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Effects of ceftriaxone on chronic ethanol consumption: a potential role for xCT and GLT1 modulation of glutamate levels in male P rats

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Abstract

Alterations in glutamatergic neurotransmission have been suggested to affect many aspects of neuroplasticity associated with alcohol/drug addiction. We have previously shown that ceftriaxone, a β -lactam antibiotic known to upregulate glutamate transporter 1 (GLT1), reduced ethanol intake after five weeks of free-choice ethanol drinking paradigm in male alcohol-preferring (P) rats. Evidence suggests that differential effects involving alterations of glutamatergic neurotransmission occur after long term ethanol consumption. In this study, we tested whether the efficacy of administration of ceftriaxone persists after 14 weeks of free access to 15% and 30 % ethanol in male P rats. After 14 weeks of ethanol consumption, male P rats were administered ceftriaxone (100 mg/kg, i.p.) or saline vehicle for five days. We found that ceftriaxone treatment resulted in a significant reduction in ethanol intake starting from Day 2 (48 hours after the first i.p. injections of ceftriaxone) through Day 14, **10 days after final injection**. Western blot analysis of brain samples from animals euthanized 24 h after treatment with the last dose of ceftriaxone revealed a significant upregulation of cystine/glutamate exchanger (xCT) and GLT1 levels in prefrontal cortex, nucleus accumbens and amygdala as compared to saline vehicle-treated group. These findings demonstrated the effectiveness of ceftriaxone in attenuating ethanol intake in a chronic consumption paradigm. These might be due in part through upregulation of both xCT and GLT1 levels in brain reward regions. Thus, the drug has a potential therapeutic action for the treatment of alcohol dependence.

Keywords

glutamate; alcohol-preferring rats; chronic ethanol exposure; xCT; GLT1

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Conflict of interest:

The authors declare no conflict of interest.

Introduction

The glutamatergic system has been suggested as one of the neurotransmitter systems responsible for the development of drug abuse. Among drugs of abuse, **the reinforcing/rewarding properties of** cocaine (Sari et al., 2009, Li et al., 2010, Moussawi et al., 2011, Lu et al., 2012), heroin (Bossert et al., 2012) and ethanol (Obara et al., 2009, Cannady et al., 2011, Sari et al., 2011, Bahi et al., 2012) have been shown to interact with the glutamatergic system at various brain reward regions.

Alcohol consumption can lead to a marked change in the extracellular glutamate levels in central reward brain regions [For review, see ref. (Rao and Sari, 2012)]. Increase in extracellular glutamate levels in the brain reward regions has been observed following administration of ethanol (Dahchour et al., 2000, Kapasova and Szumlinski, 2008, Ding et al., 2012). Alternatively, increased extracellular glutamate levels were observed in the nucleus accumbens (NAc) during ethanol withdrawal (Dahchour et al., 1998). It is noteworthy that extracellular glutamate levels are regulated by several glutamate transporters distributed in neurons and glia (Gegelashvili and Schousboe, 1997, Seal and Amara, 1999, Anderson and Swanson, 2000). Among these transporters, glutamate transporter 1 (GLT1), its human homolog is excitatory amino acid transporter 2 (EAAT2), has been suggested to regulate the removal of the majority of extracellular glutamate (Ginsberg et al., 1995, Rothstein et al., 1995, Danbolt, 2001, Mitani and Tanaka, 2003). We have recently reported that ethanol consumption for five weeks induced downregulation of GLT1 level in NAc (Sari and Sreemantula, 2012). Similarly, GLT1 level was downregulated in the NAc following cocaine self-administration (Knackstedt et al., 2010). Downregulation of GLT1 expression was reversed by treatment with ceftriaxone, a β -lactam antibiotic known to upregulate GLT1 level (Rothstein et al., 2005), in NAc and prefrontal cortex (PFC) in both cocaine and ethanol exposure animal models (Sari et al., 2009, Knackstedt et al., 2010, Sari et al., 2011).

Studies have demonstrated the role of the system x_c^- , which is a cystine/glutamate exchanger (xCT), in several drug addiction models (Baker et al., 2003, Knackstedt et al., 2010, Moussawi et al., 2011). Exposure to cocaine, for example, results in reduction of system x_c^- activity while increasing the activity of system x_c^- **prevented** reinstatement (Baker et al., 2003, Madayag et al., 2007). Similarly, nicotine self-administration in rats **reduced** expression of the catalytic subunit of the cystine-glutamate exchanger, xCT, in NAc and VTA (Knackstedt et al., 2010). Following cocaine self-administration in rats, ceftriaxone has been shown to both upregulate xCT expression in NAc and reestablish system x_c^- activity thereby **restoring** glutamate homeostasis (Knackstedt et al., 2010, Trantham-Davidson et al., 2012).

Recent studies from our lab using a five-week free choice ethanol drinking paradigm **revealed** an association of attenuation of ethanol intake and GLT1 upregulation, with either ceftriaxone or neuroimmunophilin GPI-1046, in NAc and PFC (Sari et al., 2011, Sari and Sreemantula, 2012). In the present study, we aimed to determine the efficacy of ceftriaxone treatment on ethanol consumption in P rats after 14 weeks of ethanol consumption, which is considered a chronic ethanol exposure paradigm. P rats were selected as the suitable animal

model for the present study and previous studies in our lab because of their preference to free choice ethanol exposure (Murphy et al., 2002, Bell et al., 2006). It is important to note that long term exposure to ethanol induced alterations of the glutamatergic system and other neurotransmitter systems, including dopaminergic and cholinergic systems (Syvalahti et al., 1988, Engleman et al., 2002, Thielen et al., 2004, Sari et al., 2006, Zhou et al., 2006, Szumlinski et al., 2008). We examined the efficacy of ceftriaxone in ethanol intake during treatment and post treatment that lasted 10 days after the last i.p. injection of the drug. Importantly, **we** also investigated **xCT** and **GLT1** levels in PFC, NAc and amygdala (AMG) of ceftriaxone-treated group as compared to the saline-treated group, **24h after the last injection**.

Materials and Methods

Animals

Male P rats were obtained from the Indiana **University** School of Medicine (Indianapolis, IN) breeding colonies. After habituation to the vivarium in the laboratory animal facility at The University of Toledo, animals were single housed in wood-chip bedded plastic cages in a temperature (21°C) and humidity (50%) controlled vivarium maintained on a 12/12 hour light/dark cycle. At the beginning of the experiment, all animals were at least 90 days old, and the average **weight was** 417 ± 5 g (mean $\pm$ SEM). Rats had *ad lib* access to food and water throughout the duration of the study. Protocols used in this study were approved by the Institutional Animal Care and Use Committee of The University of Toledo, Health Science Campus, Toledo, OH. These protocols were designed based on the guidelines set forth by the Institutional Animal Care and Use Committee of the National Institutes of Health and the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, Commission on Life Sciences, 1996). Animals were divided into two groups at the time of saline vehicle or drug treatment: 1) saline vehicle-treated group (n=6) and 2) Ceftriaxone (100 mg/kg, i.p.) treated group (n=10). Ceftriaxone was administered as a solution made in physiological saline. Both groups of animals had access to free choice ethanol (15% and 30%), water and food.

Ethanol consumption

All P rats had free access to two concentrations of ethanol, 15% and 30% in distilled water for the entire duration of the study. Animals were provided free choice ethanol, water and food for 14 consecutive weeks. This is an established model of ethanol drinking consisting of multiple choice of ethanol (15% and 30%) that is known to increase ethanol intake in P rats (Rodd-Henricks et al., 2001, Sari et al., 2006). Starting week 11, ethanol intake, water consumption, and body weight of all animals were measured three times per week (Monday, Wednesday, and Friday). After the completion of 14 weeks **of ethanol access**, animals were randomly divided into saline and ceftriaxone treatment groups. Ceftriaxone was injected once daily for five days around noon at the time when the bottles were weighed. We have chosen to treat the animals for 5 days based **on** previous and recent studies from our lab and others demonstrating that this period is effective in upregulating GLT1 level (Rothstein et al., 2005, Miller et al., 2008, Sari et al., 2009, Sari et al., 2011). The baseline value for the three parameters, body weight, water intake, and ethanol consumption, was based on the

average of the data from the last two weeks prior to saline vehicle or drug treatment (weeks 13 and 14). Ethanol measurements were taken to the nearest 10th of a gram by subtraction of the weight of the bottle from its previous weight. Importantly, animals with a baseline ethanol intake of less than 4 g/day were not included in this study, and they were subsequently euthanized. Following exposure to free choice ethanol for 14 weeks, each animal was i.p. injected with either saline or ceftriaxone (100 mg/kg) for 5 consecutive days. After i.p. injections, rats were monitored daily for consumption of ethanol and water for additional 10 days followed by euthanization on Day 14. A subset of ceftriaxone treated P rats (n=4) were sacrificed 24 h after the last injection of the drug (Day 6) to determine xCT and GLT1 levels in the NAc, PFC, and AMG at this time point.

Brain tissue extraction

Ten days after the last injection, Day 14, the remaining animals were euthanized by exposure to CO₂ inhalation and further decapitated. Brains were immediately removed and stored at -70 ° C. Brain regions (NAc, PFC and AMG), were isolated and stereotaxically micropunched stereotaxically using a cryostat apparatus maintained at -20°C. The extraction site coordinates were in accordance with the Paxinos and Watson Atlas of the rat brain. The brain regions were then kept frozen at -70°C until Western blot analysis (Paxinos and Watson, 2007).

Western blot analysis

Western blot analysis was performed on the brain tissue samples to determine the level of GLT1 expression employing a previously described procedure (Sari et al., 2009, Sari et al., 2010, Sari et al., 2011). In brief, brain regions (NAc, AMG, and PFC) were homogenized in lysis buffer, and the total proteins were quantified (Bio-Rad, Hercules, CA, USA). Once extracted, proteins in equal amounts from both groups (saline vehicle- and ceftriaxone-treated groups) were loaded on 10-20% glycine gel (Invitrogen). After completion, proteins were transferred electrophoretically from the gel onto a PVDF membrane. Subsequently, the membrane was blocked with 3% milk in Tris-Buffered Saline Tween 20 at room temperature. Guinea pig anti-GLT1 (Millipore; 1:5000 dilution) or rabbit anti-xCT antibody (Novus; 1:1000 dilution) was then added to the blocking buffer, and the membrane was incubated overnight at 4°C. Horseradish peroxidase labeled anti-guinea pig or anti-rabbit secondary antibody at a 1:5000 dilution were used in this study. The Kodak BioMax MR film was used to capture the chemiluminescent signal from the HRP. The film was further developed using a SRX-101A machine. GAPDH antibody was used as a loading marker. Immunoblots were digitized using the MCID system, and the data were reported as the ratio of GLT-1/GAPDH and xCT/GAPDH.

Statistical analyses

We used general linear model (GLM) repeated measures for statistical analysis (SPSS) of data related to ethanol consumption, water intake, and rat body weight. Furthermore, to observe the day-wise effect of treatment, data were re-analyzed employing one-way ANOVA (SPSS). GLT1/GAPDH and xCT/GAPDH ratio data were statistically analyzed

using independent t-test for comparison between saline vehicle (Day 14) and ceftriaxone-treated (Day 6) groups. All statistical tests were based on $p < 0.05$ level of significance.

Results

Effect of ceftriaxone on ethanol intake

The effect of ceftriaxone treatment on ethanol consumption was monitored for 14 days. Figure 1 represents the average ethanol consumption by P rats (g/kg/day) treated with either ceftriaxone (100 mg/kg) or saline vehicle. Baseline value represents the average ethanol consumed over the two weeks preceding the first injection. A GLM repeated measures analysis comparing ethanol consumption between the two groups revealed a significant main effect of Day [$F(1,14)=10.81$, $p < 0.05$] and a significant Day $\times$ Treatment interaction effect [$F(1,14)=4.11$, $p < 0.05$]. One-way ANOVA revealed that the ceftriaxone treatment induced reduction in the ethanol intake was statistically significant ($p < 0.05$) from Day 2 through Day 14.

Effect of ceftriaxone on water consumption

Figure 2 represents the average water intake by the two groups of P rats during the course of the study. After treatment with ceftriaxone, P rats were found to consume significantly higher amounts of water when compared to saline treated animals. A significant main effect of Day was observed [$F(1,14)=8.5$, $p < 0.05$] along with a significant Day $\times$ Treatment interaction effect [$F(1,14)=6.4$, $p < 0.05$]. Results from one-way ANOVA showed that the significantly higher water consumption lasted from Day 3 through Day 12.

Effect of ceftriaxone on body weight of P rats

Body weights of P rats were monitored throughout the study (Figure 3). A GLM repeated measures analysis comparing body weights of animals between the two groups revealed a significant main effect of Day [$F(1,14)=2.85$, $p < 0.05$] along with a significant Day $\times$ Treatment interaction effect [$F(1,14)=12.6$, $p < 0.05$]. However, one-way ANOVA did not reveal any significant differences in body weights between the two groups either during the treatment or at the end of the study ($p > 0.05$).

Effect of ceftriaxone on expression of xCT and GLT1

We examined the effects of ceftriaxone on GLT1 and xCT levels on Day 6 in the PFC, NAc and AMG as compared to saline vehicle-treated rats. Figure 4 and Figure 5 (upper panel) show immunoblots for GLT1 and xCT along with the loading marker in saline and ceftriaxone-treated groups for the three brain regions. Statistical analysis using student **t-test** showed significant difference in the expression of GLT1 and xCT between the ceftriaxone-treated group and the saline-treated group (Figure 4 and Figure 5, lower panel). The mean percentage upregulation of GLT1 following ceftriaxone treatment in NAc ($p=0.0004$), PFC ($p=0.019$), and AMG ($p=0.016$) was found to be 51%, 59%, and 52%, respectively, compared to the control group. Furthermore, xCT expression was found to be upregulated by 36%, 19%, and 44% in the NAc ($p=0.031$), PFC ($p=0.012$), and AMG ($p=0.029$), respectively.

Discussion

We report in this study that after 14 weeks of chronic ethanol intake, ceftriaxone treatment (100 mg/kg) was able to reduce daily ethanol intake in male P rats. The effectiveness of the treatment lasted up to 10 days after the last i.p. injection of ceftriaxone. **At the same time**, ceftriaxone did not cause any change in the body weight of the treated animals as compared to the control group (saline vehicle). However, animals treated with ceftriaxone were found to consume significantly higher amounts of water post treatment. As it was found in a recent study that used the five-week ethanol drinking paradigm (Sari et al., 2011), the increase in water consumption might be a compensatory mechanism for the reduction in ethanol intake. Along with a long lasting effect in the reduction of ethanol intake, ceftriaxone treatment led to a statistically significant upregulation of xCT and GLT1 levels in PFC, NAc and AMG **24h after the last injection** as compared to the saline group. It is important to note that changes on the levels of xCT and GLT1 were observed in the mesocorticolimbic dopamine reward circuit such as the NAc and PFC and its associated brain region (AMG) of the extended amygdala.

Drug reinforcement is attributed to various brain reward regions, in particular the PFC (Goldstein and Volkow, 2002), NAc (Childress et al., 1999, Obara et al., 2009), and AMG (Zarrindast et al., 2010, Sinclair et al., 2012, Christian et al., 2013). While it is well known that dopamine is one of the major neurotransmitters responsible for **the** development of drug dependence (Anderson and Swanson, 2000, Ito et al., 2004), studies focusing on these brain reward centers have clearly demonstrated **an** underlying relationship between glutamatergic transmission and ethanol abuse (Carrara-Nascimento et al., 2011, Gass et al., 2011, Mishra et al., 2012). Studies targeting the underlying causes of disruption of glutamate homeostasis around reward centers of the brain have revealed **that** changes in expression of two key proteins expressed on the glial cells, xCT (Baker et al., 2003, Knackstedt et al., 2010) and GLT1 (Rothstein et al., 2005, Sari et al., 2009, Sari et al., 2011, Sari and Sreemantula, 2012), **are associated with drug abuse**.

Reduced activity/expression of xCT has been shown in several drug abuse models. Studies have linked cocaine abuse with **reduced** levels of xCT in NAc and in turn reduced levels of xCT **have** been shown to promote drug-seeking behavior (Baker et al., 2003, Kau et al., 2008). It has been shown that xCT may regulate the levels of nonsynaptic glutamate levels, which stimulate the inhibitory presynaptic **receptor** mGluR_{2/3} (Moran et al., 2005). We suggest here that increased xCT levels in brain reward regions following ceftriaxone treatment would **modulate excitatory** glutamatergic transmission and thereby **reduced** ethanol intake.

Furthermore, regulation of extracellular glutamate levels, via changes in expression of GLT1, has been previously demonstrated as an effective strategy for treating alcohol dependence (Sari et al., 2011, Sari and Sreemantula, 2012). In the five-week paradigm, ceftriaxone or GPI-1046 treatment caused a marked change in expression of GLT1 in both NAc and PFC. Note that we recently found that ethanol consumption for five weeks resulted in downregulation of GLT1 level in NAc (Sari and Sreemantula, 2012). The downregulation

of GLT1 level in NAc was antagonized by GPI-1046 treatment, which is a drug known to upregulate GLT1 expression (Ganel et al., 2006, Sari and Sreemantula, 2012).

Long term exposure to ethanol **induces** alterations **in the activity** of various neurotransmitters, including **the** glutamatergic system (Syvalahti et al., 1988, Weiss et al., 1993, Weiss et al., 1996, Engleman et al., 2002, Thielen et al., 2004, Sari et al., 2006, Zhou et al., 2006, Szumlinski et al., 2008). In this study, we tested whether ceftriaxone might be beneficial in **restoring glutamate** homeostasis through regulation of xCT and GLT1 expression after chronic exposure to ethanol. Thus, the 14-week chronic ethanol drinking paradigm was used to determine the effectiveness of ceftriaxone. Evidently, ceftriaxone was effective in reducing ethanol intake in **this** chronic ethanol consumption paradigm. We suggest that the increased levels of xCT and GLT1 in PFC, NAc, and AMG with ceftriaxone treatment may restore extracellular glutamate **homeostasis**, which lead to **an** attenuation of ethanol intake. Reduced levels of xCT and GLT1 have been directly implicated in precipitation of addiction-like behavior (Reissner and Kalivas, 2010). **Therefore** increases in xCT and GLT1 levels may directly **implicate a reduction** in extracellular glutamate levels, which consequently **leads to a reduction** in ethanol intake. These observations further **support the pharmacological** mechanism of action for ceftriaxone mediated reduction in ethanol-**drinking** behavior. Further studies, focusing on the molecular mechanisms involved in the upregulation of GLT1 and xCT levels following ceftriaxone treatment, would provide information about novel molecular targets for treating alcohol dependence. In addition, dose dependent effects of ceftriaxone on the levels of GT1 and xCT are warranted investigation to evaluate the efficacy of this drug.

We conclude here that even after extensive (14-week) exposure to ethanol, ceftriaxone **attenuates** ethanol drinking behavior in P rats. The upregulation of xCT and GLT1 levels in NAc, PFC, and AMG following ceftriaxone administration appears to be associated in part with **these reductions** of ethanol intake. These findings provide **support for** the efficacy and long lasting effects of ceftriaxone **on** ethanol intake that might be mediated in part through upregulation of two important glial proteins such as xCT and GLT1. Thus, ceftriaxone may be considered **a** potential drug for the treatment of alcohol dependence.

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Figure 1.

Daily ethanol intake by P rats following treatment with saline vehicle (n=6) or ceftriaxone (100 mg/kg, n=6). Graph represents average ethanol intake during treatment (Days 1-5) and post treatment (Days 6-14). Baseline value depicts the average ethanol intake during the two-week period preceding treatment. Based on GLM repeated measures, it was found that ceftriaxone treatment resulted in a significant reduction in ethanol consumption compared to saline vehicle-treated control group. Data are expressed as mean $\pm$ SEM. (*: $p < 0.05$)

Figure 2.

Daily water intake of male P rats treated with saline vehicle (n=6) or ceftriaxone (100 mg/kg, n=6). Graph represents average daily water consumption in both groups. Baseline value depicts the average water intake during the two-week period preceding treatment. Statistical analysis (GLM repeated measures) revealed that the ceftriaxone-treated group consumed higher amounts of water per day compared to saline vehicle-treated. Data are expressed as mean $\pm$ SEM. (*: $p < 0.05$)

Figure 3.

Daily body weight of male P rats following treatment with saline vehicle (n=6) or ceftriaxone (100 mg/kg, n=6). Graph represents average body weight during treatment (Days 1-5) and post treatment (Days 6-14). Baseline value depicts the average body weight during the two-week period preceding treatment. One-way ANOVA did not reveal any significant difference ($p>0.05$) in body weights between saline vehicle- and ceftriaxone-treated groups during the treatment or at the end of the study. Data are expressed as mean $\pm$ SEM.

Figure 4.

Effect of ceftriaxone (CEF, 100 mg/kg, n=4) treatment (**24h post-treatment**) compared to saline- treated group on the expression of GLUT1 in the prefrontal cortex (PFC), nucleus accumbens (NAC) and amygdala (AMG) compared to the saline-treated control animals (Day 14). (A) Immunoblot for GLUT1 and GAPDH as loading control marker for the corresponding brain regions. (B) Quantitative analysis of the immunoblots revealed significant difference in the ratio of GLUT1/GAPDH in all brain regions in ceftriaxone-

treated group as compared to saline-treated animals (100%). Data are expressed as mean $\pm$ SEM. (* $p < 0.05$; *** $p < 0.001$)

Figure 5.

Effect of ceftriaxone (CEF, 100 mg/kg, n=4) treatment (**24h post-treatment**) on the expression of xCT in the prefrontal cortex (PFC), nucleus accumbens (NAc) and amygdala (AMG) compared to the saline treated control group (Day 14). (A) Immunoblot for xCT and GAPDH as loading control marker for the corresponding brain regions. (B) Quantitative analysis of the immunoblots revealed significant difference in the ratio of xCT/GAPDH in

all brain regions in ceftriaxone-treated group as compared to saline-treated animals (100%). Data are expressed as mean $\pm$ SEM. (*p<0.05)