Blockade of glutamate transporters facilitates cerebellar synaptic long-term depression

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Excitatory amino acid transporters (EAATs) are believed to limit extracellular glutamate concentrations with specific roles poorly understood. At cerebellar climbing fiber-Purkinje cell synapse, EAAT4 and metabotropic glutamate receptor 1 (mGluR1) are closely expressed in surrounding postsynaptic locations, suggesting that EAAT4 may regulate mGluR1 activation. We examined the actions of EAAT4 on synaptic plasticity by applying blockers of glutamate transporters, DL-threo-β-benzyloxyaspartic acid and D-aspartate. Inhibition of EAAT4 markedly prolonged AMPA receptor-mediated excitatory postsynaptic currents evoked by stimulating climbing fibers. Impairing glutamate uptake facilitated mGluR1-dependent climbing fiber-Purkinje cell synaptic long-term depression (LTD). Glutamate uptake blockers also sufficiently rescued climbing fiber-Purkinje cell synaptic LTD that failed to be induced by a weaker tetanus. Our results suggest that neuronal glutamate transporters strongly influence mGluR1-dependent cerebellar LTD. *NeuroReport* **20:502–507 © 2009 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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Introduction

Most glutamate transporters (termed as EAAT1–4), except EAAT5, are distributed in molecular layer of adult cerebellum [1,2], especially at the excitatory synapses in Purkinje cells, where glutamate transporters play more essential roles in synaptic signaling. It is widely proposed that glutamate reuptake facilitates chemical synaptic transmission by limiting receptor activation and by recycling released neurotransmitter molecules. In fact, rapid clearance of released glutamate has profound influence on the kinetics of excitatory postsynaptic currents (EPSCs), such as AMPA currents, at several synapses [3–5] and amplitude of metabotropic glutamate receptor (mGluR) currents in both parallel fiber and climbing fiber synapses [6,7].

Unlike AMPA receptors, mGluR1 shows highest densities at the edge of the postsynaptic density and shares an overlapping pattern with EAAT4 in the cerebellum [8]. MGluR1 are required for distinct forms of synaptic plasticity expressed at parallel fiber and climbing fiber synapses, such as parallel fiber long-term depression (LTD) [9], climbing fiber LTD [10,11] and a short-term presynaptic depression of climbing fiber synapses [12], and the pruning of multiple climbing fiber inputs to Purkinje cell during development [13]; however, the mechanisms are unclear.

Functional interplay between neuronal glutamate transporters and mGluR1 supports the hypothesis that postsynaptic EAAT4 may tightly control the activation of mGluR1 and synaptic plasticity. Indeed, Brasnjo and Otis [6] found that neuronal glutamate transporters regulate the activation of postsynaptic mGluRs and increased activation of mGluRs facilitates induction of LTD at parallel fiber synapses. Dzubay and Otis [7] found mGluR activation at climbing fiber synapses. Thus, it is of interest to investigate possible roles of EAAT4 in climbing fiber-Purkinje cell LTD.

In this study, by using whole-cell patch clamp technique combining pharmacologically applying DL-threo-β-benzyloxyaspartic acid (TBOA), a broad subtype-non-selective inhibitor of glutamate transporters [6] in external saline and D-aspartate (aspartate), an exclusive blocker to EAAT4 current in internal saline, we aimed to focus on the contribution of EAAT4 on properties of EPSC and mGluR-mediated slow currents, and LTD of EPSC by inhibiting EAAT4 expression in Purkinje cells. The results highlight the importance of EAAT4 in shaping EPSCs and regulating the activation of mGluR1 and induction of mGluR-dependent LTD.

Methods

All animal experiments were performed under the guidance of Animal Experimentation Ethics Committees of Zhejiang University. The experimental procedures used herein were modified from those of Shen and Linden [11]. P17–20 Sprague–Dawley rats were anesthetized by 4% halothane in oxygen and decapitated and the

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brain was removed into ice-cold artificial cerebrospinal fluid (aCSF) containing (in mM): 124 NaCl, 5 KCl, 1.25 Na_2HPO_4, 2 $MgSO_4$, 2 $CaCl_2$, 26 $NaHCO_3$ and 10 D-glucose bubbled with 95% O_2 and 5% CO_2. All the experiments were specifically designed in order to minimize the sacrifice of animals. Sagittal slices of the cerebellar vermis (250 μm thick) were prepared using a vibratome (Leica VT1000S, Germany). After a recovery period of 30 min at 37°C and at least 1 h at room temperature (25°C), the slices were placed in a submerged chamber that was perfused at a flow rate of 2 ml/min with room-temperature aCSF supplemented with 10 μM GABAzine to block γ-amino-butyric acid A ($GABA_A$) receptors. The recording electrodes (resistance 2–3 MΩ) were filled with a solution containing (in mM): 135 CsMes, 10 CsCl, 10 HEPES, 4 Na_2ATP, 0.4 Na_3GTP, and 0.3 EGTA (pH 7.2). While studying internal block of EAAT4, 20 mM aspartate was added and 135 CsMes and 10 CsCl were replaced by 130 mM CsMes to keep the ionic balance. Recording pipettes were typically 1.5–3 MΩ, and uncompensated series resistances were less than 5 MΩ.

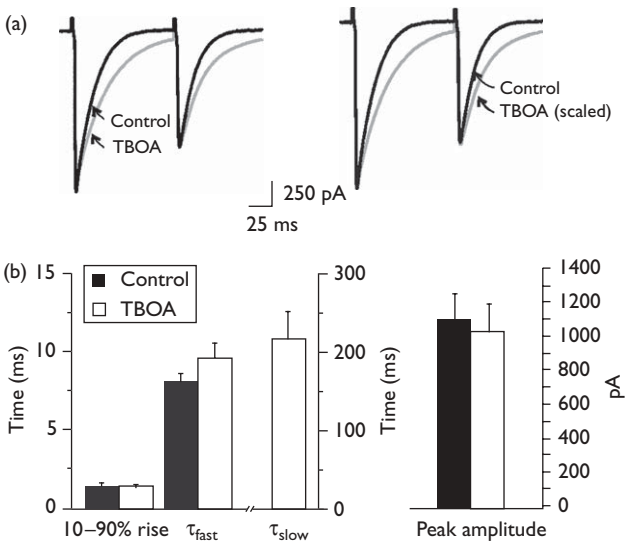
Purkinje cells were visualized using an upright Zeiss Axioskop 2FS microscope (Carl Zeiss, Jena, Germany) with a 40× water immersion objective and equipped with infrared-differential interference contrast enhancement. All whole-cell recordings were obtained with an Axopatch 200B or MultiClamp 700B amplifiers (Molecular Device Inc., Foster City, California, USA). Currents were filtered at 1 kHz and digitized at 10 kHz. All drugs were purchased from Sigma (St Louis, Missouri, USA), except for GABAzine, TBOA, NBQX, CPCCOEt (Ascent Scientific, UK). Climbing fibers were activated by applying constant current steps (20–200 μA/100 μs) to either a patch pipette or a bipolar electrode fashioned from theta pipette glass. The electrode was positioned in the granule cell layer close to the vicinity of the recorded neuron, and stimulus intensity and electrode position were adjusted so that all-or-none climbing fiber responses were elicited. For AMPA receptor-mediated EPSC, recordings were performed at holding potentials of −10 mV. Data analysis was performed using Excel 2003 (Microsoft, Chicago, USA), Clampfit 10 (Molecular Device Inc.), and Igor Pro (Wavemetrics Inc., Lake Oswego, Oregon, USA). All group data are shown as mean ± SEM. Student's *t*-tests were used to determine *P* values. Cells were excluded from the study if series resistance or input resistance varied by more than 15% over the course of the experiments.

Results

Activation of the climbing fiber input gave rise to a large EPSC, which was evoked in an all-or-none manner distinguished by a sharp stimulus threshold [10,11], as stimulation strength was gradually increased to bring the one innervating climbing fiber to threshold. To avoid large EPSC escape, the clamp control was improved by setting V_{hold} to −10 mV instead of −70 mV so as to reduce the driving force of EPSCs. Figure 1a shows a representative response to a paired-pulse stimulation recorded in a Purkinje cell. When bath application of 300 μM TBOA slightly decreased the peak amplitude of EPSC1 by 6.8 ± 0.4% (control: 1202.4 ± 40.6 pA; TBOA: 1125.4 ± 57.8 pA; $P > 0.05$, $n = 15$; Fig. 1b), it markedly prolonged EPSC. The 10–90% rising times were comparable between control and TBOA groups ($\tau = 1.3 \pm 0.2$ and 1.3 ± 0.1 ms, respectively; $n = 15$). The decay phase of control group was fitted by a single exponential ($\tau = 8.2 \pm 0.6$ ms; $n = 15$); however, it required two exponentials in TBOA group ($\tau_{fast} = 9.7 \pm 0.9$ ms; $\tau_{slow} = 215.6 \pm 34.5$ ms; $n = 15$), consistent with the results by Takayasu [5]. To show the difference, we hereby superimposed two EPSC by normalizing the EPSC amplitudes in the absence and presence of TBOA in Fig. 1. For EPSC2, all the parameters were very similar to EPSC1 (data now shown). TBOA is not transportable to induce any possible inward current [3].

We then investigated the roles of glutamate transporter blockers in climbing fiber-Purkinje cell synaptic LTD by a tetanus protocol of 5 Hz stimulation for 30 s [10,11]. Two different manipulations were made to clarify the functions of EAAT4 in climbing fiber-Purkinje cell LTD.

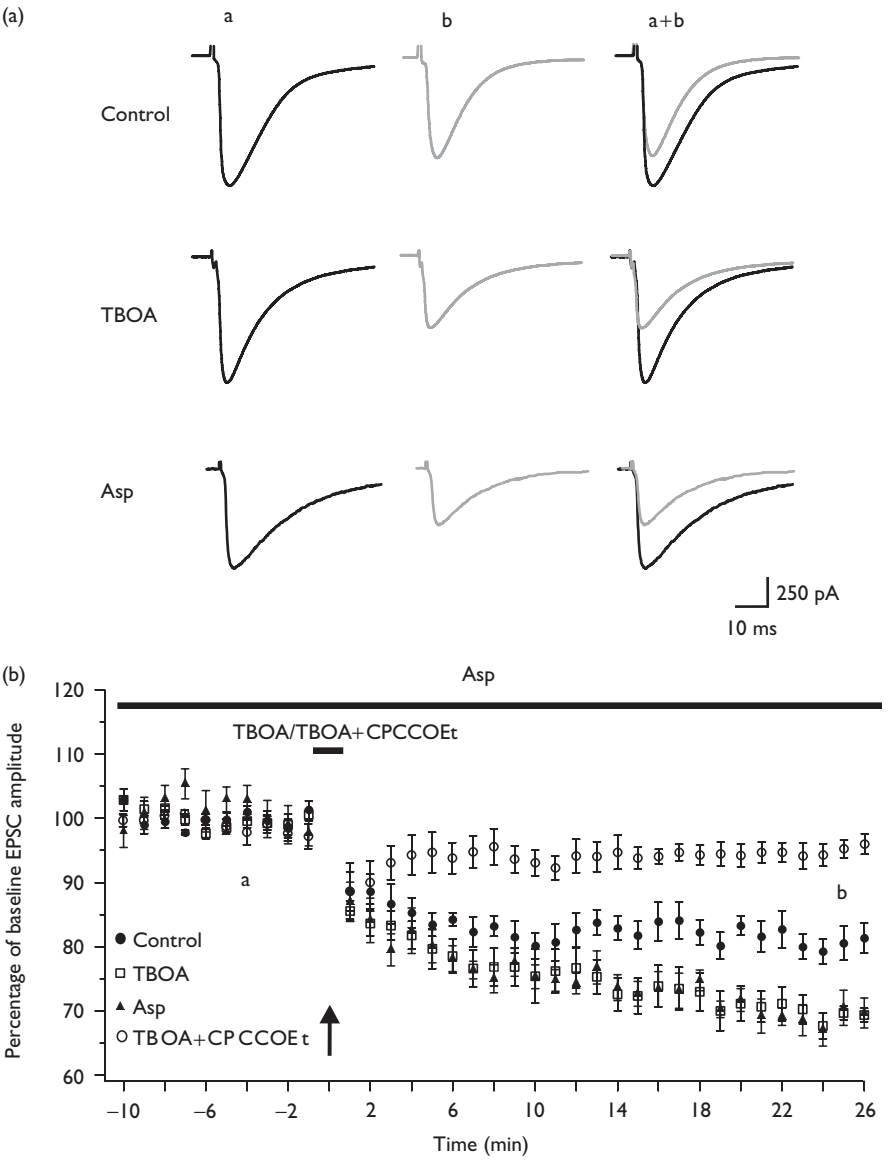
Fig. 1



Effects of inhibiting glutamate transporters by DL-threo-β-benzyloxyaspartic acid (TBOA) at climbing fiber-Purkinje cell synapses. (a) A typical response of Purkinje cell evoked by a paired-pulse stimulation. The responses obtained from the same cell are indicated by black (control) and grey (TBOA) in the absence (control) and the presence (TBOA) of 300 μM TBOA, respectively. Traces are then superimposed together in the right to show the difference of decay. (b) Statistics of decay time courses and amplitudes of excitatory postsynaptic currents 1 in control and TBOA groups. The decay was fit with two exponentials in TBOA group, revealing a larger increase in the slow component of offset ($n = 15$).

First, we applied 300 μM TBOA during the tetanus period in the background perfusion. As shown in Fig. 2, we found that LTD induced in the presence of TBOA was much enhanced compared with control LTD at the time point of 25 min (control peak amplitude 80.5 ± 2.7% of baseline, $n = 9$; TBOA peak amplitude 69.7 ± 1.0%, $n = 11$; $P < 0.01$ for comparison). To confirm that LTD observed in response to TBOA application is mGluR dependent, we coapplied 100 μM CPCCOEt, a group I mGluR1 antagonist, with TBOA during the tetanus. LTD was blocked under this condition (Fig. 2b, open circle; TBOA+CPCCOEt peak amplitude 95.2 ± 1.4% of baseline, $n = 4$). Second, we included 20 mM aspartate in the internal saline to internally block EAAT4 function. LTD under this condition was also much enhanced at the time point of 25 min (Asp peak amplitude 70.4 ± 2.7%, $n = 11$;

Fig. 2

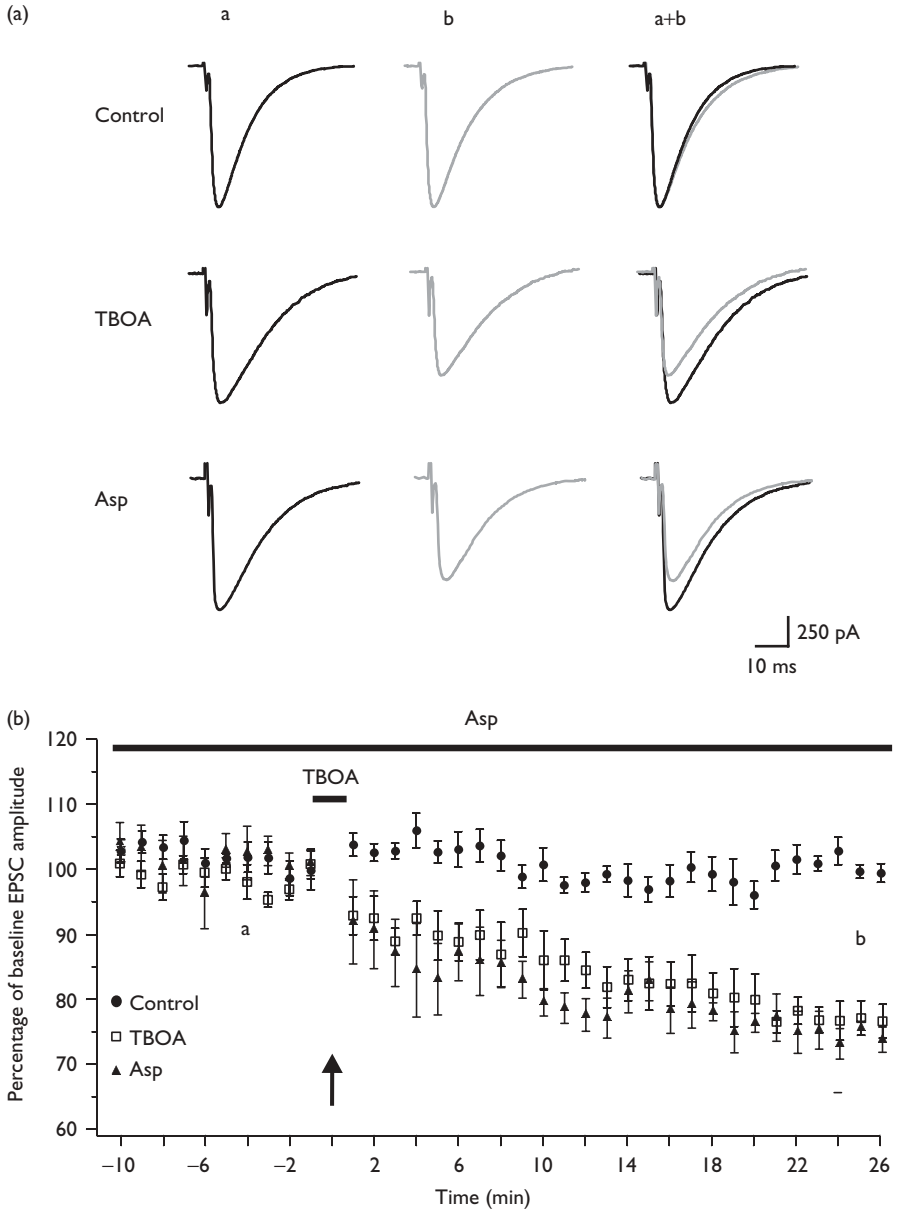


Inhibiting glutamate transporters facilitated climbing fiber long-term depression (LTD). (a) Example traces before and after low-frequency stimuli to induce climbing fiber-Purkinje cell LTD. Sample currents shown by a or b were collected at the times indicated on (b), which illustrated the effects of three manipulations: control, treatments of 300 μM DL-threo-β-benzyloxyaspartic acid (TBOA), and 20 mM Asp, respectively. (b) Comparison of three groups of neurons recorded. Mean peak amplitudes of climbing fiber-evoked excitatory postsynaptic currents (EPSC) are displayed versus time of control (filled circles, $n = 9$), 300 μM TBOA (open squares, $n = 11$), 20 mM Asp (filled triangles, $n = 11$), 300 μM TBOA plus 100 μM CPCCOEt (open circles, $n = 4$), respectively. After a baseline recording period, LTD was induced at $t = 0$ min. TBOA (open square) or TBOA plus CPCCOEt (open circle) was added 2 min before induction and immediately washed out after 30-s induction time window. Therefore, 2-min recordings of EPSCs before and after the induction, respectively, were discarded to ensure that they were not contaminated by TBOA. Tetanic stimulation is indicated by the upward arrow.

$P < 0.01$ for comparison). Like control group, LTD in TBOA and aspartate groups were not associated with apparent changes in EPSC kinetics (data not shown). Thus, blockade of EAAT4 significantly enhanced the mGluR-dependant LTD in climbing fiber-Purkinje cell synapses.

If EAAT4 inhibition did facilitate climbing fiber LTD, one can propose that it may also be able to induce climbing fiber LTD that could not be formed under a weaker tetanus. We investigated this possibility by changing induction protocol. After a baseline recording of climbing fiber EPSC, we delivered the tetanus stimuli of 3 Hz for 30 s. As shown in Fig. 3, this stimulus failed to induce climbing fiber LTD in Purkinje cell with EPSC amplitude unchanged (99.7 ± 1.1% of baseline, $n = 9$) at the time point of 25 min. Two subsequent manipulations were made to observe roles of glutamate transporter

Fig. 3



Inhibiting glutamate transporters rescued climbing fiber-Purkinje cell long-term depression. (a) Example traces before and after weak stimuli. Sample currents shown by a or b were collected at the times indicated on (b) with group treatments as same Fig. 2. (b) Comparison of three groups of neurons recorded. Mean peak amplitudes of climbing fiber-evoked excitatory postsynaptic currents (EPSC) are displayed versus time of control (filled circles, $n = 9$), 300 μM DL-threo-β-benzyloxyaspartic acid (TBOA) (open squares, $n = 10$) and 20 mM Asp (filled triangles, $n = 10$), respectively. After a baseline recording period, a weak tetanus was delivered at $t = 0$ min. TBOA was added in a time period same as Fig. 2.

blockers on this failed LTD. Inhibition of glutamate transporter by bath application of 300 μM TBOA during tetanus and inhibition of EAAT4 by internal 20 mM Asp could rescue failed climbing fiber-Purkinje cell LTD (76.8 ± 2.6% of baseline, $n = 10$; 74.0 ± 1.9% of baseline, $n = 10$, respectively; for both groups, $P < 0.01$ compared to control group). All these data confirmed that the glutamate transporters tightly controlled mGluR1-dependent LTD.

Discussion

This study shows that a blockade of glutamate transporters facilitate induction of LTD at climbing fiber-Purkinje cell synapses by regulating the activation of postsynaptic mGluRs, especially through neuronal glutamate transporters EAAT4 (Figs 2 and 3).

In climbing fiber and parallel fiber synapses, both mGluR1 [14] and the transporter EAAT4 [2,15] are expressed perisynaptically. Closely overlapping distributions of both neuronal glutamate transporters and mGluR1 raised a possibility of interaction between these two proteins. Similar affinities to released glutamate were found for EAAT4 (3 μM) [16] and mGluR1 (approximately 10 μM) [17]. It has shown that blockade of neuronal and glial transporters can dramatically increase the activation of mGluR1 at parallel fiber synapses [6], presumably by reducing the shielding of mGluR1 by neuronal transporters. Our data suggested that neuronal transporters at climbing fiber synapses played a similar role in shielding perisynaptic mGluR1.

Considering the tight correlation of two proteins in parallel fiber-Purkinje cell [6], climbing fiber-Purkinje cell ([7]; this study) and hippocampal CA1 region [8], these may reflect a general mechanism by which a postsynaptic neuron could control mGluR activation and mGluR-dependent plasticity. One could suggest that the prolongation of AMPA EPSCs is because of a slowed clearance of glutamate in the synaptic cleft. Brasnjo and Otis [18] estimated that about 17% of the released glutamate is cleared by EAAT4 at mature climbing fiber synapses. Combined with the data from their previous observation at parallel fiber synapses [6] and present work at climbing synapses, one implication is that glutamate reuptake inhibition by EAAT4 is a primary factor, but not the decisive factor to determine mGluR current amplitude by a possible mechanism that complex second messenger cascade of mGluR current is not saturated so that a change in single step was able to trigger a significant enhancement [6].

Glutamate reuptake changes through alteration of turnover rate or surface expression of glutamate transporters. Indeed, modulation of EAAC1 surface expression has been described in response to protein kinase C (PKC) activation [19]. Calcium influx increases glutamate transporter currents through a phospholipase A_2 activation pathway [20]. Shen and Linden [11] found that mGluR1 and PKC are required for postsynaptic EAAT4 LTP. Thus, high levels of mGluR activation in synapses could activate PKC, increase postsynaptic glutamate uptake, and further exert negative feedback on subsequent mGluR activation. One hypothesis is that this homeostatic mechanism might allow mGluR and neuronal glutamate transporter to work together to play a role during climbing fiber pruning [21]. Recently, Jin *et al.* [22] (for review see Kim and Linden [23]) reported an LTD of mGluR1-dependent slow EPSC by repeated climbing fiber-evoked depolarization of Purkinje cells. Therefore, it is worthwhile to examine functions of EAAT4 in climbing fiber pruning and LTD of mGluR1 current in future.

Conclusion

The results of this study show that impairing glutamate uptake facilitated mGluR1-dependent climbing fiber-Purkinje cell synaptic LTD. This study, together with evidence of roles of glutamate transporters at parallel fiber-Purkinje cell excitatory synapses [6], has suggested the significant functions of neuronal glutamate transporter components in input fiber innervation formation and the mGluR-dependent or mGluR plasticities in the brain.

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