J Mol Neurosci (2013) 51:779–787
DOI 10.1007/s12031-013-0064-y

Ceftriaxone Treatment Affects the Levels of GLT1 and ENT1 As Well As Ethanol Intake in Alcohol-Preferring Rats

Youssef Sari · Sai N. Sreemantula · Moonnoh R. Lee · Doo-Sup Choi

Received: 17 May 2013 / Accepted: 27 June 2013 / Published online: 27 July 2013

Abstract Studies have demonstrated that deletion of equilibrative nucleoside transporter 1 (ENT1) is associated with reduced glutamate transporter 1 (GLT1) level, and consequently increased ethanol intake. In this study, we measured changes in GLT1 and ENT1 levels in prefrontal cortex (PFC), and nucleus accumbens (NAc) core and shell associated with alcohol drinking in alcohol-preferring (P) rats. We examined, then, whether ceftriaxone (CEF) would affect both GLT1 and ENT1 levels in these brain regions. P rats were given 24-h concurrent access to 15 and 30 % ethanol, water, and food for 5 weeks. On Week 6, P rats received 100 mg/kg CEF (i.p.) or a saline vehicle for five consecutive days. Ethanol intake was measured daily for 8 days starting on the first day of injections. We found a significant reduction in daily ethanol intake in CEF-treated group, starting on Day 2 of injections. Western blot for GLT1 and binding assay for ENT1 revealed downregulation of GLT1 level, whereas ENT1 levels were increased in the NAc core and NAc shell, respectively, but not in the PFC in saline vehicle group. Importantly, CEF treatment reversed these effects in both NAc core and shell. These findings provide evidence for potential regulatory effects of CEF on both GLT1 and ENT1 expression in reducing ethanol intake.

Y. Sari (✉) · S. N. Sreemantula
Department of Pharmacology, College of Pharmacy and Pharmaceutical Sciences, University of Toledo, Toledo, OH 43614, USA
e-mail: youssef.sari@utoledo.edu

M. R. Lee · D.-S. Choi
Department of Molecular Pharmacology and Experimental Therapeutics, Mayo Clinic College of Medicine, Rochester, MN 55905, USA

Present Address:
M. R. Lee
Synapse and Neural Circuit Research Unit, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, MA 20892, USA

Keywords ENT1 · GLT1 · EAAT2 · Alcohol dependence · Glutamate

Introduction

Alcohol dependence and addiction have been suggested to involve alteration of transmission in several neurotransmitter systems. Among them, the glutamatergic system has been shown to play a key role in alcohol tolerance, dependence, and withdrawal (Krystal et al., 2003). Glutamate transport is found altered by ethanol intake (Othman et al., 2002; Smith, 1997; Smith and Weiss, 1999). It has been shown that repeated ethanol exposure for several days induced a decrease in glutamate uptake in nucleus accumbens (NAc) in rats (Melendez et al., 2005). In addition, chronic ethanol exposure was associated with downregulation of glutamate transport in the cerebral cortex (Schreiber and Freund, 2000). Glutamate transporter 1 (GLT1) is responsible for the removal of most of the extracellular glutamate (Danbolt, 2001; Mitani and Tanaka, 2003; Rothstein, 1995; Rothstein et al., 1995; Ginsberg et al., 1995).

We have recently demonstrated that upregulation of GLT1 in NAc and prefrontal cortex (PFC) by i.p. administration of ceftriaxone (CEF) attenuated cue-induced cocaine relapse in a dose-dependent manner (Sari et al., 2009). In accordance, Kalivas et al. found similar effects regarding cocaine relapse with CEF treatment (Knackstedt et al., 2010). CEF is a β-lactam antibiotic known to upregulate GLT1 (Miller et al., 2008; Rothstein et al., 2005; Sari et al., 2010; Sari et al., 2011). Furthermore, we recently found that CEF treatment induced a dose-dependent reduction in ethanol intake in male and female P rats compared to saline vehicle-treated rats (Sari et al., 2011; Sari et al., 2013). We have also reported that CEF attenuates relapse like to ethanol-drinking behavior (Qrunfleh et al., 2013).

It is important to note that the deletion of equilibrative nucleoside transporter 1 (ENT1) protein has been revealed to alter the extracellular adenosine and consequently induced reduction of GLT1 levels, which can lead to increased extracellular glutamate (Nam et al., 2011; Wu et al., 2010; Wu et al., 2011). ENT1 expression was suggested to be correlated with GLT1 expression and also glutamate activity (Wu et al., 2010). Thus, in this study, we aimed to investigate potential changes in ENT1 and GLT1 expressions in PFC and NAc core and shell in chronically ethanol exposed P rats. Importantly, we also tested the effects of CEF on ENT1 and GLT1 levels in the PFC as well as the NAc core and shell.

Methods and Materials

Animals

Male alcohol-preferring P rats were used in this study as an established model of alcoholism. P rats were procured from the breeding colonies of the Indiana University School of Medicine and Indiana Alcohol Research Center (Indianapolis, IN, USA) at the age of 21–30 days. They were housed in standard plastic tubs in the Department of Laboratory Animal Resources (DLAR, University of Toledo, Health Science Campus) and acclimatized to the vivarium. The plastic tubs had corn cob bedding, and a temperature of 21 °C and 50 % humidity were maintained in the room with a 12-h light/dark cycle. All P rats had ad libitum access to food and water throughout the experimental procedures. These animal procedures were approved by the Institutional Animal Care and Use Committee of The University of Toledo, Health Science Campus, Toledo, OH, USA. The program at The University of Toledo is accredited by the Association of the Assessment and Accreditation of Laboratory Animal Care International (AAALACI). The approved experimental procedures in this study are in accordance with the guidelines of the Institutional Animal Care and Use Committee of the National Institutes of Health.

P rats were divided at the age of 3 months and single housed in the bedded plastic cages. We have tested three groups as follows: (1) naïve (water) control group (exposed to water and food only and received i.p., injections of saline vehicle solution, (n=6)), (2) saline vehicle control group (ethanol control group) received i.p. injections of saline vehicle solution (n=8), and (3) CEF-treated group at dose of 100 mg/kg, i.p. (n=8). Ethanol control group (saline vehicle control group) and CEF-treated groups had access to free choice ethanol (15 and 30 %), water, and food throughout the experiments. Note that naïve (water) control group had access to water and food only throughout the experiments.

Ethanol and Water Drinking Measurements

Male P rats were given free access to food, water, and ethanol (15 and 30 %) for a period of 5 weeks. Ethanol was prepared by diluting 190 proof (95 %) ethanol with deionized water to make 15 and 30 % concentrations. Ethanol and water were replaced three times a week, and the bottles were weighed before and after consumption. The amount of water and ethanol consumed was determined to the nearest tenth of a gram by subtraction of the measured bottle weights from their initial weights containing water or ethanol. Furthermore, animals were weighed three times a week to report water intake in milliliters or ethanol intake in grams per kilogram of body weight. We have used a densitometry formula to convert the actual grams of ethanol consumed per kilogram of body weight of animals. All animals met the requirement of drinking ≥4 g of ethanol per kilogram of body weight per day consistently for at least 2 weeks before saline or CEF treatment. This requirement has been adopted in recent studies (Sari et al., 2011; Sari and Sreemantula, 2012). The average consumption during the last 2 weeks of the 5-week drinking paradigm before treatment was taken as the baseline for the drinking behavior measurements. On the first day of Week 6, P rats were administered i.p. CEF 100 mg/kg of body weight and saline vehicle daily for five consecutive days. Naïve (water) control group was also administered saline vehicle daily for 5 days. Water intake, ethanol intake, and animal body weight were measured daily for 8 days (3 days post-treatment).

Brain Region Harvesting

Animals were euthanized by exposure to isoflurane and decapitated on Day 8 (3 days after the last CEF or saline i.p. injections). Brains were removed and stored at −70 °C until use. Further, PFC, NAc core, and shell were microdissected stereotaxically using a cryostat machine. Extractions of the brain regions were performed using stereotaxic coordinates for the rat brain atlas established by Paxinos and Watson (2007). As described recently, the NAc core and shell were identified at the appearance of anterior commissure (Sari and Sreemantula, 2012). The medial part of the PFC was dissected at the same level of NAc core and shell. We used surgical blades to puncture all these identified brain reward regions. These brain regions were extracted and frozen at −70 °C for Western blot procedure to examine GLT1 levels and tritiated NBTI binding for ENT1 levels.

Western Blot for Determination of GLT1 Level

Extracted brain regions (PFC, NAc core, and NAc shell) were processed for Western blot to determine the level of

GLT1 after CEF treatment group as compared to saline (ethanol) vehicle and naïve (water) control groups. We used a Western blot protocol as reported recently (Sari et al., 2010; Sari et al., 2011; Sari et al., 2009; Sari and Sreemantula, 2012). Brain regions from all groups were homogenized in lysis buffer (n=6 for each group). Proteins were extracted and quantified using Bio-Rad Chemicals (Bio-Rad, Hercules, CA, USA). Equal amounts of proteins from control and treated groups for each brain region were separated in 10–20 % glycine gel (Life Technologies, Grand Island, NY, USA). Separated proteins were then transferred using an electrophoresis apparatus onto nitrocellulose membranes. These membranes were then preincubated for 30 min in blocking media containing 3 % milk in tris-buffered saline Tween 20 (50 mM tris–HCl; 150 mM NaCl, pH7.4; 0.1 % Tween 20). Membranes were further incubated with guinea pig anti-GLT1 primary antibody (Millipore Bioscience Research Reagents) overnight at 4 °C at a dilution 1:5,000. Membranes were washed and incubated with horseradish peroxidase (HRP)-labeled anti-guinea pig secondary antibody at dilution 1:5,000. We used β-tubulin as the loading control protein. After incubation with secondary antibodies, membranes were washed, dried, and incubated with a chemiluminescent kit (SuperSignal West Pico; Pierce) for detection of protein. Membranes were then exposed to Kodak BioMax MR films (Thermo Fisher Scientific). Films were developed using SRX-101A apparatus. Blots in the films corresponding to GLT1 or β-tubulin proteins were digitized and quantified using MCID system. Data are calculated as ratios of GLT1/β-tubulin.

[^{3}H] NBTI Binding

To measure ENT1 protein level, ENT1 binding was measured using [^{3}H] NBTI binding assay as described recently (Choi et al., 2004; Kim et al., 2011). Brain tissues (PFC, NAc core, and NAc shell) were homogenized in 50 mM tris–HCl (pH 7.5) containing protease inhibitor cocktail (Roche) and then centrifuged at 25,000g for 30 min. The pellet was resuspended in the same buffer and centrifuged again at 25,000g for 30 min and resuspended in the same buffer. [^{3}H] NBTI binding assays were performed at room temperature in 50 mM tris–HCl (pH 7.5) with a concentration of [^{3}H] NBTI (20 nM), and total binding was determined as fentomoles per milligram of protein.

Statistical Analyses

Two-way mixed analyses of variance (ANOVA) were used for statistical analyses of the body weight, ethanol, and water intake data as performed in recent studies (Sari et al., 2011; Sari and Sreemantula, 2012). When a significant main effect of day and interaction effect (Day×Treatment) was found, one-way ANOVA analyses between saline vehicle control (ethanol control group) and CEF-treated groups were followed by a post hoc Dunnett's test. Western blot for GLT1 and binding assay for ENT1 data were analyzed using one-way ANOVA, and posthoc Newman–Keuls's test was used for comparison between naïve (water) control group, saline vehicle (ethanol) control group, and CEF-treated (CEF-100) group. Statistical tests were based on $p<0.05$ level of significance.

Results

Effects of CEF Treatment on Ethanol Intake, Water Intake, and Body Weight

Daily average of ethanol consumption (grams per kilogram of body weight per day) was measured for eight consecutive days (starting 24 h after the first injection, Day 1) in P rats treated with saline-vehicle control and CEF-treated (100 mg/kg) groups (Fig. 1a). The baseline was estimated as an average ethanol intake for the last 2 weeks prior to saline vehicle or CEF i.p. injections. A 2×8 (Day×Treatment) two-way ANOVA performed on ethanol intake, followed by Dunnett's t test (two-tailed), demonstrated a significant main effect of Day [$F(1, 8)=25.86$, $p<0.001$] and a significant Day×Treatment interaction effect [$F(2, 16)=10.87$, $p<0.001$]. One-way ANOVA analyses for each day demonstrated significant difference ($F>4.63$, $p<0.03$) between saline vehicle and CEF groups from Day 2 through Day 8. Dunnett's t test analyses revealed significant reduction in ethanol intake in CEF group at Day 2 ($p<0.01$) and from Days 3 through 8 ($p<0.001$) as compared to saline groups.

Daily average of water intake (milliliters per kilogram of body weight per day) was measured for a period of 8 days (starting 24 h after the first injection, Day 1) in P rats treated with saline vehicle control and/or CEF (100 mg/kg) (Fig. 1b). A 2×8 (Day×Treatment) two-way ANOVA performed on water intake, followed by Dunnett's t test (two tailed), showed a significant main effect of Day [$F(1, 8)=21.002$, $p<0.001$] and a significant Day×Treatment interaction effect [$F(2, 16)=12.45$, $p<0.001$]. One-way ANOVA analyses for each day revealed a significant difference ($F>8.002$, $p<0.01$) in water intake between saline and CEF groups from Day 1 through Day 8. Dunnett's t test analyses showed a significant increase in water intake from Day 1 through Day 8 (Fig. 1b; *: $p<0.05$, **: $p<0.001$) in the CEF-treated group.

The effects of CEF were also evaluated on body weight. A 2×8 (Day×Treatment) two-way ANOVA performed on body weight, which was followed by Dunnett's t test (two tailed), revealed a significant main effect of Day [$F(1, 8)=8.96$, $p<0.001$], but no significant Day-by-Treatment interaction effect was found [$F(2, 16)=0.62$, $p=0.53$] (Fig. 1c). In addition, one-way ANOVA analyses for each day did not reveal any significant differences between naïve (water) control,

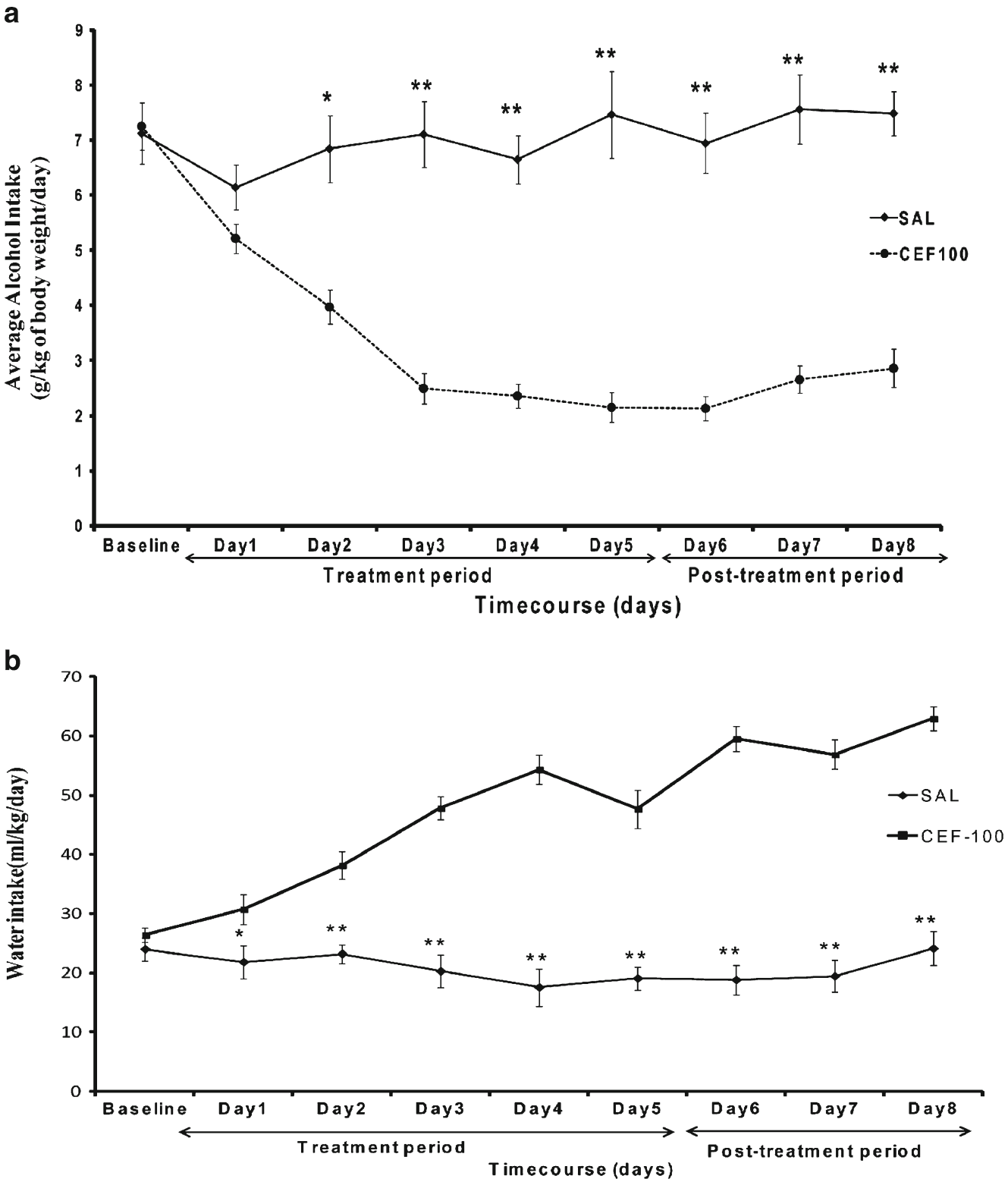


Fig. 1 **a** Daily ethanol intake of male P rats treated for 5 days with 100 mg/kg (i.p.) CEF (n=8) or saline vehicle (n=8). *Graph* represents average daily ethanol intake during the treatment (days 1–5) and post-treatment periods (days 6–8). Baseline was estimated as an average ethanol intake for the last 2 weeks prior to saline or CEF injections. One-way ANOVA analyses revealed significant differences among control and treatment groups. Dunnett's t test analyses revealed a significant reduction in ethanol intake with CEF from Day 2 through Day 8 as compared to saline groups. **b** *Graph* represents average daily water intake during the treatment (days 1–5) and post-treatment periods (days 6–8). One-way ANOVA analyses revealed significant differences among control and treatments groups from Day 1 through Day 8. Dunnett's t test analyses revealed a significant increase in water intake from Day 1 through Day 8 with CEF treatment. **c** *Graph* represents average daily body weight during the treatment (days 1–5) and post-treatment periods (days 6–8). CEF did not affect the body weight across the 8 days. All data are expressed as mean±SEM. (*: $p<0.05$, **: $p<0.001$)

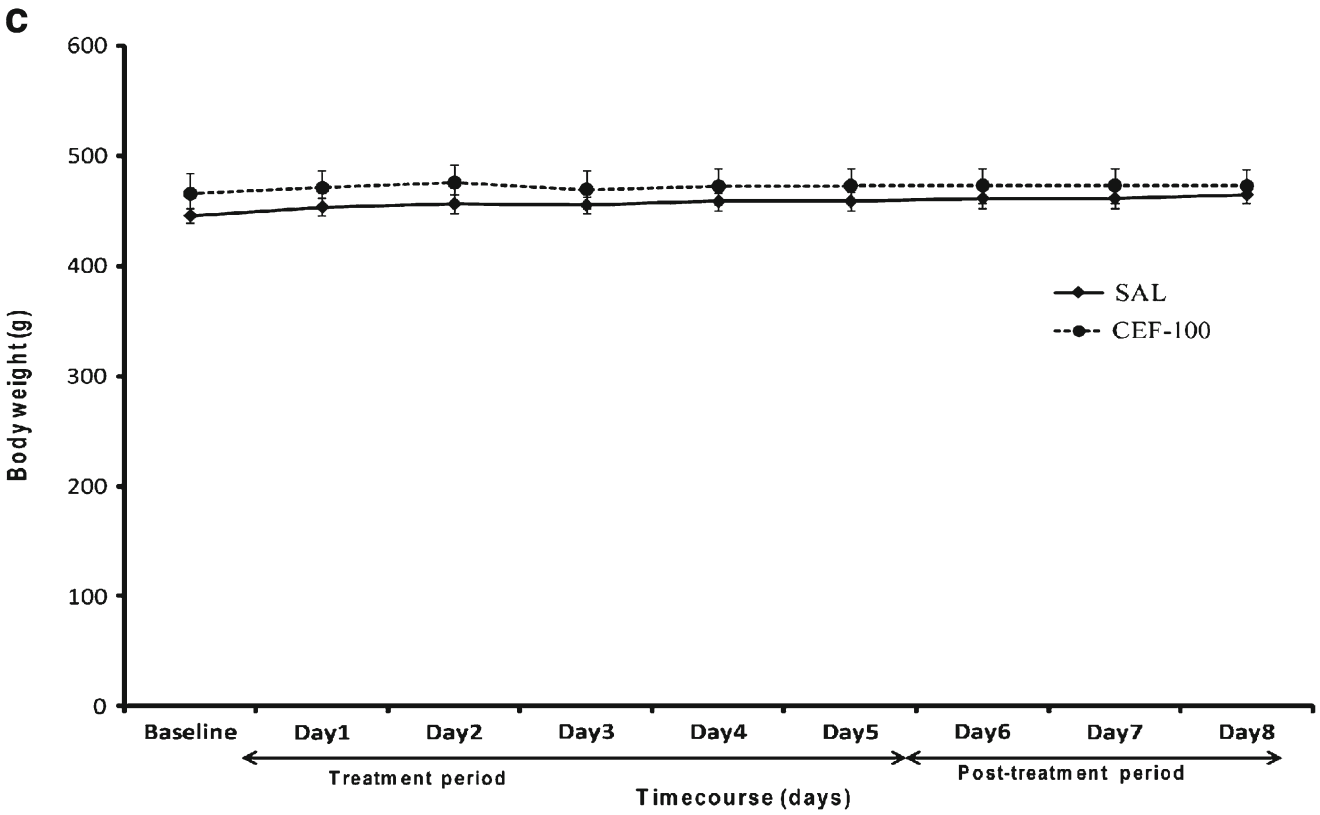


Fig. 1 (continued)

saline vehicle, and CEF-treated groups ($F<0.27$, $p>0.5$) in body weight. These data demonstrate that CEF did not alter body weight similar to our recent report (Sari et al., 2011).

Effects of CEF on GLT1 Expression in the PFC, and the NAc Shell and Core

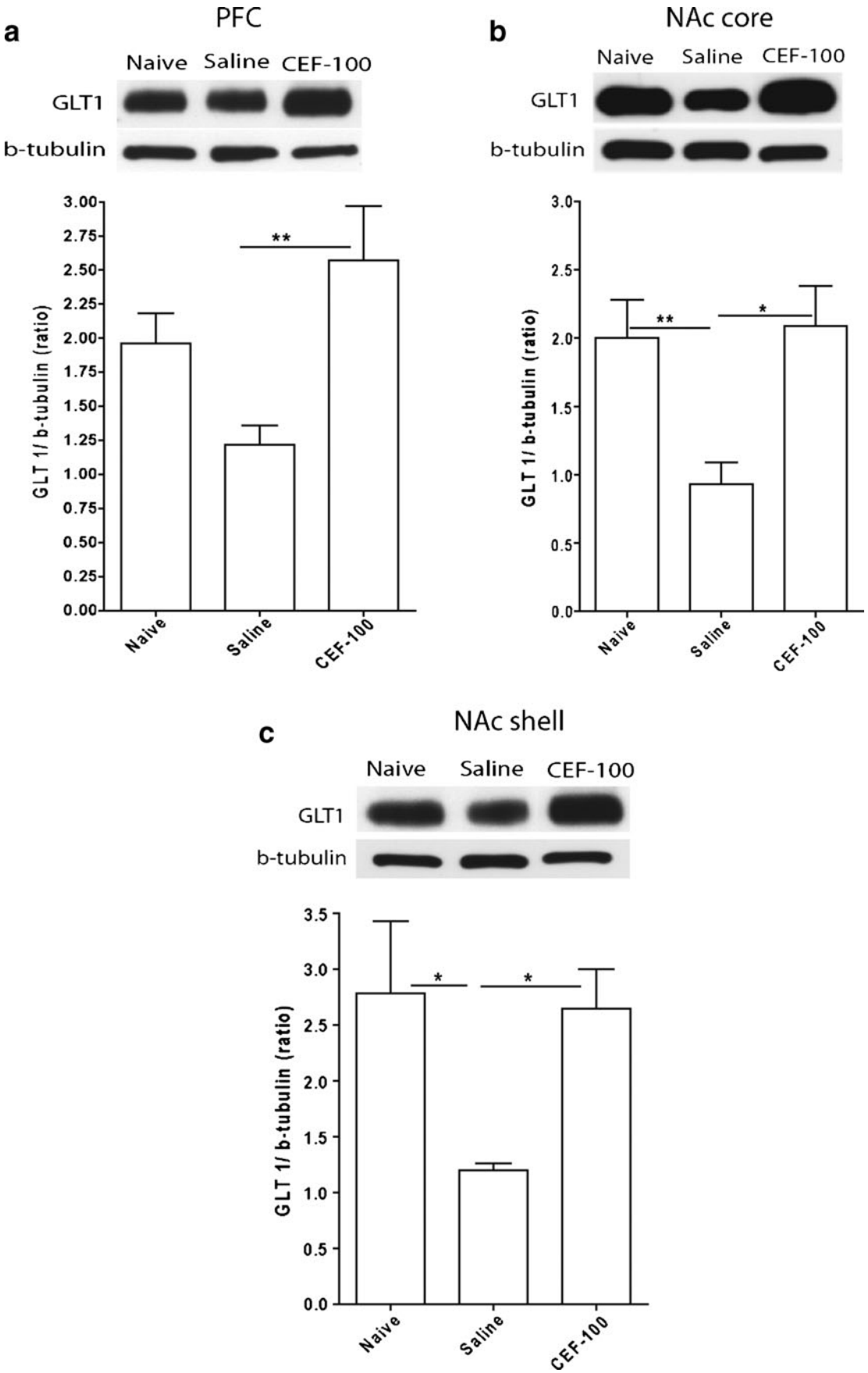
We determined next the effects of CEF treatment on GLT1 expression in PFC using Western blot analysis (Fig. 2a, upper panel). One-way ANOVA analyses revealed a significant main effects among all groups [$F(2, 17)=6.08$, $p<0.05$]. Newman–Keuls's test analyses showed significant upregulation of GLT1 level in PFC in the CEF-100-treated group (100 mg/kg, i.p.) ($p<0.01$) compared to saline vehicle group (Fig. 2a, lower panel). There were also no significant differences between CEF-100 and naïve (water) control groups. β-Tubulin, used as loading control, did not show any significant differences among all groups.

We also determined the effects of CEF treatment on GLT1 expression in NAc core using Western blot (Fig. 2b, upper panel). One-way ANOVA analyses showed a significant main effect between all groups [$F(2, 17)=6.52$, $p<0.01$]. Newman–Keuls's test analyses showed significant downregulation of GLT1 level in saline vehicle group ($p<0.01$) compared to naïve (water) group (Fig. 2b, lower panel). These results demonstrated that chronic ethanol intake decreased GLT1 level in NAc shell compared to naïve (water) control group. Furthermore, statistical analyses demonstrated upregulation of GLT1 level in NAc core in CEF-100-treated group (100 mg/kg, i.p.) ($p<0.05$) compared to the saline vehicle group (Fig. 2b, lower panel).

We also have examined the level of GLT1 in NAc shell using Western blot (Fig. 2c, upper panel). One-way ANOVA analyses demonstrated a significant main effect between all groups [$F(2,17)=4.19$, $p<0.05$]. Newman–Keuls's test demonstrated significant downregulation in GLT1 level in saline group ($p<0.05$) compared to naïve group (Fig. 2c, lower panel). These results demonstrated that chronic ethanol intake decreased the level of GLT1 in the NAc shell as compared to the naïve group. Moreover, Newman–Keuls's test analyses revealed significant increase in GLT1 level in NAc shell in the CEF-100-treated group (100 mg/kg, i.p.) ($p<0.05$) compared to the saline vehicle group (Fig. 2c, lower panel).

Effects of Ethanol Consumption and CEF Treatment on ENT1 Expression

A binding assay was performed to determine the effect of ethanol intake on the level of ENT1 in PFC, NAc core, and NAc shell. One-way ANOVA analyses did not show a


a
PFC
Naive Saline CEF-100
GLT1
b-tubulin
GLT 1/ b-tubulin (ratio)
3.00
2.75
2.50
2.25
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
**
Naive
Saline
CEF-100
b
NAc core
Naive Saline CEF-100
GLT1
b-tubulin
GLT 1/ b-tubulin (ratio)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
**
*
Naive
Saline
CEF-100
c
NAc shell
Naive Saline CEF-100
GLT1
b-tubulin
GLT 1/ b-tubulin (ratio)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
*
Naive
Saline
CEF-100

◀ **Fig. 2** **a** Effects of CEF at 100 mg/kg (CEF-100, $n=6$), saline vehicle control (ethanol control) ($n=6$), and naïve (water) control ($n=6$) groups on GLT1 expression in prefrontal cortex (PFC). (*Upper panel*) Each panel presents immunoblots for β-tubulin, which was used as a control loading protein, and GLT1. (*Lower panel*) Quantitative analysis revealed a significant increase in the ratio of GLT1/β-tubulin in CEF-100 group as compared to the saline vehicle group (ethanol control group). **b** Effects of CEF on GLT1 expression in nucleus accumbens core (NAc core). (*Upper panel*) Each panel presents immunoblots for β-tubulin, which was used as a control loading protein, and GLT1. (*Lower panel*) Quantitative analysis revealed a significant increase in the ratio of GLT1/β-tubulin in CEF-100 group as compared to the saline vehicle group. In addition, a significant downregulation of GLT1 expression was revealed in saline group as compared to naïve (water) control group. **c** Effects of CEF on GLT1 expression in nucleus accumbens shell (NAc shell). (*Upper panel*) *Each panel* presents immunoblots for β-tubulin, which was used as a control loading protein, and GLT1. (*Lower panel*) Quantitative analysis revealed a significant increase in the ratio of GLT1/β-tubulin in CEF-treated group as compared to saline vehicle group (ethanol control group). Also, statistical analyses revealed significant downregulation of GLT1 expression in saline vehicle group as compared to naïve (water) control group. All data are expressed as mean±SEM. (*$p<0.05$; **$p<0.01$)

significant main effect in the binding site for ENT1 in PFC between naïve (water) control, saline (ethanol) vehicle, and CEF-treated groups (Fig. 3a). Furthermore, CEF treatment did not induce any changes in ENT1 levels as well.

We determined next the level of ENT1 in subregions of NAc (core and shell). One-way ANOVA analyses demonstrated a significant main effect between all groups in NAc core [$F(2,8)=7.34$; $p<0.05$]. Newman–Keuls's test analyses revealed significant increase of ENT1 level in NAc core in saline (ethanol) vehicle group as compared to naïve (water) control and CEF-treated groups ($p<0.05$) (Fig. 3b). In addition, one-way ANOVA analyses revealed significant main effect between all groups in NAc shell [$F(2,8)=11.14$, $p<0.01$]. Newman–Keuls's test analyses revealed significant increase in ENT1 level in NAc shell in saline (ethanol) vehicle group as compared to naïve (water) control ($p<0.01$) and CEF-treated ($p<0.05$) groups (Fig. 3c).

Discussion

We report in this study that ethanol consumption for 5 weeks induced differential regulation of the levels of GLT1 and ENT1, which are highly expressed in striatal astrocytes and responsible for regulating synaptic glutamate and adenosine levels, respectively, in rats. Interestingly, GLT1 level was downregulated, whereas ENT1 level was increased in both NAc core and shell in saline vehicle (ethanol) group compared to naïve (water) control group. We did not see any effect on either GLT1 or ENT1 levels in the PFC in saline vehicle (ethanol) group as compared to naïve (water) group. Importantly, CEF administration upregulated GLT1 level and downregulated ENT1 level in both NAc core and shell compared to saline vehicle (ethanol) group. However, there were no significant differences in ENT1 level in the PFC among all groups. These findings suggest that GLT1 and ENT1 are inversely affected as a consequence of ethanol consumption; this suggests that neuroadaptative mechanisms are involving these proteins in both NAc core and shell, but not in the PFC.

Although the neurocircuitry of the glutamatergic system is not fully understood, it is suggested that glutamate within the PFC (Goldstein and Volkow, 2002) and the NAc (Childress et al., 1999) play a critical role in drug reinforcement. These regions receive substantial input from midbrain dopaminergic neurons, and most of the major drugs of abuse, including alcohol, increase dopamine transmission in the forebrain (Berridge and Robinson, 1998; Kalivas, 2004). The importance of the glutamatergic projections from the PFC to the NAc and the VTA have been observed in neuroimaging studies conducted during craving in different paradigms, for commonly abused drugs such as alcohol, cocaine, nicotine,

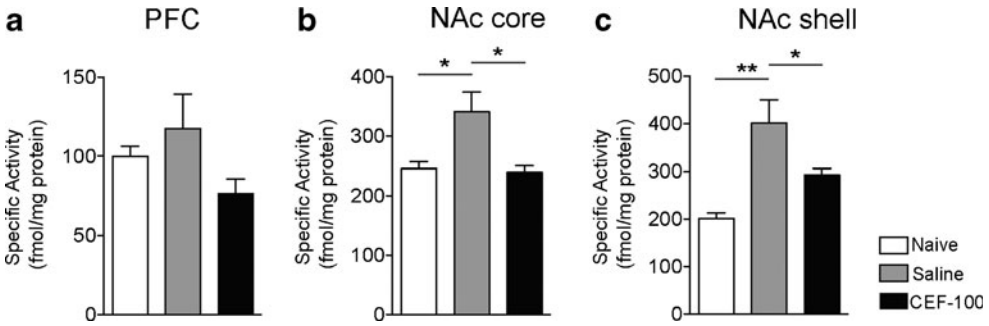


Fig. 3 Effects of CEF at 100 mg/kg (CEF-100, $n=6$), saline vehicle control (ethanol control) ($n=6$), and naïve (water) control ($n=6$) groups on ENT1 level in prefrontal cortex (PFC) and nucleus accumbens core (NAc core) and shell (NAc shell). **a** ENT1 expression is not changed after ethanol treatment (saline group) in the prefrontal cortex of alcohol-preferring rats, and ceftriaxone (CEF-100 group) did not alter ENT1 expression levels. **b**, **c** Statistical analyses demonstrated significant increase in ENT1 expression in both the **b** nucleus accumbens core and **c** shell areas in saline vehicle group (ethanol control group) as compared to naïve group. Importantly, ceftriaxone treatment was able to significantly to reverse the effect of ethanol consumption in ENT1 level back to that of the naïve group level in both NAc core and NAc shell. All data are expressed as mean±SEM. (*$p<0.05$; **$p<0.01$)

methamphetamine, and heroin (Childress et al., 1999; Goldstein and Volkow, 2002). Moreover, glutamatergic projections from the PFC to the NAc are also critical in the expression of addictive behaviors [for review, see ref. (Kalivas, 2004)]. Thus, we investigated changes in GLT1 and ENT1 levels in NAc and PFC brain reward regions.

ENT1 is a glial bidirectional nucleoside transporter that regulates the level of adenosine, which has an inhibitory effect on glutamate release [for review, see ref. (Nam et al., 2012)]. In the present study, we demonstrated that ethanol intake reduced GLT1 level in both NAc core and shell. The upregulation of ENT1 levels in these regions might be associated directly or indirectly with excess extracellular glutamate as a consequence of GLT1 downregulation. Our data suggest that there is potential interaction between both GLT1 and ENT1 in NAc core and shell. It is important to note that we did not see this neuroadaptative effect on ENT1expression in the PFC nor were there changes found in GLT1 levels in this region. The neuroadaptative mechanism may involve regulation of the extracellular adenosine levels in order to alleviate its inhibitory action on glutamate release. It has been shown that adenosine inhibits glutamate release through presynaptic adenosine A1 receptors in NAc (Harvey and Lacey, 1997). Acute exposure to ethanol may lead to a reduction of ENT1 activity, downregulation of adenosine levels, and the consequent reduction in glutamate release [for review, see ref. (Nam et al., 2012)]. However, chronic ethanol exposure may lead to downregulation of ENT1 expression and lower adenosine levels, resulting in decreased activity of A1 receptors, and a consequent increase in glutamate release (Harvey and Lacey, 1997). In contrast, our present study demonstrated that ethanol intake upregulated ENT1 levels at least in the NAc core and shell. This may result from differences in the period of ethanol consumption, the amount of ethanol consumed, strains tested, and possibly other unknown factors that were different between studies.

Studies from Choi et al. showed that mice lacking ENT1 increased their ethanol intake as compared to wild type counterparts (Choi et al., 2004). However, neuronal overexpression of ENT1 led to an increase in the intoxication effect of ethanol in these transgenic mice (Parkinson et al., 2009). Moreover, the ENT1 knockout was associated with a reduction in the level or function of GLT1 resulting in increased in extracellular glutamate (Choi et al., 2004; Nam et al., 2011; Wu et al., 2010; Wu et al., 2011). Therefore, studies have suggested that increased levels of extracellular glutamate are associated with increased ethanol intake in mice lacking ENT1 [for review, see ref. (Nam et al., 2012)]. Although transgenic mice exhibit many compensatory mechanisms as consequences of lacking or overexpressing ENT1, the study related to overexpression of ENT1 might be in accordance with our present findings demonstrating correlation between increased in ENT1 levels and ethanol intake (Parkinson et al., 2009).

We conclude in this study that ethanol consumption for 5 weeks induced downregulation of GLT1 that is associated, in part, with upregulation of ENT1 in NAc core and shell. The upregulation of ENT1 level may account for neuroadaptative mechanism to compensate for the reduction in GLT1 level in these two brain reward regions. Importantly, CEF-induced reduction in ethanol intake was associated with an upregulation of GLT1 in all examined brain regions. We suggest that this drug overcomes the upregulatory effect of ENT1 level at least in NAc core and shell. These findings provide information about possible regulatory effects of CEF in both GLT1 and ENT1 proteins and reduction of ethanol intake.

Acknowledgments This work was supported by Award Number R01AA019458 (Y.S.) and R01AA018779 (D-S. C.) from the National Institutes on Alcohol Abuse and Alcoholism. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute on Alcohol Abuse and Alcoholism or the National Institutes of Health. The authors would like to thank Mrs. Charisse Montgomery for editing this manuscript.

Conflict of interest The authors declare no conflict of interest.

References

Berridge KC, Robinson TE (1998) What is the role of dopamine in reward: hedonic impact, reward learning, or incentive salience? Brain Res Brain Res Rev 28:309–369

Childress AR, Mozley PD, McElgin W, Fitzgerald J, Reivich M, O'Brien CP (1999) Limbic activation during cue-induced cocaine craving. Am J Psychiatry 156:11–18

Choi DS, Cascini MG, Mailliard W, Young H, Paredes P, McMahon T, Diamond I, Bonci A, Messing RO (2004) The type 1 equilibrative nucleoside transporter regulates ethanol intoxication and preference. Nat Neurosci 7:855–861

Danbolt NC (2001) Glutamate uptake. Prog Neurobiol 65:1–105

Ginsberg SD, Martin LJ, Rothstein JD (1995) Regional deafferentation down-regulates subtypes of glutamate transporter proteins. J Neurochem 65:2800–2803

Goldstein RZ, Volkow ND (2002) Drug addiction and its underlying neurobiological basis: neuroimaging evidence for the involvement of the frontal cortex. Am J Psychiatry 159:1642–1652

Harvey J, Lacey MG (1997) A postsynaptic interaction between dopamine D1 and NMDA receptors promotes presynaptic inhibition in the rat nucleus accumbens via adenosine release. J Neurosci 17:5271–5280

Kalivas PW (2004) Glutamate systems in cocaine addiction. Curr Opin Pharmacol 4:23–29

Kim K, Lee SG, Kegelman TP, Su ZZ, Das SK, Dash R, Dasgupta S, Barral PM, Hedvat M, Diaz P, Reed JC, Stebbins JL, Pellecchia M, Sarkar D, Fisher PB (2011) Role of excitatory amino acid transporter-2 (EAAT2) and glutamate in neurodegeneration: opportunities for developing novel therapeutics. J Cell Physiol 226:2484–2493

Knackstedt LA, Melendez RI, Kalivas PW (2010) Ceftriaxone restores glutamate homeostasis and prevents relapse to cocaine seeking. Biol Psychiatry 67:81–84

Krystal JH, Petrakis IL, Mason G, Trevisan L, D'Souza DC (2003) *N*-Methyl-D-aspartate glutamate receptors and alcoholism: reward,

dependence, treatment, and vulnerability. Pharmacol Ther 99:79–94

Melendez RI, Hicks MP, Cagle SS, Kalivas PW (2005) Ethanol exposure decreases glutamate uptake in the nucleus accumbens. Alcohol Clin Exp Res 29:326–333

Miller BR, Dorner JL, Shou M, Sari Y, Barton SJ, Sengelaub DR, Kennedy RT, Rebec GV (2008) Up-regulation of GLT1 expression increases glutamate uptake and attenuates the Huntington's disease phenotype in the R6/2 mouse. Neuroscience 153:329–337

Mitani A, Tanaka K (2003) Functional changes of glial glutamate transporter GLT-1 during ischemia: an in vivo study in the hippocampal CA1 of normal mice and mutant mice lacking GLT-1. J Neurosci 23:7176–7182

Nam HW, Lee MR, Zhu Y, Wu J, Hinton DJ, Choi S, Kim T, Hammack N, Yin JC, Choi DS (2011) Type 1 equilibrative nucleoside transporter regulates ethanol drinking through accumbal *N*-methyl-D-aspartate receptor signaling. Biol Psychiatry 69:1043–1051

Nam HW, McIver SR, Hinton DJ, Thakkar MM, Sari Y, Parkinson FE, Haydon PG, Choi DS (2012) Adenosine and glutamate signaling in neuron–glial interactions: implications in alcoholism and sleep disorders. Alcohol Clin Exp Res 36:1117–1125

Othman T, Sinclair CJ, Haughey N, Geiger JD, Parkinson FE (2002) Ethanol alters glutamate but not adenosine uptake in rat astrocytes: evidence for protein kinase C involvement. Neurochem Res 27:289–296

Parkinson FE, Xiong W, Zamzow CR, Chestley T, Mizuno T, Duckworth ML (2009) Transgenic expression of human equilibrative nucleoside transporter 1 in mouse neurons. J Neurochem 109:562–572

Paxinos G, Watson C (2007) The rat brain in stereotaxic coordinates, 6th edn. Academic, New York

Qrunfleh AM, Alazizi A, Sari Y (2013) Ceftriaxone, a beta-lactam antibiotic, attenuates relapse-like ethanol-drinking behavior in alcohol-preferring rats. J Psychopharmacol 27:541–549

Rothstein JD (1995) Excitotoxicity and neurodegeneration in amyotrophic lateral sclerosis. Clin Neurosci 3:348–359

Rothstein JD, Patel S, Regan MR, Haenggeli C, Huang YH, Bergles DE, Jin L, Dykes Hoberg M, Vidensky S, Chung DS, Toan SV, Bruijn LI, Su ZZ, Gupta P, Fisher PB (2005) Beta-lactam antibiotics offer neuroprotection by increasing glutamate transporter expression. Nature 433:73–77

Rothstein JD, Van Kammen M, Levey AI, Martin LJ, Kuncl RW (1995) Selective loss of glial glutamate transporter GLT-1 in amyotrophic lateral sclerosis. Ann Neurol 38:73–84

Sari Y, Franklin KM, Alazizi A, Rao PS, Bell RL (2013) Effects of ceftriaxone on the acquisition and maintenance of ethanol drinking in peri-adolescent and adult female alcohol-preferring (P) rats. Neuroscience 241:229–238

Sari Y, Prieto AL, Barton SJ, Miller BR, Rebec GV (2010) Ceftriaxone-induced up-regulation of cortical and striatal GLT1 in the R6/2 model of Huntington's disease. J Biomed Sci 17:62

Sari Y, Sakai M, Weedman JM, Rebec GV, Bell RL (2011) Ceftriaxone, a beta-lactam antibiotic, reduces ethanol consumption in alcohol-preferring rats. Alcohol Alcohol 46:239–246

Sari Y, Smith KD, Ali PK, Rebec GV (2009) Upregulation of GLT1 attenuates cue-induced reinstatement of cocaine-seeking behavior in rats. J Neurosci 29:9239–9243

Sari Y, Sreemantula SN (2012) Neuroimmunophilin GPI-1046 reduces ethanol consumption in part through activation of GLT1 in alcohol-preferring rats. Neuroscience 227C:327–335

Schreiber R, Freund WD (2000) Glutamate transport is downregulated in the cerebral cortex of alcohol-preferring rats. Med Sci Monit 6:649–652

Smith AD, Weiss F (1999) Ethanol exposure differentially alters central monoamine neurotransmission in alcohol-preferring versus -nonpreferring rats. J Pharmacol Exp Ther 288:1223–1228

Smith TL (1997) Regulation of glutamate uptake in astrocytes continuously exposed to ethanol. Life Sci 61:2499–2505

Wu J, Lee MR, Choi S, Kim T, Choi DS (2010) ENT1 regulates ethanol-sensitive EAAT2 expression and function in astrocytes. Alcohol Clin Exp Res 34:1110–1117

Wu J, Lee MR, Kim T, Johng S, Rohrback S, Kang N, Choi DS (2011) Regulation of ethanol-sensitive EAAT2 expression through adenosine A1 receptor in astrocytes. Biochem Biophys Res Commun 406:47–52