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ENT1 Regulates Ethanol-Sensitive EAAT2 Expression and Function in Astrocytes

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Abstract

Background—Equilibrative nucleoside transporter 1 (ENT1) and excitatory amino acid transporter 2 (EAAT2) are predominantly expressed in astrocytes where they are thought to regulate synaptic adenosine and glutamate levels. Because mice lacking ENT1 display increased glutamate levels in the ventral striatum, we investigated whether ENT1 regulates the expression and function of EAAT2 in astrocytes, which could contribute to altered glutamate levels in the striatum.

Methods—We examined the effect of ENT1 inhibition and overexpression on the expression of EAAT2 using quantitative real-time PCR and measured glutamate uptake activity in cultured astrocytes. We also examined the effect of 0 to 200 mM ethanol doses for 0 to 24 hours of ethanol exposure on EAAT2 expression and glutamate uptake activity. We further examined the effect of ENT1 knockdown by a specific siRNA on ethanol-induced EAAT2 expression.

Results—An ENT1-specific antagonist and siRNA treatments significantly reduced both EAAT2 expression and glutamate uptake activity while ENT1 overexpression up-regulated EAAT2 mRNA expression. Interestingly, 100 or 200 mM ethanol exposure increased EAAT2 mRNA expression as well as glutamate uptake activity. Moreover, we found that ENT1 knockdown inhibited the ethanol-induced EAAT2 up-regulation.

Conclusions—Our results suggest that ENT1 regulates glutamate uptake activity by altering EAAT2 expression and function, which might be implicated in ethanol intoxication and preference.

Keywords

Excitatory Amino Acid Transporter 2 (EAAT2); Equilibrative Nucleoside Transporter 1 (ENT1); Glutamate Uptake; Adenosine Uptake

Glutamate is the primary excitatory neurotransmitter in the brain (Monaghan et al., 1989), and alterations in glutamatergic neurotransmission are thought to be involved in several psychiatric disorders, including alcoholism (Spanagel et al., 2005; Vengeliene et al., 2008). One of the important roles of astrocytes, which are the most abundant cell type in the mammalian central nervous system, is to uptake glutamate in the synaptic cleft to prevent neurons from overexcitement by glutamatergic neurotransmission (Miguel-Hidalgo, 2009). Glutamate uptake is mainly mediated by excitatory amino acid transporters (EAATs; also known as glutamate transporters). EAATs are sodium- and potassium-dependent members

of solute carrier family 1 (SLC1) and are found widely distributed throughout the mammalian brain (Amara and Fontana, 2002). Among the five mammalian EAATs, EAAT1 and EAAT2 are most abundantly expressed in astrocytes and are responsible for clearance of glutamate during neurotransmission (Amara and Fontana, 2002). While EAAT1 (GLAST) is expressed throughout the central nervous system (Danbolt, 2001), EAAT2 (GLT1) is expressed abundantly in the forebrain region with moderate expression throughout the remaining central nervous system (Gegelashvili and Schousboe, 1997). EAAT3 is a predominant subtype in peripheral tissues and found in both astroglia and neurons (Kanai and Hediger, 1992). EAAT4 is found predominantly in the Purkinje cells of the cerebellum (Fairman et al., 1995) while EAAT5 is primarily expressed in the retina (Arriza et al., 1997).

Because extracellular adenosine levels in the brain are known to mediate the acute and chronic effects of ethanol, adenosine transportation across the plasma membrane is one of the main mechanisms in regulating the effects of alcohol (Diamond and Gordon, 1994; Dunwiddie and Masino, 2001). Nucleoside transport across the plasma membrane is one of several factors that regulate extracellular adenosine concentrations, which, under basal conditions, are in the range of 25 to 250 nM in the brain (Dunwiddie and Masino, 2001). *In vitro*, acute ethanol treatment increases extracellular adenosine because of selective inhibition of one subtype of nucleoside transporter, ENT1, in S49 mouse lymphoma and NG108-15 neuroblastoma × glioma cells (Nagy et al., 1989, 1990). Recent studies demonstrated that astrocytes regulate adenosine levels in the synapse (Boison, 2008a). Adenosine uptake into astrocytes is mainly mediated by nucleoside transporters (Boison, 2008b). Sodium-independent equilibrative nucleoside transporters (ENTs) include four ENT subtypes (ENT1-4). ENTs are one of two main membrane nucleoside transporter families, mediating nucleoside transport bidirectionally across the plasma membrane (Anderson et al., 1999). ENT1 is sensitive to nM concentrations of nitrobenzylmercaptapurine riboside (NBMPR; also known as nitrobenzylthioionosine, NBTI), whereas ENT2 is resistant to NBMPR even up to mM concentrations (Citron et al., 1997; Coe et al., 1997; Crawford et al., 1998). Both ENT1 and ENT2 are widely expressed in astrocytes (Peng et al., 2005).

Previously, our laboratory reported that ENT1 null mice displayed a higher glutamate tone in striatal slices when compared to wild-type mice (Choi et al., 2004). Because ENT1 and EAAT2 are predominantly expressed in astrocytes, we hypothesized that the expression and function of EAAT2 may be regulated by ENT1 activity in astrocytes. Using a selective and water-soluble ENT1 inhibitor, NBMPR-P, and ENT1-selective siRNA, we investigated the effect of ENT1 inhibition in astrocytes. Our study provides a possible functional correlation between ENT1 and EAAT2 in response to ethanol in astrocytes.

MATERIALS AND METHODS

Astrocyte Culture

The astrocytic cell line C8-D1A, which was originally cloned from the mouse cerebellum (Alliot and Pessac, 1984), was obtained from ATCC (American Type Culture Collection, VA). Cells were maintained in Dulbecco's modified Eagle medium containing glucose, 10% heat-inactivated fetal bovine serum (FBS; ATCC, American Type Culture Collection, VA), 1% L-glutamine (Gibco, Auckland, New Zealand), and 1% Antibiotic-Antimycotic (Invitrogen, Carlsbad, CA). Monolayers were cultured at 37°C in the presence of 5% CO₂/95% O₂ (normoxia) in a fully humidified atmosphere with medium replacement every 2 to 3 days.

Real-Time RT-PCR

To measure mRNA levels, real-time quantitative RT-PCR was performed with the iCycler IQ Real-Time PCR detection system (Bio-Rad Laboratories, Inc., Hercules, CA) using QuantiTect SYBR Green RT-PCR Kit (Qiagen, CA). Gene-specific primers for ENT1, EAAT1, EAAT2, GAPDH, and alpha-tubulin were purchased from Qiagen (Qiagen, CA), and the following real-time RT-PCR protocol was used for all genes: reverse transcription step for 30 minutes at 50°C, denaturation at 95°C for 15 minutes to activate the HotStart enzyme followed by an additional 45 cycles of amplification and quantification (15 seconds at 94°C; 10 seconds at 55°C; 30 seconds at 72°C) each with a single fluorescence measurement. The mRNA expressions of the genes were normalized by GAPDH or alpha-tubulin as housekeeping genes. Fold or percentage changes were calculated by subtracting mean GAPDH Ct values from Ct values for the gene of interest using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001).

Adenosine Uptake Assay

[³H] adenosine uptake assay was conducted in phosphate-buffered saline (PBS) containing 1 mCi [³H] nucleoside/ml at room temperature for 15 minutes in the presence or absence of ENT1-specific inhibitor, NBMPR-P (nitrobenzylthioinosine 5'-monophosphate). The disodium salt NBMPR-P is water-soluble form (Gati and Paterson, 1997) and was kindly provided by Dr. W. Gati (University of Alberta, Canada). The NBMPR-P is rapidly converted to NBMPR by ecto 5-nucleotidase in cultured cells (Ogbunude et al., 1984). Nucleoside uptake was terminated by adding ice-cold PBS containing 10 μM NBMPR-P twice and then solubilizing the cells in 1% Triton X-100. Radioactivity was determined inside the cells using a liquid scintillation counter (LS65000 Beckman Coulter). The level of adenosine uptake was calculated by using the ratio of radioactivity to protein quantity of cell extracts.

Glutamate Uptake Assay

Uptake activity of astrocytes using L-[G-³H]-glutamic acid (specific radioactivity: 29 Ci/mmol, concentration: 1.0 mCi/ml; Amersham Bioscience, Arlington Heights, IL) was measured as previously described (Mysona et al., 2009). Glutamate uptake was initiated by the addition of 250 μl uptake buffer containing 2.5 μM glutamate with 2.0 μCi/ml radiolabeled [³H]-glutamate in the presence or absence of NBMPR-P and after ethanol treatments (0, 50, 100, and 200 mM) for 24 hours. Cells were incubated for 15 minutes at 37°C, the buffer was removed, and cells were washed twice with ice-cold uptake buffer. Cells were then solubilized with 1% Triton X-100, radioactivity was determined by liquid scintillation counter, and proteins were quantified using the Bio-Rad protein assay reagent (Hercules, CA). The relative level of glutamate uptake was calculated using the ratio of radioactivity to protein quantity of cell extracts.

ENT1 Knockdown in the Astrocytes

The target sequences of Slc29a1-1 siRNA and Slc29a1-3 siRNA for ENT1 are 5'-CAGGACAGGTATAAGGCAGTA-3' and 5'-AAGATTGTGCTCATCAATTCA-3', respectively. siRNAs for Slc29a1 or control siRNA (5 nM) were transfected into 10⁵ astrocytes in a 24-well plate using HiPerFect transfection reagent (Qiagen). Forty-eight hours after the transfection, total RNA was isolated using RNAeasy-Mini kit (Qiagen), and the expression levels of ENT1 mRNA and EAAT2 mRNA were measured by real-time RT-PCR.

ENT1 Overexpression in Astrocytes

pCMV-SPORT6 ENT1 (clone ID: 2631728, Open Biosystem) was used to overexpress mouse ENT1 in the astrocyte C8-D1A. One micrograms DNA constructs (pCMV-SPORT6 or pCMV-SPORT6 ENT1) were transfected into 10^5 astrocytes in a 24-well plate using 4 μ l Lipofectamine 2000 (Invitrogen). Twenty-four hours after the transfection, total RNA was isolated using RNAeasy-Mini kit (Qiagen), and the expression levels of ENT1 mRNA and EAAT2 mRNA were measured by real-time RT-PCR.

Statistical Analysis

All data were expressed as mean $\pm$ SEM (standard error mean) and were analyzed by two-tailed *t*-test or one-way ANOVA followed by Tukey post hoc test for individual comparisons. Results of comparisons were considered significantly different if the *p* value was <0.05 .

RESULTS

Inhibition of ENT1 Activity Reduced the Expression of EAAT2 in Astrocytes

Because ENT1, EAAT1, and EAAT2 are highly expressed in astrocytes, we investigated whether ENT1 inhibition directly alters expression or function of EAAT1 or 2 in astrocytes. First, we examined whether ENT1, EAAT1, or EAAT2 are actually expressed in astrocytes. As shown in Fig. 1A, both ENT1 and EAAT2 mRNAs are highly expressed in astrocytes, but EAAT1 mRNA expression appears very low in the astrocytic cell line C8-D1A. We validated primers using mouse cerebellar extracts as a positive control (Fig. S1).

Next, we examined whether pharmacological inhibition of ENT1 activity alters the expression of EAAT2 using a series of NBMPR-P concentrations from 1 nM to 10 μ M (1 nM, 10 nM, 100 nM, 1 μ M, and 10 μ M) for 24 hours. As shown in Fig. 1B, EAAT2 mRNA levels were significantly reduced after the inhibition of ENT1 at doses of 0.1, 1, and 10 μ M NBMPR-P. One-way ANOVA analysis showed that NBMPR-P treatment had a significant effect on the reduction in EAAT2 expression ($F_{5,10} = 11.21$, $p < 0.001$) at 0.1, 1.0, and 10 μ M NBMPR-P. At 10 μ M NBMPR-P, as shown in Fig. 1C, EAAT2 mRNA expression was significantly reduced at 3, 9, and 24 hours when cells were exposed to NBMPR-P for 24 hours. One-way ANOVA analysis revealed that NBMPR-P treatment had a significant effect on the reduction in EAAT2 expression ($F_{3,6} = 16.02$, $p = 0.003$) at 3, 9, and 24 hours. These results suggest that inhibition of ENT1 may down-regulate EAAT2 expression in the astrocytes.

Inhibition of ENT1 Activity Decreased Glutamate Uptake Activity in Astrocytes

Next, we examined whether ENT1 activity regulates glutamate uptake activity. As shown in Fig. 2A, NBMPR-P treatment for 24 hours significantly reduced adenosine uptake (one-way ANOVA; $F_{5,18} = 9.380$, $p < 0.001$), indicating that ENT1 activity is inhibited by NBMPR-P in astrocytes. As shown in Fig. 2B, NBMPR-P treatment also significantly reduced glutamate uptake (one-way ANOVA; $F_{5,18} = 13.65$, $p < 0.001$). Thus, together with mRNA expression, these results suggest that ENT1 expression is correlated with EAAT2 expression and glutamate uptake activity.

Knockdown of ENT1 Expression by siRNA Down-Regulated EAAT2 Expression in Astrocytes

Here, we further examined whether decreased ENT1 expression by siRNA regulates EAAT2 expression. As shown in Fig. 3A, siRNA treatments for 48 hours significantly decreased ENT1 mRNA levels compared to those of control siRNA (unpaired two-tailed *t*-test; $p <$

0.05). As shown in Fig. 3B, siRNA for ENT1 also significantly reduced EAAT2 mRNA levels (unpaired two-tailed *t*-test; $p < 0.05$). Thus, these results showed that ENT1 expression is causally related to EAAT2 expression in astrocytes, which is consistent with our finding that ENT1 inhibition by NBMPPR-P reduced the expression of EAAT2 in astrocytes.

ENT1 Overexpression Up-Regulated EAAT2 Expression and Glutamate Uptake Activity in Astrocytes

We further examined whether ENT1 overexpression regulates EAAT2 expression. As shown in Fig. 4A, ENT1 mRNA levels were significantly elevated 24 hours after the transfection of cloned mouse ENT1 gene compared to the control (unpaired two-tailed *t*-test; $p < 0.05$). As shown in Fig. 4B, EAAT2 mRNA levels were also significantly elevated along with ENT1 overexpression (unpaired two-tailed *t*-test; $p < 0.05$). In contrast to inhibition of ENT1 (Fig. 2B), overexpression increased glutamate uptake activity (unpaired two-tailed *t*-test; $p < 0.05$) (Fig. 4C). We validated the mRNA expression of ENT1 and EAAT2 using an additional housekeeping control gene, alpha-tubulin (Fig. S2). Taken together with NBMPPR-P and siRNA experiments, these results indicate that ENT1 expression is positively correlated with EAAT2 expression and glutamate uptake activity in astrocytes.

Ethanol Up-Regulates EAAT2 Expression and Function in Astrocytes

Because glutamate levels in the synapses play an important role in ethanol intoxication and the pathogenesis of alcohol dependence (Tsai et al., 1995), and a genetic variation of the EAAT2 gene confers vulnerability to risk-taking behavior in alcoholics (Sander et al., 2000), we first examined the effect of ethanol (0, 50, 100, and 200 mM) for 24 hours on EAAT2 mRNA expression. As shown in Fig. 5A, EAAT2 mRNA levels were significantly increased by ethanol (one-way ANOVA; $F_{3,8} = 101.5$, $p < 0.001$). Also, we further examined the temporal effects of ethanol on the expression of EAAT2 mRNA using quantitative real-time RT-PCR. As shown in Fig. 5B, 100 mM ethanol treatment appears to increase the EAAT2 mRNA expression levels (one-way ANOVA; $F_{4,10} = 13.13$, $p < 0.001$). Similarly, 200 mM ethanol treatment also increases the EAAT2 mRNA expression levels (one-way ANOVA; $F_{4,15} = 43.12$, $p < 0.001$) (Fig. 5C).

Next, we investigated whether ethanol exposure alters glutamate uptake activity. As shown in Fig. 5D, 100 or 200 mM ethanol treatment significantly induced glutamate uptake (one-way ANOVA; $F_{3,5} = 11.45$, $p < 0.05$), indicating that ethanol-induced increase in EAAT2 mRNA expression is positively correlated with augmented glutamate uptake activity in astrocytes.

Knocking Down ENT1 Expression Inhibited Ethanol- Induced Up-Regulation of EAAT2 Expression in Astrocytes

Because ethanol up-regulates EAAT2 expression and glutamate uptake activity in astrocytes (Fig. 5) and ENT1 inhibition/knockdown significantly reduces EAAT2 expression (Figs. 2 and 3), we decided to examine whether knockdown of ENT1 by siRNA inhibits the ethanol-induced up-regulation of EAAT2 mRNA expression. As shown in Fig. 6, ethanol-induced EAAT2 mRNA expression was completely inhibited when ENT1 is deficient by siRNA at 100 mM or 200 mM ethanol treatment for 24 hours (unpaired two-tailed *t*-test; $p < 0.05$). To validate the altered expression of EAAT2, we examined the mRNA expression using alpha-tubulin as a control, which showed similar results (Fig. S3). Taken together, these results suggest a possible correlation between ENT1 and ethanol-induced EAAT2 expression in astrocytes.

DISCUSSION

Glutamate-mediated excitatory pathways play an important role in the pathogenesis of alcohol dependence (Tsai et al., 1995), and genetic variation of the EAAT2 gene confers vulnerability to risk-taking behavior in alcoholics (Sander et al., 2000). Our findings demonstrate that ENT1 regulates ethanol-sensitive EAAT2 mRNA expression and function in astrocytes. When we incubated the astrocytic cells with an ENT1-specific antagonist, EAAT2 mRNA expression and glutamate uptake activity were significantly reduced. These results suggest that reduced EAAT2 expression is correlated with decreased glutamate uptake activity in cells deficient of ENT1 expression or function. Decreased glutamate uptake activity might contribute to increased extracellular glutamate levels and thereby activate glutamate receptor-mediated signaling. Consistently, our previous data show increased glutamate release in the nucleus accumbens of ENT1 null mice (Choi et al., 2004). We also reported that ENT1 null mice consumed significantly more ethanol compared to wild-type littermates in a two-bottle drinking experiment (Choi et al., 2004). Recently, we found that ENT1 null mice are resistant to the ataxic effect of NMDA- or AMPA-receptor antagonists in a rotarod test and seek more ethanol in an operant chamber experiment compared to wild-type littermates (Chen et al., 2010). Therefore, our findings suggest that increased glutamate levels or signaling, which is possibly attributed to decreased EAAT2 expression in astrocytes, may contribute to increased ethanol preference.

Because adenosine is an inhibitory neurotransmitter (Dunwiddie and Masino, 2001), increasing excitatory glutamate levels could be a homeostatic response to maintain cellular excitability of astrocytes. Consistently, acute ethanol exposure at 200 mM is shown to decrease adenosine uptake and increase adenosine levels in NG-108 cells, neuroblastoma × glioma hybrid cells (Sapru et al., 1994), which may increase EAAT2 mRNA expression. However, we observed decreased adenosine tones in the ENT1 null mice (Choi et al., 2004), which contribute to decreased levels of ethanol response and excessive alcohol consumption along with increased glutamate neurotransmission (Choi et al., 2004). Because ENT1 is a bidirectional transporter for nucleosides including adenosine (Dunwiddie and Masino, 2001), ablation of the ENT1 gene would significantly slow down the dynamics of adenosine exchange across the plasma membrane, which may result in low levels of extracellular adenosine in vivo. However, paradoxically decreased uptake activity of ENT1 by pharmacological inhibition in cultured cells could have an opposite effect as in ENT1 null mice.

In the central nervous system, although both EAAT1 and EAAT2 are mainly responsible for glutamate uptake (Amaral, 2002), EAAT2 and its splice variants are known to regulate about 90% of glutamate levels in the glial cells (Shachnai et al., 2005). Interestingly, our recent proteomic studies revealed that EAAT2 protein levels were reduced in the striatum of ENT1 null mice compared to wild-type mice, whereas EAAT1 protein expressions were not altered between genotypes (Nam HW and Choi DS, manuscript submitted). Therefore, C8-D1A cells appear to be useful to examine the functional relationship between ENT1 and EAAT2. Furthermore, several studies showed that EAAT2 has been implicated in alcoholism. Mouse EAAT2 (*Slc1a2*) gene is located in a central part of chromosome 2 (E2) (Kirschner et al., 1994), near a quantitative trait loci that modulate neuroexcitability and seizure frequency in mouse models of alcohol withdrawal and epilepsy (Crabbe and Belknap, 1993). Also, a genetic variant of human EAAT2, G603A, seems to be associated with antisocial alcoholics (Sander et al., 2000) and cirrhotic alcoholics (Foley et al., 2004). However, it has not been explored whether the G603A allele in EAAT2 is implicated in its expression or function. Based on our electrophysiological study (Choi et al., 2004), elevated glutamate levels in ENT1 null mice may consistently activate glutamate signaling, which is positively associated with increased alcohol-seeking behavior (Spanagel and Kiefer, 2008).

Thus, one possibility is that alcoholic patients who carry the EAAT2 G603A allele might have decreased EAAT2 activity, which causes elevated glutamate levels in the brain.

Interestingly, we found that both expression and function of EAAT2 were significantly up-regulated in astrocytes in response to ethanol exposure. Our findings suggest that increased glutamate uptake may be associated with EAAT2 expression levels in astrocytes. These results are consistent with other investigators' findings (Othman et al., 2002; Smith, 1997). Othman and colleagues (2002) reported that prolonged treatment of 50 mM ethanol for 3 days increased glutamate uptake by affecting PKC modulation of transporter activity in rat cortical astrocytes. Also, Smith and colleagues (1997) found that glutamate uptake was increased in astrocytes in response to ethanol exposure for 4 days while the expressions of EAAT1 or EAAT2 were not significantly changed. Consistent with our findings, Zink and colleagues (2004) found that both EAAT1 and EAAT2 mRNA and protein expressions were significantly increased in rat organotypic slices. While these studies presented EAAT2 expression and function separately in response to ethanol, our findings revealed that EAAT2 mRNA expression is correlated with glutamate uptake activity in response to ethanol. Nevertheless, our current study using cultured cells as well as primary or organo-cultures has some limitations. First, experiments carried out using in vitro or ex vivo conditions await validation through in vivo experiments. Second, because EAAT1 expression seems very low in the astrocyte C8-D1A cell line, we were not able to examine the role of EAAT1, which is also known to regulate glutamate levels (Spanagel et al., 2005). Thus, further in vivo studies would illuminate the physiologically relevant roles of ENT1 and EAAT2 in astrocytes or neuron-glia interaction in ethanol-regulated glutamate signaling.

Acute ethanol exposure increases EAAT4 activity in *Xenopus oocytes* (Park et al., 2008). Because EAAT4 is predominantly expressed in the cerebellar Purkinje cells (Fairman et al., 1995), whereas EAAT2 is expressed in both cerebellar and forebrain astroglial cells, the effect of ethanol on cerebellar function, such as ethanol-mediated ataxia, could be mediated by a synergistic effect of EAAT2 and EAAT4 depending on the ethanol dose. On the other hand, EAAT2 or EAAT1 is likely responsible for the effect of ethanol on forebrain regions. Together, increasing glutamate uptake might be related to ethanol-mediated glutamate reduction, which results in increased ethanol intoxication in vivo. Therefore, if ENT1 inhibition down-regulates EAAT2 expression and function, it may cause increased synaptic glutamate levels and thereby mitigate intoxicating effects of ethanol including ataxia.

EAAT2 expression seems to be regulated by CREB in primary cultured cells (Schluter et al., 2002). Because we have found that CREB activity is reduced in the striatum of ENT1 null mice (Nam HW and Choi DS, manuscript submitted), it is possible that reduced CREB activity, in response to the ablation of ENT1, may contribute to a decrease in EAAT2 expression. However, a recent study showed that transcription factors, NF- κ B (Lee et al., 2008) and kappa B-motif binding phosphoprotein (KBBP) (Yang et al., 2009), regulate EAAT2 expression. Interestingly, pituitary adenylate cyclase-activating polypeptide (PACAP) is known to promote astrocytic EAAT2 expression (Figiel and Engele, 2000). Also, some growth factors including FGF2 and TGF α increase EAAT2 expression in primary cultured cells from rats (Figiel et al., 2003). Thus, further studies are needed to reveal signaling mechanisms of ENT1-dependant EAAT2 expression.

In summary, we demonstrate a possible correlation of ENT1 expression and function with EAAT2-regulated glutamatergic signaling in astrocytes. Because the glutamatergic system is involved in alcohol use disorders, the current study will be helpful to understand the etiology of alcohol use disorders in humans as well as to develop novel therapeutic methods.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

EAAT2 expression was down-regulated by the inhibition of ENT1. **(A)** Expressions of ENT1 and EAAT2 mRNA levels were validated by quantitative RT-PCR. **(B)** EAAT2 mRNA expression was decreased by the inhibition of ENT1 at 0.1, 1, and 10 μ M NBMPR-P for 24 hours. **(C)** EAAT2 mRNA expression was decreased by the inhibition of ENT1 via 10 μ M NBMPR-P from 3 hours until 24 hours. *GAPDH* was used as an internal normalization control. $n = 3$; $*p < 0.05$ compared to saline-treated cells by Tukey post hoc tests. All data were expressed as mean $\pm$ SEM.

Fig. 2.

Inhibition of ENT1 function down-regulated glutamate uptake activity. **(A)** Adenosine uptake activity was inhibited by NBMPR-P, a potent ENT1 antagonist. **(B)** Glutamate uptake activity was inhibited by NBMPR-P. *GAPDH* was used as an internal normalization control. $n = 4$; $*p < 0.05$ compared to control (saline-treated) cells by Tukey post hoc test. All data were expressed as mean $\pm$ SEM.

Fig. 3. Knockdown of ENT1 expression decreased EAAT2 mRNA expression in astrocytes. **(A)** ENT1 mRNA levels were significantly reduced after ENT1 siRNA transfection for 48 hours. **(B)** EAAT2 mRNA levels were reduced by ENT1 knockdown by ENT1-specific siRNA. The effects of 5 nM nonsilencing siRNA or *ENT1* siRNAs (Slc29a1-1 and Slc29a1-3) on *ENT1* or EAAT2 mRNA expressions were determined by quantitative RT-PCR using *GAPDH* as an internal control. $n = 3-4$; * $p < 0.05$ compared to nonsilencing siRNA-treated transfected cells by unpaired two-tailed *t*-test. All data were expressed as mean $\pm$ SEM.

Fig. 4.

ENT1 overexpression up-regulated EAAT2 and glutamate uptake activity in astrocytes. (A) ENT1 mRNA levels were elevated after pCMV-SPORT6 ENT1 transfection for 24 hours. (B) EAAT2 mRNA levels were increased by ENT1 overexpression for 24 hours. (C) Glutamate uptake activity was elevated by ENT1 overexpression for 24 hours. The effect of 1 μ g control vector or pCMV-SPORT6 ENT1 construct on ENT1 or EAAT2 mRNA expressions in 24-well plates or on glutamate uptake activity in 12-well plates. GAPDH was used as an internal normalization control. $n = 3-4$; $*p < 0.05$ compared to control vector-transfected cells by unpaired one or two-tailed t -test. All data were expressed as mean $\pm$ SEM.

Fig. 5. Ethanol-induced up-regulation of EAAT2 mRNA expression and elevation of glutamate uptake activity. **(A)** EAAT2 mRNA levels were increased in response to 100 mM or 200 mM ethanol exposure for 24 hours. **(B)** At a 100 mM ethanol dose, EAAT2 mRNA levels were increased at 24-hour exposure. **(C)** At a 200 mM ethanol dose, EAAT2 mRNA levels were increased at 4-hour or 24-hour exposure. **(D)** Glutamate uptake activity was elevated in response to 100 mM or 200 mM ethanol exposure for 24 hours. $n = 3$; $*p < 0.05$ compared to control (saline-treated) cells by Tukey post hoc test. All data were expressed as mean $\pm$ SEM.

Fig. 6.

Knockdown of ENT1 expression by siRNA inhibited ethanol-induced EAAT2 expressions at ethanol doses of 100 mM (A) or 200 mM (B) ethanol treatment. GAPDH was used as an internal control. $n = 3$; $*p < 0.05$ compared to nonsilencing siRNA-transfected cells without ethanol exposure by t -test. All data were expressed as mean $\pm$ SEM.