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Effects of beta-lactam antibiotics on cystine/ glutamate exchanger transporter and glutamate transporter 1 isoforms as well as ethanol drinking behavior in male P rats

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A Thesis

Entitled

**Effects of Beta-Lactam Antibiotics on Cystine /Glutamate Exchanger Transporter
and Glutamate Transporter 1 Isoforms as well as Ethanol Drinking Behavior in
Male P Rats**

By

Fawaz Alasmari

Submitted to the Graduate Faculty as a partial fulfillment of the requirement for the
Master of Science Degree in Pharmacology and Toxicology

Dr. Youssef Sari, Committee Chair

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College of Graduate Studies

The University of Toledo

August 2015

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An Abstract of

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Evidence demonstrated that glial cells, mainly astrocytes, regulate glutamate uptake, which is regulated by several glutamate transporters. Among these glutamate transporters, glutamate transporter 1 (GLT-1, its human homolog is excitatory amino acid transporter-2) is responsible for the majority of glutamate uptake by glial cells. Cystine-glutamate antiporter (xCT) is another glial protein that regulates glutamate transmission. It has been previously shown that a β -lactam antibiotic, ceftriaxone, upregulated GLT-1 and xCT expression levels in prefrontal cortex (PFC) and nucleus accumbens (NAc), and consequently reduced ethanol intake and relapse-like ethanol intake in alcohol-preferring (P) rats. It has been shown that found recently that β -lactam antibiotics (ampicillin, cefazolin, and cefoperazone) upregulated GLT-1 expression in the prefrontal cortex

(PFC) and nucleus accumbens (NAc) and consequently reduced ethanol intake in alcohol-preferring (P) rats.

In this study, we investigated the effects of ampicillin, cefazolin, and cefoperazone on the expression levels of xCT and GLT-1 isoforms (GLT-1a and GLT-1b) as well as on GLAST expression using western blot assay. We found that these compounds reduced alcohol intake as compared to saline treated group. In addition, we found that ampicillin, cefazolin and cefoperazone induced upregulation of GLT-1a and GLT-1b expression levels in both PFC and NAc, but no significant effects in glutamate/aspartate transporter, GLAST, expression were induced. We also found that ampicillin and cefazolin increased xCT expression in both NAc and PFC. However, cefoperazone increased xCT expression only in the NAc. Additionally, we found that cefoperazone prevented relapse like-ethanol intake. Our findings provide additional information about the potential uses of β -lactam antibiotics as target drugs for the treatment of alcohol dependence.

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List of Abbreviations

| | |
|---------------|---|
| alphaPKC..... | Alpha protein kinase C |
| Amp..... | Ampicillin |
| AMPA..... | α -Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid |
| BSA..... | Bovine serum albumin |
| CPZ..... | Cefoperazone |
| CSF | Cerebrospinal fluid |
| CZN..... | Cefazolin |
| dSTR..... | Dorsal striatum |
| EAAT..... | Excitatory amino-acid transporters |
| GLAST..... | Glutamate/ aspartate transporter |
| GLT-1 | Glutamate Transporter 1 |
| GPI-1046..... | (3-(3-pyridyl)-1-propyl (2S)-1-(3,3-dimethyl-1,2-dioxopentyl)-2-pyrrolidinecarboxylate) |
| mGluRs..... | Metabotropic glutamate receptors |
| mPFC..... | Medial prefrontal cortex |
| MS-153..... | ((R)-(-)-5-methyl-1-nicotinoyl-2-pyrazoline). |

NAc..... Nucleus Accumbens
nAChRsNicotinic acetylcholine receptors
NMDA..... N-Methyl-D-aspartate
NP..... Alcohol non-preferring (P) rats

PFC..... Prefrontal Cortex
P rats.....Alcohol-preferring (P) rats

TBST..... Tris-buffered saline Tween-20

VGLUTs..... Vesicular glutamate transporters
VTA.....Ventral tegmental area

xCT..... Cystine-glutamate antiporter

HAD..... High-alcohol-drinking

Chapter 1

Introduction

1.1. Overview

Drug dependence is defined as chronic relapsing condition characterized by high tendency to take drug, no control to limit drug consumption, developing several emotional conditions (e.g. dysphoria, and anxiety) and developing withdrawal symptoms (Koob and Volkow, 2010). Pre-clinical studies showed that glutamate and dopamine play an important role in the neuroplastic changes (Wolf et al., 2004, Kalivas and O'Brien, 2008, Thomas et al., 2008). Changes in dopamine release and neuroadaptation in amygdala, striatum, prefrontal cortex (PFC), and orbitofrontal cortex are involved in drug addiction (Koob and Volkow, 2010). In rewarding effects of drug addiction, glutamate plays a critical role in the modulation of dopamine release in the nucleus accumbens (NAc) (Kalivas and Volkow, 2005). Alcoholism is defined as chronic alcohol consumption, which can lead to social, mental and physical problems. This disease is also called alcohol dependence (Bush et al., 1987). Several health problems are developed by alcohol dependence depending on the amount of alcohol consumption and pattern of

drinking (Anderson et al., 1993). According to National Institute of Alcohol Abuse and Alcoholism (NIAAA), alcohol develops health problems in the body (Table 1.1).

Table 1.1. Effects of Alcohol on the Body (National Institute of Alcohol Abuse and Alcoholism (NIAAA)).

| Organs | Effects |
|----------------------|--|
| Brain | Brain performance and appearance, Mood Changes, Behavioral Changes, Thinking Problems |
| Heart | Heart Damage, Cardiomyopathy, Arrhythmias, Stroke, Hypertension |
| Liver | Liver Cancer, Fibrosis, Cirrhosis, Alcoholic Hepatitis, and Steatosis or Fatty Liver |
| Pancreas | Pancreatitis, Blood vessels swelling or inflammation in pancreas |
| Immune System | Immune system weakness, alcoholism is more likely to have a disease like pneumonia and tuberculosis |
| Breast | Breast Cancer |

| | |
|------------------|-------------------------|
| Throat | Throat Cancer |
| Esophagus | Esophagus Cancer |
| Mouth | Mouth Cancer |

Most of drugs of abuse increase dopamine release in the central nervous system. A study investigated the reinforcing effect of alcohol – nicotine co-abuse in dopamine release within the ventral tegmental area (VTA) through stimulation of nicotinic acetylcholine receptors (nAChRs)(Tizabi et al., 2002). This study reported that dopamine release in VTA was higher in rats that were given 0.5 g/kg ethanol plus 0.25 mg/ kg nicotine than those who were given ethanol or nicotine alone. Furthermore, dopamine is significantly higher in groups that received only nicotine or ethanol as compared to saline group (Tizabi et al., 2002). Moreover, it was shown that i.p. injections of alcohol increased dopamine concentration in the NAc and dorsal striatum (dSTR) (Melendez et al., 2003).

Importantly, ethanol withdrawal rats (four to ten hours after last ethanol intake) showed a higher extracellular glutamate concentration in the NAc as compared to ethanol naïve group (Saellstroem Baum et al., 2006). Studies demonstrated that N-methyl-D-aspartate (NMDA) receptors, which is ionotropic glutamate receptor, play a critical role

on enhancing the effect of nicotine on ethanol administration (Ford et al., 2013). It has been shown that NMDA and 1-a-amino-3-hydroxy-5- methylisoxazole-4-propionic acid hydrate (AMPA) are involved in the mechanism of ethanol and nicotine interaction (Al-Rejaie and Dar, 2006).

1.2. Mesolimbic pathway to produce addictive and rewarding effects

Several studies investigated extensively the role of the mesolimbic pathway (dopaminergic, glutaminergic and GABAergic) in drug addiction (Adinoff, 2004, Russo and Nestler, 2013).

1.2.1. Nucleus Accumbens (NAc)

Several studies examined the role of NAc in drug addiction (Wise and Rompre, 1989, Di Ciano and Everitt, 2004). It is well known that NAc receives glutamatergic projections from amygdala and PFC inducing drug-seeking behavior (Kalivas et al., 2009). It is well known that alcohol and nicotine administration increased dopamine concentration in NAc (Yoshimoto et al., 1992, Tizabi et al., 2002, Melendez et al., 2003, Di Chiara et al., 2004). Our lab has shown that GLT-1 is downregulated in the NAc in rats exposed to ethanol for five weeks as compared to ethanol naïve group (Alhaddad et al., 2014a, Alhaddad et al., 2014b). Glutamate transmission in the NAc is important to

produce an addictive effect. Alternatively, cocaine- seeking behavior and reinstatement of cocaine taking are linked to changes in glutamate transmission in the NAc. In addition, it has been suggested that AMPA receptor antagonist could be considered as a pharmacological target for the treatment of drug addiction (Cornish and Kalivas, 2000).

1.2.2. Prefrontal Cortex (PFC)

PFC is one of the mesocorticolimbic regions that sends glutamatergic projections into the NAc (Kalivas et al., 2009). It has been shown that prelimbic cortex hypoactivity is developed with compulsive cocaine- dependent rats (Chen et al., 2013). Moreover, activation of the prelimbic cortex could be used to reduce compulsive cocaine- seeking behavior. Therefore, PFC can be a target to treat drug dependence (Chen et al., 2013). Several studies from our lab showed that ceftriaxone, MS-153 and GPI-1046 attenuated alcohol drinking in part by upregulatory effects of GLT-1, GLT-1 isoforms and xCT expression levels in the PFC (Sari and Sreemantula, 2012, Alhaddad et al., 2014a, Alhaddad et al., 2014b, Rao and Sari, 2014). It has been reported that nicotine applied on medial prefrontal pyramidal cells could lead to increase glutamate concentration in these cells in rats (Lambe et al., 2003). The medial prefrontal cortex (mPFC) plays a critical role in heroin self-administration (Doherty et al., 2013). Several studies showed that alcohol intake decreased the activity of neurons in PFC (Kahkonen et al., 2003, Tu et al., 2007, Goldstein and Volkow, 2011)

1.2.3. Striatum

It has been demonstrated that alcohol administration increased dopamine concentration in both NAc and striatum in a Wister rats (Melendez et al., 2003). In addition, cocaine exposure increased dopamine concentration in the dorsal striatum (Volkow et al., 2006). Inhibition of cue to cocaine-induced dopamine release in the striatum is a novel pharmacological target to treat cocaine addiction (Volkow et al., 2006). Importantly, dopaminergic and glutamatergic pathways in the striatum have a critical role in cocaine addiction. Intradorsal striatal infusion of dopamine antagonist and AMPA receptor antagonist, but not NMDA receptor antagonist, decreased cocaine self-administration in rats (Vanderschuren et al., 2005). Alternatively, chronic methamphetamine injection decreased glutamate receptors expression in the striatum by changing in DNA methylation and hydroxymethylation (Jayanthi et al., 2014). It is suggested that cocaine dependence developed a disruption in glutamate transmission in the dorsal striatum (Parikh et al., 2014). Ceftriaxone, beta- lactam antibiotic, reduced cocaine intake and this effect might be mediated through activation/upregulation of GLT-1 in the striatum as well as in the PFC and NAc (Sari et al., 2009, Knackstedt et al., 2010, Parikh et al., 2014).

1.2.4. Amygdala

The role of the amygdala in drug addiction has been investigated extensively (Di

Ciano and Everitt, 2004). Amygdala plays a significant role in memory (learning), anxiety, and emotional behavior conditions (Lalumiere, 2014). Moreover, glutamate release from the PFC and amygdala into the NAc is important in the sensitization and drug-seeking behaviors (Kalivas et al., 2009). It is shown that dopamine antagonist in amygdala reduces cocaine seeking (Di Ciano and Everitt, 2004). Moreover, dopamine receptor plays a critical role in nicotine addiction. Dopamine (D3) receptor antagonist reduced cue-induced reinstatement nicotine – seeking behavior in the Amy (Khaled et al., 2014). The role of amygdala in heroin addiction was examined. Blocking amygdala reduced heroin – seeking behavior in conditioned cues induced reinstatement (Rogers et al., 2008). It is known that metabotropic glutamate receptor 5 (mGluR5) is an important receptor, which is distributed in different brain areas, including NAc and amygdala. Inhibition of mGluR5 in amygdala reduced cue-induced reinstatement of ethanol - seeking behavior (Sinclair et al., 2012). Furthermore, synaptic glutamate concentration is increased in rats withdrawal from chronic alcohol exposure (Christian et al., 2013)

1.2.5. Hippocampus

It is well known that hippocampus plays a critical role in drug addiction and formation of memory (Adcock et al., 2006, Meyers et al., 2006, Shen et al., 2006, Hernandez-Rabaza et al., 2008, Delgado and Dickerson, 2012). In addition to PFC and amygdala, hippocampus also sends glutamatergic projections into NAc. Therefore, hippocampus has a crucial role in drug dependence (Meyers et al., 2006, Britt et al.,

2012, Papp et al., 2012). It has been shown that a decrease in hippocampal neurogenesis by irradiation led to an increase in cocaine self-administration in rats (Noonan et al., 2010). Alternatively, chronic alcohol administration decreased neurogenesis in hippocampus (Herrera et al., 2003, He et al., 2005). It is known that hippocampus is involved in relapse – like cocaine intake (Vorel et al., 2001, Fuchs et al., 2005). The expression of cocaine conditioned place preference (CPP) is affected by inhibition of dorsal hippocampus (Meyers et al., 2006).

1.2.6. Ventral Tegmental Area (VTA)

It has been shown VTA is involved in drug dependence. Dopaminergic projections from VTA into the shell of the NAc and the PFC play a key role in drug dependence (Wise and Rompre, 1989). Cocaine increased synapses of glutamatergic by inducing long term potentiation depending on the local rapid stimulation of NMDARs in the VTA (Heshmati, 2009). This action may be occurred due to stimulation of NMDA receptor and dopamine (D5) receptors cascade (Heshmati, 2009). It has been demonstrated that cocaine at low concentration may have blocking action on dopamine reuptake in the VTA (Brodie and Dunwiddie, 1990). It has been reported that nicotine binds to $\alpha 4$ and $\alpha 6$ subunits of nAChRs in dopaminergic axon terminals, which lead to stimulation of dopaminergic neurons activity in the VTA (Liu et al., 2012, Baker et al., 2013). It has been also shown that AMPA administration increased glutamate and dopamine release in amphetamine treated group as compared to saline- treated group in the VTA (Giorgetti et

al., 2001). Importantly, ethanol at low concentration binds to presynaptic dopamine (D1) receptor producing high extracellular glutamate concentrations and then high dopamine concentration (Xiao et al., 2009). Furthermore, DNQX, an AMPA receptor antagonist, reduced ethanol-induced dopamine release suggesting the important role of the VTA in alcohol addiction (Xiao et al., 2009).

1.3. Glutamate Transporters

Glutamate transporters regulate glutamate concentration released from presynaptic neurons to reduce toxic glutamate concentration. There are two major types of glutamate transporters. These transporters are the excitatory amino acid transporters (EAATs) and the vesicular glutamate transporters (VGLUTs) [for review see ref.(Shigeri et al., 2004). VGLUTs are expressed in both central and peripheral nervous system as well as peripheral non-neuronal system (Moriyama and Hayashi, 2003, Moriyama and Yamamoto, 2004). It has been shown that VGLUT 1 and VGLUT 2 are transporters of glutamatergic neurons, while VGLUT 3 is a transporter of both glutamatergic and non-glutamatergic neurons (Stornetta et al., 2002, Takamori et al., 2002). VGLUT 1, VGLUT 2, and VGLUT 3 transporters can transport glutamate from cytoplasm to storage vesicle (Takamori, 2006).

The second type of glutamate transporters is EAAT family, which contains five

subtypes [for review see ref.(Shigeri et al., 2004):

- 1- GLT-1 (human homologue is EAAT2), glutamate transporter 1
- 2- GLAST (human homologue is EAAT1), glutamate/aspartate transporter.
- 3- EAAC1 (human homologue is EAAT3), excitatory amino acid carrier type 1
- 4- EAAT4, excitatory amino acid carrier type 4
- 5- EAAT5, excitatory amino acid carrier type 5

The most important transporter expressed highly in astrocytes is glutamate transporter 1 (GLT-1, its human homolog is excitatory amino acid transporter-2, EAAT2). The majority (approximately 90%) of glutamate uptake is regulated by GLT-1. Therefore, GLT-1 can remove high concentration of glutamate to make the extracellular glutamate concentration below the toxic level (Mitani and Tanaka, 2003). There are two isoforms for GLT-1: GLT-1a and GLT-1b. It has been shown that GLT-1a is found in both neurons and astrocytes while GLT-1b is expressed only in astrocytes (Berger et al., 2005, Holmseth et al., 2009). It has been reported that the ability of one isoform to transport extracellular glutamate into glia cells is not that different than the second isoform (Holmseth et al., 2009).

GLAST is considered as the major glutamate transporter in the cerebellum (Lehre and Danbolt, 1998). It has been reported that GLAST is distributed throughout brain (Schmitt et al., 1997). However, GLAST is the most common glutamate transporter in the inner ear and the retina (Takumi et al., 1997, Lehre and Danbolt, 1998).

EAAT3 transports glutamate at post-synaptic neurons. It has been reported that neuronal activity as well as other signaling pathways (phosphatidylinositol-3-kinase (PI3K) and alpha protein kinase C (alphaPKC)) regulated EAAT3 (Nieoullon et al., 2006). Additionally, EAAT4 is also primarily expressed in neurons. It has been shown that EAAT4 as well as EAAT3 are found in neurons of hippocampus and cerebellum (Rothstein et al., 1994, Dehnes et al., 1998). Alternatively, EAAT5 is mainly expressed in the retinal bipolar cells and rod photoreceptor (Arriza et al., 1997).

Cystine-glutamate antiporter (xCT), another glial protein, plays an important role in glutamate regulation. xCT regulates glutamate transmission by exchanging extracellular cystine for intracellular glutamate (Baker et al., 2002)

1.4. Glial Proteins and Alcohol Dependence

Several studies from our laboratory showed that continuous and relapse – like ethanol intake are associated with the decrease in the expression levels of GLT-1, GLT-1 isoforms (GLT-1a and GLT-1b) and xCT in mesocorticolimbic area of male P rats (Rao and Sari, 2012, Qrunfleh et al., 2013, Alhaddad et al., 2014a, Alhaddad et al., 2014b, Rao and Sari, 2014). We focus here on investigating these selected glutamatergic transporters as well as others.

1.4.1 GLT-1 and Alcohol Dependence

It has been shown that a decrease in GLT-1 expression was found associated with increase extracellular glutamate concentration in animal models of alzheimer and ischemia (Li et al., 1997, Martin et al., 1997, Sari and Sreemantula, 2012). Moreover, glutamate transporters upregulator compound, tamoxifen, enhances uptake of extracellular glutamate concentrations into astrocyte (Lee et al., 2009, Karki et al., 2013). Several studies found that cocaine self-administration, nicotine self-administration and chronic alcohol consumption decreased GLT-1 level (Knackstedt et al., 2009, Knackstedt et al., 2010, Kryger and Wilce, 2010, Alhaddad et al., 2014a). A study from our laboratory found that chronic alcohol intake for five weeks decreased GLT-1 expression in the NAc as compared to water naïve group (Sari and Sreemantula, 2012). As compared to water naïve group, continuous ethanol drinking for five weeks downregulated GLT-1 isoforms (GLT-1a and GLT-1b) in the NAc (Alhaddad et al., 2014a).

It has been shown that ceftriaxone, a beta- lactam antibiotic, upregulated GLT-1 expression and consequently reduced reinstatement of cocaine- seeking behavior (Sari et al., 2009). Ceftriaxone also attenuated alcohol intake in male P rats in part by upregulatory effects on GLT-1 and its isoforms in the NAc and the PFC (Alhaddad et al., 2014a, Rao and Sari, 2014). It has also been reported that MS-153 and GPI-1046 were able to upregulate GLT-1 and consequently decreased alcohol intake in male P rats (Sari and Sreemantula, 2012, Alhaddad et al., 2014b).

1.4.2 xCT and Alcohol Dependence

Our lab recently reported that xCT expression is downregulated in the NAc and the PFC in P rats exposed to ethanol for five weeks as compared to water naïve group (Alhaddad et al., 2014a). In addition to upregulatory effect on GLT-1 in the NAc and the PFC, ceftriaxone increased also xCT expression in both NAc and the PFC, and consequently reduced alcohol intake and cue to cocaine -seeking behavior (Knackstedt et al., 2010, Alhaddad et al., 2014a).

1.4.3 Other Glutamate Transporters and Alcohol Dependence

It has been shown that tamoxifen, estrogen receptor antagonist, upregulated both GLT-1 and GLAST, and consequently increased glutamate uptake (Karki et al., 2013). However, GLAST expression was not affected in P rats exposed to ethanol for five weeks (Alhaddad et al., 2014a, Alhaddad et al., 2014b). It has been shown that an acute dose of ethanol did not change the levels of both GLAST and GLT-1 expression levels (Melendez et al., 2005). In addition, studies from our lab did not find any upregulatory effects on GLAST with the two compounds known as GLT-1 upregulators, Ceftriaxone and MS-153, in male P rats exposed to alcohol (Alhaddad et al., 2014a, Alhaddad et al., 2014b).

Alternatively, acute ethanol exposure increased EAAT3 activity; however, EAAT3

activity is reduced by chronic exposure to alcohol (Kim et al., 2005). However, EAAT4 activity was found reduced by acute alcohol administration (Park et al., 2008).

1.5. Alcohol-Preferring (P) rats as an Established Animal Model for Alcohol Dependence

Most of the studies examined the neurobiological mechanisms for continuous alcohol intake and relapse-like alcohol. It has been reported that P rats met the criteria for animal model that is applicable for alcohol dependence. P rats can be used to assess alcohol preference. Moreover, P rats display a robust response to the alcohol deprivation effect (ADE), therefore, P rats are animal model for relapse like-alcohol intake (Bell et al., 2006). A study showed that alcohol non-preferring rats (NP) drink less than 1 g/kg/day, while P rats consumed more than 4 g/kg/day (Li et al., 1987). It has been reviewed and suggested that P rats and high-alcohol-drinking (HAD) rats met all the criteria as animal models for alcoholism, which can be used to investigate the effects of several compounds on alcohol consumption, relapse-like alcohol intake, alcohol and nicotine co-addiction (McBride et al., 2014). In addition, it has been shown that P rats had higher preference to 10% (v/v) alcohol as compared to water (Li et al., 1993). A comparative study found that P rats consumed more alcohol after deprivation period, which makes P rats a good model to study relapse-like alcohol intake (Vengeliene et al., 2003). Furthermore, study investigated the effect of isolate housing in alcohol consumption in P and NP rats, showed that ethanol intake was higher in P rats as

compared to NP rats (Ehlers et al., 2007).

1.6. Aims and Objectives

Several studies tested the effects of several compounds on GLT-1 expression to discover a new compound, that offers neuroprotection. One of these studies found that β -lactam antibiotics were the most potent upregulators of GLT-1 (Rothstein et al., 2005). Rothstein et al (2005) tested the effect of several β -lactam antibiotics on GLT-1 expression. Among these antibiotics that stimulated GLT-1 expression are ampicillin and cefoperazone. Therefore, in this study, the effects of ampicillin (Figure 1-1) and cefoperazone (Figure 1-2) on alcohol intake were examined using alcohol preferring rat. We also have investigated the effect of cefazolin, another β -lactam antibiotic (Figure 1-3) on alcohol consumption in P rats. Ampicillin is semisynthetic penicillin β -lactam antibiotic, while cefazolin and cefoperazone are first and third generations cephalosporin β -lactam antibiotics. We also have determined the effects of selected β -lactam antibiotics in the expression of GLT-1a, GLT-1b, xCT, and GLAST in the NAc and the PFC of male P rats using western blot assay.

It has been reported that ceftriaxone, third generation cephalosporin β -lactam antibiotic, attenuated relapse-like ethanol intake (Qrunfleh et al., 2013). Therefore, we investigated the effects of cefoperazone with β -lactam structure and third generation cephalosporin similar to ceftriaxone on relapse like ethanol intake in male P rats.

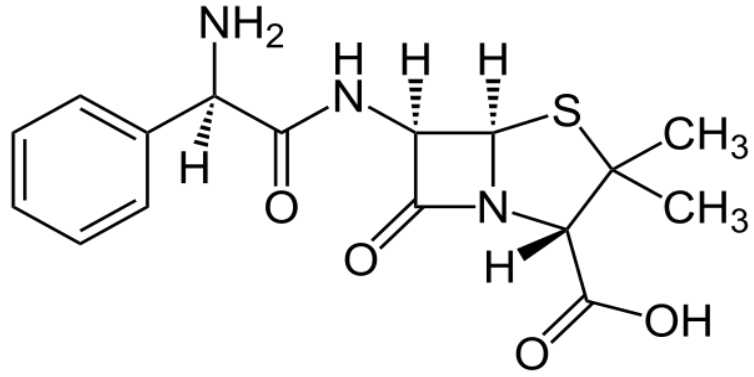


Figure 1-1 Chemical Structure of Ampicillin

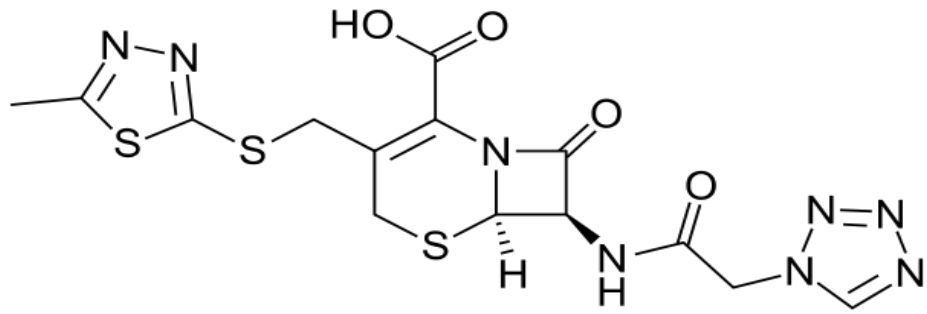


Figure 1-2 Chemical Structure of Cefazolin

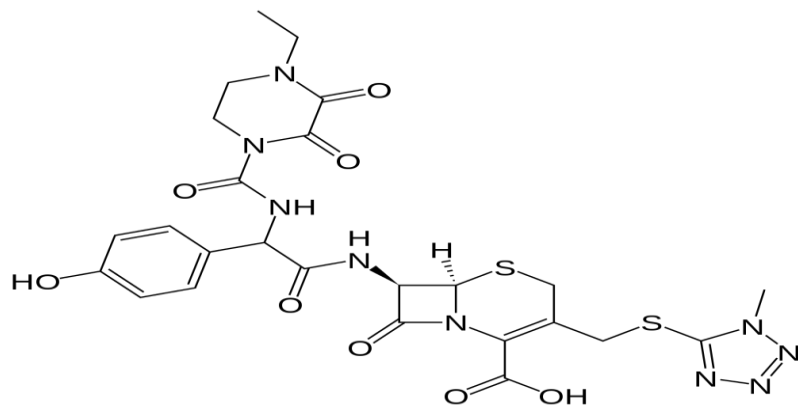


Figure 1-3 Chemical Structure of Cefoperazone

Chapter 2

Materials and Methods

2.1. Animals

Alcohol-preferring male (P) rats were received from Indiana University, School of Medicine (Indianapolis, IN, USA). Rats were housed in bedded plastic tubs and kept at 21°C, 50% humidity in the Department of Laboratory Animal Resource at The University of Toledo, Health Science Campus. The Institutional Animal Care and Use Committee of The University of Toledo approved all animal housing and experimental procedures in accordance with guidelines of 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). All rats were at age of 90 days, and they were individually housed in standard plastic cages and divided into four experimental groups: saline group received water and food and i.p. injection of 0.9 % saline solution (n=6), ampicillin group received 100 mg/kg of the drug

(i.p) (n=6), cefazolin group received 100 mg/kg of the drug (i.p) (n=6), and cefoperazone group received 100 mg/kg of the drug (i.p) (n=6). In regard to cefoperazone relapse study, 3 months old rats were divided into two experimental groups: ethanol vehicle group received water and food and i.p. injection of vehicle solution (1% DMSO in PBS) (n=6), and cefoperazone group (CPZ) received 100 mg/kg of the drug (i.p) (n=6).

2.2. Behavioral drinking paradigms

2.2.1 Beta-lactam antibiotics study

At age of 90 days, designated saline, ampicillin, cefazolin, and cefoperazone groups of rats were given free choice to food, water and two ethanol concentrations (15% and 30%, v/v) for five weeks. Body weight, water intake and ethanol intake were evaluated three times a week during the last two weeks. Densitometry formula was used to convert ethanol intake measurements to gram per kilogram of body weight of animal per day. Rats selected for the study were required to achieve at least 4 g/kg/day or more of ethanol intake. Body weight, water intake and ethanol intake were measured during Week 4 and Week 5, which served as baseline values. On Week 6, P rats were i.p. injected either saline, ampicillin (100 mg/kg), cefazolin (100 mg/kg), or cefoperazone (100 mg/kg) daily for five consecutive days. During these five days animals body weight, water intake and ethanol intake were measured every day. Rats were euthanized by CO₂ inhalation and further decapitated 24 hours after the last i.p. injections of saline or drug. Note that

ethanol preference was calculated using the following formula: (ethanol intake measurement/ total fluid consumed) x 100

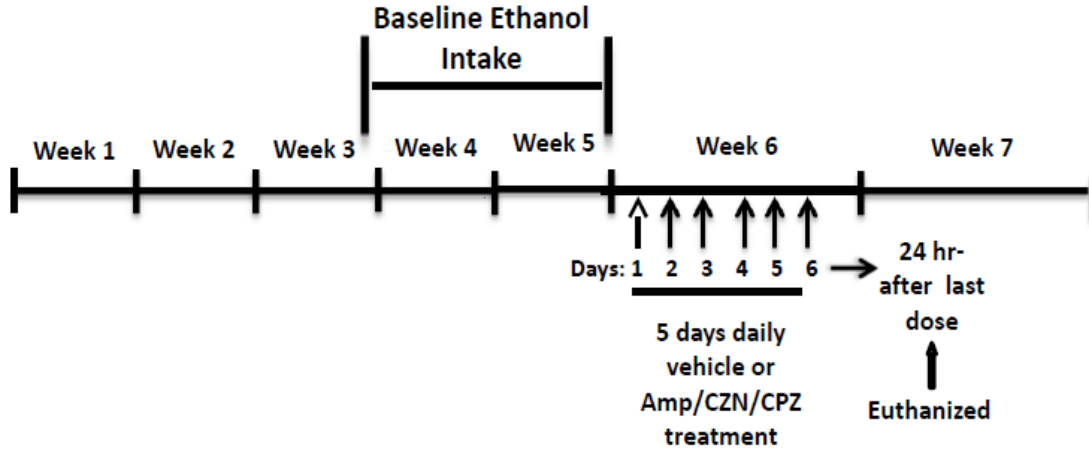


Figure 2-1 Timeline for Continuous Ethanol Drinking Paradigm

2.2.2. Cefoperazone Relapse Study

At age of 90 days, rats were given access to free choice to ethanol concentrations (15% and 30%, v/v), water and food for five weeks. We evaluated water intake, body weight, and ethanol intake three times a week during last two weeks. We also used densitometry formula to convert alcohol consumption measurements to gram per kilogram of body weight of each animal per day. Rats selected for the study were required to consume at least 4 g/kg/day or more of alcohol intake. We measured body weight, water intake and ethanol intake during Week 4 and Week 5, which served as baseline values. On week 6, P rats were deprived of ethanol for two weeks and divided

into two experimental groups. During last five days of 14 days deprivation time, P rats were i.p. injected either vehicle solution or cefoperazone (100 mg/kg) daily for five consecutive days. Twenty-four hours after last injection, all rats were re-exposed to ethanol for seven days. During these seven days, animals body weight, water intake and ethanol intake were measured every day. Twenty-four hours after these seven days, rats were euthanized by CO₂ inhalation and then rapidly decapitated. Note that ethanol preference was calculated as: (ethanol intake measurement / total fluid consumed) x 100.

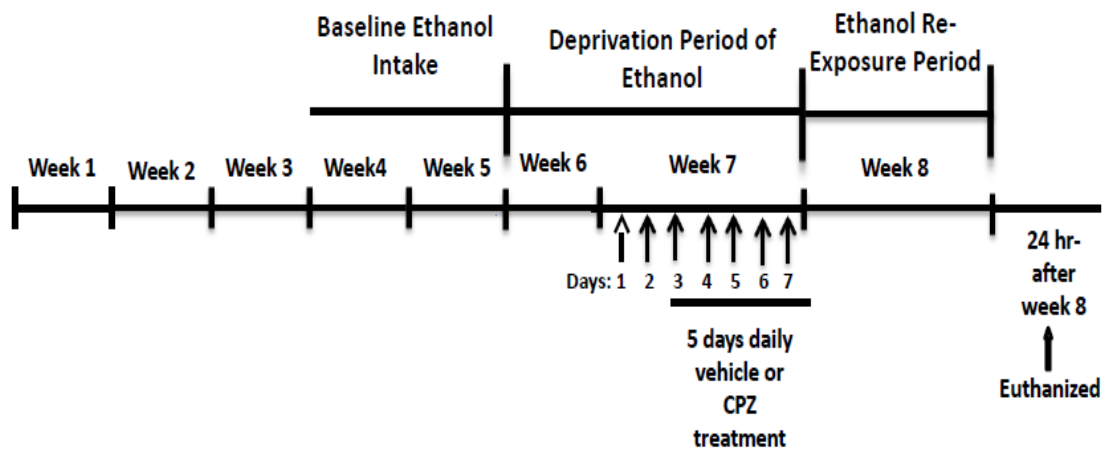


Figure 2-2 Timeline for Relapse-Like Ethanol Drinking Paradigm.

2.3. Brain tissue harvesting

Brains were removed then immediately frozen on dry ice and stored at -80°C . Brains regions (NAc and PFC) were microdissected with Leica cryostat apparatus using stereotaxic coordinates from the rat brain Atlas (Paxinos and Watson, 2007). These brain regions were then immediately stored at -80°C for further immunoblot testing.

2.4. Protein Quantification Assay

2.4.1 Beta-lactam antibiotics study

After all brain samples were lysed using buffer containing protease and phosphatase inhibitors, we used Lowry protein quantification assay to determine the exact amount of proteins in each sample. We then determined regression line and standard curve using bovine serum albumin (BSA) (New England Bio labs). Further, $1\mu\text{L}$ from each sample was added into $4\mu\text{L}$ of lysis buffer in four well in 96 well plates. Then, we added $25\mu\text{L}$ of mixture contains 3 ml of reagent A and $60\mu\text{L}$ of reagent S (Both reagents are purchased from BioRad Laboratories) into each well. After that, $200\mu\text{L}$ of reagent B (BioRad Laboratories) was added into each well, then plates were kept at room temperature in a dark place for 15 minutes. Multiskan FC spectrophotometer (Thermo Scientific) was used to measure the absorbance of all samples at 750 nm. Finally, samples protein concentrations were determined using standard curve and line regression.

Therefore, equal amount of proteins were used for Western Blot Assay.

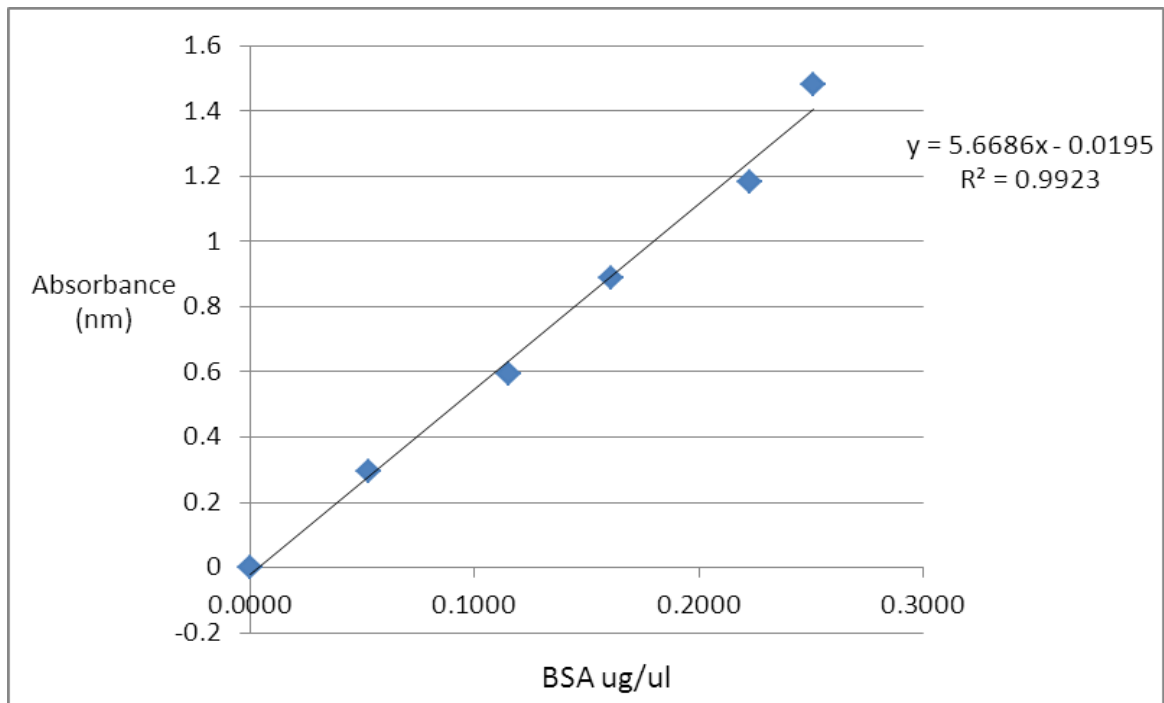


Figure 2-3 Standard curve generated for NAc (Saline (n =4), ampicillin (n =4), cefazolin (n =4), and cefoperazone (n =4))

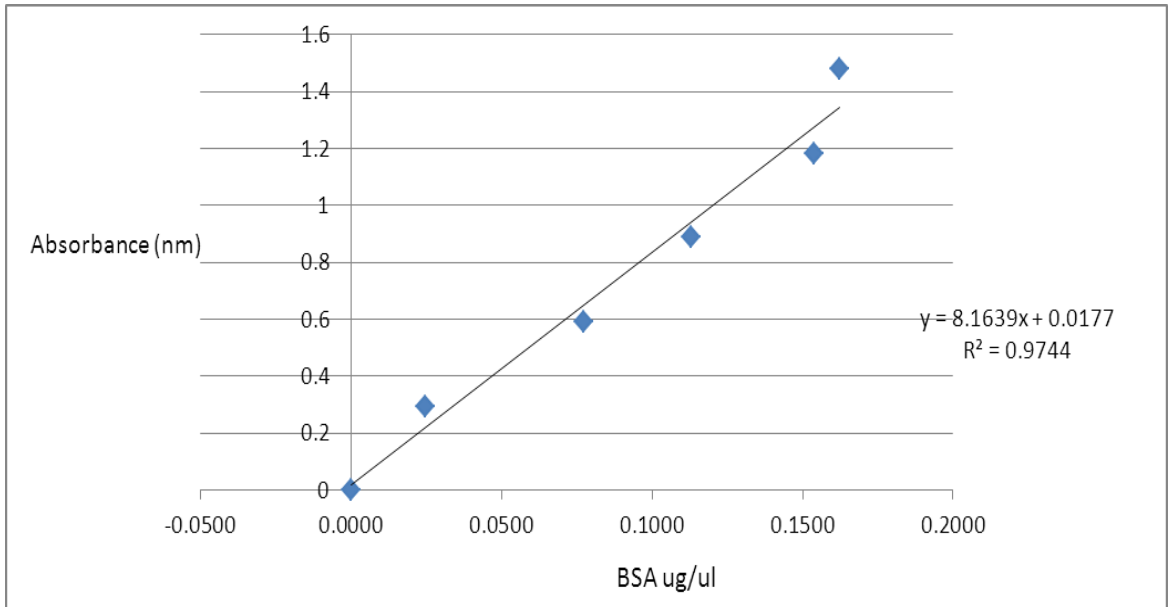


Figure 2-4 Standard curve generated for PFC (Saline (n =4), ampicillin (n =4), cefazolin (n =4), and cefoperazone (n =4))

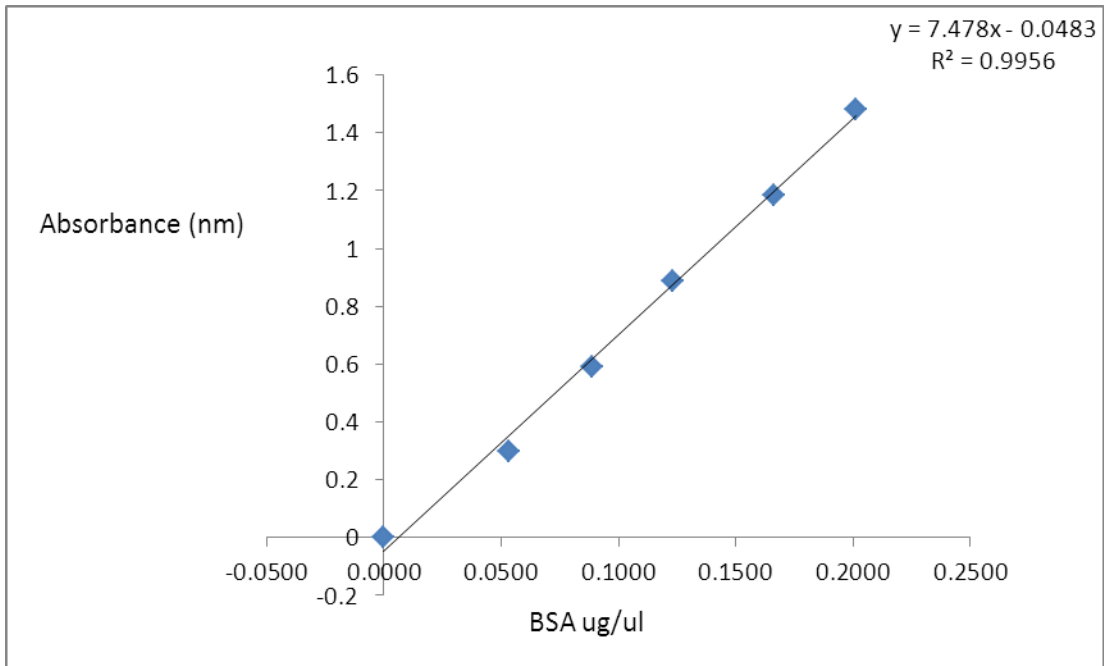


Figure 2-5 Standard curve generated for NAc and PFC (Saline (n =4), ampicillin (n =4), cefazolin (n =4), and cefoperazone (n =4))

2.5. Western blot protocol

2.5.1 Beta- lactam antibiotics study

2.5.1.1. Gel Preparation

We used 10-20% polyacrylamide gel for western blot assay. We mixed different reagents to prepare the separating and stacking gels. Those reagents were used in specific concentrations based on the number of gels that we prepare. The reagents using in this study are as follows: 1.5 M Tris Buffer pH 8.8, 10 % SDS, 30 % Acrylamide/Bis solution (BioRad), 10% Ammonium Persulfate (APS, Fisher Scientific), Deionized water (DI water, and TEMED (N,N,N',N'- tetramethylethylenediamine, BioRad). We added the separating mixture into specific apparatus (BioRad), then we immediately added DI water in the same apparatus to avoid the dryness of separating gel. After separating gel was solidified, we removed DI water and immediately added the stacking gel. Thirty minutes after the gels are prepared; we used them for western blot assay.

2.5.1.2. Western blot protocol for detection of GLT-1a, GLT-1b, xCT and GLAST

Changes in GLT-1a, GLT-1b, xCT, GLAST and GAPDH expression levels in NAc and PFC were determined using Western Blot technique as described previously (Sari et al., 2011, Alhaddad et al., 2014a, Rao and Sari, 2014). Brain tissues were homogenized in

lysis buffer containing protease inhibitor, and the total protein were quantified using BioRad kit. Equal amount of lysed NAc or PFC from both groups were loaded on 10-20% polyacrylamide gel. Proteins were then transferred electrophoretically onto PVDF membrane using transfer apparatus. The membranes were blocked in 3% milk in Tris-buffered saline Tween-20 (TBST) for 30 minutes at room temperature, then incubated overnight at 4°C with one of the following antibodies: rabbit anti-GLT-1a (1:5,000 gift from Dr. Jeffery Rothstein, Johns Hopkins University), rabbit anti-GLT-1b (1:5,000 gift from Dr. Paul Rosenberg, Harvard Medical School University), rabbit anti-xCT (1:1,000 Abcam), rabbit anti- GLAST (1:5,000 Abcam) and mouse anti-GAPDH(1:5,000, Millipore). The membranes were washed next day with TBST, and then blocked with 3% milk in TBST for 30 minutes at room temperature. Immunoblotting membranes were then incubated with Anti-rabbit IgG (1:3000) or anti-mouse IgG (1:3000) for 90 minutes. After washing, membranes were dried and incubated with chemiluminescent kit (Super Signal West Pico, Pierce Inc.) for one minute. Membranes were then further dried and exposed to HyBlot CL Film (Thermo Fisher Scientific). Films were developed using SRX-101A Film processor and digitized blots were quantified with MCID software. Data for GLT-1a, GLT-1b, xCT and GLAST expression levels were represented as ratio of GAPDH expression in NAc and PFC.

2.6. Statistical analysis

2.6.1 Beta -Lactam Antibiotics Study

Two-ways (mixed) ANOVA with repeated measures were performed to analyze behavioral statistical data (daily ethanol intake, average body weight, daily water intake, and daily ethanol preference). We also used ordinary one-way ANOVA followed by Dunnett's multiple comparison test to determine the effect of ampicillin cefazolin and cefoperazone treatments on each day. Quantitative t-test was used to analyze western blot analysis data for comparisons between treatment (ampicillin, cefazolin and cefoperazone) and saline groups.

2.6.2 Cefoperazone Relapse Study

Two way (mixed) ANOVA with repeated measures, followed by Bonferroni multiple comparisons, was used for analysis of ethanol intake, water intake, ethanol preference, and body weight. All statistical analyses were based on $p < 0.05$ level of significance.

Chapter 3

Effects of β -lactam antibiotics treatment on xCT, GLT-1 isoforms, GLAST expression levels as well as ethanol drinking in male P rats

3.1. Introduction

Ethanol dependence is a public health issue. Existing treatments for ethanol addiction are limited, and finding a neurotransmitter system as a therapeutic target is important (Heilig et al., 2011). Among the neurotransmitters involved, the glutamatergic system is now well known for its important role in drug abuse, including ethanol (Kalivas et al., 2009, Sari et al., 2009, Rao and Sari, 2012, Sari, 2014). Glutamatergic inputs from the prefrontal cortex (PFC) into the nucleus accumbens (NAc) are critical in ethanol dependence [for review see refs (Rao and Sari, 2012, Sari, 2014)].

Glutamate transmission is regulated by several glutamate transporters, glutamate transporter-1 (GLT1, its human homolog excitatory amino acid transporter 2, EAAT2) is considered as the major glutamate transporter responsible for regulating the majority of glutamate uptake (Greene et al., 1979, Tanaka et al., 1997, Grewer et al., 2000, Bunch et al., 2009, Vandenberg and Ryan, 2013, Jensen et al., 2014).

Importantly, GLT-1 is expressed in the mammalian brain primarily in two isoforms, GLT-1a and GLT-1b (Chen et al., 2002, Chen et al., 2004, Berger et al., 2005). However, GLT-1c isoform is less expressed in the brain but highly expressed in the retina (Chen et al., 2002, Chen et al., 2004, Berger et al., 2005, Sogaard et al., 2013). It has been reported that GLT-1a is expressed in both neurons and astrocytes, however GLT-1b is expressed only in astrocytes (Berger et al., 2005, Holmseth et al., 2009). Changes in the expression levels of these isoforms may vary among different diseases. Thus, in amyotrophic lateral sclerosis disease it was found downregulation of GLT-1a expression and upregulation of GLT-1b expression (Maragakis et al., 2004). We have investigated in this study the effects of ampicillin in the expression levels of GLT-1a and GLT-1b on association with ethanol intake. We have also determined the expression of cysteine/glutamate exchanger transporter (xCT) as another glial glutamate transporter. xCT system transports anionic cystine inside astrocytes in exchange with glutamate (Bannai, 1986, Melendez et al., 2005). xCT was found to be downregulated in P rats exposed to free choice ethanol (15% and 30%) for 5 weeks (Alhaddad et al., 2014a). In addition, studies have shown also downregulation of xCT in cocaine seeking behavior (Knackstedt et al., 2010). xCT has been found to be associated with cocaine, nicotine

and ethanol seeking behaviors (Baker et al., 2003, Knackstedt et al., 2009, Knackstedt et al., 2010, Alhaddad et al., 2014a, Rao et al., 2015b). Together, these studies provide ample information about the important role of GLT-1 and xCT in drug abuse, including ethanol.

Studies from our lab demonstrated that administration of compounds that upregulated GLT-1 with its isoforms (GLT-1a and GLT-1b) and xCT reduced ethanol intake and relapse-like ethanol intake in P rats (Sari et al., 2011, Sari and Sreemantula, 2012, Qrunfleh et al., 2013, Sari, 2013, Alhaddad et al., 2014a, Alhaddad et al., 2014b, Rao and Sari, 2014, Aal-Aaboda et al., 2015). These compounds are as follows: ceftriaxone, β -lactam antibiotic, neuroimmunophilin GPI-1046 (3-(3-pyridyl)-1-propyl (2S)-1-(3,3-dimethyl-1,2-dioxopentyl)-2-pyrrolidinecarboxylate), and MS-153 ((R)-(-)-5-methyl-1-nicotinoyl-2-pyrazoline).

In this study, using male P rats, we focused on testing the effects of ampicillin, cefazolin and cefoperazone, other β -lactam antibiotics, with a β -lactam structure similar to ceftriaxone on the expression levels of xCT, GLT-1a, GLT-1b, and glutamate aspartate transporter (GLAST) as another glial glutamate transporter. The rationale for testing selected β -lactam antibiotics is based in recent findings from our lab showing that these antibiotics upregulated GLT-1 (Rao et al., 2015a). However, it is unclear about the effects of ampicillin, cefazolin and cefoperazone on the expression levels of xCT and GLAST as well as on the expression of GLT-1 isoforms (GLT-1a and GLT-1b). Furthermore, in contrast to ceftriaxone, ampicillin has clinical relevance, as it has the potential to be administered orally. P rats were exposed for five weeks to free choice ethanol (15% and

30% v/v) as an established drinking paradigm, water and food. Selected β -lactam antibiotics and saline vehicle were administered on Week 6 for five consecutive days. Ethanol intake, water intake, ethanol preference and body weight were measured for comparison between groups. We further examined the effects of ampicillin, cefazolin and cefoperazone on ethanol intake and determined whether there are any upregulatory effects on xCT, GLT-1a, GLT-1b, and GLAST expression levels.

3.2. Results

3.2.1 Effect of β -lactam antibiotics on ethanol intake, water intake, ethanol preference and body weight

We examined the effect of ampicillin, cefazolin and cefoperazone on ethanol and water intakes as well body weight. Ordinary one way ANOVA followed by Dunnett's multiple comparison test demonstrated a significant reduction on ethanol intake in selected β -lactam antibiotics treated groups compared to saline treated group on day 2 to day 5 ($p < 0.0001$). Moreover, mixed ANOVA demonstrated a significant main effect of day [$F(1, 5) = 41.02, p < 0.0001$] and a significant day x treatment interaction [$F(3, 15) = 3.472, p < 0.0001$] of ethanol intake (Table 3.1). Furthermore, ordinary one-way ANOVA followed by Dunnett's multiple comparison tests showed a significant increase in water intake in ampicillin treated group compared to saline treated group started on day 2 through day 4 ($p \leq 0.01$) and on Day 5 ($p < 0.05$). Alternatively, cefazolin treatment

increased water intake significantly in P rats only on day 3 ($p < 0.01$) and day 4 ($p < 0.05$) compared to saline-treated animals. Cefoperazone resulted in higher water intake on day 2 ($p < 0.01$), day 4 ($p < 0.001$) and day 5 ($p < 0.01$). Additionally, a significant main effect of day [$F(1, 5) = 6.992, p < 0.0001$] and a significant day x treatment interaction [$F(3, 15) = 2.791, p = 0.0010$] of water intake were found using mixed ANOVA analysis (Table 3.2). Furthermore, ordinary one-way ANOVA followed by Dunnett's multiple comparisons test measures demonstrated that ampicillin and cefazolin treatments reduced ethanol preference significantly as compared to saline treated group started on day 2 through day 5. Additionally, cefoperazone treatment resulted in a significant lower ethanol preference started on day 2 through day 5 except day 3. Mixed ANOVA revealed a significant main effect of day [$F(1, 5) = 6.212, p < 0.0001$] and a non-significant day x treatment interaction [$F(3, 15) = 1.623, p > 0.05$] of ethanol preference (Table 3.3). However, ordinary one-way ANOVA followed by Dunnett's multiple comparisons test did not reveal any significant effect on body weight between control and all treatment groups. Moreover, mixed ANOVA did not show any significant main effect of day [$F(1, 5) = 0.6930, p = 0.6297$] and day x treatment interaction [$F(3, 15) = 0.03184, p = 1.0000$] of average body weight (Table 3.4.).

Table 3.1. Effects of ampicillin, cefazolin and cefoperazone treatments on ethanol consumption (g/kg/day) in male P rats exposed to five weeks of continuous free choice of ethanol and water.

| | Ethanol Drinking (g/kg/day) | | | |
|-----------------|---------------------------------------|-------------------|------------------|---------------------|
| | SALINE | AMPICILLIN | CEFAZOLIN | CEFOPERAZONE |
| Baseline | 5.41±0.35 | 5.00±0.35 | 4.85±0.24 | 5.40±0.38 |
| Day 1 | 3.88±0.64 | 3.09±0.58 | 2.24±0.46 | 2.84±0.72 |
| Day 2 | 4.93±0.51 | 1.31±0.08# | 1.76±0.19# | 1.55±0.07# |
| Day 3 | 4.37±0.35 | 1.19±0.03# | 1.32±0.09# | 1.33±0.10# |
| Day 4 | 4.83±0.71 | 1.33±0.12# | 1.53±0.11# | 1.57±0.22# |
| Day 5 | 4.43±0.39 | 1.42±0.13# | 1.70±0.21# | 1.62±0.18# |

Significant difference between treatment groups # ($p < 0.0001$). Data are shown as mean \pm SEM; (n= 6 for each group).

Table 3.2. Effects of ampicillin, cefazolin and cefoperazone treatments on water intake (g/kg/day) in male P rats exposed to five weeks of continuous free choice of ethanol and water.

| | Water Intake (g/kg/day) | | | |
|-----------------|-----------------------------------|---------------------|---------------------|----------------------|
| | SALINE | AMPICILLIN | CEFAZOLIN | CEFOPERAZONE |
| Baseline | 13.50±0.90 | 12.50±0.40 | 12.30±0.80 | 14.70±1.30 |
| Day 1 | 13.10±2.60 | 17.10±3.90 | 15.30±0.70 | 15.60±3.20 |
| Day 2 | 11.10±1.40 | 26.10±2.10** | 18.60±2.70 | 24.50±3.60** |
| Day 3 | 9.90±1.80 | 24.00±1.70** | 24.90±1.50** | 17.60±4.70 |
| Day 4 | 10.40±1.50 | 25.70±1.50** | 20.60±1.50* | 31.10±5.00*** |
| Day 5 | 12.30±0.90 | 20.40±2.20* | 17.40±1.70 | 23.30±2.70** |

Significant difference between treatment groups *(p<0.05); **(p<0.01); ***(p <0.001).

Data are shown as mean ± SEM; (n= 6 for each group).

Table 3.3. Effects of ampicillin, ceftazolin and cefoperazone treatments on daily ethanol preference (%) in male P rats exposed to five weeks of continuous free choice of ethanol and water.

| | Ethanol Preference (%) | | | |
|----------|------------------------|-------------|--------------|---------------|
| | SALINE | AMPICILLIN | CEFAZOLIN | CEFOPERAZONE |
| Baseline | 29.11.5±2.77 | 28.52±2.26 | 28.78±2.72 | 27.68±3.53 |
| Day 1 | 25.70±6.97 | 24.87±12.41 | 12.66±2.78 | 26.68±13.06 |
| Day 2 | 30.30±3.85 | 4.88±0.46# | 11.99±4.65** | 8.128±2.95*** |
| Day 3 | 31.10±5.22 | 4.85±0.40* | 5.12±0.48* | 22.64±10.98 |
| Day 4 | 30.43±4.87 | 4.98±0.52# | 7.13±0.78# | 6.45±2.48# |
| Day 5 | 26.88±2.88 | 6.88±0.91# | 9.92±2.57# | 7.23±1.82# |

Significant difference between treatment groups *(p<0.05); **(p<0.01); ***(p<0.001); # (p<0.0001). Data are shown as mean ± SEM; (n= 6 for each group).

Table 3.4. Effects of ampicillin, cefazolin and cefoperazone treatments on average body weight in male P rats exposed to five weeks of continuous free choice of ethanol and water.

| | Average Body Weight (g /day) | | | |
|-----------------|------------------------------|------------|------------|--------------|
| | SALINE | AMPICILLIN | CEFAZOLIN | CEFOPERAZONE |
| Baseline | 439.3±11.0 | 454.3±13.9 | 433.3±9.8 | 446.1±13.5 |
| Day 1 | 451.9 ±15.8 | 472.4±15.2 | 450.0±10.0 | 462.7±16.6 |
| Day 2 | 452.3±14.7 | 476.0±15.7 | 449.3±9.0 | 461.4±17.6 |
| Day 3 | 449.3±17.1 | 474.2±14.6 | 445.1±10.6 | 459.0±20.2 |
| Day 4 | 447.8±19.1 | 475.0±14.9 | 452.2±8.9 | 453.9±22.4 |
| Day 5 | 449.8±19.0 | 474.4±14.4 | 450.6±8.8 | 460.7±18.3 |

Significant difference between treatment groups. Data are shown as mean ± SEM; (n= 6 for each group).

3.2.2. Effects of ampicillin on GLT-1a expression in NAc and PFC

Analysis of immunoblots (Fig. 3-1A) revealed a significant main effect of ampicillin treatment on GLT-1a expression in NAc and PFC. Independent t-test analysis of immunoblots demonstrated a significant increase in GLT-1a/GAPDH ratios (100% saline control-value) in NAc ($p < 0.05$) and PFC ($p < 0.05$) in ampicillin treated group as compared to saline treated group (Fig. 3-1B).

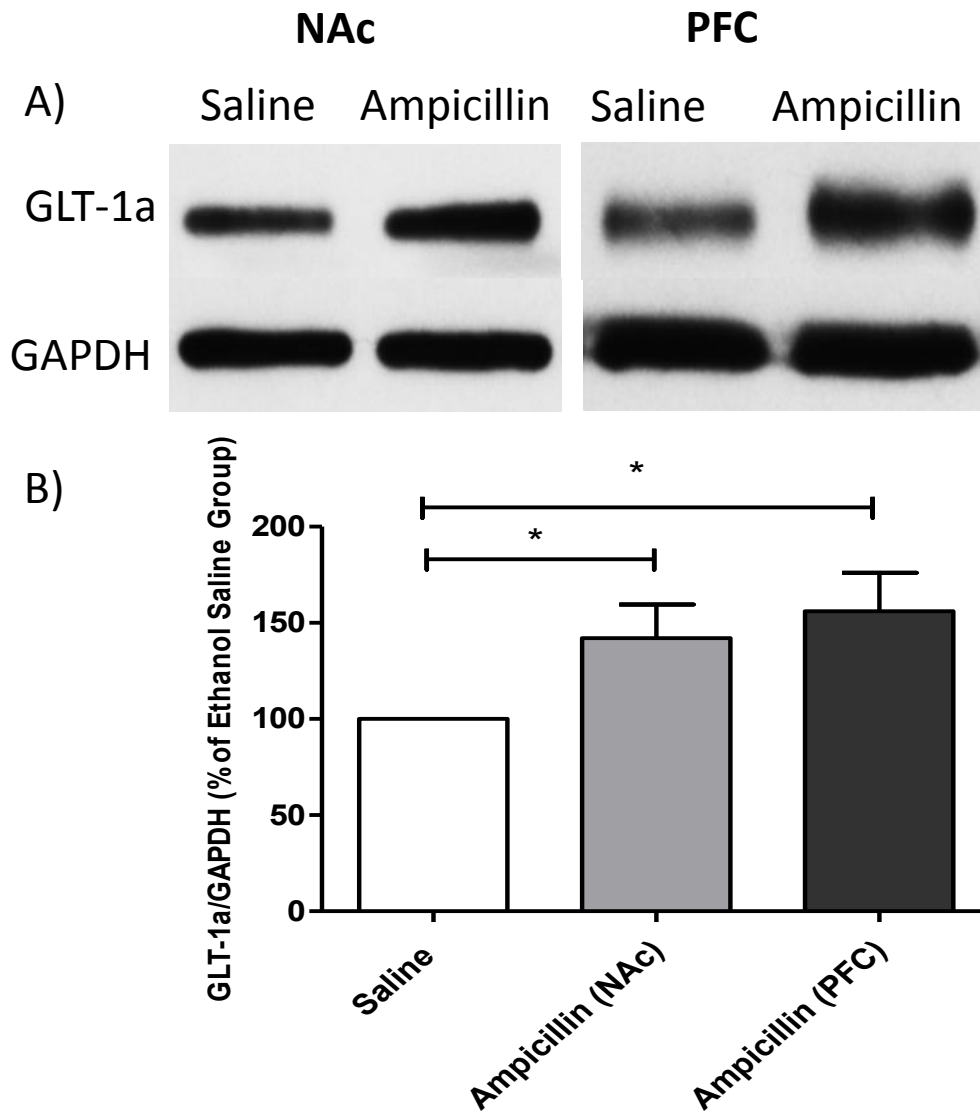


Figure 3-1. Effect of ampicillin on GLT-1a expression in NAc and PFC.

A) Immunoblots for GLT-1a expression and GAPDH as control loading protein in NAc and PFC.

B) Quantitative t-test analysis of immunoblots showed that ampicillin increased significantly the % ratio of GLT-1a/GAPDH in NAc and PFC as compared to saline control group (100% control-value).

Data are shown as mean \pm SEM; (n= 6 for each group); (*p<0.05).

3.2.3. Effects of ampicillin on GLT-1b expression in NAc and PFC

We further investigated GLT-1b expression in NAc and PFC in ampicillin treated group. Analysis of immunoblots (Fig. 3-2A) showed a significant main effect among ampicillin group on GLT-1b expression in both NAc and PFC. Independent t-test revealed a significant increase in GLT-1b/GAPDH ratios (100% saline control-value) in ampicillin treated group NAc (p<0.05) and PFC (p<0.05) (Fig. 3-2B).

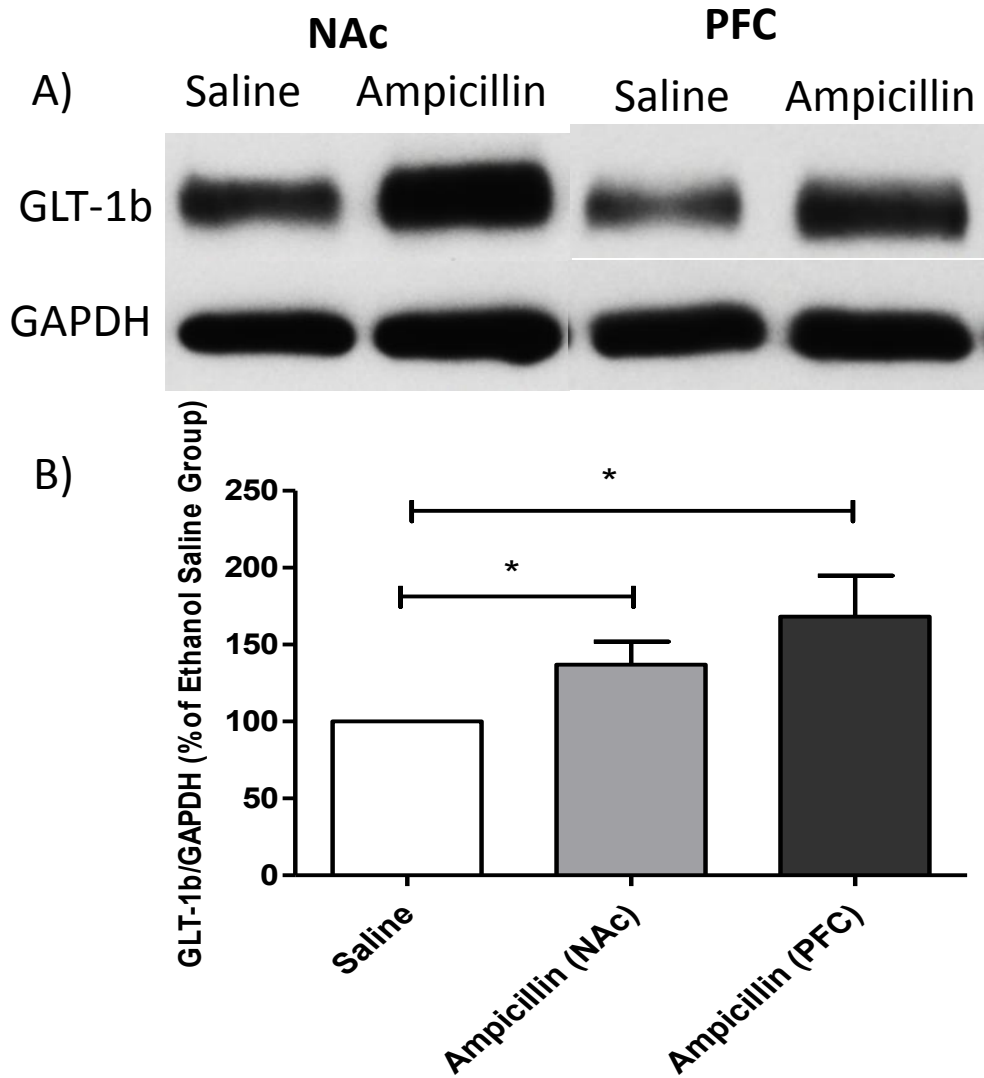


Figure 3-2. Effect of ampicillin on GLT-1b expression in NAc and PFC.

A) Immunoblots for GLT-1b expression and GAPDH as control loading protein in NAc and PFC.

B) Quantitative t-test analysis of immunoblots revealed that ampicillin increased significantly the % ratio of GLT-1b/GAPDH in NAc and PFC as compared to saline control group (100% control-value).

Data are shown as mean \pm SEM; (n= 6 for each group); (*p<0.05).

3.2.4. Effects of ampicillin on xCT expression in NAc and PFC

We investigated also the effect of ampicillin on xCT expression (Fig. 3-3A). An independent t-test analysis of immunoblots revealed increased in xCT/GAPDH ratios in NAc ($p < 0.05$) and PFC ($p < 0.05$) in ampicillin treated group as compared to saline treated group (Fig. 3-3B).

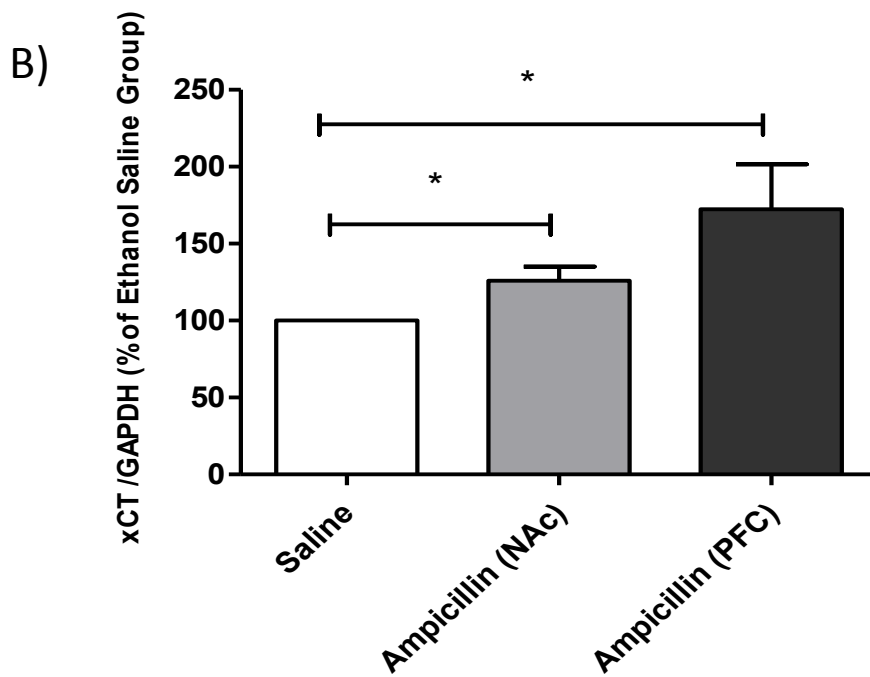
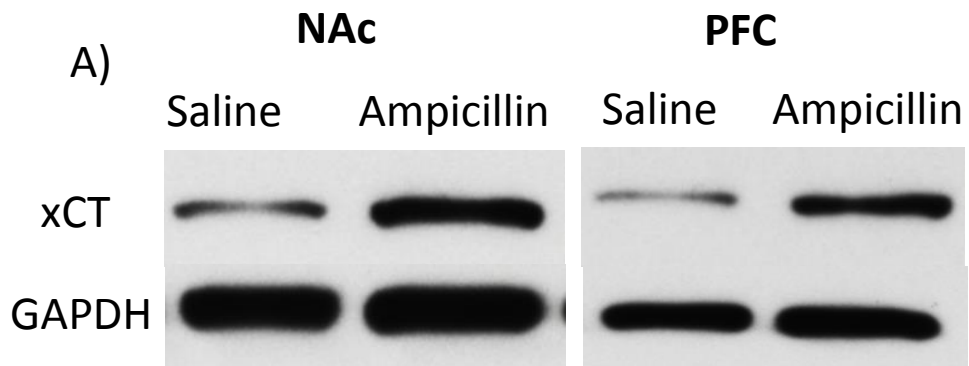


Figure 3-3. Effect of ampicillin on xCT expression in NAc and PFC.

- A)** Immunoblots for xCT expression and GAPDH as control loading protein in NAc and PFC.
- B)** Quantitative t-test analysis of immunoblots showed that ampicillin increased significantly in the expression of the % ratio of xCT/GAPDH in NAc and PFC as compared to saline control group (100% control-value).
Data are shown as mean \pm SEM; (n= 6 for each group); (*p<0.05).

3.2.5. Effects of ampicillin on GLAST expression in NAc and PFC

We then determined GLAST expression in both NAc and PFC. We did not observe any changes in GLAST expression between control and ampicillin treated groups in both NAc and PFC (Fig. 3-4A). An independent t- test analysis did not show any significant effect between control and ampicillin treated groups in NAc ($p > 0.05$) and PFC ($p > 0.05$) (Fig. 3-4B).

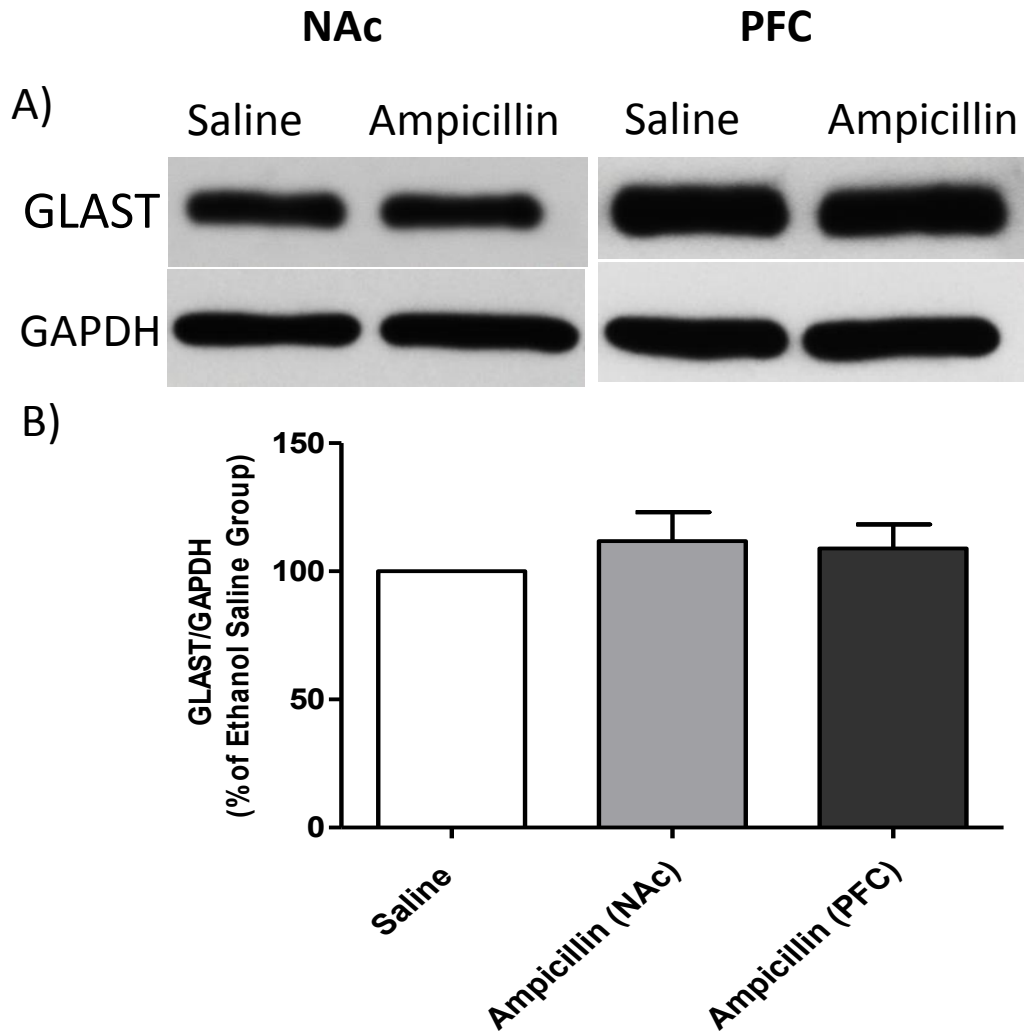


Figure 3-4. Effect of ampicillin on GLAST expression in in NAc and PFC.

A) Immunoblots for GLAST expression and GAPDH as a control loading protein in NAc and PFC.

B) Quantitative t-test analysis of immunoblots showed no significant increase in the % ratio GLAST/GAPDH in NAc and PFC saline control group (100% control value) and treatment group. Data are shown as mean \pm SEM; (n= 6 for each group) (*p<0.05).

3.2.6. Effects of cefazolin and cefoperazone on GLT-1a expression in NAc and PFC

Immunoblots showed a significant increase in GLT-1a expression following treatment of both cefazolin and cefoperazone in both NAc and PFC (n=6 in each group) (Figure 2, 3; Upper Panel) and (Figure 3-5, 3-6; Upper Panel). As compared to saline-treated group, independent t-test analyses of immunoblots demonstrated a significant increase in GLT-1a/GAPDH ratio in the NAc with cefazolin- ($p < 0.05$) and cefoperazone- ($p < 0.05$) treated groups and also in the PFC following treatment of cefazolin ($p < 0.05$) and cefoperazone ($p < 0.05$) (Figure 3-5, 3-6; Lower Panel).

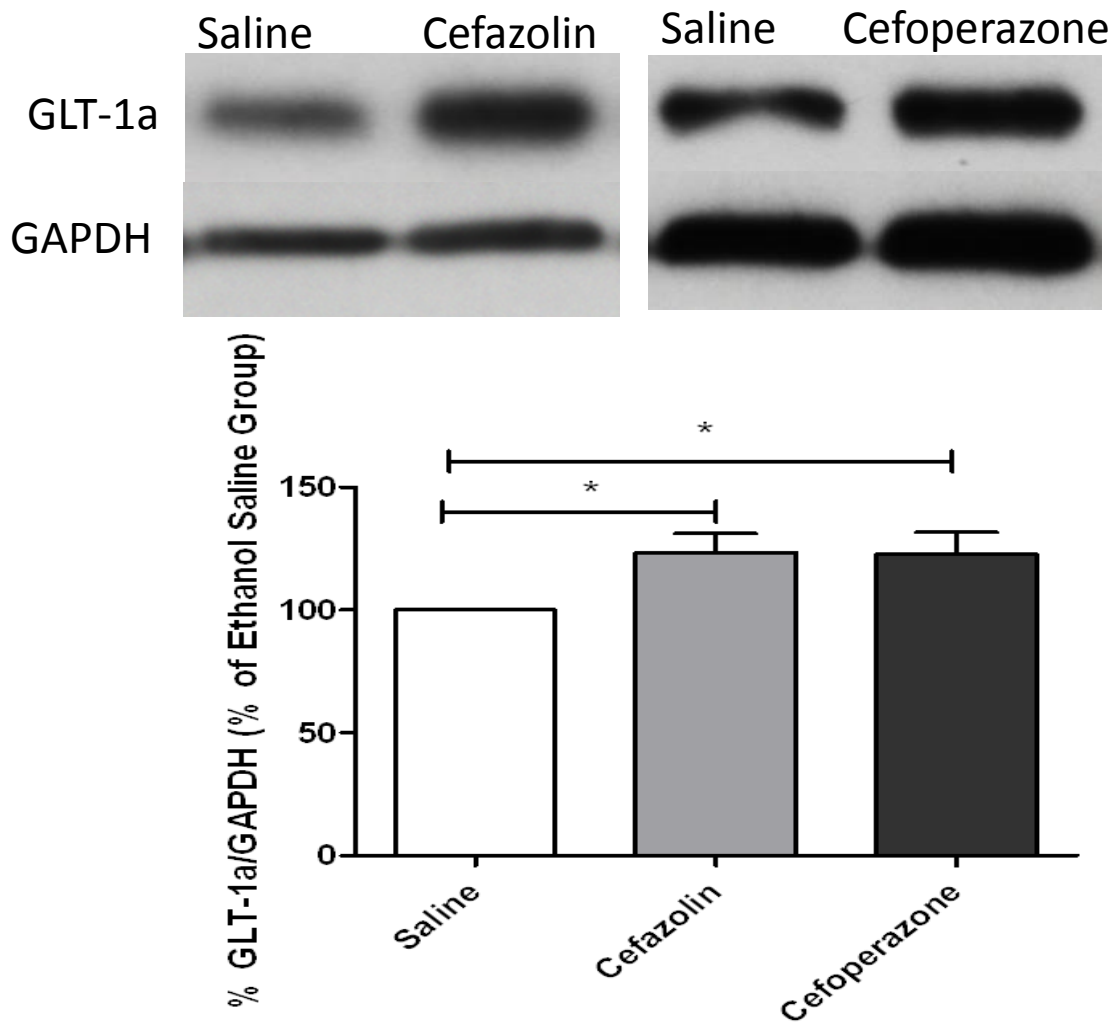


Figure 3-5. Effect cefazolin and cefoperazone on GLT-1a expression in NAc.

Upper Panel) Immunoblots for GLT-1a expression and GAPDH as control loading protein in NAc

Lower Panel) Quantitative t-test analysis of immunoblots showed that cefazolin and cefoperazone increased significantly the % ratio of GLT-1a/GAPDH in NAc as compared to saline control group (100% control-value). Data are shown as mean \pm SEM; (n= 6 for each group); (*p<0.05).

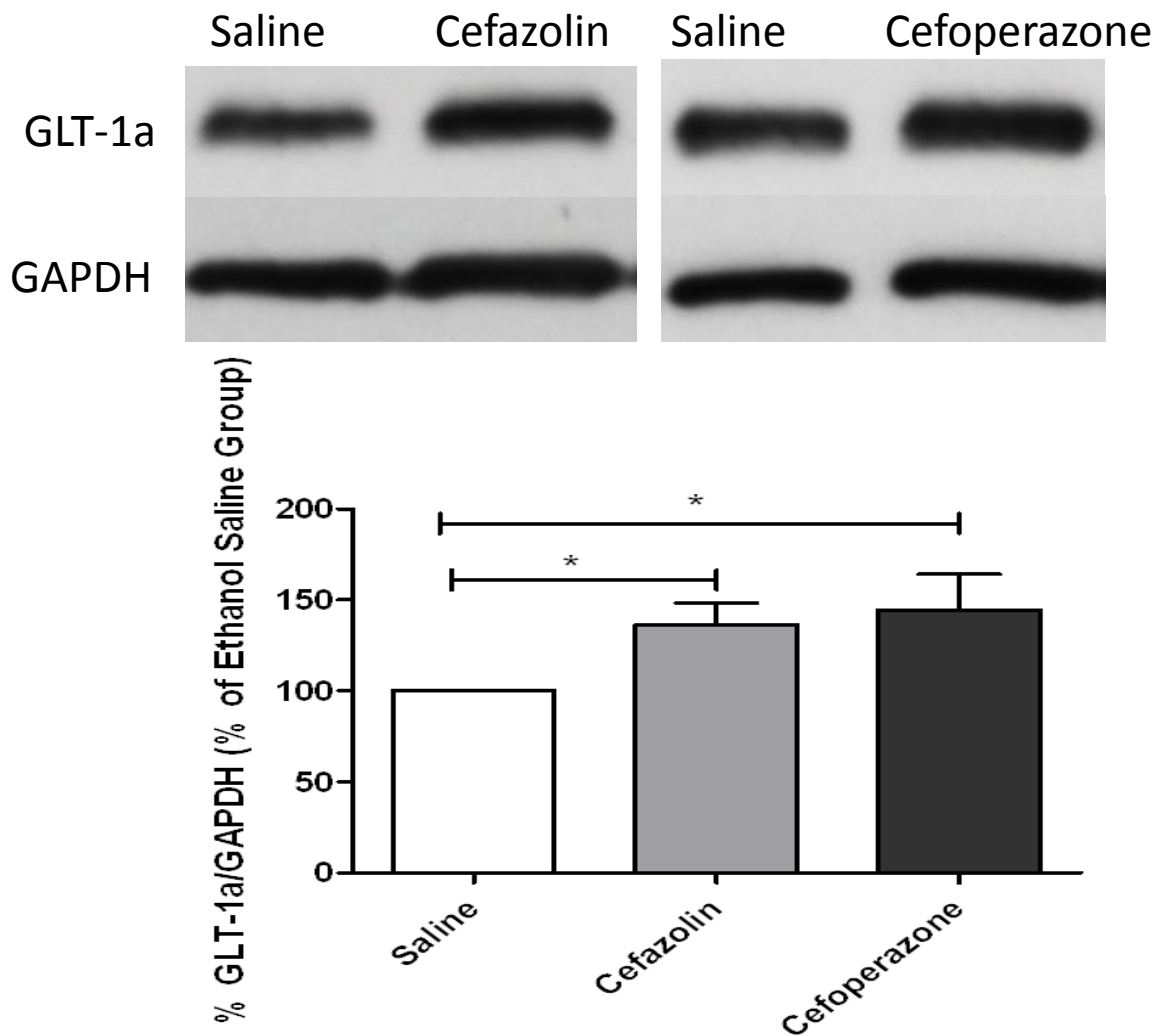


Figure 3-6. Effect cefazolin and cefoperazone on GLUT-1a expression in PFC.

Upper Panel) Immunoblots for GLUT-1a expression and GAPDH as control loading protein in PFC.

Lower Panel) Quantitative t-test analysis of immunoblots showed that cefazolin and cefoperazone increased significantly the % ratio of GLUT-1a/GAPDH in PFC as compared to saline control group (100% control-value).

Data are shown as mean \pm SEM; (n= 6 for each group);(*p<0.05)

3.2.7. Effects of cefazolin and cefoperazone on GLT-1b expression in NAc and PFC

Next, we further investigated GLT-1b expression in the NAc and PFC following treatment of cefazolin and cefoperazone. An increase in GLT-1b expression in the NAc and PFC were shown in both cefazolin- and cefoperazone-treated groups (n=6 in each group) (Figure 3-7, 3-8; Upper Panel). As normalized to GAPDH, an independent t-test analyses of the immunoblots demonstrated a significant increase in GLT-1b expression in NAc and PFC following treatment of cefazolin ($p<0.05$) and cefoperazone ($p<0.05$) as compared to saline-treated group (Figure 3-7, 3-8; Lower Panel).

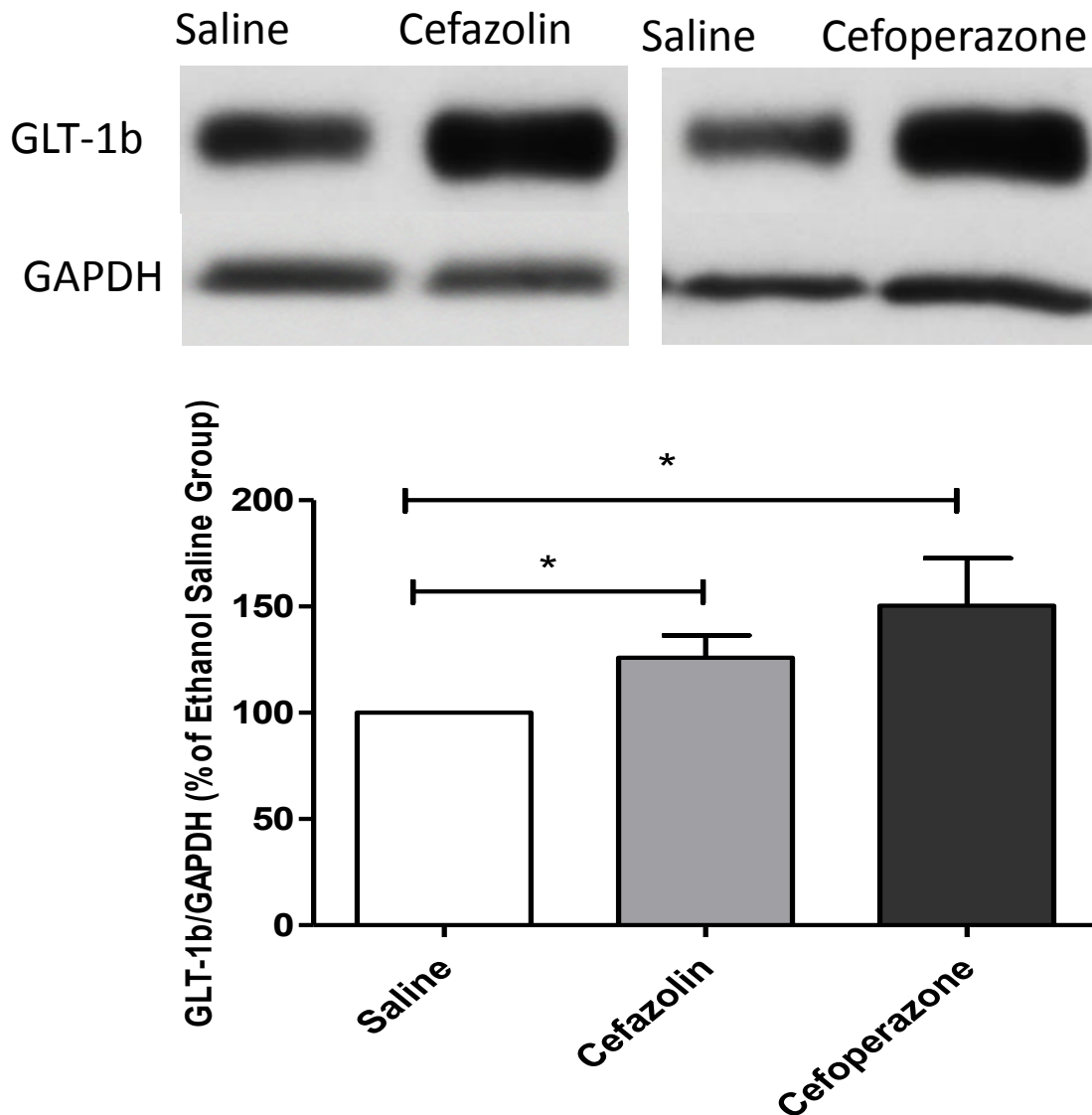


Figure 3-7. Effect cefazolin and cefoperazone on GLT-1b expression in NAc.

Upper Panel) Immunoblots for GLT-1b expression and GAPDH as control loading protein in NAc.

Lower Panel) Quantitative t-test analysis of immunoblots showed that cefazolin and cefoperazone increased significantly the % ratio of GLT-1a/GAPDH in NAc as compared to saline control group (100% control-value). Data are shown as mean \pm SEM; (n= 6 for each group); (*p<0.05).

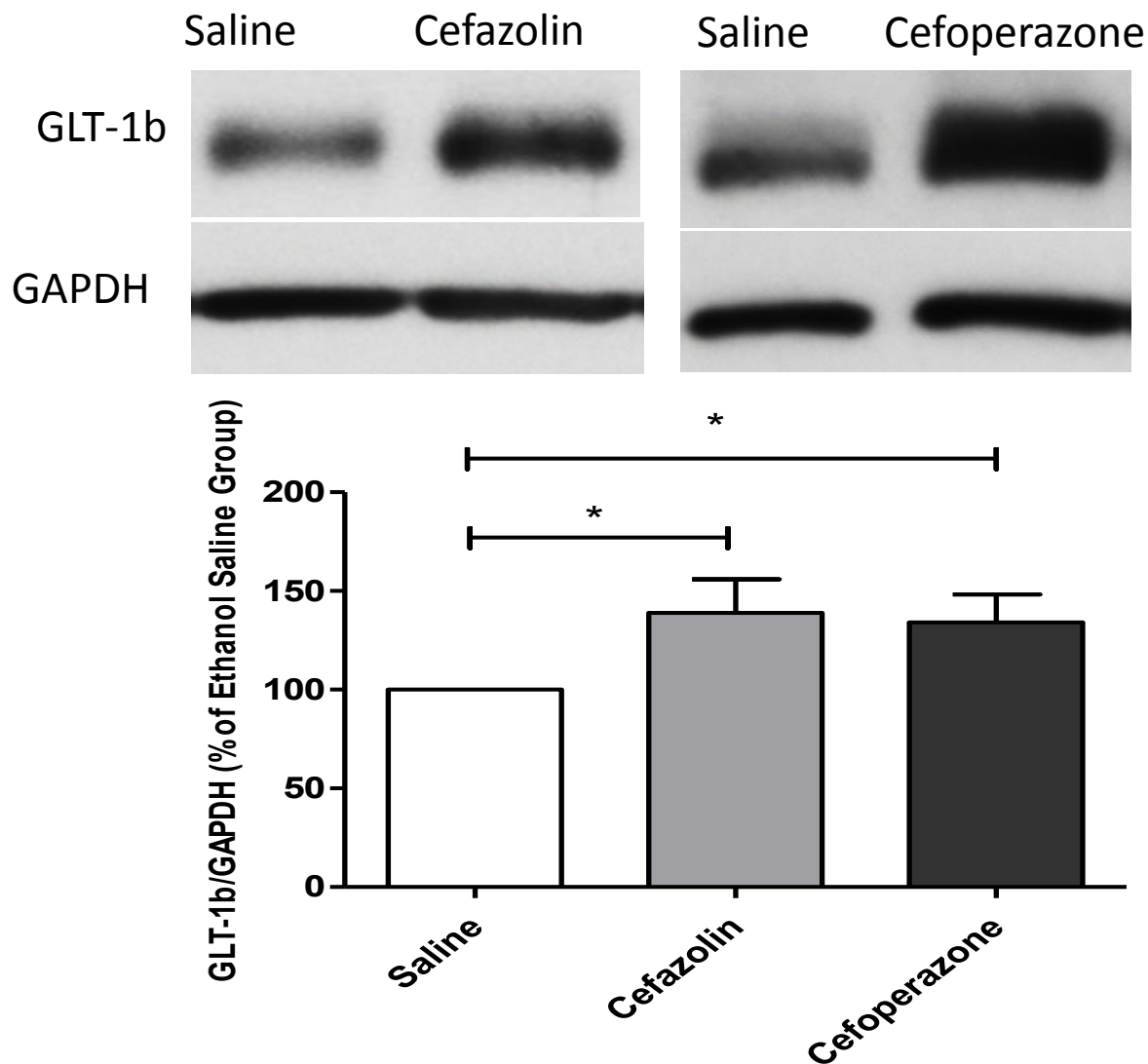


Figure 3-8. Effect cefazolin and cefoperazone on GLT-1b expression in PFC.

Upper Panel) Immunoblots for GLT-1b expression and GAPDH as control loading protein in PFC.

Lower Panel) Quantitative t-test analysis of immunoblots showed that cefazolin and cefoperazone increased significantly the % ratio of GLT-1a/GAPDH in PFC as compared to saline control group (100% control-value).

Data are shown as mean \pm SEM; (n= 6 for each group); (*p<0.05).

3.2.8. Effects of cefazolin and cefoperazone on xCT expression in NAc and PFC

Next, we tested the effect of cefazolin and cefoperazone in xCT expression, another important glial glutamate transporter in the brain. Western blot assay showed an upregulation in xCT expression in cefazolin-treated group in the NAc and PFC, while cefoperazone upregulated xCT expression only in the NAc (n=5-6 in each group) (Figure 3-9, 3-10; Upper Panel). Moreover, a quantitative t-test analyses of immunoblots showed a significant increase in xCT/GAPDH ratio in the NAc after treatment of cefazolin ($p < 0.05$) and cefoperazone ($p < 0.05$) as compared to saline-treated group. However, only cefazolin treatment increased xCT/GAPDH ratio in the PFC ($p < 0.05$) (Figure 3-9, 3-10; Lower Panel).

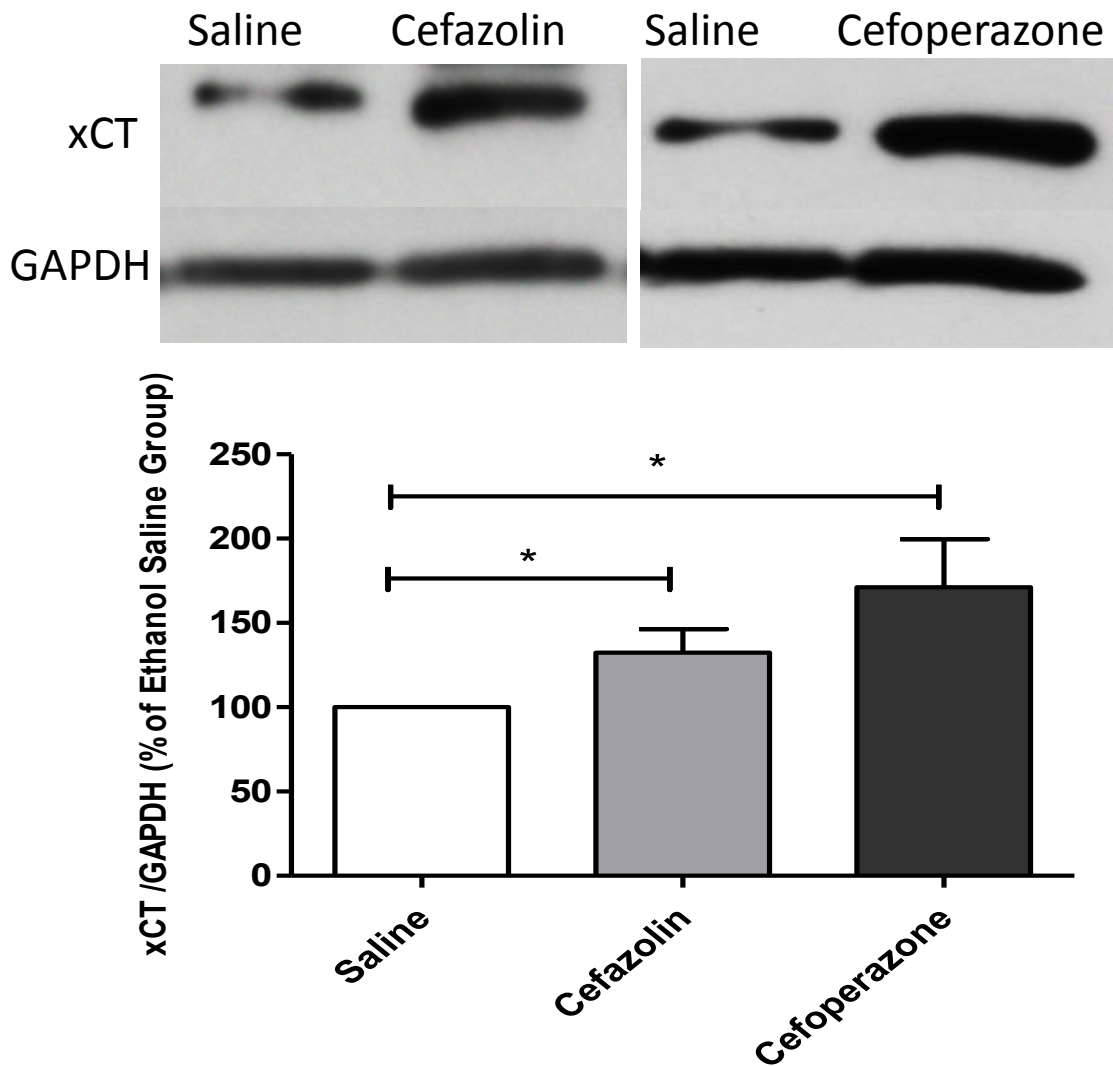


Figure 3-9. Effect cefazolin and cefoperazone on xCT expression in NAc.

Upper Panel) Immunoblots for GLT-1b expression and GAPDH as control loading protein in NAc.

Lower Panel) Quantitative t-test analysis of immunoblots showed that cefazolin and cefoperazone increased significantly the % ratio of GLT-1a/GAPDH in NAc as compared to saline control group (100% control-value). Data are shown as mean \pm SEM; (n= 5-6 for each group); (*p<0.05).

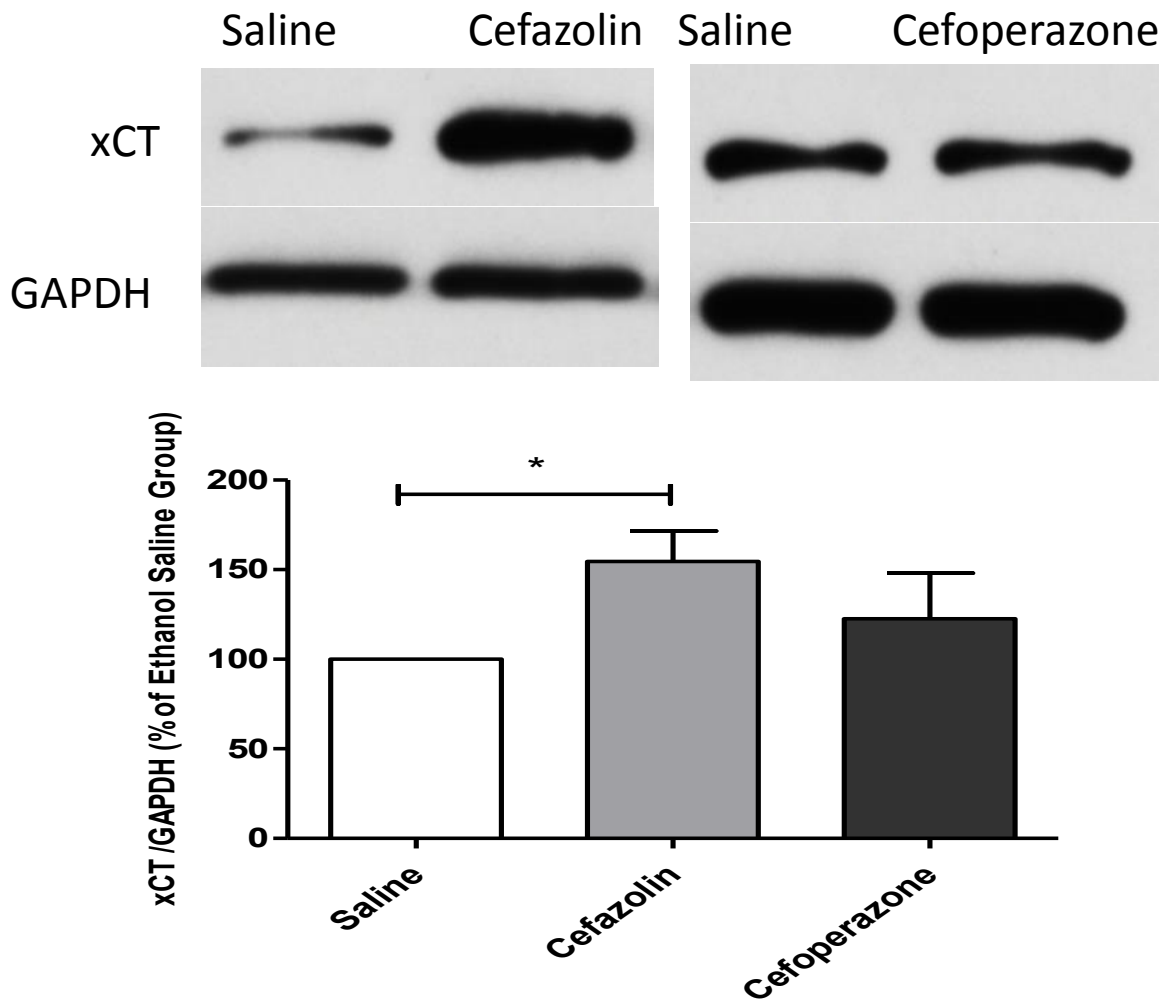


Figure 3-10. Effect cefazolin and cefoperazone on xCT expression in PFC.

Upper Panel) Immunoblots for GLT-1b expression and GAPDH as control loading protein in PFC.

Lower Panel) Quantitative t-test analysis of immunoblots showed that cefazolin increased significantly the % ratio of GLT-1a/GAPDH

in PFC as compared to saline control group (100% control-value).

Data are shown as mean \pm SEM; (n= 6 for each group); (*p<0.05).

3.2.9. Effects of cefazolin and cefoperazone on GLAST expression in NAc and PFC

We did not observe any significant effect of cefazolin and cefoperazone treatment on GLAST expression using western blot assay in both NAc and PFC (n=6 in each group) (Figure 3-11, 3-12; Upper Panel). Additionally, an independent t-test analyses of immunoblots did not reveal any significant increase in GLAST/GAPDH ratio in the NAc and PFC in cefazolin- ($p>0.05$) and cefoperazone- ($p>0.05$) treated groups as compared to saline treated group (Figure 3-11, 3-12; Lower Panel).

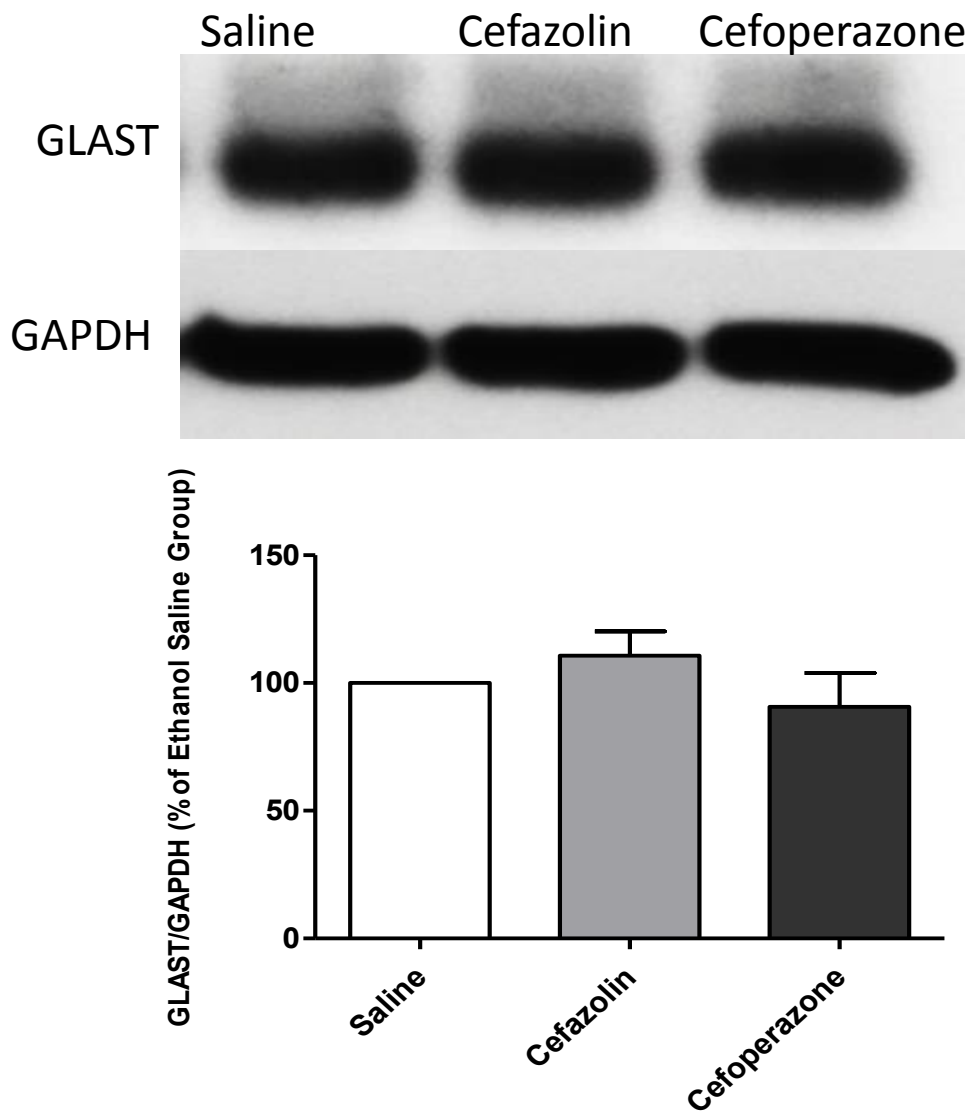


Figure 3-11. Effect of cefazolin and cefoperazone on GLAST expression in in NAc.

Upper Panel) Immunoblots for GLAST expression and GAPDH as a control loading protein in NAc.

Lower Panel) Quantitative t-test analysis of immunoblots showed no significant increase in the % ratio GLAST/GAPDH in NAc as compared to saline control group (100% control-value) and treatment group. Data are shown as mean \pm SEM; (n= 6 for each group); (*p<0.05).

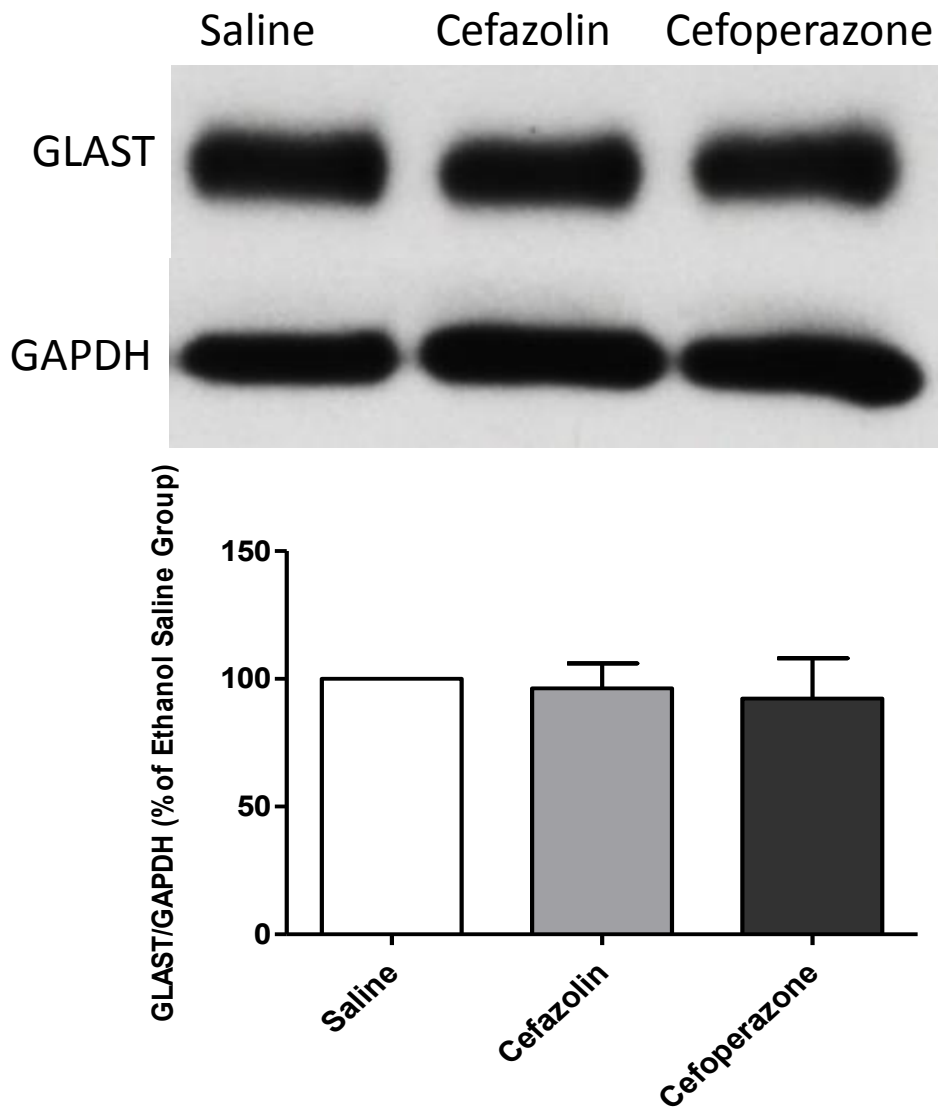


Figure 3-12. Effect of cefazolin and cefoperazone on GLAST expression in in PFC.

Upper Panel) Immunoblots for GLAST expression and GAPDH as a control loading protein in PFC.

Lower Panel) Quantitative t-test analysis of immunoblots showed no significant

increase in the % ratio GLAST/GAPDH in PFC as compared to saline control group (100% control-value) and treatment group.

Data are shown as mean \pm SEM; (n= 6 for each group); (*p<0.05).

3.3. Discussion

Studies from our lab have shown that treatment with ceftriaxone decreased ethanol intake and relapse-like ethanol drinking (Sari et al., 2011, Qrunfleh et al., 2013, Alhaddad et al., 2014a, Rao et al., 2015b). Additionally, we have recently shown that ampicillin, cefazolin and cefoperazone treatments reduced ethanol intake and upregulated in part GLT-1 expression in PFC and NAc (Rao et al., 2015a). However, the effects of ampicillin, cefazolin and cefoperazone on the expression levels of xCT, GLAST and GLT-1 isoforms have not been investigated. Thus, we focused in this study to investigate these important proteins that have critical role in regulating extracellular glutamate.

It is well known that ethanol consumption can lead to a marked increase in the extracellular glutamate concentrations in mesocorticolimbic brain regions (Kapasova and Szumlinski, 2008, Ward et al., 2009, Ding et al., 2012, Rao and Sari, 2012, Ding et al., 2013). It has been reported that ceftriaxone-induced attenuation of ethanol intake and relapse-like ethanol drinking in male P rats is associated in part through upregulation of GLT-1 and its isoforms (GLT-1a and GLT-1b) in the NAc and PFC (Sari et al., 2011, Qrunfleh et al., 2013, Alhaddad et al., 2014a, Rao et al., 2015b). The upregulatory effects in GLT-1 could be associated with decrease in extracellular glutamate concentrations that may lead to reduction in ethanol intake. In our earlier study, we found that ampicillin, cefazolin and cefoperazone treatments successfully reduced ethanol consumption in male P rats, presumably through induction of GLT-1 expression in NAc and PFC (Rao et al., 2015a). As an extension of our previous work, in the present study, we report here that

selected β -lactam antibiotics treatment upregulated GLT-1 isoforms in the NAc and PFC, and consequently reduced ethanol intake. Although GLT-1a and GLT-1b are expressed differentially Although GLT-1a and GLT-1b are expressed differentially (Berger et al., 2005, Holmseth et al., 2009), ampicillin, cefazolin and cefoperazone treatments found to increase the expression of both GLT-1 isoforms in astrocytes and neurons possibly by similar mechanism.

We have also investigated the effects of ampicillin in the expression of xCT, which is considered as an exchanger transporter of cystine and glutamate. xCT has a role in neuroprotection by modulating glutathione supply in the brain through cystine/glutamate exchange (Shih et al., 2006). It has been shown that synaptic glutamate release is increased with downregulation of the expression of xCT. Therefore, glutamate released through xCT can bind to metabotropic glutamate receptor 2/3 (mGluR2/3), and consequently reduced synaptic glutamate release (Shih et al., 2006). Several studies from our lab reported that the increases in xCT as well as GLT-1 expression levels are linked to the attenuation in ethanol consumption in male P rats (Alhaddad et al., 2014a, Alhaddad et al., 2014b, Rao and Sari, 2014, Rao et al., 2015b). In this study, we also tested for changes in the expression of xCT in both NAc and PFC with β -lactam antibiotics treatment. It is noteworthy that previous study in our lab found that ceftriaxone treatment reduced ethanol intake possibly through upregulation of xCT expression in the NAc, PFC, and amygdala in male P rat (Alhaddad et al., 2014a, Rao and Sari, 2014). Ceftriaxone also was able to attenuate relapse-like cocaine and ethanol

intake at least in part through upregulation of xCT expression (Knackstedt et al., 2010, Alhaddad et al., 2014a). It is important to note that chronic consumption of ethanol led to downregulation of the expression of xCT in the NAc and PFC (Alhaddad et al., 2014a). In accordance, downregulation of xCT was also observed in NAc in cocaine seeking animal model (Knackstedt et al., 2010). Importantly, we reported here that ampicillin and cefazolin has the ability to normalize the expression of xCT in both NAc and PFC. However, cefoperazone increased xCT expression only in the NAc. This normalization of xCT may play a key factor in regulating extracellular glutamate and consequently contributed to the reduction in ethanol intake.

We further tested for the effect of ampicillin on GLAST expression, which is co-localized with GLT-1 in astrocytes. We did not observe an upregulatory effect on GLAST expression with β -lactam antibiotics treatments. This effect is in accordance with a recent finding demonstrating that ceftriaxone treatment did not induce an upregulatory effect on GLAST expression (Alhaddad et al., 2014a, Rao et al., 2015b). Together, these findings suggest the selective upregulatory effects on xCT and GLT-1 isoforms. The upregulatory effects of selected β -lactam antibiotics on GLT-1 isoforms and xCT expression levels may play a critical role on regulating extracellular glutamate concentrations in central reward brain regions.

In summary, the present findings suggest that ampicillin, cefazolin and cefoperazone reduced alcohol intake significantly, at least in part through upregulation of xCT, GLT-1a and GLT-1b expression in both the NAc and PFC. The upregulatory effects of selected β -lactam antibiotics on xCT and GLT-1 isoforms may normalize extracellular glutamate concentrations in these brain regions. These data provide ample evidence about the potential therapeutic implications of β -lactam antibiotics for the treatment of alcohol dependence.

A worth mentioning that one of the adverse effects associated with the use of cefoperazone but not ampicillin and cefazolin is the disulfiram like-reaction (Fromtling and Gadebusch, 1983, Rao et al., 2015a), which means that the drug could act centrally in the brain and well as peripherally in liver in reducing of alcohol intake. Cefoperazone could work through several mechanisms, apart from modulating the glutamatergic neurotransmission, it may inhibit the enzyme aldehyde dehydrogenase in the liver, which could offer another possible mechanism for cefoperazone effect on alcohol consumption (Rao et al., 2015a)

It is important to note that ampicillin is a drug that can be given orally, thus it has clinical relevance for its use in alcohol dependence. Studies are warranted to determine the effects of oral administration of this compound on ethanol intake as well as on the expression levels of xCT, GLT-1, GLT-1 isoforms and GLAST.

Chapter 4

Effects of Cefoperazone Treatment on Relapse-Like Ethanol Intake

4.1 Introduction

Glutamate is taken up into astrocytes by specific transporters. There are two major types of glutamate transporters that normally transport glutamate into synaptic vesicles. These transporters called the Excitatory Amino Acid Transporters (EAATs) and the Vesicular Glutamate Transporters (VGLUTs) (Shigeri et al., 2004, Thompson et al., 2005). Glutamate transporter-1 (GLT-1, its human homolog is excitatory amino acid transporter-2) is considered the major transporter in astrocytes. It transports majority of extracellular glutamate into astrocytes. It is responsible for removing high extracellular glutamate concentrations to below the toxic level (Tanaka et al., 1997). Cystine-glutamate antiporter (xCT) is considered the regulator for glutamate neurotransmission. It exchanges cysteine which is found outside the cell for intracellular glutamate (Baker et

al., 2002).

Glutamine transmission is involved in alcohol addiction and drug abuse. It is noteworthy to mention that continuous and relapse-like ethanol drinking affect the glutamine-glutamate system (Backstrom and Hyytia, 2004, Besheer et al., 2010, Rao and Sari, 2012). Chronic alcohol consumption can lead to alcohol dependence partially by increasing extracellular glutamate concentrations [for review see ref. (Rao and Sari, 2012)]. Ceftriaxone decreased cue to cocaine-seeking behavior and attenuated relapse-like ethanol intake, in part, through upregulation of GLT-1 and xCT expression levels (Sari et al., 2009, Knackstedt et al., 2010, Trantham-Davidson et al., 2012, Qrunfleh et al., 2013). Moreover, it has been shown that xCT played an important role in relapse-like cocaine behavior, relapse-like ethanol intake and also in nicotine self-administration (Knackstedt et al., 2009, Knackstedt et al., 2010, Alhaddad et al., 2014a). In addition, it has been reported that glutamate uptake is restored following treatment of ceftriaxone by increasing the expression of xCT in reinstatement of cocaine-seeking behavior animal model (Knackstedt et al., 2010, Trantham-Davidson et al., 2012). Ceftriaxone did not upregulate GLAST in relapse like-ethanol drinking in P rats (Alhaddad et al., 2014a). Therefore, we have investigated the effects of cefoperazone on ethanol intake in male P rats.

4.2 Results

4.2.1 Effect of Cefoperazone on relapse-like ethanol intake in male P rats

Two way ANOVA with repeated measures followed by Bonferroni multiple comparisons demonstrated a significant reduction on ethanol intake in cefoperazone-treated group compared to saline-treated group on day 2 to day 7 (* $p \leq 0.05$; ** $p \leq 0.01$). Moreover, mixed ANOVA demonstrated a significant main effect of day [F (1, 7) = 4.070, $p \leq 0.001$] and a non-significant day x treatment interaction [F (1, 7) = 1.803, $p > 0.05$] of ethanol intake (Fig. 1A).

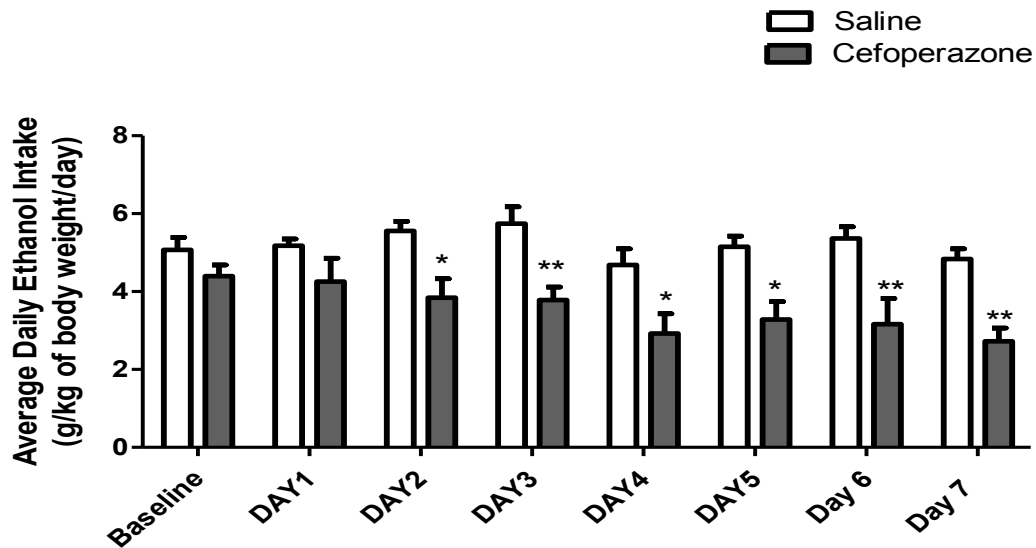


Figure 1. (A) Effects of cefoperazone treatment on ethanol consumption (g/kg/day) in male P rats exposed to five weeks of continuous free choice of ethanol and water. Two way ANOVA followed by Bonferroni multiple comparisons revealed that cefoperazone decreased significantly ethanol consumption from day 2 through day 7 compared to control saline vehicle group. Data are shown as mean \pm SEM; (n= 6 for each group); (* $p \leq 0.05$; ** $p \leq 0.01$).

4.2.2 Effects of cefoperazone on water intake in male P rats

Two way ANOVA with repeated measures followed by Bonferroni multiple comparisons showed a significant increase in water intake in cefoperazone-treated group compared to saline treated group on day 1 to day 7. Additionally, a significant main effect of day [$F(1, 7) = 4.090, p \leq 0.001$] and a significant day x treatment interaction [$F(1, 7) = 6.279, p \leq 0.0001$] of water intake were found using mixed ANOVA analysis (Fig. 1B).

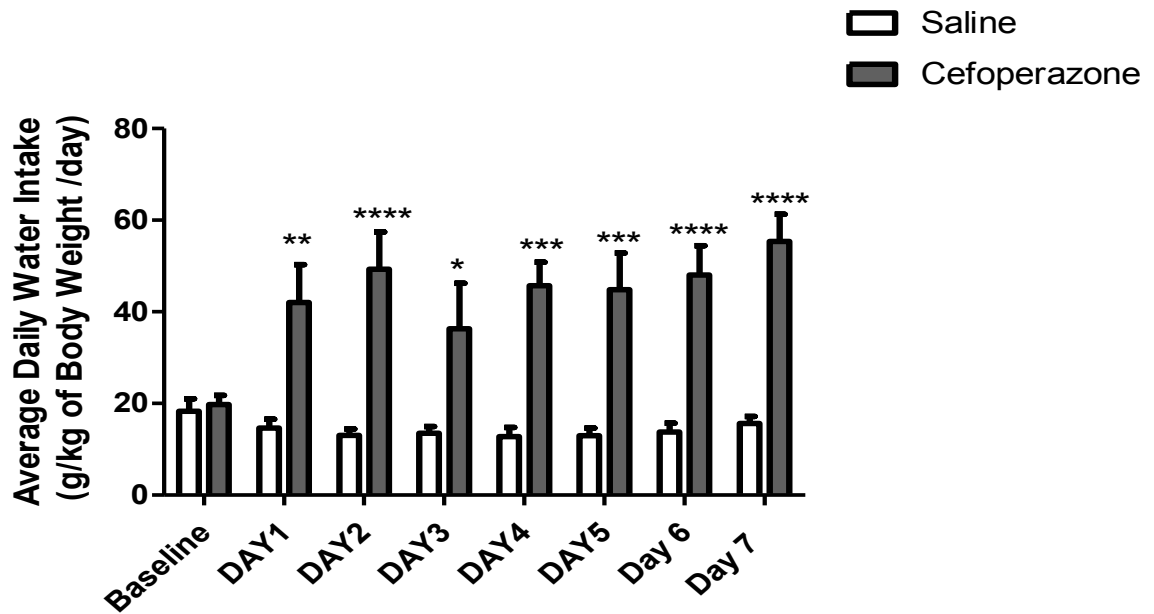


Figure 1. (B) Effects of cefoperazone treatment on water consumption (g/kg/day).

Two way ANOVA followed by Bonferroni multiple comparisons showed cefoperazone increased significantly water intake from day 1 through day 7 as compared to control saline vehicle group. Data are shown as mean \pm SEM; (n= 6 for each group); (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; # $p \leq 0.0001$).

4.2.3 Effects of cefoperazone on daily ethanol preference (%) in male P rats

Repeated measures demonstrated that cefoperazone treatment reduced ethanol preference significantly as compared to saline-treated group started on day 1 to day 7 ($p < 0.0001$). Mixed ANOVA revealed a significant main effect of day [$F(1, 7) = 2.694$, $p \leq 0.05$] and a significant day x treatment interaction [$F(1, 7) = 5.637$, $p \leq 0.0001$] of ethanol preference (Fig. 1C).

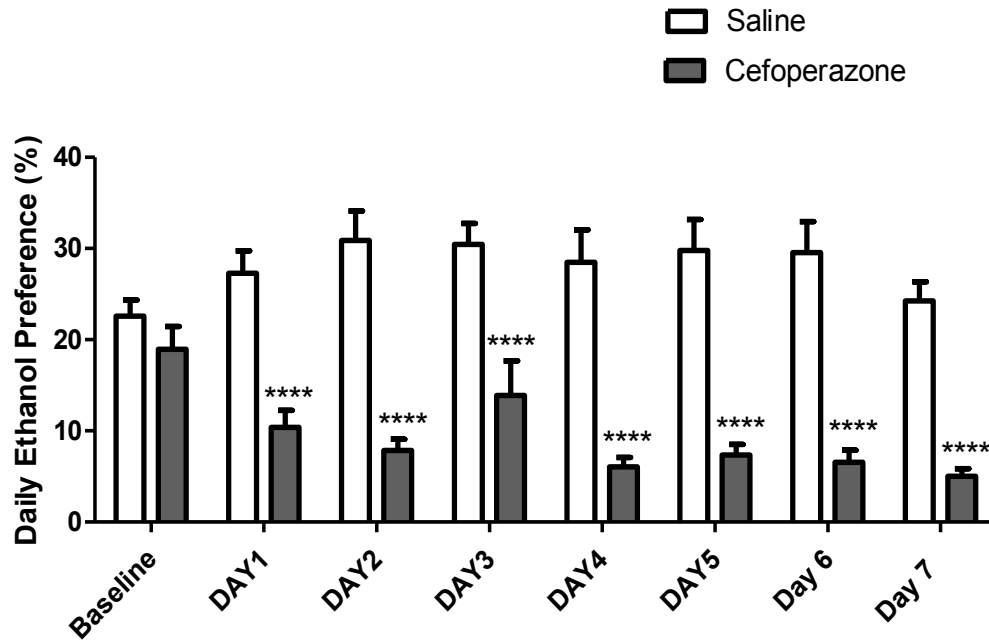


Figure 1. (C) Effects of cefoperazone treatment on ethanol preference (%).

Two way ANOVA followed by Bonferroni multiple comparisons showed cefoperazone decreased significantly the % of ethanol preference from day 1 through day 7 as compared to control saline vehicle group.

Data are shown as mean \pm SEM; ($n = 6$ for each group);

(# $p \leq 0.0001$).

4.2.4 Effects of cefoperazone on average body weight in male P rats

Two-way ANOVA with repeated measures did not reveal any significant effect on body weight between control and treated groups. Moreover, mixed ANOVA showed a significant main effect of day [F (1, 7) = 12.51, $p \leq 0.0001$] and day x treatment interaction [F (1, 7) = 3.786, $p \leq 0.01$] of average body weight (Fig. 1D).

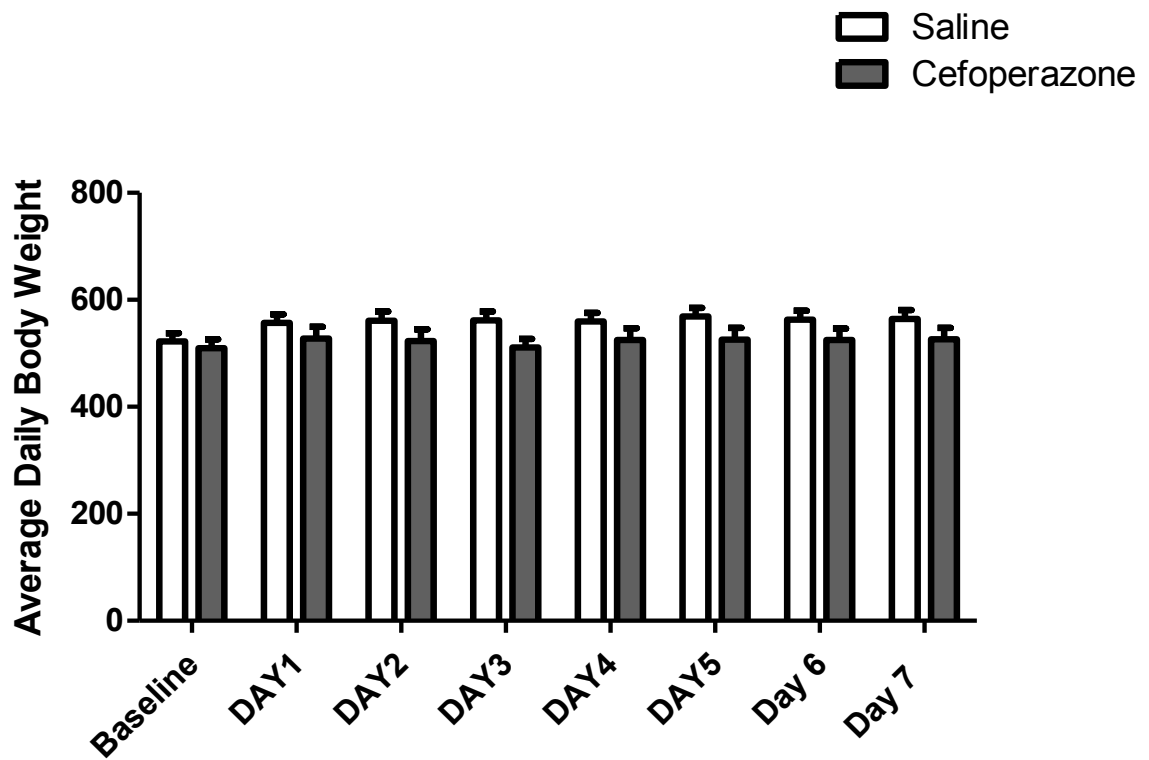


Figure 1. (D) Effects of cefoperazone treatment on body weight (g/day).

Two way ANOVA followed by Bonferroni multiple comparisons demonstrated no significant effect on body weight between control and treatment groups.

Data are shown as mean \pm SEM; (n= 6 for each group);

4.3 Discussion

The effect of cefoperazone treatment on relapse to alcohol in P rats was examined in this study.

Previous studies from our lab demonstrated that β -lactam antibiotic, ceftriaxone, decreased continuous ethanol intake and relapse-like alcohol intake (Sari et al., 2011, Qrunfleh et al., 2013, Alhaddad et al., 2014a). In this study, we found that cefoperazone treatment reduced relapse-like ethanol intake significantly in male P rats starting on day 2 through day 7. We also reported that cefoperazone treatment increased water intake significantly from day 1 through day 7. Therefore, the increase in water intake could be a compensatory mechanism for decreasing alcohol consumption. However, we did not observe any significant changes in body weight following treatment of cefoperazone as compared to saline treated group in male P rats. Our findings are in accordance with a recent findings revealing that ceftriaxone treatment reduced relapse-like ethanol intake, increased water intake and did not change body weight of male P rats (Qrunfleh et al., 2013, Alhaddad et al., 2014a).

Rothstein and colleagues found that cefoperazone upregulated GLT-1 expression (Rothstein et al., 2005). It has been reported that ceftriaxone attenuated continuous and relapse-like ethanol drinking in male P rats, in part, through upregulation of GLT-1 levels in NAc and PFC regions (Sari et al., 2011, Qrunfleh et al., 2013, Rao and Sari, 2014, Rao et al., 2015b). Our lab reported recently that GLT-1 isoforms (GLT-1a and GLT-1b) may

have an important role in the attenuation of relapse-like ethanol consumption following treatment of ceftriaxone (Alhaddad et al., 2014a).

xCT is an important glial protein, which plays a role in the exchange between intracellular glutamate with extracellular cysteine. Several studies demonstrated that ceftriaxone attenuates alcohol intake in male P rats at least in part by increasing xCT and GLT-1 expression levels in mesocorticolimbic brain regions (Alhaddad et al., 2014a, Rao and Sari, 2014, Rao et al., 2015b). A previous study in our laboratory found that ceftriaxone treatment reduced ethanol intake, in part, through upregulation of xCT expression in the NAc, the PFC, and amygdala in male P rat (Rao and Sari, 2014). Ceftriaxone also attenuated relapse-like cocaine and ethanol intake at least in part by upregulation of xCT in rats (Knackstedt et al., 2010, Alhaddad et al., 2014a).

In summary, we showed here that cefoperazone treatment reduced relapse-like ethanol consumption and preference in male P rats. We will further test the effect of cefoperazone on GLT-1, xCT and GLAST expression levels in mesocorticolimbic brain regions to determine whether the behavioral effects are associated in part with upregulation of GLT-1 and xCT expression levels.

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