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Glutamate transporter type 3 regulates mouse hippocampal GluR1 trafficking

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ABSTRACT

Background: Rapid trafficking of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) to the plasma membrane is considered a fundamental biological process for learning and memory. GluR1 is an AMPAR subunit. We have shown that mice with knockout of excitatory amino acid transporter type 3 (EAAT3), a neuronal glutamate transporter, have impaired learning and memory. The mechanisms for this impairment are not known and may be via regulation of AMPAR trafficking.
Methods: Freshly prepared 300 μm coronal hippocampal slices from wild-type or EAAT3 knockout mice were incubated with or without 25 mM tetraethylammonium for 10 min. The trafficking of GluR1, an AMPAR subunit, to the plasma membrane and its phosphorylation were measured.
Results: Tetraethylammonium increased the trafficking of GluR1 and EAAT3 to the plasma membrane in the wild-type mouse hippocampal slices but did not cause GluR1 trafficking in the EAAT3 knockout mice. Tetraethylammonium also increased the phosphorylation of GluR1 at S845, a protein kinase A (PKA) site, in the wild-type mice but not in the EAAT3 knockout mice. The PKA antagonist KT5720 attenuated tetraethylammonium-induced GluR1 phosphorylation and trafficking in the wild-type mice. The PKA agonist 6-BNz-cAMP caused GluR1 trafficking to the plasma membrane in the EAAT3 knockout mice. In addition, EAAT3 was co-immunoprecipitated with PKA.
Conclusions: These results suggest that EAAT3 is upstream of PKA in a pathway to regulate GluR1 trafficking.
General significance: Our results provide initial evidence for the involvement of EAAT3 in the biochemical cascade of learning and memory.

1. Introduction

A fundamental cellular process for learning and memory is the trafficking of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) to the plasma membrane in the neurons, which increases synaptic strength [1–4]. A mechanism for this AMPAR trafficking is via phosphorylation [2,3,5]. For example, phosphorylation of GluR1, an AMPAR subunit, at serine 845 increases GluR1 trafficking to the plasma membrane [2]. Thus, activation of the signaling pathway glutamate receptor-protein kinase-AMPAR has been proposed as an initial biochemical event for learning and memory [1]. Interestingly, fear conditioning learning is associated with the trafficking of glutamate transporter (also named excitatory amino acid transporter, EAAT) type 3 (EAAT3), a neuronal EAAT, to the plasma membrane of hippocampus in mice [6]. EAAT3$^{-/-}$ mice have learning and memory impairment [7,8]. Thus, it is possible that EAAT3 is involved in learning and memory and their underlying biochemical processes, such as GluR1 trafficking. To explore this possibility, we used tetraethylammonium (TEA) to induce GluR1 trafficking in the hippocampal slices prepared from wild-type and EAAT3$^{-/-}$ mice.

2. Materials and methods

The animal protocol was approved by the Institutional Animal Care and Use Committee of the University of Virginia (Charlottesville, VA, USA). All animal experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publications number 80–23) revised in 2011.

2.1. Animals

8- to 12-week old male EAAT3$^{-/-}$ mice and their wild-type CD1 littermates were used in this study. The EAAT3 knockout mice whose exon 1 of the EAAT3 gene was disrupted by a neomycin resistance cassette were confirmed to have no EAAT3 protein [9–11]. These mice were backcrossed with wild-type CD-1 wild-type mice for more than 10 generations to produce an EAAT3$^{-/-}$ mouse line before our study.

Abbreviations: ACSF, artificial cerebrospinal fluid; AMPAR, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; DMSO, dimethyl sulfoxide; EAAT, excitatory amino acid transporter; PKA, protein kinase A; PP2A, protein phosphatase 2A

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They were also backcrossed with wild-type CD1 mice at least once every 8 generations to prevent genetic drift as recommended from the Banbury Conference [12]. The CD1 wild-type mice used for backcrossing were from Charles River Laboratories (Wilmington, MA, USA). The CD1 wild-type mice used in this study are colonies generated from the littermates of EAAT3$^{-/-}$ mice during the backcrossing of every 8 generations.

2.2. Hippocampal slice preparation

As we reported before [13,14], mice were euthanized by 5% isoflurane. To determine whether the short isoflurane exposure during euthanasia affected the response of hippocampal slices to TEA, some mice were sacrificed by cervical dislocation without isoflurane exposure. Their brains were removed rapidly and placed in ice-cold artificial cerebrospinal fluid (ACSF) containing 116 mM NaCl, 26.2 mM $NaHCO_3$, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.9 mM $MgCl_2$, 0.9 mM NaH_2PO_4 and 5.6 mM glucose (pH 7.4). Hippocampal slices at 300 μm thickness were prepared using a vibrating tissue slicer (Microslicer DTK 1500E, TED Pella, Inc., Redding, CA) in cold cutting solution containing 260 mM sucrose, 26.2 mM $NaHCO_3$, 3 mM KCl, 1.2 mM NaH_2PO_4, 5 mM $MgCl_2$ and 9 mM glucose (pH 7.4) that was bubbled with 5% CO_2 and 95% O_2. The hippocampal slices were maintained for 0.5 h in 4 °C ACSF bubbled with 5% CO_2 and 95% O_2 before they were used for experiments.

2.3. Hippocampal slice incubation

Freshly prepared 300 μm coronal hippocampal slices were incubated in ACSF containing or not containing 25 mM TEA in the presence or absence of 2 μM KT5720 for 10 min at 37 °C. Some hippocampal slices from wild-type and EAAT3$^{-/-}$ mice were incubated with 400 μM N^6-benzoyl-cAMP (6-BNZ-cAMP) for 10 min at 37 °C. The ACSF was bubbled with 5% CO_2 and 95% O_2 during the incubation. KT5720 and 6-BNZ-cAMP were initially dissolved in dimethyl sulfoxide (DMSO) and then diluted with ACSF. The highest DMSO concentrations in the final incubation buffer containing KT5720 and 6-BNZ-cAMP were 1% and 2%, respectively. This amount of DMSO was presented in the corresponding vehicle experiments.

2.4. Biotinylation

The procedure was similar to that described before [13,15]. To biotinylate cell surface proteins, hippocampal slices were incubated in ACSF containing 1 mg/ml sulfo-NHS-SS-biotin (sulfosuccinimidyl-2-(biotinamido)ethyl-1,3-dithiopropionate; Thermo Scientific, Rockford, IL) with gentle shaking during the exposure to TEA or incubation buffer only. After the 10-min incubation, unreacted reagent was removed by quenching with ice-cold phosphate buffered saline (PBS)-Ca^{2+}/Mg containing 100 mM glycine for 10 min twice. The slices were sonicated and resuspended in a lysis buffer (PBS-Ca^{2+}/Mg) containing 0.1% sodium deoxycholate, 1% Triton X-100, protease inhibitors and phosphatase inhibitors (phosSTOP Phosphatase Inhibitor Cocktail Tablets; Roche, Nutley, NJ) for 1 h. The total lysates were centrifuged at 20,000*g* for 10 min at 4 °C to remove nuclei and debris. An aliquot of the homogenate (100 μl) was kept aside for Western blotting of phospho-protein kinase A (PKA) and phospho-GluR1. The remaining sample was incubated with equal volumes of immobilized NeutrAvidin beads (300 μl bead suspension to 300 μl lysates) in a Pierce centrifuge column (Thermo Scientific) with gentle shaking overnight at 4 °C. The mixture was centrifuged at 1000*g* for 3 min at 4 °C. The column was washed three times, each time with 500 μl PBS containing 1% NP40 and centrifugation at 1000*g* for 3 min. The beads pellet that contained the biotinylated proteins was re-suspended in 200 μl of 2× Laemmli buffer for 30 min at 4 °C to dissolve the biotinylated proteins. The biotinylated proteins then were collected in the supernatant after a centrifugation of 1000*g* for 5 min and used in Western blotting. A control experiment that omitted sulfo-NHS-SS-biotin in the incubation buffer was performed.

2.5. Immunoprecipitation

This procedure was performed as we did before [13,15]. The hippocampal slices of wild-type CD1 mice were prepared and treated with TEA as described above. They were lysed in 300 μl lysis buffer containing 50 mM Tris–HCl, 1% NP40, 0.5% sodium deoxycholate, 50 mM NaCl and complete protease inhibitors for 1 h at 4 °C. The lysates were centrifuged at 14,000*g* for 10 min. The supernatants were incubated overnight with 2 μg affinity-purified polyclonal rabbit anti-EAAT3 antibody (Alpha Diagnostics International, San Antonio, TX) at 4 °C and then incubated with 40 μl Protein A/G Plus-Agarose beads with gentle shaking for 1 h at 4 °C. The sample was centrifuged at 500*g* for 2 min at 4 °C. The pellet was washed four times with the lysis buffer. The immune complexes bound to beads were eluted by incubation with 100 μl of Laemmli buffer at 90 °C to 95 °C for 5 min. The samples were then used for Western blotting. A control experiment was performed in the same way but replacing the incubation with the anti-EAAT3 antibody by an incubation with 2 μg rabbit IgG.

2.6. Western blotting

About 30–50 μg proteins per lane were subjected to Western blotting. The membrane was incubated overnight with the following primary antibodies: rabbit polyclonal anti-EAAT3 at 0.5 μg/ml, goat polyclonal anti-GluR1 at 1:1000 dilution, goat polyclonal anti-phospho-GluR1-845 at 1:500 dilution, goat polyclonal anti-phospho-GluR1-831 at 1:500 dilution, rabbit polyclonal anti-phospho-PKA antibody at 1:1000 dilution, rabbit polyclonal anti-PKA at 1:2000 dilution, mouse monoclonal anti-PP2A at 1:2000 dilution or rabbit polyclonal anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) at 1:3000 dilution. The anti-GluR1, anti-phospho-GluR1 and anti-protein phosphatase 2A (PP2A) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-PKA and anti-phospho-PKA antibodies were from Cell Signaling (Danvers, MA) and Abcam Inc. (Cambridge, MA), respectively. The anti-GAPDH antibody was from Sigma-Aldrich (St. Louis, MO, USA). After being incubated with a horseradish peroxidase-conjugated goat anti-rabbit (1:4000 dilution), donkey anti-goat (1:4000 dilution) or goat anti-mouse IgG secondary antibody (1:2000 dilution), the protein bands were finally visualized by the enhanced chemiluminescence method and quantified using the G:Box equipped with Gene tools analysis software (Syngene, Frederick, MD). The densities of protein bands were normalized to those of GAPDH or EAAT3 (the immunoprecipitation experiments) in the same sample and then normalized by the same day control results. The hippocampal slices for the control and various experimental conditions on the same day were from the same mice.

2.7. Phosphatase activity assays

The hippocampal slices from wild-type CD1 mice were treated with or without TEA for 10 min. They were sonicated and placed in 300 μl lysis buffer for 30 min at 4 °C. The lysates were centrifuged at 14,000*g* for 10 min. The supernatants were used to detect protein phosphatase activity according to the protocol of an assay kit (Cat: 786453; G-Biosciences, St Louis, MO).

2.8. Statistics analysis

Results are presented as mean ± S.D. ($n \geq 4$). The n indicates the number of animals used in each condition. Statistical analysis was performed by paired t-test or one-way ANOVA followed by Tukey test for *post hoc* comparison as appropriate. A $P < 0.05$ was considered statistically significant.

3. Results

To determine the specificity of our method to harvest the biotinylated proteins, some hippocampal slices from wild-type mice were not incubated with sulfo-NHS-SS-biotin but were subjected to the rest of procedure outlined in Section 2.4. Omitting the incubation resulted in no protein bands corresponding to EAAT3, GluR1 and GAPDH in the sample, suggesting the specificity of the method to collect the biotinylated proteins in our system. Three protein bands at ~65, 130 and 200 kDa were visualized when the anti-EAAT3 antibody was used for the Western blotting of the biotinylated proteins. These bands may represent singlet and multimer of EAAT3. A single protein band at 106 kDa was revealed with the use of the anti-GluR1 antibody on these samples (Fig. 1A).

TEA increased the amount of EAAT3 in the biotinylated fraction/plasma membrane no matter whether the singlet or singlet and multimer were quantified and analyzed (Fig. 1B). The brief isoflurane exposure used to euthanize the animals did not appear to affect TEA-induced increase of EAAT3 in the plasma membrane (1.45 ± 0.35 of the control for isoflurane-euthanized mice vs. 1.45 ± 0.47 of the control for cervical dislocation mice, n = 4, P = 0.996). Different euthanizing methods also did not change the amount of EAAT3 in the plasma membrane under control conditions (arbitrary unit: 0.46 ± 0.26 for isoflurane-euthanized mice vs. 0.41 ± 0.23 for cervical dislocation mice, n = 4, P = 0.761). Thus, we used isoflurane to euthanize the animals for all other experiments.

We used GAPDH data to normalize the results of EAAT3. GAPDH is known to be present in the plasma membrane [16,17] and is a housekeeping protein with stable expression [18]. TEA did not change the expression of GAPDH (1.04 ± 0.27 of the control, n = 6, P = 0.704). The wild-type and EAAT3$^{-/-}$ mice had a similar level of GAPDH proteins in their hippocampi (1.00 ± 0.12 vs. 1.05 ± 0.17, n = 5, P = 0.571). TEA still increased EAAT3 in the plasma membrane even if the results were not normalized by GAPDH data in the wild-type mice (1.39 ± 0.37 of the control, n = 6, P = 0.049). Thus, the increased EAAT3 by TEA appears to be a specific effect and not to be caused by changes in GAPDH expression.

TEA also increased GluR1 in the plasma membrane of wild-type mice but not in the EAAT3$^{-/-}$ mice (Fig. 1C). Similarly, TEA increased phospho-GluR1 at S845 in the wild-type mice but not in the EAAT3$^{-/-}$ mice. There was no change in phospho-GluR1 at S831 in both wild-type and EAAT3$^{-/-}$ mice (Fig. 1D and E).

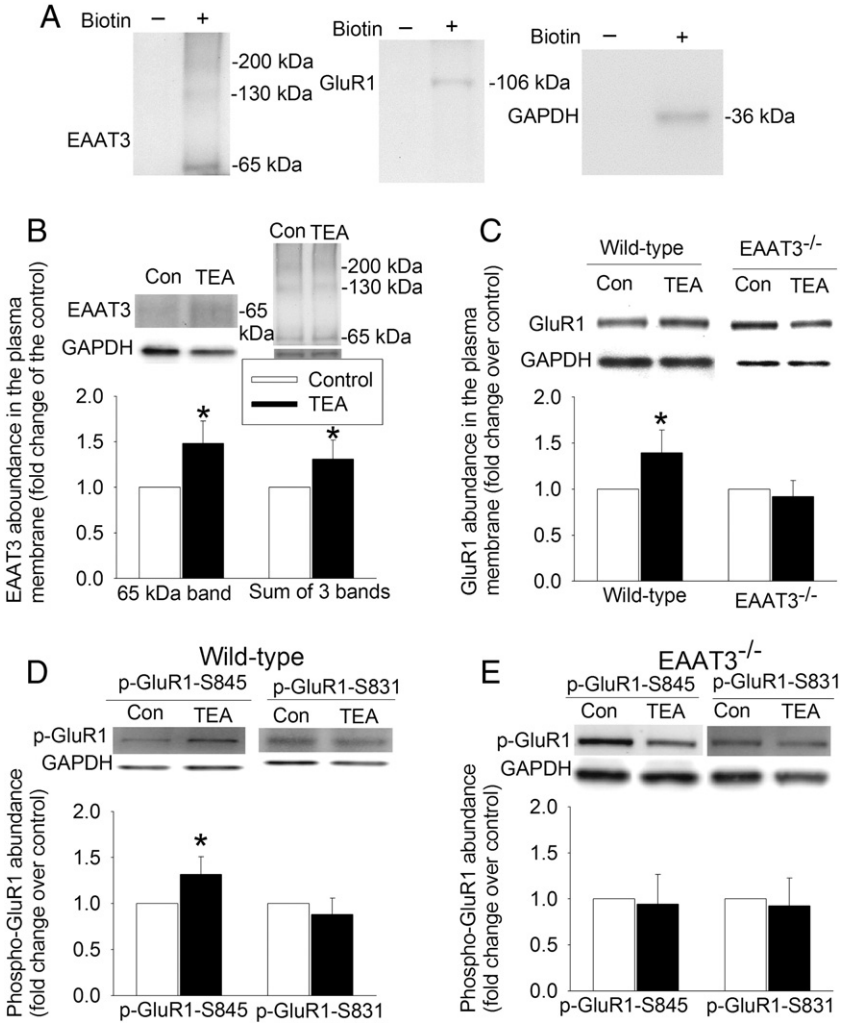

Fig. 1. TEA induces EAAT3 and GluR1 trafficking to the plasma membrane and GluR1 phosphorylation in the CD1 wild-type mouse hippocampus but not in the EAAT3$^{-/-}$ mice. Freshly prepared 300 μm coronal hippocampal slices from 8 to 12 week old CD1 male mice and their litter-mate EAAT3$^{-/-}$ mice were incubated with or without 25 mM TEA in the presence or absence of 1 mg/ml sulfo-NHS-SS-biotin, a biotinylation reagent, for 10 min at 37 °C. The biotinylation reagent was quenched immediately after the incubation. The homogenates of the hippocampal slices were used for Western blotting of phospho-GluR1. The biotinylated fraction was used for Western blotting of EAAT3 and GluR1. A: hippocampal slices of wild-type mice were incubated with or without sulfo-NHS-SS-biotin and then subjected to the rest of the procedure for harvesting biotinylated proteins, B: biotinylated EAAT3, C: biotinylated GluR1, D: phospho-GluR1 in wild-type mice, and E: phospho-GluR1 in EAAT3$^{-/-}$ mice. Results are mean ± SD (n = 6 for panels B, C and E, n = 5 for panel D). *P < 0.05 compared with corresponding control. Con: control, GAPDH: glyceraldehyde 3-phosphate dehydrogenase.

TEA increased phospho-PKA at T197 in the wild-type mice but not in the EAAT3$^{-/-}$ mice (Fig. 2A). The increase of phospho-GluR1 at S845 and GluR1 in the plasma membrane of the wild-type mice was blocked by KT5720, a PKA inhibitor (Fig. 2B and C). The PKA activator 6-BNZ-cAMP increased GluR1 in the plasma membrane of both wild-type and EAAT3$^{-/-}$ mice (Fig. 2D).

The omission of the incubation with the anti-EAAT3 antibody in the immunoprecipitation experiment using hippocampal slices of wild-type mice resulted in no detection of a protein band corresponding to EAAT3, PKA or PP2A (Fig. 3A), suggesting the dependence of the immunoprecipitate formation on the antibody. The immunoprecipitates prepared by the anti-EAAT3 antibody from the wild-type mouse hippocampal slices stimulated by TEA contained more PKA and PP2A than those from control slices (Fig. 3B); whereas TEA did not affect the amount of EAAT3 immunoprecipitated (0.96 ± 0.11 of control, $n = 5$, $P = 0.41$). Interestingly, TEA did not change the activity of protein phosphatase in these slices (Fig. 3C).

4. Discussion

We have shown that a short (minutes) or long (hours) exposure of isoflurane can increase the amount of EAAT3 in the plasma membrane or the total amount of EAAT3 in cells, respectively [13,19]. We used isoflurane to euthanize animals. This short exposure (minutes) was followed by at least 45 min of isoflurane-free period before the hippocampal slices were used in the experiments. The isoflurane effects on EAAT3 trafficking may have been disappeared by that time. Consistent with this possibility, hippocampal slices from wild-type mice euthanized by isoflurane had the same amount of EAAT3 in the plasma membrane as those from mice sacrificed by cervical dislocation. The short isoflurane exposure also did not affect the response of the hippocampal slices to TEA stimulation for EAAT3 increase in the plasma membrane.

To determine a potential role of EAAT3 in the biochemical cascade for learning and memory, we used TEA that has been shown previously to induce long-term potentiation [20] in freshly prepared hippocampal slices from CD1 wild-type mice. As expected, TEA also increased the trafficking of GluR1 to the plasma membrane. The phosphorylation of GluR1 at S845 but not at S381 was also increased by TEA. These TEA effects on GluR1 trafficking and phosphorylation did not occur in the hippocampal slices of EAAT3$^{-/-}$ mice that have a CD1 mouse gene background. We have shown previously that these mice do not express EAAT3 in the brain [9,10]. These results suggest a critical role of EAAT3 in TEA-induced GluR1 phosphorylation and trafficking.

S845 in GluR1 is a PKA phosphorylation site [2] and S831 in the molecule is a Ca^{++}/calmoduline-dependent protein kinase phosphorylation site [21,22]. The phosphorylation of GluR1 at S845 and S831 can enhance the trafficking of GluR1 to the plasma membrane [1,2]. Consistent with the increase of phospho-GluR1 at S845 by TEA, TEA also increased the phosphorylation of PKA at T197, an active form of PKA, in the hippocampus of CD1 mice. Inhibition of PKA by KT5720 abolished TEA-induced increase of phospho-GluR1 at S845 and the trafficking of GluR1 to the plasma membrane. These results suggest that TEA activates PKA, which then leads to GluR1 phosphorylation and trafficking in the CD1 wild-type mice. On the other hand, TEA did not increase the phospho-PKA at T197 in the EAAT3$^{-/-}$ mouse hippocampus. However, activation of PKA by 6-BNZ-cAMP could enhance GluR1 trafficking to

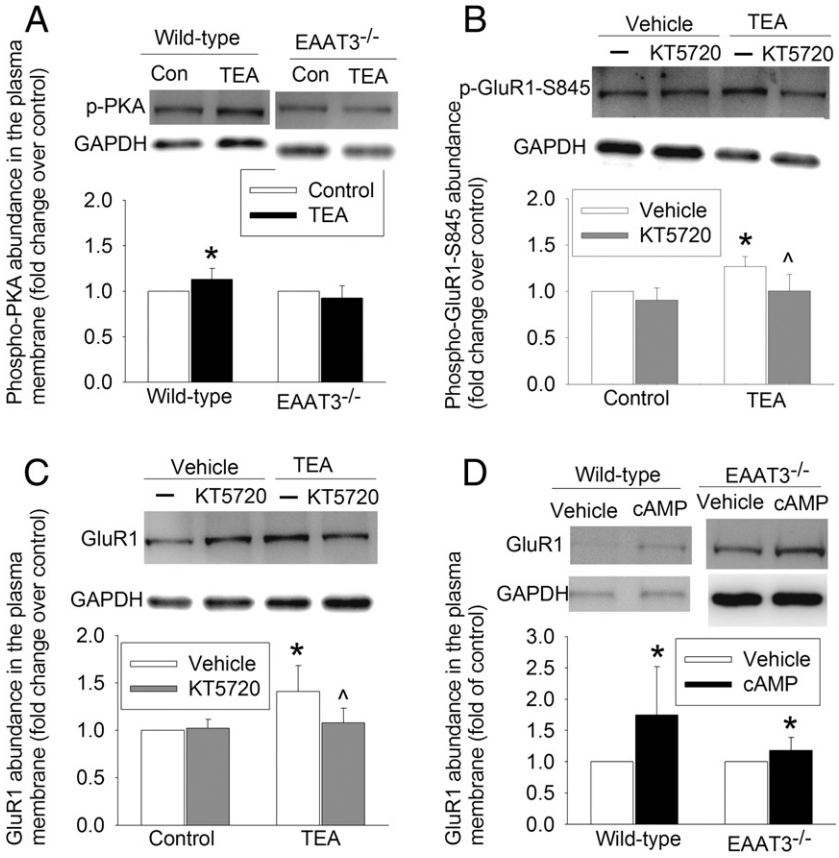

Fig. 2. TEA induces a PKA-dependent GluR1 phosphorylation and trafficking in the hippocampus of CD1 wild-type mice. Freshly prepared 300 μm coronal hippocampal slices from 8 to 12 week old CD1 male mice and their litter-mate EAAT3$^{-/-}$ mice were incubated with or without 25 mM TEA in the presence of 1 mg/ml sulfo-NHS-SS-biotin for 10 min at 37 °C. Some incubation buffers contained 2 μM KT5720 or 400 μM 6-BNZ-cAMP. Since KT5720 and 6-BNZ-cAMP were initially dissolved in the dimethyl sulfoxide and then diluted in the incubation buffer, the buffer for corresponding control experiment also contained the same final concentrations of dimethyl sulfoxide (vehicle group). The homogenates of the hippocampal slices were used for Western blotting of phospho-PKA and phospho-GluR1. The biotinylated fraction was used for Western blotting of GluR1. A: phospho-PKA, B: phospho-GluR1, and C and D: biotinylated GluR1. Results are mean ± SD ($n = 6$ for panels A, B and C, $n = 8$ for panel D). *$P < 0.05$ compared with corresponding control. ^$P < 0.05$ compared with TEA stimulation only. cAMP: 6-BNZ-cAMP, Con: control, GAPDH: glyceraldehyde 3-phosphate dehydrogenase.

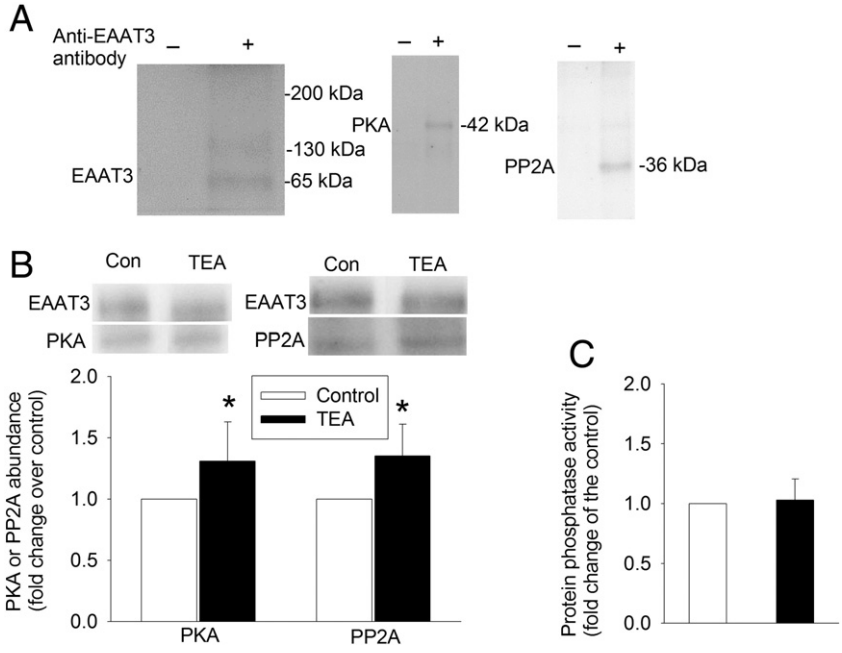

Fig. 3. TEA increased the amount of PKA and PP2A in the immunoprecipitates prepared by an anti-EAAT3 antibody. Freshly prepared 300 μm coronal hippocampal slices from 8 to 12 week old CD1 male mice were incubated with or without 25 mM TEA for 10 min at 37 °C. The slices were immediately prepared for immunoprecipitation by an anti-EAAT3 antibody or the homogenates of the slices were used for measuring protein phosphatase activity using para-nitrophenyl phosphate as the substrate. A: homogenates of wild-type mouse hippocampal slices were incubated with or without the anti-EAAT3 antibody and then subjected to the rest of immunoprecipitation procedure, B: PKA and PP2A abundance in the immunoprecipitates, and C: protein phosphatase activity. Results are mean ± SD (n = 5–7 for panel B, n = 8 for panel C). *P < 0.05 compared with corresponding control. Con: control.

the plasma membrane in the hippocampal slices prepared from EAAT3$^{-/-}$ mice and wild-type mice. These results suggest that the mechanisms to induce PKA activation after TEA stimulation have been altered in the EAAT3$^{-/-}$ mice.

To determine how EAAT3 may be involved in TEA-induced PKA activation, we performed an immunoprecipitation assay. The TEA-treated hippocampal slices of the CD1 mice had more PKA and PP2A in the immunoprecipitates prepared by an anti-EAAT3 antibody than control slices, suggesting that EAAT3 may directly or indirectly interact with PKA. Also, the immunoprecipitates prepared from TEA-treated slices had an increased PP2A. However, the activity of protein phosphatases in the slices was not changed by TEA. These results suggest that dephosphorylation of PKA and GluR1 by protein phosphatases may not be a target for TEA-induced PKA and GluR1 phosphorylation in the CD1 wild-type mice.

Overall, our results suggest that EAAT3 may help transmit TEA stimulation to intracellular signaling molecules that regulate GluR1 trafficking. EAAT3 can bind and transport extracellular glutamate to regulate glutamate neurotransmission [23]. This effect may limit the spill-over of glutamate molecules in one synapse to its neighboring synapses [24]. A previous study has shown that EAAT3 can limit recruitment of perisynaptic NR2B containing N-methyl-D-aspartic acid receptors to facilitate the formation of long-term potentiation induced by short bursts of high frequency stimulation in mouse hippocampal slices [25]. Our study helps identify the intracellular signaling mechanisms for this effect. Together, these findings provide initial evidence for EAAT3 as a critical player in the biochemical cascade of learning and memory.

One confounding issue is that EAAT3, in addition to transporting glutamate, can take up cysteine, a major substrate for the synthesis of glutathione in the mature neurons [8,26]. Lack of EAAT3 in the EAAT3$^{-/-}$ mice may result in a decreased neuronal glutathione level, which can lead to oxidative injury to neurons. This effect may then cause lack of TEA-induced GluR1 trafficking in these mice. However, direct activation of PKA by 6-BNZ-cAMP induced GluR1 trafficking to the plasma membrane in the EAAT3$^{-/-}$ mouse hippocampus, suggesting that the signaling process from PKA to GluR1 is preserved in these mice.

Our results suggest that EAAT3 can regulate GluR1 trafficking in the hippocampus. One unsolved issue is how EAAT3 can affect PKA activation. Our results showed that PKA was co-immunoprecipitated with EAAT3 after TEA stimulation, suggesting that PKA and EAAT3 are in the same protein complex. This physical arrangement may facilitate the interaction between EAAT3 and PKA. Future studies are needed to determine whether EAAT3 interacts directly or indirectly with PKA to regulate its activation.

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