 1995). EAAT4 and EAAT5 expressed in the cerebellum and retina, respectively, have specialized functions and mediate substrate-activated chloride conductance that is uncoupled from substrate translocation. Selective and potent inhibitors are needed to identify the physiological roles of transporters in the regulation of synaptic transmission or in the pathogenesis of neurological diseases. Selective inhibitors such as *threo*-β-hydroxyaspartate (THA) or pyrrolidine dicarboxylate (PDC) derivatives (*trans*-2,4-PDC, *trans*-2,3-PDC, and 2,4-methano-PDC) have served as important tools to address the function of the glutamate transporters (Bridges et al., 1999;

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**ABBREVIATIONS:** THA, *threo*-β-hydroxyaspartate; PDC, pyrrolidine dicarboxylate; TFB-TBOA, (2*S*,3*S*)-3-{3-[4-(trifluoromethyl)benzoylamino]benzyloxy}aspartate; L-TBOA, L-*threo*-β-benzyloxyaspartate [(2*S*,3*S*)-3-benzyloxyaspartate]; EAAT, human excitatory amino acid transporter; TBOA, *threo*-β-benzyloxyaspartate; A-TBOA, (2*S*,3*S*)-3-(3-aminobenzyloxy)aspartate; PivA-TBOA, (2*S*,3*S*)-3-[3-(pivaloylamino)-benzyloxy]aspartate; MDCK, Madin-Darby canine kidney; CGP39653, (*E*)-2-amino-4-propyl-5-phosphono-3-pentenoic acid ; KA, kainate; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; NMDA, *N*-methyl-D-aspartate; CHO, Chinese hamster ovary; DMSO, dimethyl sulfoxide; BzA-TBOA, (2*S*,3*S*)-3-{3-[(substituted benzoylamino)]benzyloxy}aspartate; PMB-TBOA, (2*S*,3*S*)-3-[3-(4-methoxybenzoylamino)benzyloxy]aspartate; CNB-TBOA, (2*S*,3*S*)-3-[3-(4-cyanobenzoylamino)benzyloxy]aspartate; mGluR, metabotropic glutamate receptor; DHKA, dihydrokainate.

Balcar 2002). We reported previously that introduction of a substituent on the hydroxyl group of THA effectively blocked the glutamate transport (Lebrun et al., 1997; Shimamoto et al., 1998). Among the THA derivatives, those possessing a bulky substituent such as benzyl (DL- or L-TBOA) or naphthyl group function as nontransportable blockers for all subtypes of EAATs (Shimamoto et al., 2000; Shigeri et al., 2001). However, a derivative with a small substituent such as methyl group acts as a partial substrate for EAAT1, EAAT3, or EAAT4 but blocks EAAT2 or EAAT5. These results demonstrate that a large substituent is required to block EAAT1, EAAT3, and EAAT4. Bulkier analogs would be expected to block ligand transport more efficiently in all EAATs. Therefore, we introduced substituents on the benzene ring of L-TBOA. We describe herein the characterization of substituted TBOA analogs, which were more potent than TBOA. Among them, (2*S*,3*S*)-3-{3-[4-(trifluoromethyl) benzoylamino]benzyloxy}aspartate (TFB-TBOA) was about 50 to 1500-fold more potent than L-TBOA in the uptake assay with EAAT1–3. Electrophysiological studies on *Xenopus laevis* oocytes expressing EAAT1–5 and behavioral experiments in mice and neonatal rats revealed potent blockade by new TBOA analogs.

## Materials and Methods

**Materials.** L-TBOA and its derivatives shown in Fig. 1 were synthesized as optically pure forms from a commercially available epoxide (2*S*,3*R*)-[3-(benzyloxymethyl)oxiranyl]methanol *para*-nitrobenzoate (Fluka Chemie AG, Buchs, Switzerland), and the structure and purity (>95%) of the compounds were confirmed by 400 MHz NMR and/or high-performance liquid chromatography. Details of the syntheses will be published elsewhere. Stock solutions (10 mM) were prepared in DMSO as trifluoroacetic acid salt except for L-TBOA, A-TBOA, PrA-TBOA, and PivA-TBOA, which were dissolved in water as salt-free form (10 mM). Stock solutions were stored at −20°C until the day of the experiments and were confirmed to be stable for more than 3 months. DL-TBOA was used in the behavioral experiments because L-TBOA was not available at the time when the experiment was conducted. Radiolabeled ligands were purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). All other chemicals were commercially available at highest purity from Nacalai Tesque (Kyoto, Japan), Sigma (St. Louis, MO), or Tocris Cookson (Bristol, UK).

**Glutamate Uptake Assay.** COS-1 cells were transfected and cultured as described previously (Shimamoto et al., 1998). Madin-Darby canine kidney (MDCK) cells stably expressing EAAT2 or EAAT3 were seeded onto 96-well plates and cultured in Dulbecco's modified Eagle's medium containing 10% dialyzed fetal bovine serum and antibiotics (G418) for 2 days before the assay (Takaoka et al., 2003). The uptake assay was performed as described previously (Shimamoto et al., 1998). The relative specific uptake of [$^{14}$C]glutamate (1 $\mu$M) was determined from triplicate experiments. Dose-response curves were fitted to the Hill equation using Origin software (OriginLab Corp, Northampton, MA), and $IC_{50}$ values were obtained from the dose-response curves and presented as mean ± S.E.M. from at least three experiments.

**Electrophysiology.** Capped RNAs transcribed from linearized pOTV-EAAT1, -2, -3, -4, or -5 using T7 RNA polymerase (mMessage mMachine; Ambion, Austin, TX) were microinjected into defollicu-

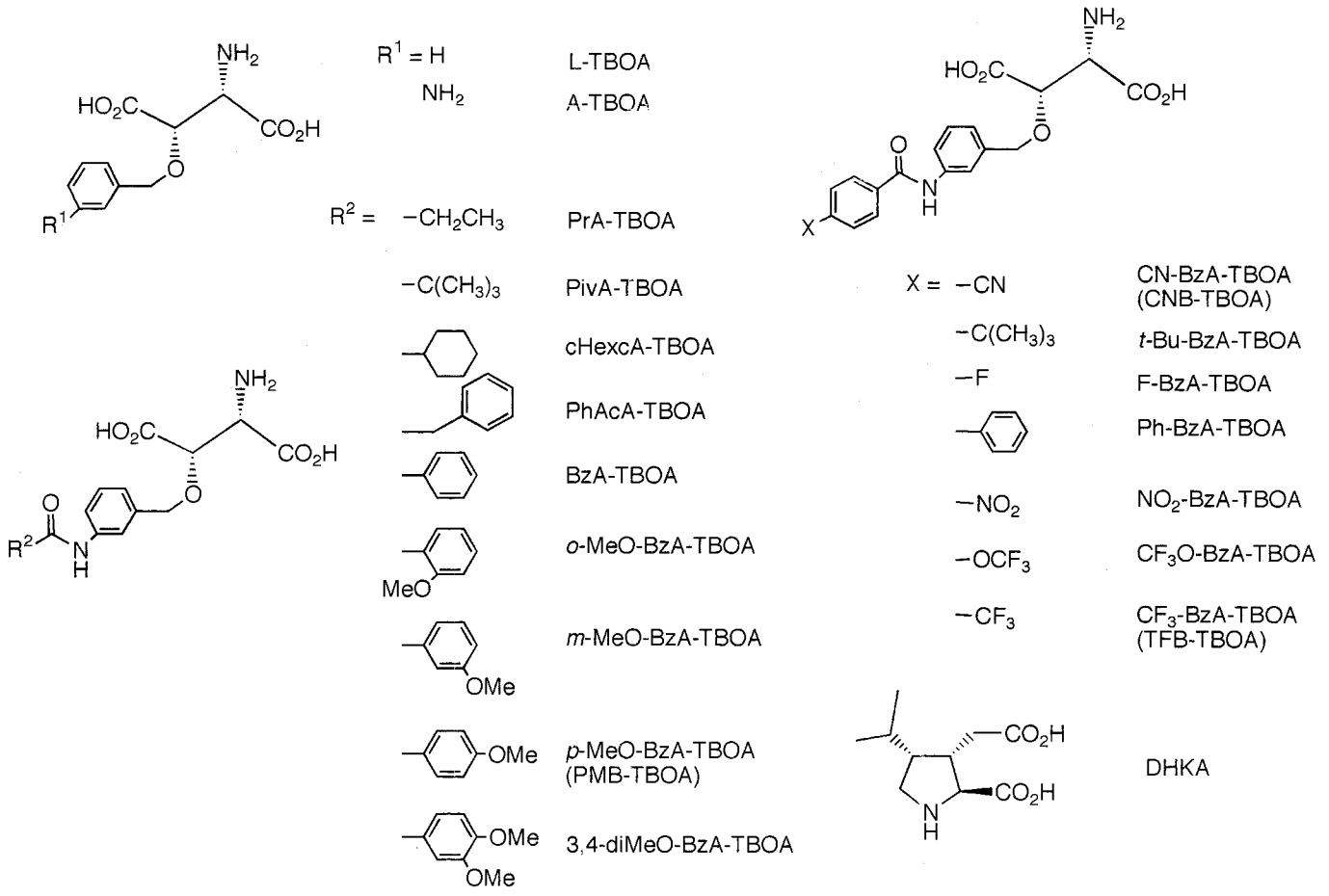


**Fig. 1.** Structures of L-TBOA, its analogs, and DHKA.

lated stage IV or V oocytes (~10 ng/oocyte). The oocytes were kept at 18°C for 2 to 6 days. Two-microelectrode voltage-clamp recordings were performed using a GeneClamp 500B amplifier with a Digidata 1322A interface (Axon Instruments, Foster City, CA) as described previously (Shimamoto et al., 1998; Shigeri et al., 2001). The pClamp8 (Axon Instruments) and Power Lab 4SP (AD Instruments, Castle Hill, NSW, Australia) were used to control stimulation parameters and data acquisition. Oocytes were usually clamped at −60 mV and continuously superfused with ND96 or with solutions containing test compounds, which flowed by gravity from one of several reservoirs. Switching from one reservoir to another was accomplished by Iso-Lach valves (Parker Hannifin Corporation, Fairfield, NJ) to control the application of test compounds. Substrates were usually applied to oocytes for 30 s in the presence or absence of the test compounds. Total currents were baseline-subtracted and calculated using Clampfit 9 (Axon Instruments) or Chart 4 for Windows (AD Instruments).

**Receptor Binding Assay.** Ionotropic glutamate receptor binding assays using rat brain synaptic membranes were conducted as described previously (Shimamoto et al., 2000). [$^3$H]CGP39653, [$^3$H]KA, and [$^3$H]AMPA were used as the radiolabeled ligands for NMDA, KA, and AMPA receptors, respectively (London and Coyle, 1979; Murphy et al., 1987; Sills et al., 1991). Binding assays for the other receptors and transporters reported under *Results* as well as glutamic acid decarboxylase assay were performed according to the procedures of MDS Pharma Services (Bothell, WA).

**Metabotropic Glutamate Receptor Assay.** CHO cells stably expressing mGluR1, -2, -4, or -5 were kindly donated by Prof. S. Nakanishi (Kyoto University, Kyoto, Japan). Agonist and antagonist activities for mGluR1 or mGluR5 were determined by intracellular $Ca^{2+}$ concentration measurements in Fura2-loaded CHO cells in the absence or presence of glutamate (10 $\mu$M), respectively, as described previously (Kawabata et al., 1996). Agonist activities for mGluR2 or mGluR4 were examined by measuring the inhibition of forskolin-induced cAMP formation as described previously (Hayashi et al., 1992). Antagonist activities were evaluated by measuring levels of prevention of glutamate (10 $\mu$M)-induced inhibition of forskolin-induced cAMP formation. A cAMP enzyme immunoassay kit (Amersham Biosciences, Piscataway, NJ) was used for cAMP measurement.

**Behavioral Experiments in Mice.** Male ddY mice (4–6 weeks old, 15.4 ± 4.0 g; Owada Experimental Animals, Iwate, Japan) were used. Intracerebroventricular (i.c.v.) administrations were performed according to a method described previously (Laursen and Belknap, 1986; Sakai et al., 2001). Mouse behavior was observed for 1 h. Convulsant behaviors were graded by severity as follows (Tasker et al., 1991): normal = 0; hypoactive = 1; rigid posture or occasional head scratching = 2; violent episodes including jumping, running or scratching accompanied by rigidity in hind and forelimbs = 3; whole body convulsion = 4; death after whole body convulsion = 5; and immediate death within 1 min = 6. The most severe symptom observed in the session was assigned as the behavioral score for each mouse. At least three animals were tested at each dose. Total number of mice used for each experiment is indicated in Table 3. Dose-response curves were generated with Origin computer software. All the animals were maintained according to the National Research Council's Guide for the Care and Use of Laboratory Animals, and the experiments were approved by the Animal Welfare, Care and Use Committee at Kitasato University.

**Neonatal Rat Models.** Litters of Sprague-Dawley rats were removed from dams at 8 days (17.3 ± 1.5 g for TFB-TBOA, 20.5 ± 1.0 g for DL-TBOA, 19.0 ± 3.0 g for DMSO; $n$ = 3) and at 16 days old (34.0 ± 2.5 g; $n$ = 3) (Schoepp et al., 1990). DMSO (10%) solution of TBOA or TFB-TBOA diluted with water for final volume of 0.5 ml (final concentration of DMSO was about 4%) were administrated intraperitoneally. Behaviors of the animals were observed for 2 h.

**Radiolabeled Glutamate Uptake Assay.** First, the effects of substituted analogs were compared with effects of nonsubstituted L-TBOA (Table 1, run 1–7). Inhibition of glutamate uptake by the analogs was assessed in MDCK cells stably expressing EAAT2 (glial transporter) or EAAT3 (neuronal transporter) (Takaoka et al., 2003). The potency of A-TBOA (run 2), an amino-substituted analog, was virtually equivalent to that of L-TBOA for EAAT2 but slightly less potent for EAAT3. Acylation of the amino group of A-TBOA en-

TABLE 1

Inhibition of [$^{14}$C]glutamate uptake in MDCK cells stably expressing EAAT2 or EAAT3

$IC_{50}$ values were obtained from the dose-response inhibition curves. Values are presented as mean ± S.E.M. from at least three independent experiments.

| Run | Compound | $IC_{50}$ | | Ratio ($IC_{50EAAT3}/IC_{50EAAT2}$) |
|---|---|---|---|---|
| | | EAAT2 | EAAT3 | |
| | | *nM* | | |
| 1 | L-TBOA | 1300 ± 120 | 1300 ± 120 | 1.0 |
| 2 | A-TBOA | 2100 ± 100 | 7900 ± 760 | 3.8 |
| 3 | PrA-TBOA | 1200 ± 100 | 4100 ± 390 | 3.4 |
| 4 | PivA-TBOA | 980 ± 130 | 3500 ± 380 | 3.6 |
| 5 | cHexcA-TBOA | 244 ± 12 | 288 ± 8.0 | 1.2 |
| 6 | PhAcA-TBOA | 143 ± 7.9 | 529 ± 32 | 3.7 |
| 7 | BzA-TBOA | 55 ± 5.0 | 729 ± 98 | 13 |
| 8 | *o*-MeO-BzA-TBOA | 145 ± 2.2 | 1000 ± 42 | 7.0 |
| 9 | *m*-MeO-BzA-TBOA | 37 ± 2.5 | 356 ± 12 | 9.6 |
| 10 | PMB-TBOA | 12 ± 0.5 | 266 ± 20 | 22 |
| 11 | 3,4-MeO-BzA-TBOA | 49 ± 3.8 | 917 ± 30 | 19 |
| 12 | CNB-TBOA | 36 ± 2.9 | 1400 ± 99 | 39 |
| 13 | *t*-Bu-BzA-TBOA | 25 ± 0.5 | 182 ± 11 | 7.3 |
| 14 | F-BzA-TBOA | 22 ± 2.7 | 473 ± 13 | 22 |
| 15 | Ph-BzA-TBOA | 21 ± 2.2 | 34 ± 19 | 1.6 |
| 16 | $NO_2$-BzA-TBOA | 14 ± 1.4 | 278 ± 32 | 20 |
| 17 | $CF_3O$-BzA-TBOA | 7.0 ± 0.5 | 128 ± 12 | 18 |
| 18 | TFB-TBOA | 1.9 ± 0.1 | 28 ± 1.6 | 15 |
| 19 | DHKA | 56 ± 9.7 $\mu$M | 1000 ± 100 $\mu$M | 18 |

PrA-TBOA, (2*S*,3*S*)-3-[3-(propionylamino)benzyloxy]aspartate; *c*HexcA-TBOA, (2*S*,3*S*)-3-[3-(cyclohexylcarbonylamino)benzyloxy]aspartate; PhAcA-TBOA, (2*S*,3*S*)-3-{3-[(phenylacetyl)amino]benzyloxy}aspartate; BzA-TBOA, (2*S*,3*S*)-3-[3-(benzoylamino)benzyloxy]aspartate; *o*-MeO-BzA-TBOA, (2*S*,3*S*)-3-[3-(2-methoxybenzoylamino)-benzyloxy]aspartate; *m*-MeO-BzA-TBOA, (2*S*,3*S*)-3-[3-(3-methoxybenzoylamino)benzyloxy]aspartate; *p*-MeO-BzA-TBOA (PMB-TBOA), (2*S*,3*S*)-3-[3-(4-methoxybenzoylamino)benzyloxy]aspartate; 3,4-MeO-BzA-TBOA, (2*S*,3*S*)-3-[3-(3,4-methoxybenzoylamino)benzyloxy]aspartate; CNB-TBOA, (2*S*,3*S*)-3-[3-(4-cyanobenzoylamino)benzyloxy]aspartate; *t*-Bu-BzA-TBOA, (2*S*,3*S*)-3-[3-(4-*tert*-butylbenzoylamino)benzyloxy]aspartate; F-BzA-TBOA, (2*S*,3*S*)-3-[3-(4-fluorobenzoylamino)benzyloxy]aspartate; Ph-BzA-TBOA, (2*S*,3*S*)-3-[3-(4-phenylbenzoylamino)benzyloxy]aspartate; $NO_2$-BzA-TBOA, (2*S*,3*S*)-3-[3-(4-nitrobenzoylamino)benzyloxy]aspartate; $CF_3O$-BzA-TBOA, (2*S*,3*S*)-3-{3-[4-(trifluoromethoxy)benzoylamino]benzyloxy}aspartate; TFB-TBOA, (2*S*,3*S*)-3-{3-[4-(trifluoromethyl)benzoylamino]benzyloxy}aspartate.

TABLE 2
Inhibition of [$^{14}$C]glutamate uptake in COS-1 cells transiently expressing EAAT1, EAAT2, or EAAT3

$IC_{50}$ values were obtained from the dose-response inhibition curves. Values are presented as mean ± S.E.M. from at least three independent experiments.

| Compound | $IC_{50}$ | | |
|---|---|---|---|
| | EAAT1 | EAAT2 | EAAT3 |
| | | *nM* | |
| L-TBOA | 33,000 ± 3700 | 6200 ± 490 | 15,000 ± 1300 |
| PivA-TBOA | 44,000 ± 3500 | 1000 ± 120 | 23,000 ± 1200 |
| CNB-TBOA | 286 ± 45 | 105 ± 5.1 | 5300 ± 230 |
| PMB-TBOA | 116 ± 4.5 | 59 ± 6.1 | 2400 ± 280 |
| TFB-TBOA | 22 ± 0.4 | 17 ± 1.0 | 300 ± 45 |
| DHKA | >1,000,000 | 196,000 ± 56,000 | >1,000,000 |

hanced the inhibitory activity for both EAAT2 and EAAT3. Acyl groups possessing aromatic moieties (phenylacetyl and benzoyl groups) were more effective than acyl groups with aliphatic moieties (propionyl, pivaloyl, and cyclohexylcarbonyl groups). The benzoyl derivative BzA-TBOA (run 7), was 25-fold more potent ($IC_{50}$ = 55 ± 5.0 nM) than L-TBOA ($IC_{50}$ = 1300 ± 120 nM) at inhibiting glutamate uptake by EAAT2. Both potency and selectivity were improved by the introduction of a benzoyl group (ratio of $IC_{50EAAT3}$/ $IC_{50EAAT2}$ = 13). Thus, we investigated the effects of various substituted-benzoyl analogs (X-BzA-TBOAs, run 8–18). Among the synthetic analogs, TFB-TBOA (run 18: X = $CF_3$) was the most potent inhibitor of glutamate uptake by both EAAT2 and EAAT3 ($IC_{50EAAT2}$ = 1.9 ± 0.1 nM, $IC_{50EAAT3}$ = 28 ± 1.6 nM, $IC_{50EAAT3}$/ $IC_{50EAAT2}$ = 15). Although PMB-TBOA (run 10: X = $OCH_3$) ($IC_{50EAAT2}$ = 12 ± 0.5 nM, $IC_{50EAAT3}$ = 266 ± 20 nM, $IC_{50EAAT3}$/ $IC_{50EAAT2}$ = 22) was slightly less potent than TFB-TBOA, it could still be a useful inhibitor if its methoxy group was labeled with a radioactive isotope. On the other hand, CNB-TBOA (run 12: X = CN) was the most selective compound for EAAT2 and EAAT3 ($IC_{50EAAT2}$ = 36 ± 2.9 nM, $IC_{50EAAT3}$ = 1400 ± 99 nM, $IC_{50EAAT3}$/ $IC_{50EAAT2}$ = 39). It is noteworthy that CNB-TBOA was more selective than DHKA ($IC_{50EAAT3}$/ $IC_{50EAAT2}$ = 18), which weakly inhibited uptake at 1 mM by EAAT3 expressed in MDCK cells, although it did not affect uptake by EAAT3 expressed in COS-1 cells or *Xenopus laevis* oocytes. Therefore, we chose to further characterize TFB-TBOA, PMB-TBOA, and CNB-TBOA among the X-BzA-TBOA analogs.

To compare the effects of the analogs on the other widely distributed glial transporter EAAT1, we next examined analog inhibition of glutamate uptake by EAAT1, EAAT2, and EAAT3 transiently expressed in COS-1 cells (Table 2) (Shimamoto et al., 1998, 2000). Levels of glutamate uptake by EAAT4 or EAAT5 expressed in COS-1 cells or *Xenopuslaevis* oocytes were too low for measurement of specific uptake; reportedly, more than 95% of observed steady-state currents are attributed to chloride conductance uncoupled from substrate translocation (Bergles et al., 2002). COS-1 cells were slightly

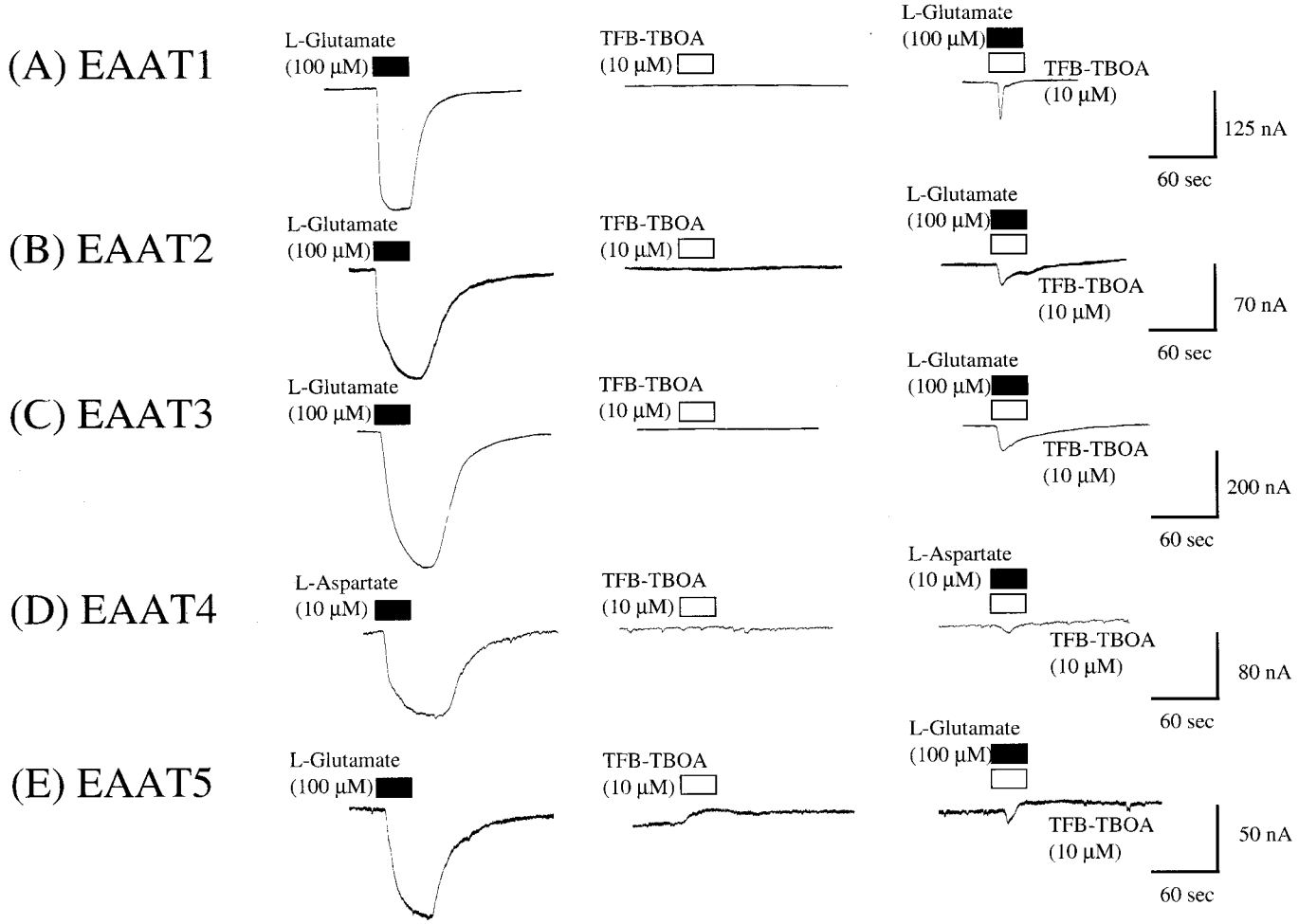


**Fig. 2.** Effects of TFB-TBOA on EAAT subtypes. The compounds were applied to EAAT-expressing oocytes voltage-clamped at −60 mV. A–E, results of EAATs 1 to 5, respectively. Left, L-Glutamate (100 μM) was used as substrate for EAAT1, -2, -3, and -5 and L-aspartate (10 μM) was used as substrate for EAAT4. They induced large inward currents. Middle, application of TFB-TBOA (10 μM) alone did not elicit any currents in EAAT1–4 but induced small outward currents in EAAT5. Right, the glutamate-induced currents were significantly attenuated by the coapplication with TFB-TBOA (10 μM). Nearly identical results were observed in four different cells.

less sensitive than MDCK cells but the rank of the ligands by potency was similar in the two cell-lines. EAAT1 was less sensitive than EAAT2 or EAAT3 to inhibition by L-TBOA. Although the $IC_{50}$ values of PivA-TBOA were only slightly higher than those of L-TBOA for EAAT 1 and EAAT3, PivA-TBOA was considerably more potent than L-TBOA at inhibiting uptake by EAAT2. In contrast, X-BzA-TBOAs were more potent inhibitors of EAAT1. TFB-TBOA ($IC_{50}$ = 22 ± 0.4 nM) was 1500-fold more potent against EAAT1 than L-TBOA ($IC_{50}$ = 33 ± 3.7 μM). The $IC_{50}$ values of X-BzA-TBOAs for EAAT1 were comparable with those for EAAT2. CNB- and PMB-TBOA also inhibited EAAT1 and EAAT2 selectively and were less potent at inhibiting uptake by EAAT3.

**Effects of TFB-TBOA on EAAT1–5.** To examine the effects of the TBOA analogs on transport currents associated with EAAT1–5, we performed electrophysiological analyses in *X. laevis* oocytes expressing each subtype of EAAT. L-Glutamate and L-aspartate elicited dose-dependent inward currents with each subtype. In the EAAT1, -2, -3, and -5 subtypes, all $I_{max}$ values with L-glutamate were larger than those with L-aspartate, whereas $K_m$ values with L-glutamate were quite similar to those with L-aspartate. In contrast, both L-glutamate and L-aspartate had 10-fold higher apparent affinities for EAAT4 compared with the other subtypes and the $I_{max}$ with L-aspartate was greater than that with L-glutamate, as noted previously (Fairman et al., 1995). Thus, unless otherwise noted, L-glutamate (100 μM) was used as a substrate for EAAT1, -2, -3, and -5, and L-aspartate (10 μM) was used as a substrate for EAAT4 (Shigeri et al., 2001).

TFB-TBOA was applied initially in the absence of substrates to oocytes expressing each subtype of EAAT. With EAAT1, -2, -3, or -4, TFB-TBOA (10 μM) alone did not elicit a detectable current at any potential (−90 ~ +80 mV). Representative currents at −60 mV are shown in Fig. 2A-D, middle. However, with EAAT5, the analog elicited small outward currents at −60 mV (Fig. 2E). Because these currents were outward at negative potentials and became small and inward at positive potentials, these currents probably reflect blockade of EAAT5-mediated leak currents, as reported previously (Arriza et al., 1997; Shigeri et al., 2001).

TFB-TBOA was then applied in the presence of substrates to oocytes expressing each subtype of EAAT. With all subtypes of EAAT, TFB-TBOA significantly attenuated substrate-induced currents at all potentials (−90 ~ +80 mV), indicating that it blocked substrate transport. Representative currents at −60 mV are shown in Fig. 2 (the right). Initial inward currents and small but sustained outward currents were observed with EAAT5 after the coapplication of L-glutamate and TFB-TBOA (10 μM) (Fig. 2E). Because TFB-TBOA elicits small outward currents at −60 mV, it probably inhibits both substrate-induced currents and leak currents associated with EAAT5. TFB-TBOA inhibited the glutamate-induced currents associated with EAAT1 in a dose-dependent fashion (Fig. 3A). The inward currents associated with EAAT1 ($I_{EAAT1}$) induced by glutamate with 10, 100, and 1 μM TFB-TBOA were 93.2 ± 1.6, 43.5 ± 4.2, and 2.2 ± 2.0% of the maximal glutamate currents, respectively (mean ± S.D., $n$ = 3). However, small inward currents continued even when higher concentrations of TFB-TBOA (10 μM) were used ($I_{EAAT1}$ =

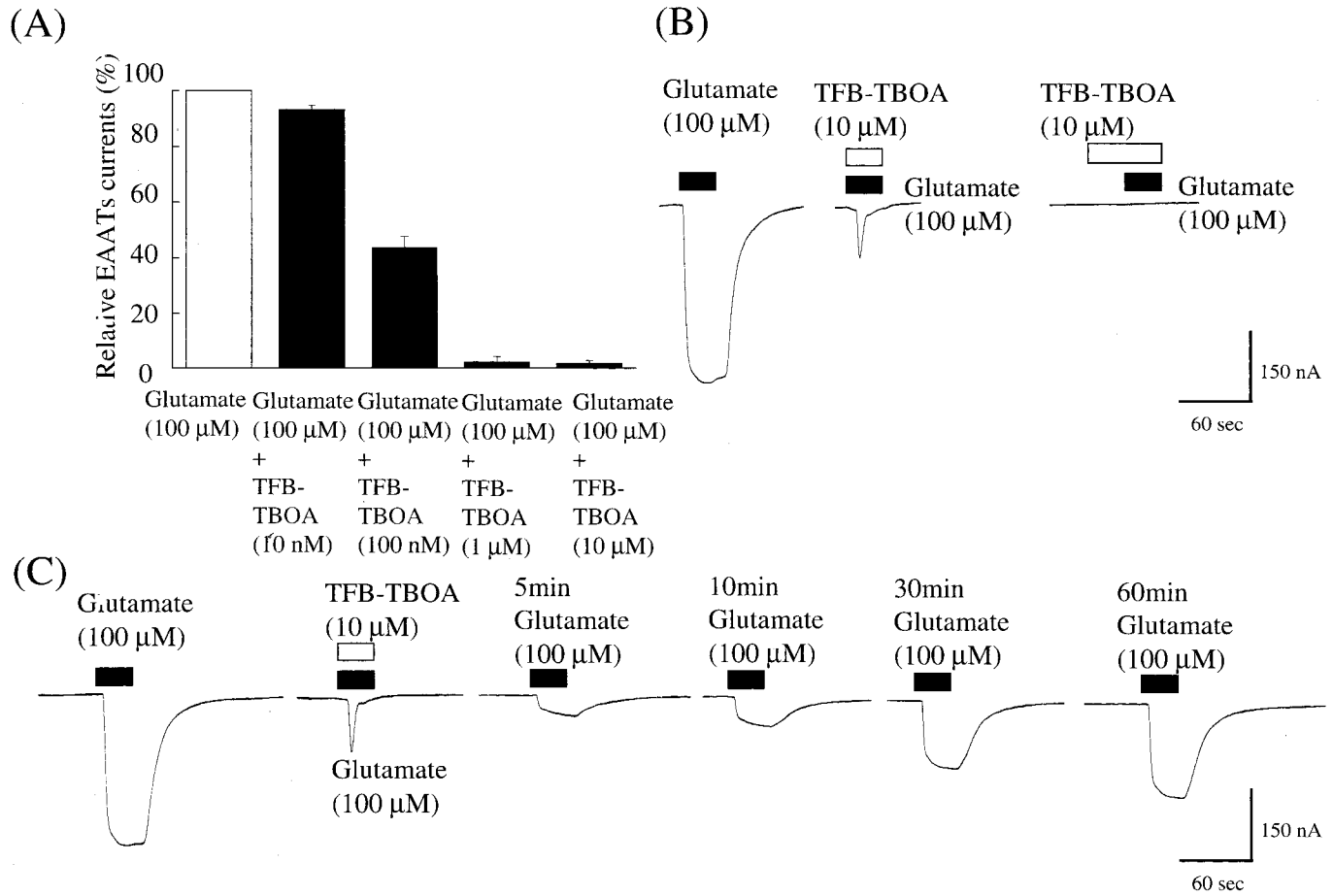


**Fig. 3.** Effects of TFB-TBOA on EAAT1-expressing oocytes voltage-clamped at −60 mV. A, TFB-TBOA (10 nM-10 μM) dose-dependently inhibited currents elicited by glutamate (100 μM). The results are expressed as the percentage compared with the control currents induced by L-glutamate (100 μM). Each column represents mean ± S.D. ($n$ = 3). B, a small inward current was observed in the coapplication of L-glutamate and TFB-TBOA (10 μM) but this current disappeared when TFB-TBOA (10 μM) was preapplied for 30 s before the coapplication. C, L-glutamate (100 μM) was applied 5, 10, 30, and 60 min after the application of TFB-TBOA (10 μM). The glutamate-induced currents were reduced in comparison with the control. Nearly identical results were observed in four different cells.

1.6 ± 1.2%, mean ± S.D., $n = 3$) (Figs. 2A and 3B). Similar currents were also observed with the other subtypes (Fig. 2, B–E). Pretreatment of cells with TFB-TBOA (10 μM) for 30 s before the coapplication of L-glutamate with TFB-TBOA (10 μM) completely abolished these currents associated with EAAT1 as well as with other EAAT subtypes (Fig. 3B).

When L-glutamate was repeatedly applied to EAAT1-expressing oocytes at 5-min intervals after the application of TFB-TBOA (10 μM), the L-glutamate-induced currents were significantly reduced and recovered slowly with time (Fig. 3C). Despite successive washing, the inhibition persisted for more than 1 h. This sustained inhibition was observed with EAAT1, EAAT2, and EAAT4, whereas the L-glutamate-induced currents for EAAT3 and EAAT5 completely recovered within 10 and 30 min, respectively. In contrast, L-glutamate-induced currents rapidly recovered after application of L-TBOA with all subtypes (data not shown).

**Effects of TBOA Analogs on EAAT1–5.** To examine the effects of other TBOA analogs on currents in EAAT1–5–expressing oocytes, L-TBOA, TFB-TBOA, and selected TBOA analogs (PivA-TBOA, CNB-TBOA, and PMB-TBOA) (10 μM) were applied in the absence of substrates to EAAT-expressing oocytes held at −60 mV. None of the TBOA analogs or L-TBOA elicited currents associated with EAAT1, -2, -3, and -4, but the analogs generated small outward currents associated with EAAT5 (data not shown). These results indicate that the TBOA analogs tested here, as well as TFB-TBOA, block leak currents associated with EAAT5. Next, the TBOA analogs were applied in the presence of substrate. Although the analogs were generally applied at the concentration of 10 μM, the analog concentrations were reduced to 1 μM with EAAT2 because of their increased potency for this subtype. As shown in Fig. 4, all TBOA analogs blocked the substrate-induced currents associated with all subtypes of EAATs. With EAAT5, PMB-TBOA as well as TFB-TBOA induced outward currents even in the presence of L-glutamate, suggesting that they block the leak current more effectively than other analogs. The rank order of the potency of TBOA analogs at inhibiting substrate-induced currents was almost identical to that in the uptake assay except that PivA-TBOA was slightly less potent than L-TBOA for EAAT2. TFB- and PMB-TBOA were more potent blockers than L-TBOA of all subtypes of EAATs. The inhibitory potency of CNB-TBOA for EAAT3 was nearly the same as that of L-TBOA.

**Effects on Glutamate Receptors and Other Neuronal Systems.** The selectivity of these analogs for glutamate receptors is critical for their practical use as transporter blockers. L-TBOA did not affect the activity of mGluRs or non-NMDA receptors, although it did show a weak binding affinity for NMDA receptors ($IC_{50} = 49 \pm 5.0$ μM for [$^3$H]CGP39653 binding) (Shimamoto et al., 2000). Introduction of a bulky substituent that interferes with the binding could be an effective means of preventing this interaction. Binding assay results revealed that X-BzA-TBOAs were essentially inactive for all subtypes of the ionotropic glutamate receptors ($IC_{50} > 100$ μM), although PivA-TBOA showed almost the same affinity for NMDA receptors ($IC_{50} = 59 \pm 5.6$ μM) as L-TBOA. None of the TBOA analogs affected either mGluRs 1 or 5 ($Ca^{2+}$ mobilization assay) or mGluRs 2 or 4 (cAMP formation monitoring assay) at 100 μM. Moreover, they did not inhibit glutamic acid decarboxylase, a GABA biosynthetic enzyme (data not shown). To further confirm the specificity, effects of L-TBOA and TFB-TBOA were examined on other representative neurotransmitter receptors and transporters using radioligand binding assays. The compounds showed no significant activity at 10 μM for the following receptors and transporters: acetylcholine (muscarinic and nicotinic), adrenergic ($\alpha_1$, $\alpha_2$, and $\beta$), dopamine $D_1$, $GABA_A$ (antagonist site), $GABA_B$, NMDA (glycine site, phencyclidine site, and polyamine site), glycine (strychnine-sensitive), histamine $H_1$, serotonin (5-$HT_1$ and 5-$HT_2$), choline transporter, dopamine transporter, GABA transporter, norepinephrine transporter, and serotonin transporter (data not shown).

**Behavioral Experiments.** Effects of TBOA analogs on mice behavior were assessed by i.p. and i.c.v. injecting DL-TBOA and selected analogs (PivA-, PMB-, and TFB-TBOA). The i.p. injection of TBOA analogs up to 20 mg/kg did not alter the general behavior of mice; however, i.c.v. injections of TBOA analogs dose-dependently induced stereotypical behaviors and seizure activity. The observed behaviors were graded in order of severity according to the scores indicated under *Materials and Methods*, and the $ED_{50}$ values for each drug were estimated on the basis of dose-response curves generated between immediately lethal and lower threshold doses (Table 3).

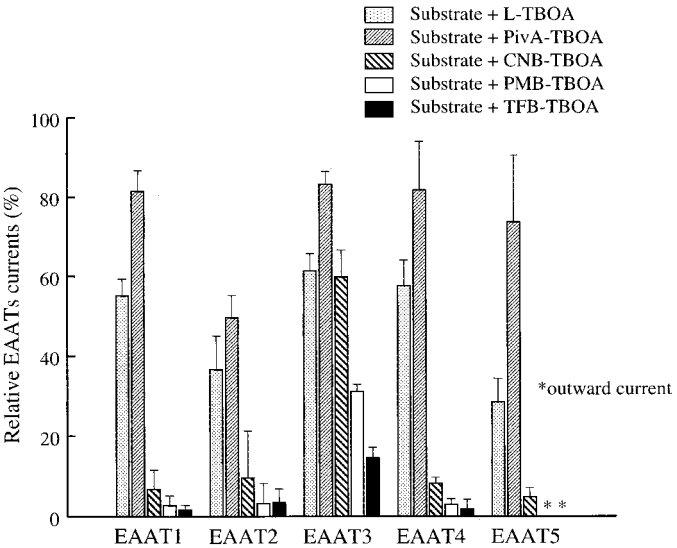


**Fig. 4.** Effects of TBOA analogs (L-TBOA, PivA-TBOA, CNB-TBOA, PMB-TBOA, and TFB-TBOA) on each EAAT subtype. L-Glutamate (100 μM) was used as substrate for EAAT1, -2, -3, and -5 and L-aspartate (10 μM) was used as substrate for EAAT4. The concentrations of TBOA analogs used were 10 μM for EAAT1, -3, -4, and -5 and 1 μM for EAAT2, respectively. The results are expressed as the percentage compared with the currents induced by L-glutamate (100 μM). Each column represents mean ± S.D. ($n = 3$). Asterisk shows the outward currents.

The behavioral patterns induced by all TBOA analogs were very similar even though the potencies varied. The most noticeable behaviors induced at higher doses were violent convulsion including running and jumping. The highest doses were almost immediately lethal to the animals. The next highest doses induced transient running-jumping behavior that progressed into severe whole body convulsions in about 10 min, resulting in loss of normal body posture and severe seizure. Most animals died within 15 min. At sublethal doses (the doses not lethal within 1 h), severe whole body convulsion lasted as long as 4 h. At nonlethal doses, severe whole body symptoms were observed for up to 20 min after the injection. Most animals recovered their body posture 30 min after drug administration but

TABLE 3

Convulsant activity of TBOA analogs in mice after i.c.v. injection

At least five doses of each compound were examined between the lowest (threshold) and the highest (immediately lethal) doses. $ED_{50}$ values were obtained from the dose-response curves and the values are presented as mean ± S.D. Total number of animals used for each compound is listed. At least three animals were administered each dose.

| | Dose Range | $ED_{50}$ | $n$ |
|---|---|---|---|
| | *nmol/mouse (μg/mouse)* | *nmol/mouse (μg/mouse)* | |
| DL-TBOA | 2.60–167 (0.63–40.0) | 11.1 ± 1.5 (2.7 ± 0.45) | 21 |
| PivA-TBOA | 1.77–59 (0.60–20) | 4.56 ± 0.92 (1.5 ± 0.31) | 24 |
| PMB-TBOA | 0.19–3.98 (0.10–2.0) | 0.65 ± 0.04 (0.33 ± 0.04) | 21 |
| TFB-TBOA | 0.09–1.39 (0.05–0.75) | 0.37 ± 0.04 (0.20 ± 0.02) | 21 |

stereotypical behaviors such as scratching then became prominent. Lower dosages induced transient jumping-running convulsion in some animals, but most animals displayed only stereotypical behaviors.

L-Glutamate, injected as a positive control, induced behavioral patterns different from those induced by the TBOA analogs. Injection of L-glutamate (1.4 μmol/mouse) induced loss of body posture accompanied by persistent scratching. Mice then displayed rigidity in the lower body and hind limbs, which was not observed after treatment with the TBOA analogs. These convulsive behaviors faded within 1 h.

Because the blood-brain barrier of the postnatal rat is incomplete for the first 11 days (Schoepp et al., 1990), the effects of DL-TBOA and TFB-TBOA on 8- and 16-day-old neonatal pups were compared. The systemic administration of TFB-TBOA (10 mg/kg, 20 μmol/kg) to 8-day-old rats resulted in severe convulsion in all tested animals. The early symptoms such as scratching and tail wagging began about 30 min after injection. The symptoms progressed to severe whole-body convulsion, including rolling and distorted body posture. Injection of DL-TBOA (10 mg/kg, 40 μmol/kg) also induced convulsant behaviors; however, the early symptoms did not begin until about 60 min after injection. Whole-body convulsion was prominent 90 min after administration, and body rolling and distorted posture occurred until the end of the experiment. In contrast, 16-day-old rats did not display any of the convulsive behaviors observed in the younger rats. Injection of the vehicle (4% DMSO) caused slight behavioral changes in the 8-day-old rats (occasional scratching) but no severe effects.

## Discussion

In this study, we characterized the functional effects of novel TBOA analogs on glutamate transporter subtypes. All of the synthetic analogs were potent inhibitors in the radiolabeled glutamate uptake assays and were confirmed as blockers for EAAT1–5 in electrophysiological studies in voltage-clamped oocytes.

Introduction of a benzoyl group dramatically increased the potency of the effects on the transporters. X-BzA-TBOAs were more selective for EAAT1 and EAAT2, the glial transporters, compared with EAAT3, the major neuronal transporter. The most potent compound, TFB-TBOA, was 1500- and 350-fold more potent at inhibiting glutamate uptake by EAAT1 and EAAT2, respectively, but only 50-fold more potent at inhibiting uptake by EAAT3 in the COS-1 cell expression system. CNB- and PMB-TBOA were slightly less potent than TFB-TBOA but more selective for EAAT1 and EAAT2. Although the observed selectivity was not sufficient for the practical discrimination of EAAT3 from the other subtypes, the finding that selectivity can be modified by the introduction of substituents suggests additional direction for the development of subtype selective blockers.

The analogs possessing an aromatic acyl group (X-BzA-TBOAs and PhAcA-TBOA) were more active than the aliphatic acyl derivatives (PrA-, PivA-, cHexcA-TBOA) in EAAT2-expressing MDCK cells. The aromatic ring enhances binding to this subtype, possibly because of a stacking effect or hydrophobic interaction with the binding site. These results are consistent with previous results demonstrating that the binding site is in a hydrophobic environment (Kanner and Borre, 2002; Leighton et al., 2002). Interestingly, introduction of a substituent on the *para*-position of the benzoyl group enhanced the potency regardless of electrostatic conditions; both electron-withdrawing groups (i.e., $CF_3$, $NO_2$, CN) and electron-donating groups (i.e., OMe, *t*-butyl, Ph) enhanced the activity. Although the reasons for this striking enhancement in binding affinity are not yet clear, these *para*-substituted benzoyl derivatives are promising lead compounds for affinity chromatography or photoaffinity label ligands.

In the electrophysiological studies, the TBOA analogs (PivA-, CNB-, PMB-, and TFB-TBOA) significantly blocked all EAAT subtypes. In addition, all the analogs inhibited substrate-induced currents and elicited apparent outward currents associated with EAAT5. These results indicate that the analogs block not only substrate-induced currents but also transporter-associated leak currents. The rank order of the potency of TBOA analogs at inhibiting substrate-induced currents was almost identical to that in the uptake assay. In the electrophysiological assay as well as the uptake assay, TFB-TBOA was the most potent inhibitor. The kinetic activity of TFB-TBOA on EAATs is very different from that of L-TBOA. A small current remained even when a high concentration of TFB-TBOA (10 μM) was applied with L-glutamate, whereas the L-glutamate-induced currents were completely attenuated by pretreatment of the cells with TFB-TBOA. Moreover, after application of TFB-TBOA (10 μM), the substrate-induced currents remained lower for longer times, and the currents did not return to the control level for more than 1 h with EAAT1, EAAT2, or EAAT4. In contrast, pretreatment with L-TBOA (10 μM) had no effects on the currents induced by the subsequent application of substrate. These results probably reflect slow association and dissociation of TFB-TBOA and suggest that TFB-TBOA binds more tightly to a site within EAATs by virtue of its bulky and hydrophobic substituent. Although this property of TFB-TBOA might be cause problems in some experiments, it is superior to L-TBOA in its potency, duration of action, and selectivity for the glutamate receptors. Therefore, TFB-TBOA would be a useful tool in experiments that require complete and/or sustained blockade of EAATs.

Bulky substituents also altered the binding affinity for NMDA receptors. X-BzA-TBOAs were inactive at 100 μM in [$^3$H]CGP39653 binding. Neither analog could displace [$^3$H]KA or [$^3$H]AMPA binding to rat brain synaptic membranes. In addition, neither of them activated or inhibited mGluRs. Although the binding affinity of L- or DL-TBOA for NMDA receptors is not as high as the affinity of receptor ligands, the $IC_{50}$ values for NMDA receptor binding are close to the $K_d$ values for EAATs. The concentrations of TBOA used in the electrophysiological studies are relatively high (several hundred micromolar) and thus the side effects must be considered even though 200 μM DL-TBOA reportedly did not activate NMDA receptors in outside-out patches from neurons (Jabaudon et al., 1999). Because the effective dose of TFB-TBOA (0.1–10 μM) was much lower than that of TBOA, and its binding affinity for glutamate receptors was weaker ($IC_{50} > 100$ μM), the activation of glutamate receptors by TFB-TBOA is probably negligible. Moreover, L-TBOA and TFB-TBOA did not have any effects on the other representative neuronal receptors, transporters, and enzymes as described under *Results*. Thus, TFB-TBOA is highly selective for glutamate transporters.

EAATs protect neurons from the excitotoxic actions of glutamate. The EAAT blockers tested here produced dose-dependent convulsive behaviors in mice. Behavioral profiles observed with the four drugs used here were similar to each other, suggesting similar modes of action. The convulsant potencies of the compounds seemed to parallel their inhibitory activities on EAATs and not their binding affinities for NMDA receptors. Thus, PMB- and TFB-TBOA, which potently inhibited EAATs but did not bind to NMDA receptors, induced convulsions at doses

much lower than DL-TBOA or PivA-TBOA. These results indicate that the convulsant behaviors induced by these analogs are probably mediated through their actions on EAATs. Although TBOA and PivA-TBOA showed affinity for NMDA receptors, they are probably too weak to elicit their actions directly through the synaptic receptors in vivo. Extracellular glutamate levels in cerebral cortical superfusates are reportedly increased by the application of DL-TBOA (Phillis et al., 2000). Thus, the blockade of glutamate transporters by TBOA analogs would result in the local accumulation of glutamate near the synapses and a sustained activation of glutamate receptors. Interestingly, the onset of the drug-induced convulsion was rapid, generally less than 10 s, and a time-dependent progression of symptoms was observed when sublethal doses were administered. Early-onset symptoms, such as running and jumping convulsion, are similar to those induced by NMDA. On the other hand, about 10 min later, whole-body convulsion followed by stereotyped behaviors were observed, which are the typical behavioral patterns elicited by non-NMDA agonists (Sakai et al., 2001). This progression may reflect a differential accumulation rate in various regions of the brain or differential threshold concentrations for activation of receptor subtypes. We observed that the behavioral profile after injection of glutamate itself was somewhat different from that induced by TBOA analogs, probably because of the the different pharmacodynamics of endogenous and exogenous glutamate. The i.c.v. administration of glutamate might simultaneously activate neurons in broad areas of the brain but the excess glutamate would be taken up gradually, resulting in reduction of the symptoms. On the other hand, the blockade of EAATs by TBOA analogs would result in the local accumulation of endogenous glutamate near glutamatergic neurons. Because the primary clearance mechanism for glutamate is inhibited, receptor activation is likely to be sustained.

Of note, TBOA analogs do not permeate the blood brain barrier in rodent models; the drugs caused convulsions in 8-day-old rats but not in 16-day-old rats or in adult mice when administered systemically.

In summary, we have developed a series of potent EAAT blockers, the X-BzA-TBOAs. TFB-TBOA, the most potent among them, is effective at low nanomolar concentrations in the glutamate uptake assay and is highly selective for EAATs. Electrophysiological analyses revealed that TFB-TBOA blocks all subtypes of EAAT. Moreover, blockade of EAATs by TFB-TBOA induced severe convulsive behaviors in mice. Taken together, novel TBOA analogs may become important tools for further elucidation of the physiological roles of EAATs and their contribution to the etiology of neuronal disorders.

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