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Ceftriaxone treatment affects the levels of GLT1 and ENT1 as well as ethanol intake in alcohol-preferring rats

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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-hour concurrent access to 15% and 30% ethanol, water, and food for five 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.

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 down-regulation of glutamate transport in the cerebral cortex (Schreiber and Freund, 2000). Glutamate

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Conflict of interest:

The authors declare no conflict of interest.

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 and colleagues 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 ceftriaxone 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 expression 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) Indiana Research Center at the age of 21–30 days. They were housed in standard plastic tubs in the DLAR (Department of Laboratory Animal Resources, 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-hour 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. 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 three months and single-housed in the bedded plastic cages. We have tested three groups: (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 ml or ethanol intake in g/kg of body weight. We have used a densitometry formula to convert the actual grams of ethanol consumed per kg of body weight of animals. All animals met the requirement of drinking 4 grams of ethanol per kg 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 two 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 5 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 regions harvesting

Animals were euthanized by exposure to Isoflurane and decapitated on Day 8 (three 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 micro-dissected stereotactically using a cryostat machine. Extractions of the brain regions were performed using stereotaxic coordinates for the rat brain atlas established by Paxinos and Watson (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). 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). Separated proteins were then transferred using an electrophoresis apparatus onto nitrocellulose membranes. These membranes were then pre-incubated for 30 minutes 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 α -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 α -tubulin proteins were digitized and quantified using MCID system. Data are calculated as ratios of GLT1/ α -tubulin.

[³H] NBTI binding

To measure ENT1 protein level, ENT1 binding was measured using [³H] 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 25,000 g for 30 min. The pellet was resuspended in the same buffer and centrifuged again at 25,000 g for 30 min and resuspended in the same buffer. [³H] NBTI binding assays were performed at room temperature in 50 mM Tris-HCl (pH 7.5) with a concentration of [³H] NBTI (20 nM), and total binding was determined as fmol/mg 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 X Treatment) was found, one-way ANOVA analyses between saline vehicle control (ethanol control group) and CEF-treated groups were followed by *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 (g/kg body weight/day) was measured for consecutive 8 days (starting 24 hrs after the first injection, Day 1) in P rats treated with saline vehicle control and CEF treated (100 mg/kg) groups (Figure 1A). The baseline was estimated as an average ethanol intake for the last two weeks prior to saline vehicle or CEF i.p. injections. A 2×8 (Day X 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 x 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 (ml/kg body weight/day) was measured for a period of 8 days (starting 24 hours after the first injection, Day 1) in P rats treated with saline vehicle control, and/or CEF (100 mg/kg) (Figure 1B). A 2×8 (Day X 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 x 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 (Figure 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 X 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 [$F(2, 16) = 0.62$, $p = 0.53$] (Figure 1C). In addition, one-way ANOVA analyses for each day did not reveal any significant differences

between naïve (water) control, 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 (Figure 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 (Figure 2A, Lower panel). There were also no significant differences between CEF-100 and naïve (water) control groups. α -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 (Figure 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 down-regulation of GLT1 level in saline vehicle group ($p < 0.01$) compared to naïve (water) group (Figure 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 (Figure 2B, Lower panel).

We also have examined the level of GLT1 in NAc shell using Western blot (Figure 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 down-regulation in GLT1 level in saline group ($p < 0.05$) compared to naïve group (Figure 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 (Figure 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 significant main effect in the binding site for ENT1 in PFC between naïve (water) control, saline (ethanol) vehicle and CEF treated groups (Figure 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 ($p < 0.05$) (Figure 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 (Figure 3C).

Discussion

We report in this study that ethanol consumption for five 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 may involve neuroadaptive mechanisms 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, 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 neuroadaptive effect on ENT1 expression in the PFC, nor were there changes found in GLT1 levels in this region. The neuroadaptive 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 our group 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 five 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 neuroadaptive 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.

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Figure 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 $\pm$ SEM. (*: $p<0.05$, **: $p<0.001$)

Figure 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 down-regulation 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 down-regulation of GLT1 expression in saline vehicle group as compared to naïve (water) control group. All data are expressed as mean $\pm$ SEM. (* $p < 0.05$) (** $p < 0.01$)

Figure 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 $\pm$ SEM. (* $p < 0.05$)