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Effects of ß-lactam compounds on GLT1 and xCT expression levels as well as ethanol intake in alcohol-preferring rats

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A Thesis

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Effects of β-lactam Compounds on GLT1 and xCT Expression levels as well as Ethanol Intake in Alcohol-Preferring Rats

By

Alqassem Hakami

Submitted to the Graduate Faculty as a partial fulfillment of the requirement for the

Master of Science Degree in Pharmaceutical Sciences

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An Abstract of

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Drug abuse is associated with deficits in glutamate uptake and impairment of glutamate homeostasis. Glutamate transporters are the key players in regulating extracellular glutamate concentrations. Considering the importance of glutamate transporters, pharmacological management of the transporter functions can be used as very promising therapeutic targets. Ceftriaxone (beta-lactam antibiotic) has been shown to attenuate ethanol consumption and cocaine-seeking behavior in part by restoring glutamate homeostasis in mesocorticolimbic regions. Furthermore, recent studies from our lab have demonstrated the effects of amoxicillin and Augmentin on upregulating GLT-1 expression level as well as reducing ethanol consumption in male P rats. Therefore, in this project, we examined the effects of amoxicillin and Augmentin on other glutamate transporters (xCT and GLAST) expression levels in the nucleus accumbens (NAc) and prefrontal cortex (PFC). Furthermore, we also investigated the effects of clavulanic acid administration on alcohol consumption as well as GLT-1 and xCT expression levels in NAc. Additionally, we also determined whether oral Augmentin have any effect in

reducing alcohol intake in male P rats. Rats were exposed to free choice of ethanol (15% and 30%), water, and food for a period of five weeks. During week six, rats were given five consecutive daily i.p. injections of saline vehicle, 100 mg/kg amoxicillin injections or 100 mg/kg Augmentin injections. Both compounds significantly increased xCT expression level in NAc. Augmentin also increased xCT expression level in PFC. In the clavulanic acid study, rats were given five consecutive i.p. injections of 5 mg/kg clavulanic acid for the treatment group and the saline injections for the saline group. Clavulanic acid significantly reduced ethanol consumption and significantly upregulated GLT-1 and xCT expression levels in NAc. In oral Augmentin study, oral gavage of Augmentin (100 mg/kg) significantly attenuated alcohol consumption in male P rats as compared to the water gavage group. These findings revealed that amoxicillin, Augmentin and clavulanic acid may have a potential therapeutic action for the treatment of alcohol dependence that are mediated through upregulation of GLT-1 and xCT expression levels in the mesocorticolimbic structures.

Key words: GLT-1, xCT, GLAST, Amoxicillin, Augmentin, Clavulanic Acid

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List of Abbreviations

ANOVA …….…….Analysis of Variance

BBB…….……Blood Brain Barrier

CNS…….…….Central Nervous System
CSF…….……..Cerebrospinal Fluid

GLAST…….Glutamate-Aspartate Transporter
GLT1……Glutamate Transporter 1

iGLURs.....…….…Ionotropic Glutamate Receptors
i.p…….……Intraperitoneal injection

NAc …….……Nucleus Accumbens

PFC …….……Prefrontal Cortex

xCT …….……Cystine-Glutamate Exchanger

Chapter 1

Introduction

1.1. Overview

Drug addiction is a serious worldwide problem that occurs due to the prolonged influences of addictive drugs on the brain (Leshner, 1997). Drug craving is one of the main features of drug addiction which results in compulsive drug-taking behavior. Mostly, drug craving results of repeated self-administration of this drug, which can lead to several serious health problems (Robinson and Berridge, 1993). Importantly, alcohol dependence is a common form of drug addiction, which usually is recognized by the presence of tolerance and withdrawal effects. The alcohol withdrawal effects are linked mainly to the physical dependence on ethanol, which usually disappear after ethanol intake. Alcohol tolerance is defined as the necessity to increase the alcohol dose in order to give the desired effect (Basavarajappa and Hungund, 2005).

It has been demonstrated that chronic exposure to alcohol is responsible for the alcohol withdrawal symptoms, tolerance and dependence due to changes in the function of neurotransmitters (Diana et al., 2003). Of these neurotransmitters, glutamate is one of the main neurotransmitters that have proven to be changed following alcohol dependence and drug of abuse. We have recently shown that chronic alcohol intake reduced glutamate

transporters (Qrunfleh et al., 2013, Alhaddad et al., 2014, Rao et al., 2015). These findings suggest that targeting glutamate transporters is the key for the treatment of alcohol dependence.

1.2. Glutamatergic System and Alcoholism

Glutamate is the major excitatory transmitter in the mammalian central nervous system (accounting for 80-90%) and has several receptor proteins (McKenna, 2007). Studies have suggested the important role of glutamate transmission in normal brain function, including cognition, learning and memory (Headley and Grillner, 1990). Glutamate also plays an essential role in neurodevelopment processes, including cell induction and elimination (Steinhauser and Gallo, 1996). It has been suggested that glutamate can excite the cell to a level, which may lead to their death mediated through excitotoxicity (Atlante et al., 2001). Cell death is a result of oxidative stress that caused by increasing the production of reactive oxygen and nitrogen species in response to high extracellular glutamate concentration (Adelmann et al., 1999). Studies have shown that there are no enzymes to metabolize the extracellular glutamate to any significant degree (Logan and Snyder, 1972). Additionally, ionotropic glutamate receptors (iGLURs) are only expressed in postsynaptic glutamate axon terminals. Therefore, glutamate exerts its action through binding to iGLURs in mesocorticolimbic brain regions (Tzingounis and Wadiche, 2007). It is important to note that glutamate uptake has been proven to be the only long-term mechanism to maintain low glutamate extracellular concentration

(Jabaudon et al., 1999). Glutamate uptake is regulated by several glutamate transporters located in glia or neurons. Glutamate transporters serve as the only rapid way to regulate glutamate uptake (Johnston, 1981, Danbolt, 2001).

1.3. Glutamate Transporters

Glutamate transporters are the key player in regulating extracellular glutamate concentration. The uptake process uses electrochemical gradient difference across the plasma membranes as driving force (Rothstein et al., 1994). Due to the high degree of glutamate release, inhibition of its uptake can lead to high extracellular glutamate concentrations and consequently cell toxicity (Rosenberg and Aizenman, 1989). Alcohol dependence is responsible for the inhibition of glutamatergic neurotransmission by alteration of N-methyl-D-aspartate receptors (NMDA). Moreover, glutamatergic inhibition can lead to upregulation of NMDA receptors as compensatory mechanism [for review see ref. (Sari, 2013)]. Excitatory amino acid transporters (EAATs) and vesicular glutamate transporters (vGLUTs) are two types of the glutamate transporters involved in regulating glutamate homeostasis (Sims and Robinson, 1998). EAATs use the electrochemical gradient of Na^+ and K^+ ions for their effects and these transporters are found in plasma membranes (Beart and O'shea, 2007). Glutamate transporters in plasma membranes are responsible for controlling most of the extracellular glutamate concentrations (Kanai and Hediger, 1992). Furthermore, studies have shown that increase of the expression level of a major glutamate transporter termed as glutamate transporter 1

(GLT-1) may modulate extracellular glutamate concentration (Castaldo et al., 2007, Griffin et al., 2014). Another transporter plays an important role in controlling the glutamate extracellular concentration is the cystine-glutamate exchanger (xCT) (Moran et al., 2005). It is important to note that xCT function depends on the exchange process in addition to the net uptake. Exchange is a 1:1 process where xCT exchanges internal glutamate for external cystine with the electrochemical gradient (Volterra et al., 1996, Danbolt, 2001).

1.3.1. Glutamate Transporters in Plasma Membranes

Plasma membranes glutamate transporters are divided into two groups depending on the K_m value (high and low-affinity transporters) (Gegelashvili and Schousboe, 1997). High-affinity transporters are named (sodium and potassium coupled glutamate transporters) to distinguish them from the low-affinity transporters. High-affinity transporters use Na^+ for glutamate binding and K^+ for the process of net transport (Roskoski, 1979, Szatkowski et al., 1991). Five transporter subtypes have been cloned in the high-affinity group with K_m values varying between (1-100 μM depending on the type of transporter) (Gegelashvili and Schousboe, 1998). Plasma membrane high-affinity transporters are defined as the EAATs. There are five types of high-affinity plasma membrane EAATs (Table 1-1).

Table 1.1 Glutamate transporters belong to slc (solute carrier) family (EAATs).

Approved symbols (HGNC)	Approved names	Other names
EAAT1;slc1a3	Excitatory amino acid transporter 1 Solute carrier family 1 member 3	GLAST(Storck et al., 1992)
EAAT2;slc1a2	Excitatory amino acid transporter 2 Solute carrier family 1 member 2	GLT-1(Arriza et al., 1994)
EAAT3;slc1a1	Excitatory amino acid transporter 3 Solute carrier family 1 member 1	EAAC1(Kanai and Hediger, 1992)
EAAT4;slc1a6	Excitatory amino acid transporter 4 Solute carrier family 1 member 6	(Fairman et al., 1995)
EAAT5;slc1a7	Excitatory amino acid transporter 5 Solute carrier family 1 member 7	(expressed primary in retina) (Arriza et al., 1997)

Among different glutamate transporters, GLT-1 (human homologs is EAAT2) is responsible for the uptake of 90% of the extracellular glutamate (Rothstein, 1994).

GLT-1 is predominantly expressed in astrocytes in both mature and normal brain (Danbolt et al., 1992). Considering the importance of glutamate regulation by its transporters especially GLT-1, several pathogenic processes were found as a result of glutamate dysregulation. For instance, spontaneous epilepsy was a result of the complete absence of EAAT2 (Tanaka et al., 1997) and also showed an increase in extracellular glutamate concentration (Mitani and Tanaka, 2003). However, no direct link was found between glutamate transporter expression levels and patients suffered from epilepsy (Tessler et al., 1999). Another example of extracellular glutamate accumulation associated disorders is amyotrophic lateral sclerosis (ALS) (Rothstein et al., 1992). This accumulation was accompanied by a detected loss of GLT-1 (Rothstein et al., 1995). Furthermore, studies have shown that elevation of extracellular glutamate concentration has a direct link with the alcohol dependence development (Gass and Olive, 2008, Rao and Sari, 2012, Sari et al., 2013c). Recent studies in our lab have shown that β-lactam antibiotics upregulate GLT-1 and reduced alcohol consumption in the alcohol preferring (P) rat model (Sari et al., 2011). Similarly, attenuation of relapse-like ethanol consumption behavior in P rats was found associated in part with upregulation of GLT-1 expression level (Qrunfleh et al., 2013). Thus, GLT-1 has become a target for the treatment of alcohol dependence. The role of GLT-1 also has already been tested on the drug of abuse mouse models that show dysregulation in the glutamate transmission as a result of exposure to addictive drugs. For instance, MS-153 (GLT-1 activator) has successfully upregulated GLT-1 expression level in the mouse model (Nakagawa et al.,

2005). Another study showed the effect of ceftriaxone (β-lactam antibiotic) in the prevention of cannabinoids tolerance through increasing the GLT-1 transporter expression level was conducted in mice model (Gunduz et al., 2011). Recently, a study from our lab demonstrated that increases in GLT-1 expression levels in the NAc and PFC were associated with attenuation of reinstatement to cocaine-seeking behavior in male Sprague Dawley rats (Sari et al., 2009). These data provide ample evidence that upregulating GLT-1 expression level in brain reward regions may attenuate dependence to alcohol.

Another EAAT transporter is GLAST (human homologs is EAAT1), which is expressed in astrocytes throughout the central nervous system (CNS) (Lehre et al., 1995). It is the major glutamate transporter in the cerebellum (Lehre and Danbolt, 1998). Studies have demonstrated the presence of GLAST in other peripheral organs such as the retina (Rauen et al., 1996, Lehre et al., 1997, Rauen and Wiessner, 2000), the inner ear (Furness and Lehre, 1997, Takumi et al., 1997), the circumventricular organs, and it co-expresses with GLT-1 in the membrane of astroglial cells (Berger and Hediger, 2000). Mice lacking GLAST (Watase et al., 1998) develop normally but showed symptoms of inadequate glutamate uptake in the regions where GLAST was the major transporter (Harada et al., 1998). Furthermore, the effect of GLAST deficiency on the Cochlea (inner ear organ) showed exacerbation of the noise-induced hearing loss in mice lacking this transporter. The hearing loss was due to the glutamate accumulation in the cochlea (GLAST transporter highly expressed region) (Hakuba et al., 2000). Lack of GLAST didn't lead to

spontaneous epilepsy like GLT-1 does; however, it increases the seizure severity and duration (Watanabe et al., 1999).

xCT is the main subunit of the x^-_c system along with 4F2hc subunit (Burdo et al., 2006). It is classified as sodium-independent uptake system which found mainly in glia (Makowske and Christensen, 1982). This uptake system works in contrast to the Na^+ and K^+ dependent uptake system. The physiological role of this transporter is to exchange cystine-glutamate in 1:1 ratio that transports cystine inside the cell in exchange for intracellular glutamate (Bannai, 1986). Thus, it acts as a cystine carrier that uses the gradient of high-intracellular and low-extracellular glutamate concentrations as a driving force (Bannai and Kitamura, 1980). Then, cystine uptake is inhibited by the high level of extracellular glutamate. In the other way, the uptake of cystine leads to glutamate release extracellulary (Danbolt, 2001). Therefore, xCT is important to regulate the extracellular glutamate concentration. Moreover, if this concentration is high enough, it may possibly catalyze the release of cystine. For this reason, cell death may occur due to the oxidative stress (Murphy et al., 1989, Murphy et al., 1990). This might be due to the fact that cystine is reduced into cysteine, which serves as the limiting precursor in the synthesis of glutathione, an essential antioxidant in the brain (Dringen, 2000). Thus, cystine uptake is increased when cells are exposed to an oxidative stress (Ohtsuka et al., 1988, Miura et al., 1992). These results indicate that xCT has dual mechanism such as regulating the extracellular glutamate concentrations (Baker et al., 2002a) and defense mechanism against reactive oxygen species (ROS) (Ohtsuka et al., 1988, Tan et al., 2001). The

distribution of xCT in the brain has not been fully determined yet and studies have suggested that expression level may vary in stressful situations (Sato et al., 2002).

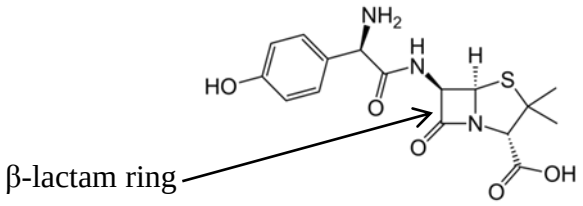
1.4. Drugs for the Treatment of Alcoholism.

Considering the importance of glutamate transporters, pharmacological management of the transporters function can be used as a very promising therapeutic target (Sheldon and Robinson, 2007) especially alcohol dependence. Rothstein and his colleagues have identified that beta-lactam antibiotics are potential targets for upregulating GLT-1 expression level after testing 1,040 FDA approved drugs (Rothstein et al., 2005). One of the most exciting discoveries so far in the treatment of alcohol addiction is the role of ceftriaxone (Sari et al., 2013b, Alhaddad et al., 2014). In this project, three compounds have been tested for the upregulation of GLT-1 expression levels and their association with alcohol intake. These compounds are amoxicillin, Augmentin, and clavulanic acid.

1.4.1. Amoxicillin (Amox)

Amoxicillin is widely used as moderate spectrum β-lactam antibiotics. Amoxicillin is susceptible to enzyme degradation by bacteria that produce β-lactamase. For this reason, it often combined with β-lactamase inhibitors (e.g. clavulanic acid). This combination can lead to the increase of antibacterial effect by reducing the susceptibility to the β-lactamase-produced from bacteria and then to decrease the resistance level

(Figure 1-1). Amoxicillin also contains the β-lactam ring, which is common structure in all β-lactam antibiotics that are suggested to have upregulatory effects in GLT-1 expression level (Rothstein et al., 2005). The drug has a good safety profile with a small percentage of gastrointestinal disturbance (diarrhea) and skin rash (majority in children) side effects (Aspin et al., 1994).



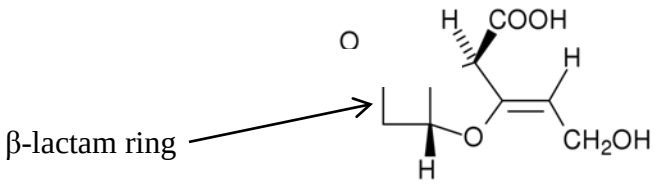
Amoxicillin

Figure 1-1 Chemical Structure of Amoxicillin

1.4.2. Clavulanic Acid

Clavulanic acid is a member of β-lactam antibiotics with no antibiotic activity of its own and has no therapeutic value as an antibacterial. It is used as beta-lactamase inhibitor in combination with penicillin, for instance, to extend the spectrum and potentiate the antibacterial activity of amoxicillin (Wüst and Wilkins, 1978, Crosby and Gump, 1982). The structure shows a similarity to cephalosporins and penicillin with a central β-lactam ring, which is the core structure in almost all compounds (Rothstein et al., 2005). Clavulanic acid has many advantages over ceftriaxone, which is proved as a

potential therapeutic target for alcohol dependence and drug of abuse (Sari et al., 2011, Qrunfleh et al., 2013, Sari et al., 2013b). First, the clavulanic acid has the central β-lactam ring, which is believed to be responsible for the ceftriaxone GLT-1 upregulation (Figure1-2). Also, clavulanic acid is considered orally active and stable, with bioavailability between 64 and 75% while ceftriaxone is a parenteral drug (Davies et al., 1985). Clavulanic acid is considered as a safe drug with minimal side effects like GI upset, including diarrhea. Clavulanic acid displayed an inverted-U shaped dose response curve when tested across a broad dose range (Kim et al., 2009). Studies have proved that clavulanic acid passes readily the blood brain barrier with a CSF/plasma ratio is around 0.25 making it a viable CNS drug (Nakagawa et al., 1994). It was recently reported that clavulanic acid is highly potent anxiolytic with a unique profile of activity in rodents and non-human primates and it may be an effective target for the treatment of anxiety disorders (Kim et al., 2009). Another study demonstrated that clavulanic acid decreases the rewarding hyperthermia effect of morphine in the rat model (Schroeder et al., 2014). Furthermore, findings demonstrated that clavulanic acid has a neuroprotective effect in animal models with CNS disorders, such as Alzheimer's and Parkinson's disease (Huh et al., 2010). All the previous studies supported that clavulanic acid may be a potential therapeutic drug target for drug addiction, including alcohol dependence with proven effect against ethanol withdrawal symptoms like anxiety.



Clavulanic acid

Figure 1-2 Chemical Structure of clavulanic acid

1.4.3. Augmentin (Aug).

Augmentin is a wide spectrum antibiotic, which is developed over 20 years ago to provide an effective treatment against resistant bacteria especially beta-lactamase producing bacteria (Kaye et al., 2001). It contains a combination of amoxicillin and clavulanic acid at a fixed ratio. Amoxicillin and clavulanic acid have a well absorption profile when given orally and peak serum level of oral dose reached after 60 to 90 min and 40 to 120 min, respectively. The elimination half-lives of both compounds are around 60 min with 60% excreted unchanged in feces and urine (White et al., 2004). Augmentin is generally well tolerated with a very good safety profile. The safety data collected from clinical trials published from 1979 and 1992, including 32,440 patients have shown a good safety profile with the most common side effect that was diarrhea (3.4%) (Neu et al., 1993) (Figure1-3)

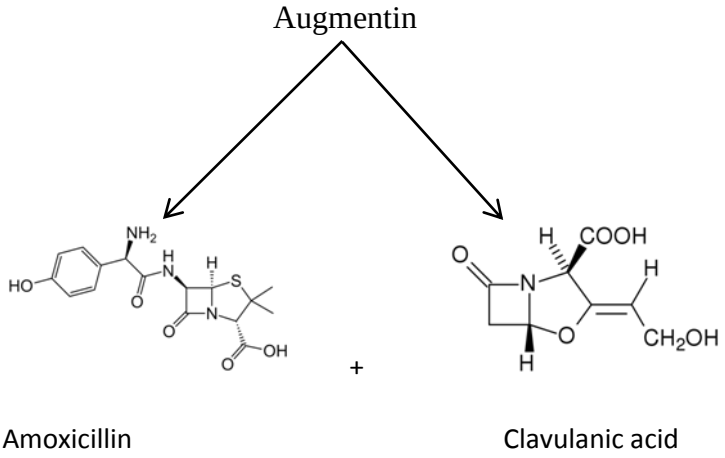


Figure 1-3 Chemical Structure of Amoxicillin + Clavulanic Acid = (Augmentin)

1.5. Alcohol Preferring (P) Rats Model for Alcoholism

Several criteria were set for an animal model of alcoholism; first, the animal should voluntary self-administer the alcohol orally to resemble the human model. Secondly, the amount of ethanol consumed should result of pharmacologically relevant alcohol level in blood. Also, chronic consumption of ethanol should cause some withdrawal effect such as dependence. Ethanol tolerance should also be a result of the chronic ethanol consumption. Further, ethanol consumption behavior should be positively reinforcing (Lester and Freed, 1973, Cicero, 1979).

According to the previous mentioned criteria, one important example of the breeding lines is the P rats from Indiana University, which were developed by a

bidirectional selective breeding of a selective mass selection from Wistar foundation stock (Lumeng et al., 1977). According to the criteria for rat model of alcoholism, P rats drink more than 5 g/kg/day of ethanol voluntarily (Li et al., 1986). Recently, studies have demonstrated P rats as an ideal animal model to satisfy the previously proposed criteria for the animal model of alcoholism including the development of dependence with a 24-hour free choice of ethanol consumption (Bell et al., 2006).

1.6. Aims and Objectives

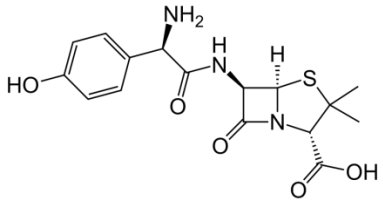
β-lactam antibiotics have been proved to increase GLT-1 expression level and as a consequence, reduced ethanol consumption (Rothstein et al., 2005, Rao and Sari, 2012, Alhaddad et al., 2014, Rao et al., 2015). We have determined in our lab the effect of amoxicillin and amoxicillin/clavulanic acid (Augmentin) on GLT-1 expression level and ethanol consumption in male P rats (Goodwani, 2014). We found that administration of amoxicillin and augmentin attenuated ethanol consumption and increased the expression level of GLT-1. Therefore, we investigated the effect of these treatments on other transporters (xCT and GLAST) and its relation with the ethanol consumption reduction in both PFC and NAc. Furthermore, we also investigated the effect of clavulanic acid administration on alcohol consumption as well as GLT-1 and xCT expression levels in NAc and PFC. We also determined whether oral treatment with Augmentin has any effect in reducing alcohol intake in male P rats.

Chapter 2

Materials and Methods

2.1. Amoxicillin

Amoxicillin was obtained from GlaxoSmithKline, France and was given as an i.p injection to the rats after the reconstitution with 0.9 % saline at the dose of 100mg/kg (Figure 2-1).

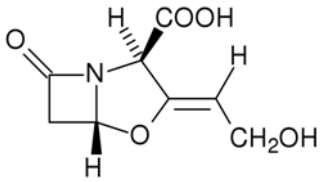


Amoxicillin

Figure 2-1 Chemical Structure of Amoxicillin

2.2. Clavulanic Acid

Clavulanic acid was purchased from SIGMA-ALDRICH in the form of potassium clavulanate VETRANAL salt. The treatment group dose was 5 mg/kg (i.p injection) (Figure 2-2).



Clavulanic acid

Figure 2-2 Chemical Structure of Clavulanic acid

2.3. Augmentin

Augmentin is combined of amoxicillin and clavulanic acid at the ratio of 1:5 (1 gm: 200 mg). It was purchased from GlaxoSmithKline, France as powder and reconstituted with 0.9% saline. The drug was i.p. injected at 100 mg/kg (Figure 2-3)

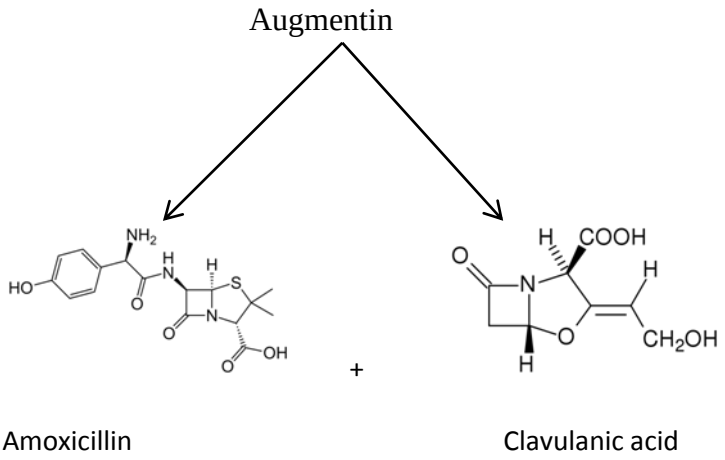


Figure 2-3 Chemical Structure of Amoxicillin + Clavulanic Acid (Augmentin)

2.4. Animals

Adult male P rats were used in this study. P rats were obtained from Indiana university medical health center (Indianapolis, IN, USA) at the age of 21-30 days and housed in the DLAR (Department of Laboratory Animal Resources, University of Toledo, HSC). At the age of 90 days, rats were individually housed and divided into two groups, ethanol group and water group. Both groups were housed in a plastic corn-cob bedding tubs and had an access to food and water ad lib. Each cage in the ethanol group had a voluntary uninterrupted access of three bottles of liquids. Two bottles were for two ethanol concentrations (15% and 30%) and one bottle for water. The room temperature was maintained at 21°C and 50% humidity with a 12-hour light-dark cycle to resemble the natural habitat throughout the experiment procedures. Alcohol and water were changed three times a week as well as food filling. All procedures in animal housing facility used in the present experiments were in compliance and approved by the Institutional Animal Care and Use committee of The University of Toledo in consonance with the 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). The association accredited the DLAR for the Assessment and Accreditation of Laboratory Animal Care, International.

2.4.1. Animals Treated i.p. with Amoxicillin and Augmentin Study

In this, rats were randomly divided into four different groups. (a) Water control group received i.p. injections of saline vehicle solution (n=6); (b) Ethanol control group received i.p. injections of saline vehicle solution (n=12); (c) Ethanol amoxicillin group (AMOX group) received 100 mg/kg, i.p. injections of amoxicillin (n=12); and (d) Ethanol Augmentin group (AUG group) received 100 mg/kg, i.p. injections of Augmentin (n=12).

2.4.2. Animals Treated i.p. with Clavulanic Acid Study

Rats were randomly divided into two groups. (a) Ethanol control group received 5 consecutive daily i.p injections of saline vehicle solution (n=8); (b) Rats in the treatment group were given 5 mg/kg i.p injections of clavulanic acid (n=8).

2.4.3. Animals treated orally with Augmentin.

Rats were randomly separated into two groups: (a) Ethanol control group received oral gavage of water (n=7); (b) Treatment group received 100 mg/kg oral gavage dose of Augmentin (n=7).

2.5. Ethanol Drinking Paradigm

Rats were exposed to free choice of ethanol 15% and 30%, water, and food for a period of five weeks. All rats in water group had a free choice of access to water and food. The alcohol preparation procedure has been made by diluting 190-proof of ethanol in distilled water in order to make 15% and 30% concentrations. The 190-proof ethanol was purchased from PHARMCO-AAPER (Shelbyville, KY). After the third week of drinking paradigm, we started to measure the ethanol intake, water drinking, and weight for two weeks. The measurement of ethanol intake was taken by subtraction of the bottle weight before and after alcohol consumption. The water measurement was done also in the same way with the weight measurement in grams. All the measurement procedures were done every Monday, Wednesday, and Friday. The ethanol intake and water drinking measurements were expressed in a gram of liquid consumption per kg of animal body weight per day. The baseline was the average of ethanol intake, water drinking and body weight of the last two weeks of the five weeks drinking paradigm. As previously done in our laboratory, animals that drank less than 4 g/kg/day of ethanol were excluded from the experiments before we started the saline vehicle or drug treatment (Sari et al., 2013c).

2.5.1. Drug treatments

Drugs were injected i.p. based on the rat weight every day for five consecutive doses during week six. The injections were administered at fixed time (2 P.M). The ethanol control and water control groups were received i.p. injections of saline for the same period as the treatment group.

In Augmentin oral gavage study, the dose for each rat was calculated according to the rat weight at 100 mg/kg Augmentin and reconstituted in water. It was administered orally through the gavage needles at 2.P.M. every day for five consecutive days. The saline control group received an oral gavage of water every day for five consecutive days also. Figure (2-4) shows the drinking paradigm of the experiment.

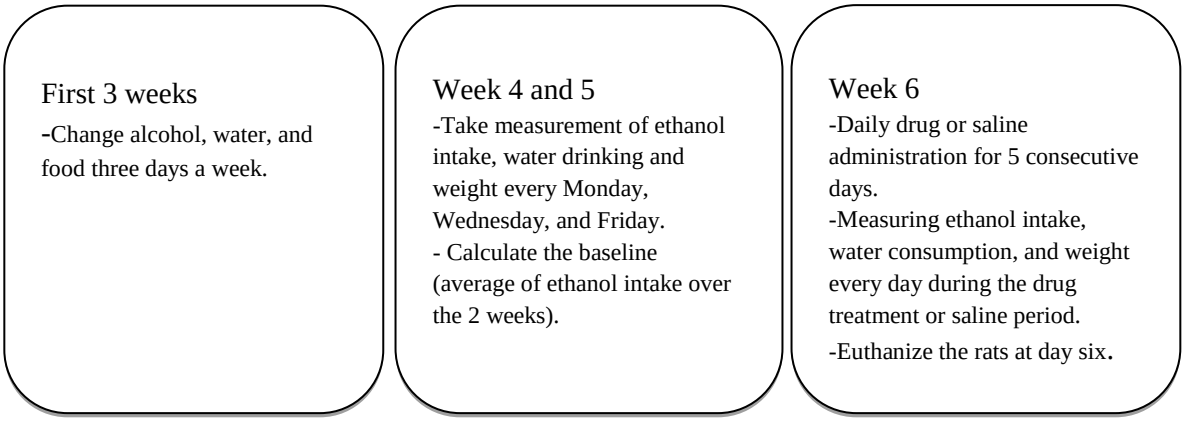


Figure 2-4 Timeline for ethanol drinking paradigm.

2.6. Brain tissue harvesting

Rats were euthanized using carbon dioxide 24 hours after last dose and directly decapitated using the guillotine. The brains were then immediately placed on dry ice and stored at -70^0 C. Brain regions were then isolated using cryostat microdissection at -20^0 C. Two brain regions were isolated [Prefrontal cortex (PFC) and nucleus accumbens (NAc)] according to the rat brain guideline (Paxinos and Watson, 1997). The Extracted regions were stored in -70^{0}C for western blot analysis and examine the protein expression levels.

2.7. Western blot protocol

The Western blot assay was conducted for two studies: 1) amoxicillin and Augmentin study; and 2) clavulanic acid study. The samples used in amoxicillin and Augmentin study were from NAc and PFC regions; however we tested only NAc region for clavulanic acid study using western blot procedure. For both studies, the brain region samples were lysed using regular filtered lysis buffer [2.5mL 1M Tris HCL, 2.5mL 3 M NaCl, 0.1mL 0.5M EDTA, 2.5mL 10% NP-40, 5mL 10% Triton, 0.5mL 10% SDS (sodium dodecyl sulfate), 5 mL of protease inhibitor solution, and 31.9 mL Millipore water] with 0.5 ml of phosphatase inhibitor. Then, we add 150-250 µL of lysis buffer to each sample in the eppendorf tube and then tissue samples were homogenized using the lysis pestle. Then, samples were placed on ice for 30 minutes with 5 seconds vortex performed every 10 minutes. Samples were then centrifuged using centrifuge 5415R, Eppendorf Inc. at 4ºC and 13,200 RPM speed for 15 minutes. The supernatant in each sample was divided into three equal aliquots. Lowry protein quantification assay was performed using one aliquot from each brain sample on 96 well plates to determine the level of protein in the samples. The following step was to prepare the 10-wells gels using the (BioRad) gel apparatus in specific proportions of reagents according to the desired gel numbers to be prepared.

On the day of Western blot assay, the samples were prepared from equal amount of extracted protein (according to the protein quantification calculations) mixed with 5 µL of 5X laemmli loading dye and then were loaded in the 10-wells gel in the perspective

order (water-saline-treatments). Then, proteins separation was performed using the gel electrophoresis method (protein running) and the gel was transferred on a PVDF membrane by the same electrophoresis concept (Bio-Rad, Hercules, CA). The membranes were then washed with distilled water once for 10 minutes then blocked with 3% blocking buffer for 30 minutes at room temperature (3 gram milk in 100 mL TBST). TBST is a mixture of 50 mM Tris HCl; 150 mM Acbl, pH7.4; 0.1% Tween 20. After the blocking period, primary antibody was added to the blocking buffer depending on the desired protein to be detected and incubated overnight on the orbital shaker at 4^0C.

The membranes were incubated with one of the following primary antibodies: rabbit anti-xCT antibody (Novus; 1:1000 dilution), rabbit anti-EAAT1 antibody (GLAST), and guinea pig anti-GLT1 (Millipore; 1:5000 dilution). Likewise, mouse anti β-tubulin was used as loading control (1:5000; Cell signaling technology).

On the next day, membranes were washed with TBST 5 times (5 minutes each) on the shaker and then were blocked with 3% blocking buffer for 30 minutes. Then, membranes were further incubated with secondary antibody for 90 minutes at room temperature. The types of secondary antibodies were anti-guinea pig (Jackson ImmunoResearch Laboratories, Inc.) and anti-rabbit at 1:5000 dilutions (thermo scientific).

After the secondary antibody incubation, the membranes were washed 5 times with TBST (5 minutes each) and then followed by drying phase on whatman papers. After that, the membranes were exposed to the SuperSignal West Pico Chemiluminescent substrate by thermo scientific for one minute. In the dark room, the membranes were exposed to Kodak BioMax MR Film (Fisher Inc.) and the films were developed on SRX-101A machine.

Upon obtaining the immunoreactive protein bands, MCID system (GE Healthcare Niagara Inc., US) was used to quantify these bands, and the results were presented as percentage of ratio of tested protein/control protein, relative to ethanol or water control groups (100% control-value).

2.8. Statistical Analysis

2.8.1. Behavioral Data in clavulanic acid and oral Augmentin Treatment Studies.

Statistical analysis using two-way (mixed) ANOVA was used to determine the main effect of day x treatment interaction on water consumption, ethanol intake, ethanol preference, and body weight comparisons between the control group (ethanol vehicle group) and treatment groups (ethanol clavulanic acid g group) and (ethanol Augmentin group). Two-way ANOVA followed by Bonferroni multiple comparisons test to

determine the daily effect of the treatment. All statistical results were based on a significance level of <0.05 P value.

2.8.2. Western Blot Data

One-way ANOVA was used to analyze western blot data (GLAST/ β-tubulin and xCT/ β-tubulin) followed by Newman-Keuls post-hoc test for comparing between the water control, ethanol vehicle and treatment groups (amoxicillin and Augmentin) in amoxicillin and Augmentin study. While, the independent unpaired t-test was used in the clavulanic acid study to analyze the data (GLT1/ β-tubulin and xCT/ β-tubulin) for comparison between ethanol vehicle group and treatment group (clavulanic acid). All results were based on a significance level of $p < 0.05$ value.

Chapter 3

Results

3.1. Amoxicillin and Augmentin Study.

3.1.1. Effect of Chronic Ethanol Consumption on xCT Expression Levels in NAc and PFC.

In the present study, we analyzed the effect of chronic ethanol consumption on xCT expression levels in NAc and PFC. Figure (3-1) and (3-2) show comparison of the effect of chronic alcohol consumption on xCT expression levels in NAc and PFC between ethanol vehicle (saline) group and ethanol-naïve vehicle (water) group. Statistical data using independent t-test showed significant downregulation of xCT expression level in ethanol vehicle group as compared to the ethanol-naïve (water) group in NAc ($p<0.05$). In PFC, an independent t-test analysis showed similar results of xCT expression level between the control and saline group ($p<0.05$).

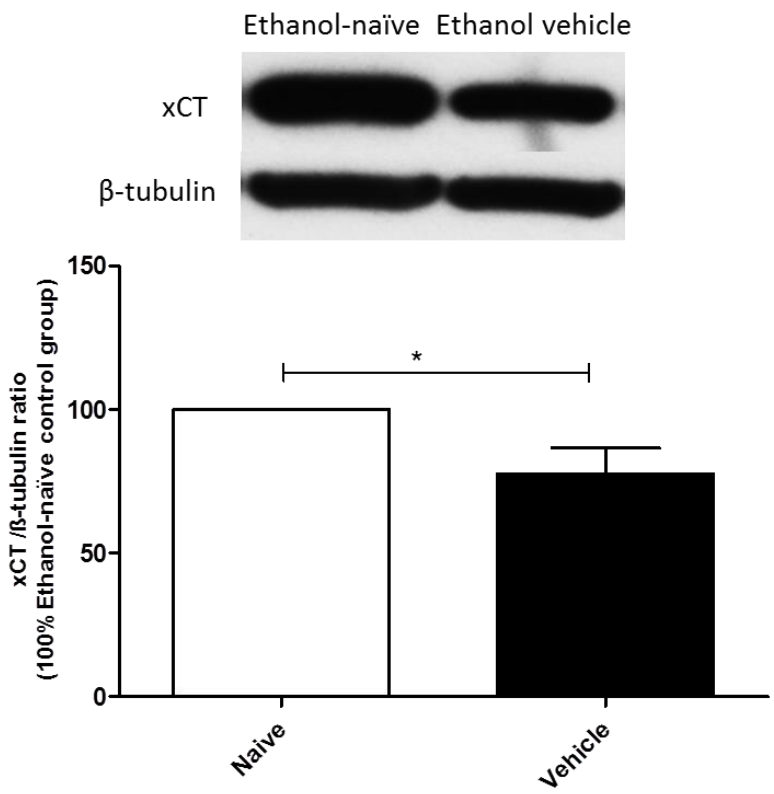


Figure (3-1) <u>The effect of chronic ethanol consumption on xCT expression level in NAc</u>

Upper panel: shows western blots for xCT and β-tubulin loading control in NAc.

Lower panel: quantitative analysis of immunoblots revealed significant reduction in xCT expression level in ethanol vehicle group following chronic alcohol consumption as compared to the ethanol-naïve group (100%). Data are shown as mean ± SEM (*$p<0.05$). (Ethanol-naïve vehicle, n=6; Ethanol Vehicle, n=6).

PFC

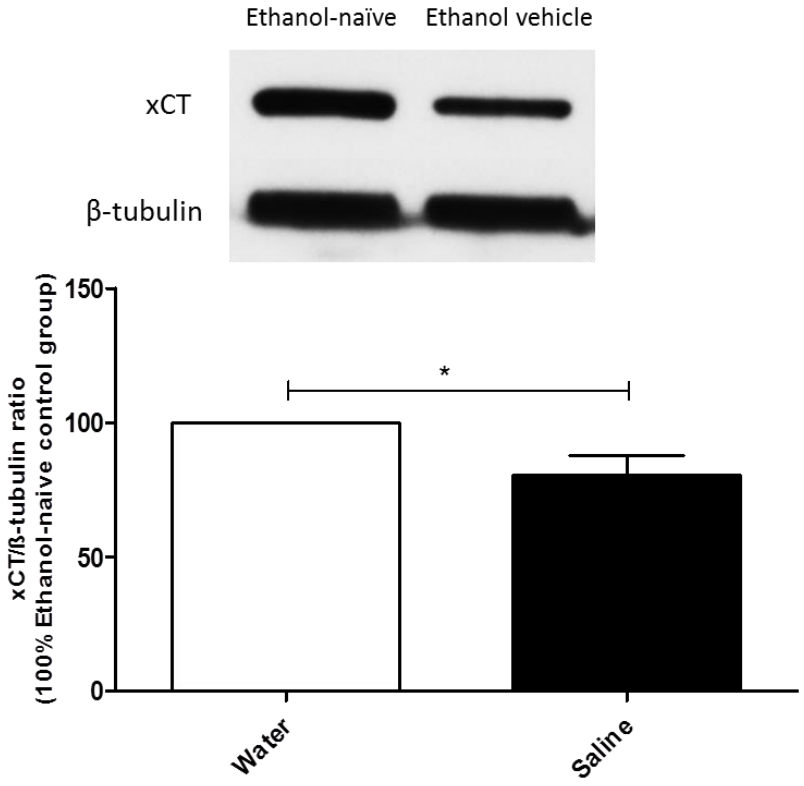


Figure (3-2) <u>The effect of chronic ethanol consumption on xCT expression level in PFC</u>

Upper panel: shows western blots for xCT and β-tubulin loading control in PFC.

Lower panel: quantitative analysis of immunoblots revealed significant reduction in xCT expression level in ethanol vehicle group following chronic alcohol consumption as compared to ethanol-naïve group (100%). Data are shown as mean ± SEM (*$p<0.05$). (Ethanol-naïve vehicle, n=6; Ethanol Vehicle, n=6).

3.1.2. Effects of Amoxicillin and Augmentin on xCT Expression Level in NAc.

Figure (3-3) shows the effects of amoxicillin and Augmentin on xCT expression level in NAc. Western blot analysis showed a significant difference between treatments and ethanol vehicle groups in NAc, [$F(2,12) = 6.821$, $p=0.0105$]. One-way ANOVA followed by Newman-Keuls multiple comparison test revealed a significant upregulation of xCT expression level in both amoxicillin ($p<0.05$) and Augmentin ($p<0.01$) treatment groups as compared to the ethanol vehicle group.

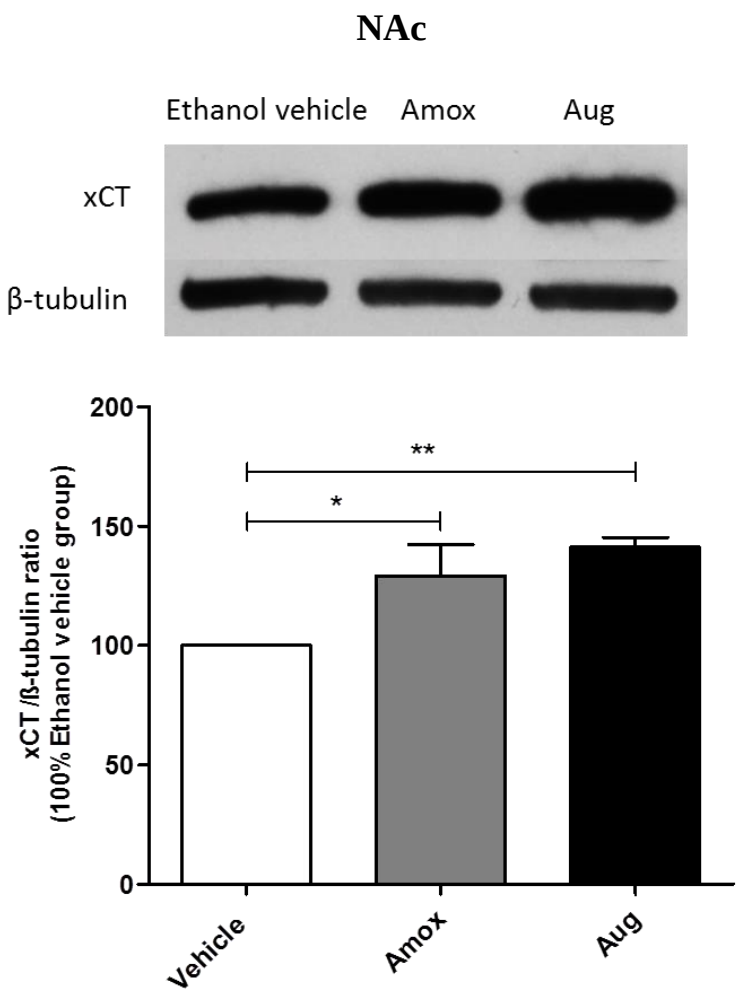


Figure (3-3) <u>The effect of amoxicillin and Augmentin on xCT expression level in NAc.</u>

Upper panel: shows western blots for xCT and β-tubulin loading control in NAc.

Lower panel: statistical analysis of immunoblots revealed significant upregulation of xCT expression level in both amoxicillin and Augmentin treated groups comparing to ethanol vehicle group (100%). Data are shown as mean ± SEM, (*$p<0.05$), (**$p<0.01$). (Ethanol Vehicle, n=5; Ethanol Amoxicillin, n=5; Ethanol Augmentin, n=5).

3.1.3. Effects of Amoxicillin and Augmentin treatment groups on xCT Expression Level in PFC.

To detect the effect of amoxicillin and Augmentin on xCT expression level in PFC, one-way ANOVA revealed a significant difference between treatments and ethanol vehicle groups (Figure 3-4), [$F(2,12) = 4.287$, $P = 0.0394$].

A Newman-Keuls multiple comparison post-hoc test did not result of any significant upregulation of xCT expression level in PFC following amoxicillin treatment as compared to the saline vehicle group (100%). However, Augmentin treated group revealed significant upregulation of xCT expression level as compared to ethanol vehicle group (100%) ($p<0.05$). Additionally, there was a significant increase in xCT expression level in Augmentin group as compared to amoxicillin group in PFC ($p<0.05$).

PFC

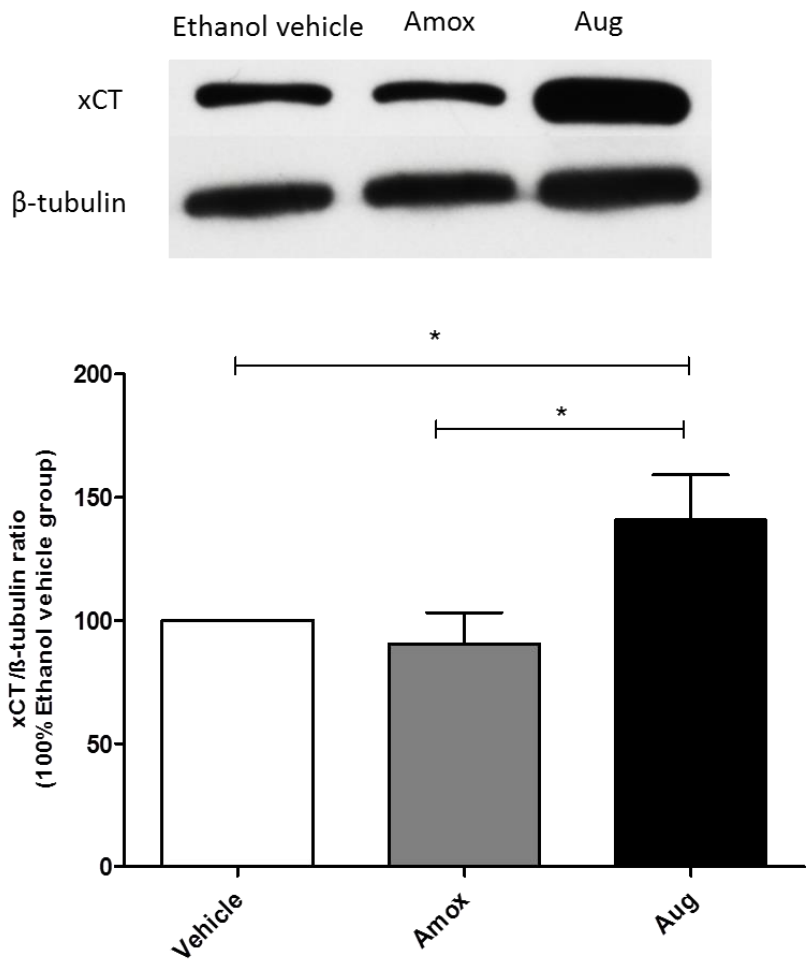


Figure (3-4) <u>The effect of amoxicillin and Augmentin on xCT expression level in PFC</u>

Upper panel: shows western blots for xCT and β-tubulin loading control in PFC.

Lower panel: statistical analysis of immunoblots revealed significant upregulation of xCT expression level in Augmentin treated group comparing to amoxicillin group and ethanol vehicle group (100%). However, no significant difference in the xCT expression level was observed between amoxicillin treated group and ethanol vehicle group (100%). Data are shown as mean ± SEM, (*$p<0.05$). (Ethanol Vehicle, n=5; Ethanol Amoxicillin, n=5; Ethanol Augmentin, n=5).

3.1.4. Effect of Amoxicillin and Augmentin on GLAST Expression Level in NAc.

Statistical analysis of GLAST immunoblots in NAc using one-way ANOVA didn't reveal any significant change between ethanol-naïve group (100%) and ethanol vehicle group. Moreover, post-hoc multiple comparison test (Newman-Keuls) revealed no significant difference between treatment and ethanol-naïve or ethanol vehicle groups, [$F(3,16)=0.2937$, $P=0.8293$], (Figure 3-5).

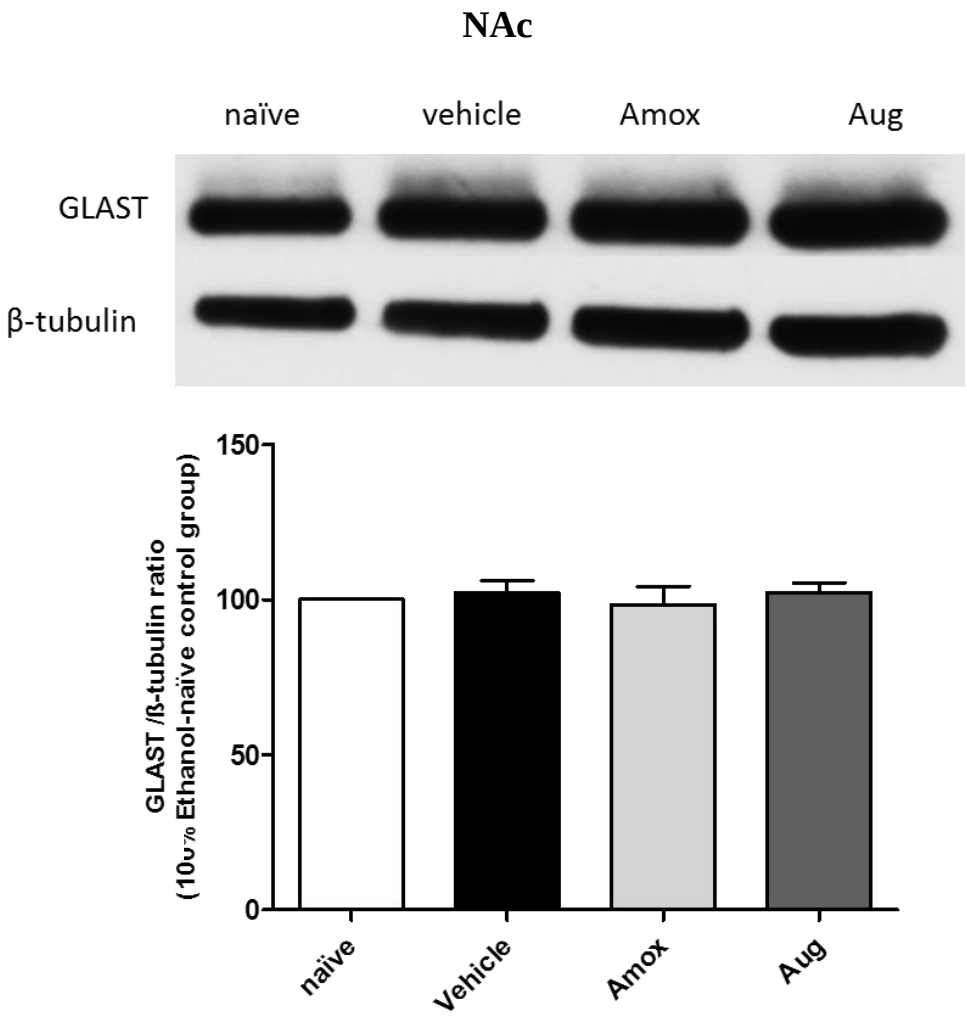


Figure (3-5) The effect of amoxicillin and Augmentin on GLAST expression level in NAc

Upper panel: shows western blots for GLAST and β-tubulin loading control in NAc.
Lower panel: statistical analysis of immunoblots revealed no significant change in GLAST expression level in NAc following Augmentin and amoxicillin administration comparing to ethanol vehicle group and ethanol-naïve groups (100%). Additionally, statistical analysis showed no significant difference between ethanol-naïve (100%) and ethanol vehicle groups in GLAST expression level. Data are shown as mean ± SEM. (ethanol-naïve, n=5; Ethanol Vehicle, n=5; Ethanol Amoxicillin, n=5; Ethanol Augmentin, n=5).

3.1.5. Effect of Amoxicillin and Augmentin on GLAST Expression Level in PFC.

Statistical analysis of GLAST immunoblots in PFC (Figure 3-6) using one-way ANOVA followed by post-hoc multiple comparison test (Newman-Keuls) revealed no significant difference between ethanol-naïve (100%), ethanol vehicle and treatment groups [$F(3,16)=0.2854, p=0.8351$].

PFC

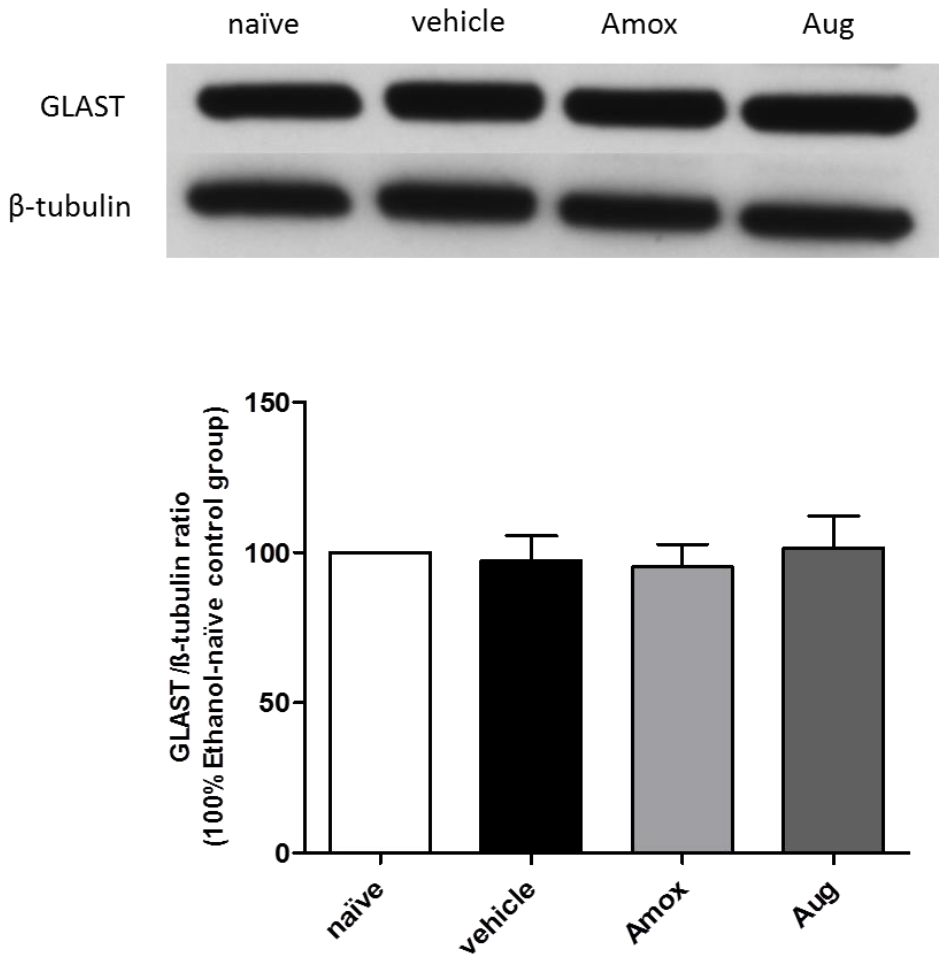


Figure (3-6) The effect of amoxicillin and Augmentin on GLAST expression level in PFC

Upper panel: shows western blots for GLAST and β-tubulin loading control in PFC.
Lower panel: statistical analysis of immunoblots revealed no significant change in GLAST expression level in PFC following Augmentin and amoxicillin administration comparing to ethanol vehicle and ethanol-naïve groups (100%). Additionally, statistical analysis showed no significant difference between ethanol-naïve (100%) and ethanol vehicle groups in GLAST expression level. Data are shown as mean ± SEM. (ethanol-naïve, n=5; Ethanol Vehicle, n=5; Ethanol Amoxicillin, n=5; Ethanol Augmentin, n=5).

3.2. Clavulanic acid Study.

3.2.1. Effect of Clavulanic Acid on Average Ethanol Intake in Male P Rats.

Figure (4-1) presents the effect of clavulanic acid i.p. injections (5 mg/kg) on alcohol consumption in male P rats over the period of 5 consecutive days (starting 24 hours after the first injection).

The average ethanol consumption over the last two weeks was considered as the baseline. P rats were then divided into two groups (ethanol vehicle and clavulanic acid groups) depending on the baseline drinking calculations.

Statistical analysis using two-way (mixed) ANOVA revealed significant main effect of Days [$F(1,5)= 3.786$, $p<0.01$] and significant Treatment x Day interaction [$F(1,5)=3.950, p<0.001$]. Two-way ANOVA followed by Bonferroni multiple comparisons test results of significant ethanol consumption reduction in the clavulanic acid group as compared to saline vehicle group started 24 hours after the first injection ($p<0.001$).

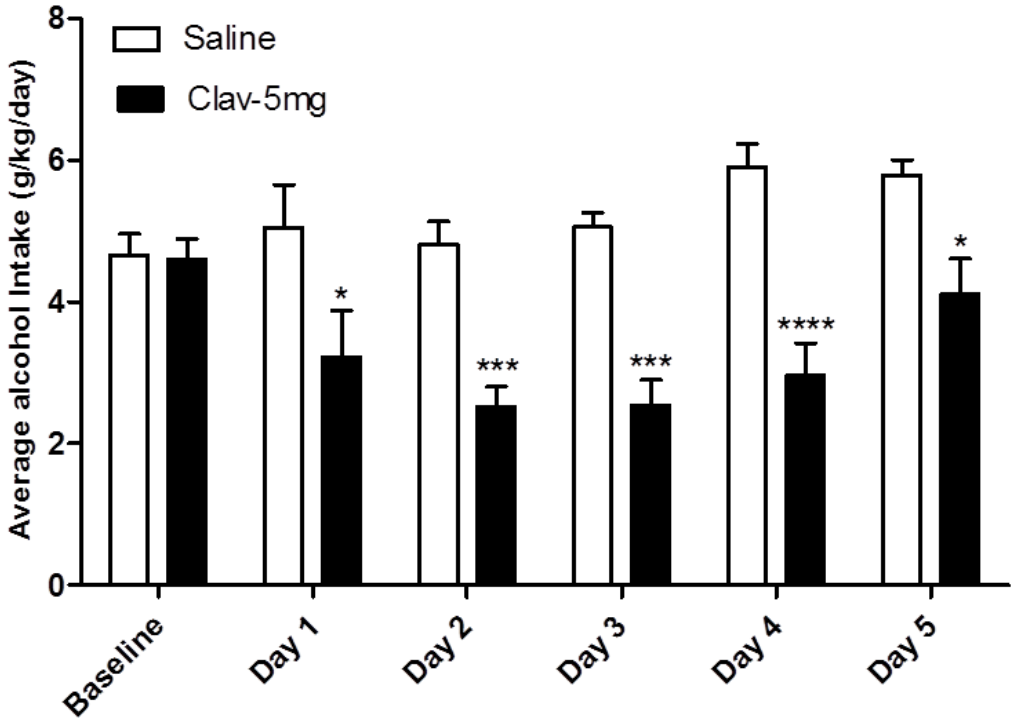


Figure (4-1) <u>Effect of clavulanic acid on average daily ethanol intake in male P rats</u>. Two-way ANOVA analyses revealed significant differences among control and treatment groups. Bonferroni multiple comparison test exhibits significant reduction in ethanol intake following clavulanic acid treatment starting Day 1 through the end of the study comparing to the ethanol vehicle group (saline). Data are expressed as mean ± SEM. (*p<0.05;***p<0.001;****p<0.0001); (Ethanol vehicle (saline), n=7; Ethanol clavulanic acid, n=7).

3.2.2. Effect of Clavulanic Acid on Water Intake in Male P Rats.

Figure (4-2) illustrates the effect of clavulanic acid i.p. injection on water intake started 24 hours after the first injection. The water intake was measured daily for each rat for 5 consecutive days in clavulanic acid (5 mg/kg) treatment group and ethanol vehicle (saline) group.

Two-way ANOVA demonstrated significant effect of Days [$F(1,5)= 7.087$, $p<0.0001$] and significant Treatment x Day interaction [$F(1,5)= 3.093$, $p<0.05$]. Bonferroni multiple comparison test following two-way ANOVA presented a significant increase in water intake in the clavulanic acid group ($p<0.01$) comparing to ethanol vehicle group. The increase in water intake in the clavulanic acid group was detected during the last week of study (treatment administration week) as compared to the saline vehicle group. However, statistical analysis revealed a significant increase in clavulanic acid treatment group water intake on days 2, 3, and 4 of the 5 days treatment period as compared to the saline vehicle group.

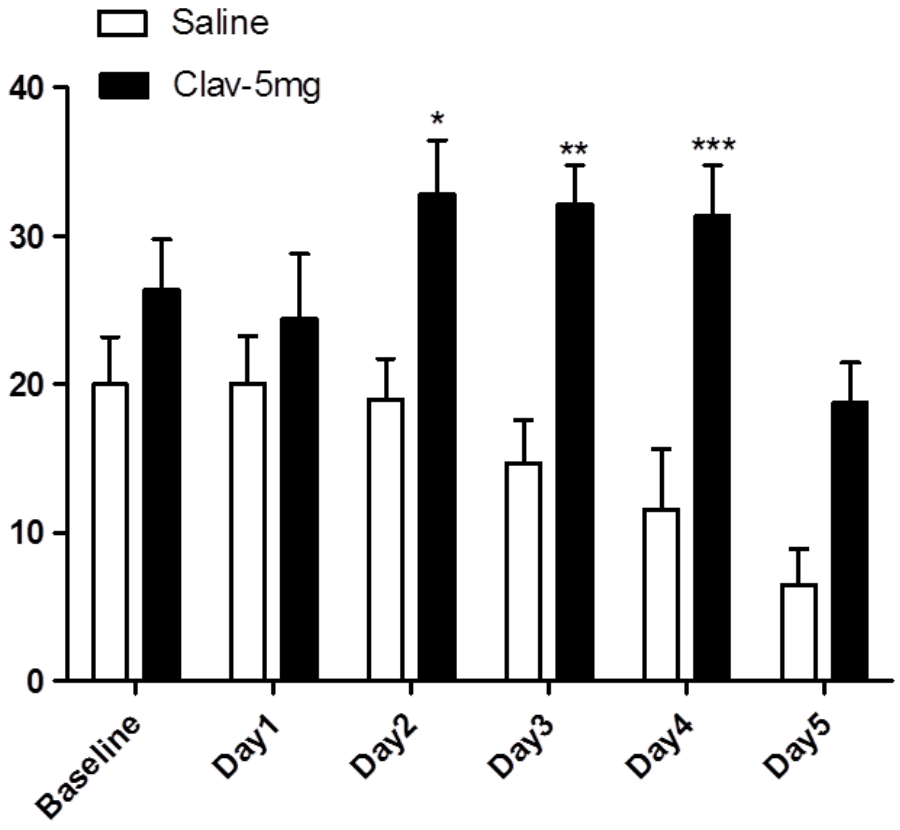


Figure (4-2) <u>Effect of clavulanic acid on average daily water intake in male P rats</u>. Two-way ANOVA analyses revealed significant differences among control and treatment groups. Bonferroni multiple comparisons exhibit a significant increase in water intake in clavulanic acid treated group starting Day 1 through the end of the study comparing to ethanol vehicle group (saline). However, a significant increase was observed in days 2, 3 and 4 in water intake between the two groups. Data are expressed as mean ± SEM. (*p<0.05;**p<0.01;***p<0.001); (Ethanol vehicle (saline), n=7; Ethanol clavulanic acid, n=7).

3.2.3. Effect of Clavulanic Acid on Body Weight.

The effect of clavulanic acid on male P rat body weight was monitored each day throughout the study (Figure 4-3). Two-way ANOVA was used to determine if there is any significant effect of clavulanic acid administration on male P rat body weight. Statistical analysis of clavulanic acid treatment and ethanol vehicle (saline) groups revealed a significant main effect of days [$F(1,5)= 7.276, p<0.0001$] and there was no significant in day x treatment interaction effect [$F(1,5)= 1.377, p>0.05$] . However, two-way ANOVA followed by Bonferroni multiple comparisons test unveiled no significant difference on the body weight throughout the treatment period between the rats treated with clavulanic acid compared to the ethanol vehicle (saline) group.

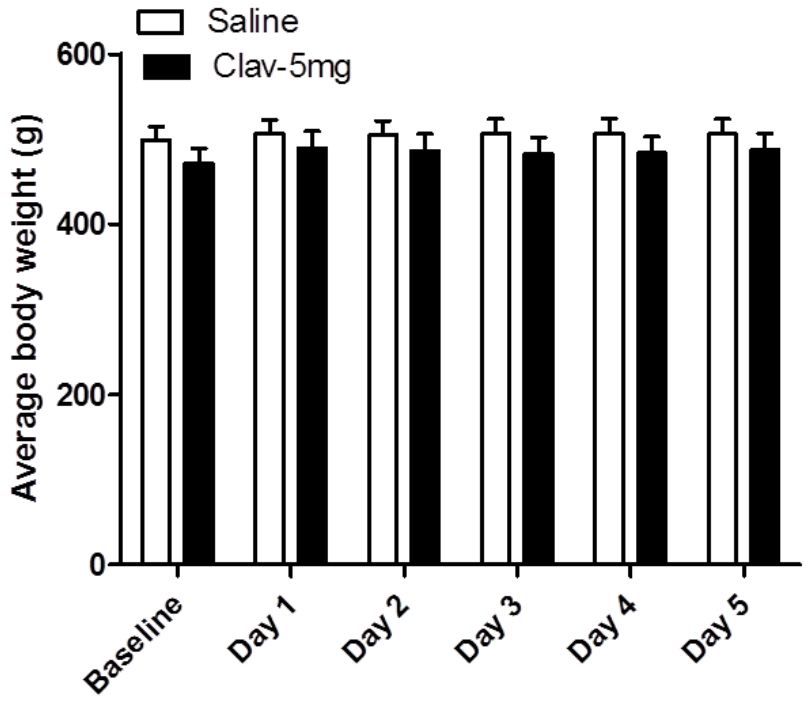


Figure (4-3) <u>Effect of clavulanic acid on average body weight in male P rats</u>. Two-way ANOVA analyses revealed no significant difference between control and clavulanic acid groups. Bonferroni multiple comparisons show no significant difference in the average body weight between clavulanic acid treated group (starting Day 1 through the end of the study) and ethanol vehicle group (saline). Data are expressed as mean ± SEM. (Ethanol vehicle (saline), n=7; Ethanol clavulanic acid, n=7).

3.2.4. Effect of Clavulanic Acid on GLT-1 Expression Level in NAc.

In the present study, we analyzed the effect of clavulanic acid administration on GLT-1 expression level in NAc. Figure (4-4) shows a significant change in GLT-1 expression level in clavulanic acid treated group as compared to ethanol vehicle group. The statistical data using independent t-test revealed a significant increase in GLT-1 expression level in the clavulanic acid group as compared to ethanol vehicle (Saline) group in NAc ($P<0.05$).

NAc

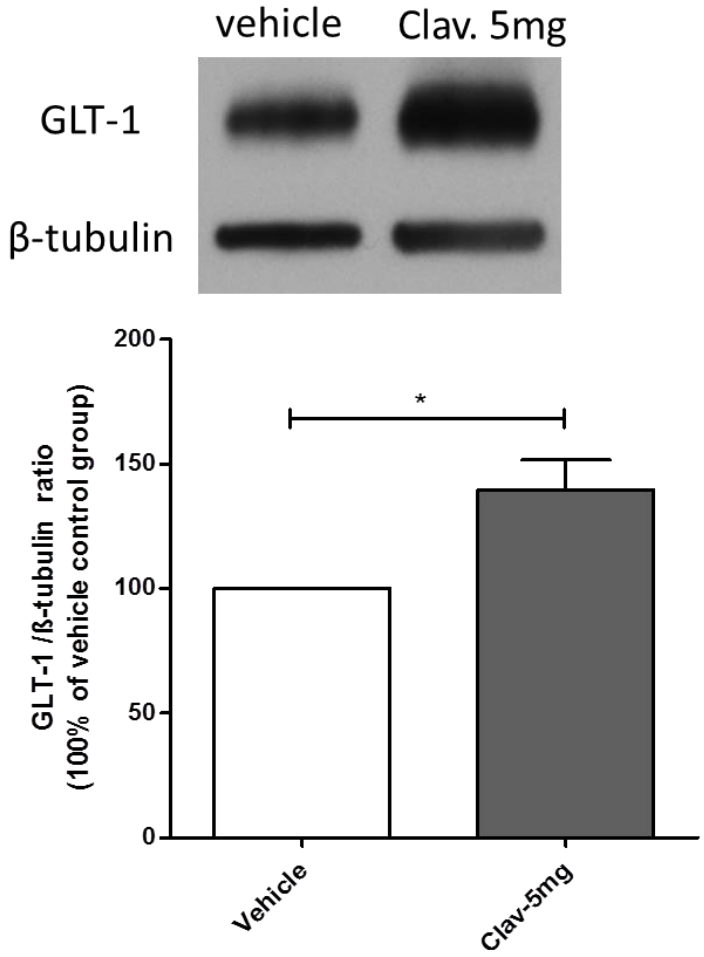


Figure (4-4) <u>Effect of clavulanic acid on GLT-1 expression level in NAc.</u>

Upper panel: shows western blots for GLT-1 and β-tubulin loading control in NAc.
Lower panel: quantitative analysis of immunoblots revealed a significant increase in GLT-1 expression level following administration of clavulanic acid (5 mg/kg) as compared to ethanol vehicle group (100%). Data are shown as mean ± SEM (*$p<0.05$). (Ethanol Vehicle, n=5; Clavulanic acid, n=5)).

3.2.5. Effect of Clavulanic Acid on xCT Expression Level in NAc.

Figure (4-5) illustrates the effect of clavulanic acid on xCT expression level in NAc. The statistical analysis of the immunoblots using independent t-test revealed a significant upregulation of xCT expression level in the clavulanic acid group as compared to ethanol vehicle group ($p<0.05$).

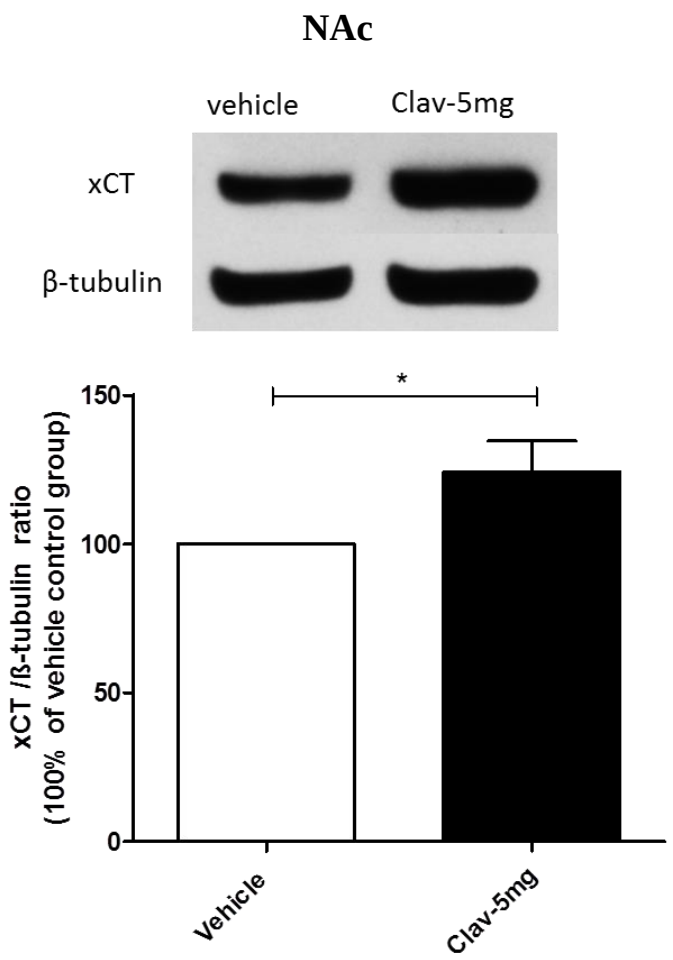


Figure (4-5) <u>The effect of clavulanic acid on xCT expression level in NAc.</u>

Upper panel: shows western blots for xCT and β-tubulin loading control in NAc.
Lower panel: quantitative analysis of immunoblots results of significant increase in xCT expression level following administration of clavulanic acid (5 mg/kg) as compared to ethanol vehicle group (100%). Data are shown as mean ± SEM (*$p<0.05$). (Ethanol Vehicle, n=5; Clavulanic acid, n=5).

3.3. Oral Augmentin Study.

3.3.1. Effect of Augmentin on Average Ethanol Intake in Male P Rats.

Figure (5-1) presents the effect of oral Augmentin dose (100 mg/kg) on alcohol consumption in male P rats over the period of 5 consecutive days.

The average ethanol consumption over the last two weeks of drinking paradigm was considered as the baseline. P rats were then divided into two groups: ethanol vehicle and oral Augmentin groups depending on the baseline drinking calculations.

Statistical analysis using two-way (mixed) ANOVA revealed a significant main effect of Days [$F(1,5)= 3.786$, $p<0.01$] and significant Treatment x Day interaction [$F(1,5)= 3.950$,$p<0.001$]. Two-way ANOVA followed by Bonferroni multiple comparisons test revealed a significant reduction in ethanol consumption in the treatment group (Augmentin group) as compared to ethanol vehicle (saline) group started 24 hours after the first day of treatment ($p<0.001$).

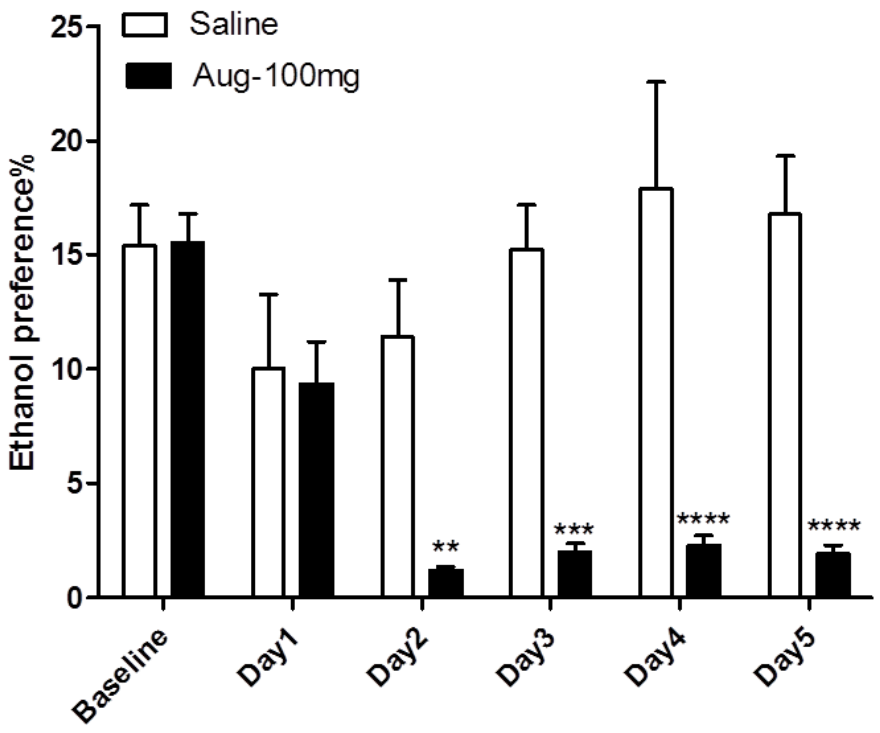


Figure (5-1) <u>Effect of oral Augmentin on average daily ethanol intake in male P rats</u>. Two-way ANOVA analyses revealed significant differences among control and treatment groups. Bonferroni multiple comparisons demonstrated a significant reduction in ethanol intake in Augmentin group starting Day 2 through the end of the study comparing to ethanol vehicle group (saline). Data are expressed as mean ± SEM. (**p<0.01;***p<0.001;****p<0.0001); (Ethanol vehicle (saline), n=7; Ethanol Augmentin, n=7).

3.3.2. Effect of Oral Augmentin on Water Intake in Male P Rats

Figure (5-2) illustrates the effect of oral Augmentin on water intake starting 24 hours after the first treatment dose. Water intake was measured daily for each rat for 5 consecutive days in Augmentin (100 mg/kg) and ethanol vehicle (saline) groups.

Two-way ANOVA demonstrated significant effect of Days [$F(1,5)= 6.325$, $p<0.0001$] and significant Treatment x Day interaction [$F(1,5)= 11.25$, $p<0.0001$]. Bonferroni multiple comparisons test following two-way ANOVA presented a significant increase in water intake in all rats treated with Augmentin ($p<0.001$) comparing to the ethanol vehicle group. This increase in water intake in Augmentin group was detected during the last week of study (treatment administration week) as compared to the saline vehicle group. However, statistical analysis revealed a significant increase in water intake from day 2 and throughout the last day of the study (day 5) as compared to the ethanol vehicle (saline) group.

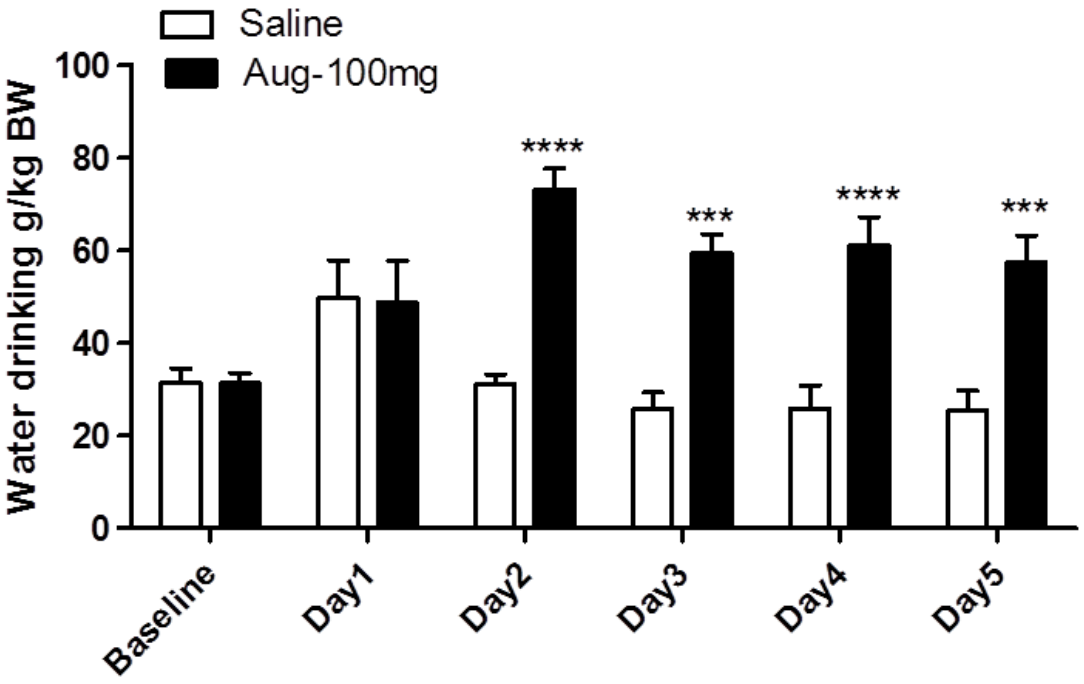


Figure (5-2) <u>Effect of oral Augmentin on average daily water intake in male P rats</u>. Two-way ANOVA analyses revealed significant differences among the control and treatment groups. Bonferroni multiple comparisons exhibit a significant increase in water intake in Augmentin treated group starting Day 2 through the end of the study comparing to ethanol vehicle group (saline). Data are expressed as mean ± SEM. (***p<0.001;****p<0.0001); (Ethanol vehicle (saline), n=7; Ethanol Augmentin, n=7).

3.3.3. Effect of Oral Augmentin on Body Weight.

The effect of oral Augmentin on male P rat's body weight was monitored each day throughout the study (Figure 5-3). Two-way ANOVA was used to determine if there is any significant effect of Augmentin oral administration on the rat's body weight. Statistical analysis in body weight revealed a significant main effect of days [$F(1,5)= 14.42, p<0.0001$] and there was no significant in day x treatment interaction effect [$F(1,5)= 2.043, p>0.05$] . Additionally, two-way ANOVA followed by Bonferroni multiple comparisons test unveiled no significant difference on the body weight during the 5 treatment days between rats treated with Augmentin as compared to the ethanol vehicle (saline) group.

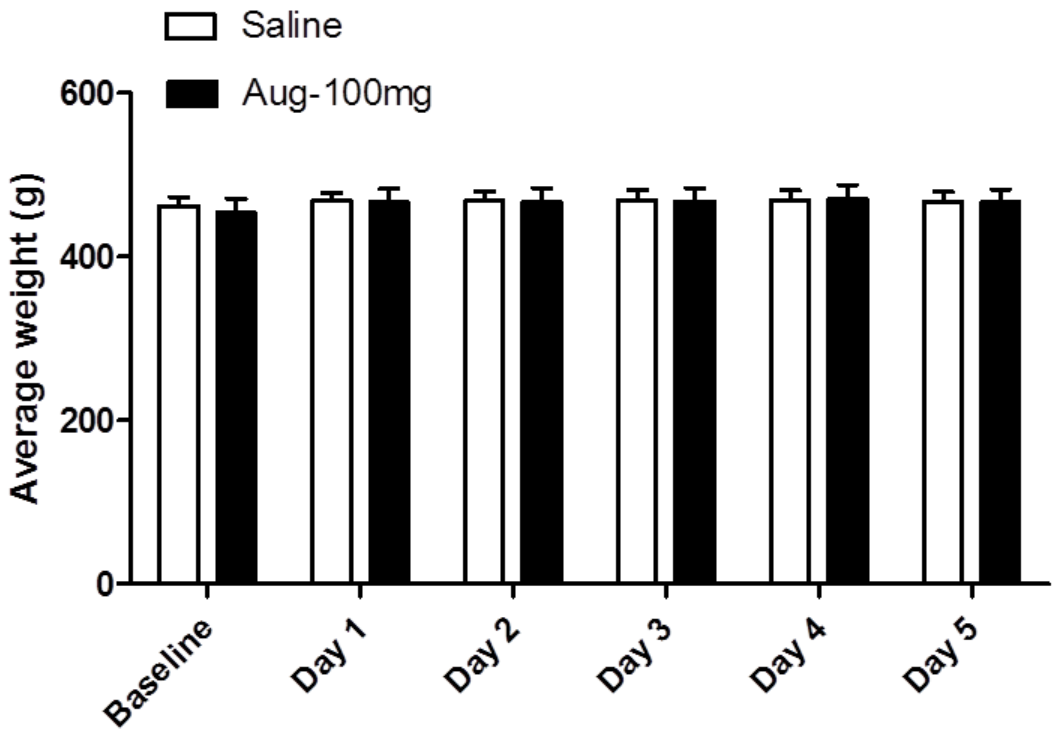


Figure (5-3) <u>Effect of oral Augmentin on average body weight in male P rats</u>. Two-way ANOVA analyses revealed no significant difference between the control and treatment groups. Bonferroni multiple comparisons test shows no significant difference in average body weight between the Augmentin group starting Day 1 through the end of the study comparing to the ethanol vehicle group (saline). Data are expressed as mean ± SEM. (Ethanol vehicle (saline), n=7; Ethanol Augmentin, n=7).

3.3.4. Effect of Oral Augmentin on Ethanol Preference in Male P Rats.

Figure (5-4) exhibits the effect of oral Augmentin on ethanol preference in male P rats. The percent of ethanol preference was calculated daily by the following equation: total alcohol consumption / total fluid consumption x 100 using the daily ethanol and water consumption of each rat as described previously (Lee et al., 2013). Two-way (mixed) ANOVA demonstrated significant main effect of Day [$F(1,5)= 4.741$, $p<0.01$] and significant Treatment x Day interaction [$F(1,5)= 6.473$, $p<0.0001$]. Bonferroni multiple comparisons test following two-way ANOVA revealed a significant difference in ethanol preference from day 2 throughout the study in Augmentin group ($p<0.001$) as compared to the ethanol vehicle (saline) group.

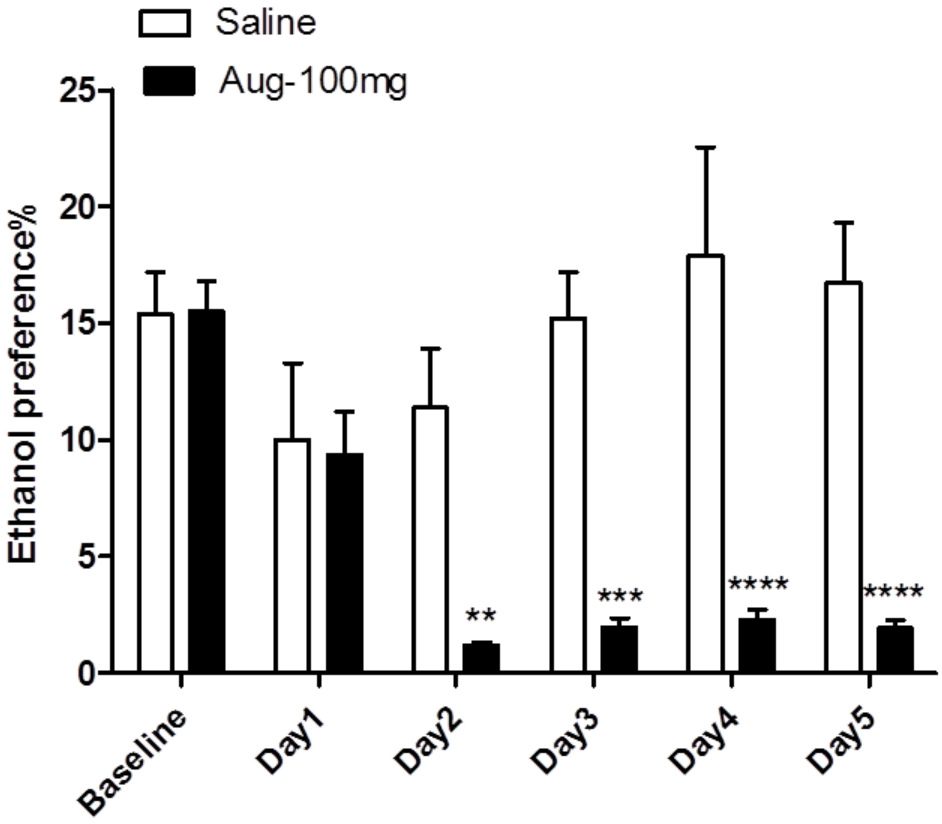


Figure (5-4) <u>Effect of oral Augmentin on percent of ethanol preference in male P rats</u>.

Two-way ANOVA demonstrated a significant difference of ethanol intake between treatment and control groups. Bonferroni multiple comparisons test following two-way ANOVA test revealed a significant decrease in ethanol preference (%) in oral Augmentin group from day 2 through the end of study as compared to the ethanol vehicle (saline) group. Data are expressed as mean ± SEM. (**p<0.01;***p<0.001;****p<0.0001); (Ethanol vehicle (saline), n=7; Ethanol Augmentin, n=7).

Chapter 4

Discussion

It has been shown that β-lactam antibiotics upregulated GLT-1 expression level in the brain (Rothstein et al., 2005, Alhaddad et al., 2014). Ceftriaxone has been found to increase GLT-1 expression level (Sari et al., 2011, Sondheimer and Knackstedt, 2011, Qrunfleh et al., 2013). It was demonstrated that alcohol consumption can be attenuated with ceftriaxone and other β-lactam antibiotics (Sari et al., 2013c, Rao et al., 2015). Additionally, several studies have demonstrated the role of xCT in controlling the extracellular glutamate concentrations (Moran et al., 2005). The role of other glutamate transporters such as xCT and GLAST in alcohol addiction has been investigated recently in our laboratory. xCT has already been suggested as a glutamate transporter with an essential role in alcohol dependence (Alhaddad et al., 2014). xCT has a crucial role in controlling the extracellular glutamate concentrations in both NAc and PFC (Baker et al., 2002b, MORAN et al., 2003). Several studies have demonstrated the important role of xCT on reinstatement to cocaine and nicotine abuse as well as seeking behavior (Baker et al., 2003, Knackstedt et al., 2009). These findings propose the potential role of xCT for the treatment of alcohol dependence.

The aim of this thesis was to test the effect of β-lactam antibiotics on the other glutamate transporters (xCT and GLAST). We also aimed to investigate the possible role of β-lactamase inhibitor (clavulanic acid) on ethanol consumption in male P rats. Furthermore, in order to determine the effect of clavulanic acid administration on glutamate transporter expression levels in NAc, we also aimed to determine whether oral treatment with Augmentin have any effect in reducing alcohol consumption in male P rats.

4.1. Amoxicillin and Augmentin Study

We have previously reported that amoxicillin and Augmentin attenuated ethanol intake in male P rats. In addition, western blot statistical analysis revealed an upregulation of GLT-1 expression level after administration of amoxicillin and Augmentin. Treatment with these compounds showed a significant upregulation of GLT-1 expression level in NAc as compare to ethanol vehicle group, however, Augmentin but not amoxicillin treatment upregulated GLT-1 expression level in PFC (Goodwani, 2014).

In this study, we also reported that the effect of chronic alcohol consumption on xCT and GLAST expression levels. In NAc and PFC, we reported that chronic alcohol consumption was found to downregulate xCT expression level in ethanol vehicle group as compared to ethanol naïve group. Interestingly, recent studies have shown a decrease

of xCT expression level after chronic cocaine self-administration (Knackstedt et al., 2010, Trantham-Davidson et al., 2012). Furthermore, studies from our laboratory have demonstrated that xCT expression level was significantly downregulated after chronic alcohol consumption in both NAc and PFC (Alhaddad et al. 2014a).

Amoxicillin showed a significant increase of xCT expression level in NAc comparing to ethanol vehicle group, however, there was no significant effect of amoxicillin on xCT in PFC. Additionally, Augmentin showed a significant upregulation of xCT expression level in both NAc and PFC comparing to ethanol vehicle group. These data suggest that the differential effects of Augmentin versus amoxicillin might be due to the fact that Augmentin contains clavulanic acid, which has also a β-lactam ring and may have a synergistic effect. Thus, we rationalized that upregulation in xCT expression level following Augmentin treatment in both PFC and NAc might be attributed to the presence of clavulanic acid in the formulation. Studies from our laboratory also demonstrated the effect of ceftriaxone on the upregulation of xCT expression level and attenuation of ethanol consumption (Alhaddad et al., 2014, Rao et al., 2015). Moreover, ceftriaxone also has been shown to increase the expression level of xCT and GLT-1 in cocaine-seeking behavior models (Knackstedt et al., 2010) (Sari et al., 2009). Our findings with Augmentin and amoxicillin may provide additional information about the important role of β-lactam in the attenuation of drug seeking behavior, including cocaine and alcohol.

Amoxicillin and Augmentin treatments revealed no significant change in GLAST expression level between ethanol vehicle group and the ethanol naïve group in both NAc and PFC. These results may support the findings that GLAST may not have an important role as a glutamate transporter in NAc and PFC. GLAST has been shown to be predominant glutamate transporter in the cerebellum (Lehre and Danbolt, 1998). GLAST also expressed in other peripheral organs such as the retina (Rauen et al., 1996, Lehre et al., 1997, Rauen and Wiessner, 2000), the inner ear (Furness and Lehre, 1997, Takumi et al., 1997), and the circumventricular organs.

This study provides convincing evidence about the efficacy of amoxicillin and Augmentin in attenuating alcohol consumption and restoring the level of GLT-1 and xCT. We have used only one dose of each compound (100 mg/kg). Thus, studies are warranted to test several doses for the determination of dose-dependent.

4.2. Clavulanic Acid Study

This study was conducted to determine whether clavulanic acid has any effect in the expression levels of GLT-1 and xCT since we have observed a better effect with Augmentin, which contains clavulanic acid, as compared to amoxicillin administered alone. Augmentin but not amoxicillin significantly upregulated GLT-1 and xCT expression levels in PFC. Clavulanic acid is a member of β-lactam antibiotics with no antibiotic activity of its own; and has no therapeutic value as an antibacterial compound. We report here that administration of clavulanic acid attenuates ethanol consumption in male P rats. The effect was observed 24 hours after the first administered dose. Although

there was a trend observed with weight reduction after clavulanic acid administration, the reduction was not significant as compared to the ethanol vehicle group. It is noteworthy that clavulanic acid has a very safe profile with mild diarrhea as a side effect (Bucher et al., 2003, Kim et al., 2009). Thus, mild diarrhea might be responsible for the slight animal's body weight reduction in the clavulanic acid treatment group.

Interestingly, we observed an increase in the water intake following the clavulanic acid treatment as compared to the ethanol vehicle group from day 1 throughout the study. The significant increase in water intake was observed on days 2, 3 and 4 of the 5 days treatment period as compared to the saline vehicle-treated group. We propose that this increase in water intake was a compensatory mechanism associated with the decrease in ethanol consumption. Previous studies from our laboratory on other drugs that attenuate ethanol consumption have displayed similar effects on water intake (Sari and Sreemantula, 2012, Sari et al., 2013a).

Intriguingly, the attenuation in ethanol consumption was associated with a significant upregulation of GLT-1 expression level in NAc. Additionally, western blot analysis showed a significant increase in xCT expression level in clavulanic acid treatment as compared to ethanol vehicle group in NAc. Previous studies from our laboratory have demonstrated that ceftriaxone was efficient in increasing the expression levels of GLT-1 and xCT in both NAc and PFC along with reduction of ethanol consumption in P rats (Alhaddad et al., 2014, Rao et al., 2015). Thus, these findings

suggest that drugs restoring glutamate homeostasis by increasing GLT1 and xCT expression levels in NAc and PFC may be a potential therapeutic target for the treatment of alcohol dependence. Moreover, β-lactam ring is a common structure between clavulanic acid, amoxicillin as well as ceftriaxone, which believed to be the responsible site for the ceftriaxone effect in GLT-1 upregulation (Rothstein et al., 2005).

Ample findings suggest that clavulanic acid is a CNS-modulating compound (Kim et al., 2009, Huh et al., 2010, Schroeder et al., 2014) Ceftriaxone has been demonstrated as a neuroprotective compound, which has been widely studied in drug abuse models. However, patient's compliance can be limited because of its parenteral route of administration. However, clavulanic acid is orally active and stable with oral bioavailability between 64 and 75%. Studies have proved that clavulanic acid passes readily the blood brain barrier with a CSF/plasma ratio is around 0.25 making it a viable CNS drug (Nakagawa et al., 1994). It was recently reported that clavulanic acid is highly potent anxiolytic with unique neuroprotection ability, which might be a promising treatment for neurodegenerative diseases (Kim et al., 2009, Huh et al., 2010). Together, these findings provide evidence that clavulanic acid can cross the blood brain barrier and might be a potential target for different CNS disorders, including alcohol addiction.

Although the present study revealed the efficacy of clavulanic acid in attenuating ethanol consumption at a single dose, it is warranted to determine the dose-dependent effect. It is important to note that clavulanic acid has an inverted U-shaped dose-response curve, which provides unpredictable dose-response curve (Kim et al., 2009). This study revealed that doses of 100 pg, 100 µg and 10 mg showed no significant effect as compared to the vehicle control group, while doses of 10 ng and 1 µg showed significant effects as compared to the vehicle control group. Further studies are required to establish a clear dose-response relationship of the drug in the attenuation of alcohol intake as well as the effects in GLT-1 and xCT expression levels.

4.3. Augmentin Oral Gavage Study

We have also studied the effect of oral Augmentin in P rats. The 100 mg/kg dose of oral Augmentin dose significantly attenuated the alcohol consumption in male P rats. The alcohol consumption attenuation started from day 2 throughout the study. Interestingly, the decrease in alcohol consumption was associated with significant increase in the water intake in Augmentin treated group as compared to the ethanol vehicle group starting on day 2 throughout the study. We suggest that this increase in water intake in Augmentin treated group was a compensatory mechanism associated with the reduction in ethanol intake. Furthermore, oral administration of Augmentin didn't reveal any significant change in rat's body weight as compared to saline vehicle-treated group. However, Augmentin administered i.p. reduced body weight significantly as compared to ethanol vehicle group (Goodwani, 2014). It might due to the fact that the

bioavailability of oral administration of Augmentin is lower than the i.p injection. Thus, studies are warranted this differential effect as well as whether there are any effect in the expression levels of GLT-1 and xCT.

In summary, these studies demonstrated the effects of amoxicillin and Augmentin on glutamate transporters, including xCT and GLAST and its association with the reduction on ethanol consumption in male P rats. Furthermore, we revealed the effect of clavulanic acid treatment on reducing ethanol consumption in P rats. This attenuation was associated with GLT-1 and xCT upregulation in NAc. In addition, we report here that oral administration of Augmentin reduced alcohol consumption in male P rats. These findings provide information about the efficacy of amoxicillin, Augmentin and clavulanic acid on attenuating ethanol consumption and restoring the expression levels of glutamate transporters. Together, these suggest the potential therapeutic role of these compounds in the treatment of alcohol dependence.

References

Adelmann G, Jonas P, Monyer H (1999) Ionotropic glutamate receptors in the CNS: Springer.

Alhaddad H, Das SC, Sari Y (2014) Effects of ceftriaxone on ethanol intake: a possible role for xCT and GLT-1 isoforms modulation of glutamate levels in P rats. Psychopharmacology 231:4049-4057.

Arriza JL, Eliasof S, Kavanaugh MP, Amara SG (1997) Excitatory amino acid transporter 5, a retinal glutamate transporter coupled to a chloride conductance. Proceedings of the National Academy of Sciences 94:4155-4160.

Arriza JL, Fairman WA, Wadiche JI, Murdoch GH, Kavanaugh MP, Amara SG (1994) Functional comparisons of three glutamate transporter subtypes cloned from human motor cortex. The Journal of neuroscience 14:5559-5569.

Aspin MM, Hoberman A, McCarty J, McLinn SE, Aronoff S, Lang DJ, Arrieta A (1994) Comparative study of the safety and efficacy of clarithromycin and amoxicillin-clavulanate in the treatment of acute otitis media in children. The Journal of pediatrics 125:136-141.

Atlante A, Calissano P, Bobba A, Giannattasio S, Marra E, Passarella S (2001) Glutamate neurotoxicity, oxidative stress and mitochondria. FEBS letters 497:1-5.

Baker D, Shen H, Kalivas P (2002a) Cystine/glutamate exchange serves as the source for extracellular glutamate: modifications by repeated cocaine administration. Amino acids 23:161-162.

Baker DA, McFarland K, Lake RW, Shen H, Tang X-C, Toda S, Kalivas PW (2003) Neuroadaptations in cystine-glutamate exchange underlie cocaine relapse. Nature neuroscience 6:743-749.

Baker DA, Xi Z-X, Shen H, Swanson CJ, Kalivas PW (2002b) The origin and neuronal function of in vivo nonsynaptic glutamate. The Journal of neuroscience 22:9134-9141.

Bannai S (1986) Exchange of cystine and glutamate across plasma membrane of human fibroblasts. The Journal of biological chemistry 261:2256-2263.

Bannai S, Kitamura E (1980) Transport interaction of L-cystine and L-glutamate in human diploid fibroblasts in culture. Journal of Biological Chemistry 255:2372-2376.

Basavarajappa BS, Hungund BL (2005) Role of the endocannabinoid system in the development of tolerance to alcohol. Alcohol and alcoholism 40:15-24.

Beart P, O'shea R (2007) Transporters for L-glutamate: An update on their molecular pharmacology and pathological involvement. British journal of pharmacology 150:5-17.

Bell RL, Rodd ZA, Lumeng L, Murphy JM, McBride WJ (2006) REVIEW: The alcohol-preferring P rat and animal models of excessive alcohol drinking. Addiction biology 11:270-288.

Berger UV, Hediger MA (2000) Distribution of the glutamate transporters GLAST and GLT-1 in rat circumventricular organs, meninges, and dorsal root ganglia. The Journal of comparative neurology 421:385-399.

Bucher HC, Tschudi P, Young J, Périat P, Welge-Lüssen A, Züst H, Schindler C (2003) Effect of amoxicillin-clavulanate in clinically diagnosed acute rhinosinusitis: a placebo-controlled, double-blind, randomized trial in general practice. Archives of internal medicine 163:1793-1798.

Burdo J, Dargusch R, Schubert D (2006) Distribution of the Cystine/Glutamate Antiporter System x− c in the Brain, Kidney, and Duodenum. Journal of Histochemistry & Cytochemistry 54:549-557.

Castaldo P, Magi S, Gaetani S, Cassano T, Ferraro L, Antonelli T, Amoroso S, Cuomo V (2007) Prenatal exposure to the cannabinoid receptor agonist WIN 55,212-2 increases glutamate uptake through overexpression of GLT1 and EAAC1 glutamate transporter subtypes in rat frontal cerebral cortex. Neuropharmacology 53:369-378.

Cicero TJ (1979) A critique of animal analogues of alcoholism. Biochemistry and pharmacology of ethanol 2:533-560.

Crosby M, Gump D (1982) Activity of cefoperazone and two beta-lactamase inhibitors, sulbactam and clavulanic acid, against Bacteroides spp. correlated with beta-lactamase production. Antimicrobial agents and chemotherapy 22:398-405.

Danbolt N, Storm-Mathisen J, Kanner B (1992) An [Na++ K+] coupledl-glutamate transporter purified from rat brain is located in glial cell processes. Neuroscience 51:295-310.

Danbolt NC (2001) Glutamate uptake. Progress in neurobiology 65:1-105.

Davies B, Coates P, Clarke J, Thawley A, Sutton J (1985) Bioavailability and pharmacokinetics of clavulanic acid in healthy subjects. International journal of clinical pharmacology, therapy, and toxicology 23:70-73.

Diana M, Brodie M, Muntoni A, Puddu MC, Pillolla G, Steffensen S, Spiga S, Little HJ (2003) Enduring effects of chronic ethanol in the CNS: basis for alcoholism. Alcoholism, clinical and experimental research 27:354-361.

Dringen R (2000) Metabolism and functions of glutathione in brain. Progress in neurobiology 62:649-671.

Fairman W, Vandenberg R, Arriza J, Kavanaught M, Amara S (1995) An excitatory amino-acid transporter with properties of a ligand-gated chloride channel.

Furness DN, Lehre KP (1997) Immunocytochemical localization of a high-affinity glutamate-aspartate transporter, GLAST, in the rat and guinea-pig cochlea. The European journal of neuroscience 9:1961-1969.

Gass JT, Olive MF (2008) Glutamatergic substrates of drug addiction and alcoholism. Biochemical pharmacology 75:218-265.

Gegelashvili G, Schousboe A (1997) High affinity glutamate transporters: regulation of expression and activity. Molecular pharmacology 52:6-15.

Gegelashvili G, Schousboe A (1998) Cellular distribution and kinetic properties of high-affinity glutamate transporters. Brain research bulletin 45:233-238.

Goodwani S (2014) Amoxicillin and Augmentin Reduce Ethanol Intake and Increase GLT1 Expression as well as AKT Phosphorylation in Mesocorticolimbic Regions. Ohiolink.

Griffin WC, 3rd, Haun HL, Hazelbaker CL, Ramachandra VS, Becker HC (2014) Increased extracellular glutamate in the nucleus accumbens promotes excessive ethanol drinking in ethanol dependent mice. Neuropsychopharmacology 39:707-717.

Gunduz O, Oltulu C, Ulugol A (2011) Role of GLT-1 transporter activation in prevention of cannabinoid tolerance by the beta-lactam antibiotic, ceftriaxone, in mice. Pharmacology, biochemistry, and behavior 99:100-103.

Hakuba N, Koga K, Gyo K, Usami SI, Tanaka K (2000) Exacerbation of noise-induced hearing loss in mice lacking the glutamate transporter GLAST. The Journal of neuroscience : the official journal of the Society for Neuroscience 20:8750-8753.

Harada T, Harada C, Watanabe M, Inoue Y, Sakagawa T, Nakayama N, Sasaki S, Okuyama S, Watase K, Wada K, Tanaka K (1998) Functions of the two glutamate transporters GLAST and GLT-1 in the retina. Proceedings of the National Academy of Sciences of the United States of America 95:4663-4666.

Headley PM, Grillner S (1990) Excitatory amino acids and synaptic transmission: the evidence for a physiological function. Trends in pharmacological sciences 11:205-211.

Huh Y, Ju MS, Park H, Han S, Bang YM, Ferris CF, Koppell GA, King JA, Kim ML, Kim DJ (2010) Clavulanic acid protects neurons in pharmacological models of neurodegenerative diseases. Drug Development Research 71:351-357.

Jabaudon D, Shimamoto K, Yasuda-Kamatani Y, Scanziani M, Gahwiler BH, Gerber U (1999) Inhibition of uptake unmasks rapid extracellular turnover of glutamate of nonvesicular origin. Proceedings of the National Academy of Sciences of the United States of America 96:8733-8738.

Johnston G (1981) Glutamate uptake and its possible role in neurotransmitter inactivation. Glutamate: Transmitter in the Central Nervous System 77-87.

Kanai Y, Hediger MA (1992) Primary structure and functional characterization of a high-affinity glutamate transporter.

Kaye CM, Allen A, Perry S, McDonagh M, Davy M, Storm K, Bird N, Dewit O (2001) The clinical pharmacokinetics of a new pharmacokinetically enhanced formulation of amoxicillin/clavulanate. Clinical therapeutics 23:578-584.

Kim DJ, King JA, Zuccarelli L, Ferris CF, Koppel GA, Snowdon CT, Ahn CH (2009) Clavulanic acid: a competitive inhibitor of beta-lactamases with novel anxiolytic-like activity and minimal side effects. Pharmacology Biochemistry and Behavior 93:112-120.

Knackstedt LA, LaRowe S, Mardikian P, Malcolm R, Upadhyaya H, Hedden S, Markou A, Kalivas PW (2009) The role of cystine-glutamate exchange in nicotine dependence in rats and humans. Biological psychiatry 65:841-845.

Lee MR, Ruby CL, Hinton DJ, Choi S, Adams CA, Kang NY, Choi D-S (2013) Striatal adenosine signaling regulates EAAT2 and astrocytic AQP4 expression and alcohol drinking in mice. Neuropsychopharmacology 38:437-445.

Lehre KP, Danbolt NC (1998) The number of glutamate transporter subtype molecules at glutamatergic synapses: chemical and stereological quantification in young adult rat brain. The Journal of neuroscience : the official journal of the Society for Neuroscience 18:8751-8757.

Lehre KP, Davanger S, Danbolt NC (1997) Localization of the glutamate transporter protein GLAST in rat retina. Brain research 744:129-137.

Lehre KP, Levy LM, Ottersen OP, Storm-Mathisen J, Danbolt NC (1995) Differential expression of two glial glutamate transporters in the rat brain: quantitative and immunocytochemical observations. The Journal of neuroscience : the official journal of the Society for Neuroscience 15:1835-1853.

Leshner AI (1997) Addiction is a brain disease, and it matters. Science 278:45-47.

Lester D, Freed EX (1973) Criteria for an animal model of alcoholism. Pharmacology Biochemistry and Behavior 1:103-107.

Li T-K, Lumeng L, McBride WJ, Murphy JM (1986) Rodent lines selected for factors affecting alcohol consumption. Alcohol and alcoholism (Oxford, Oxfordshire) Supplement 1:91-96.

Logan WJ, Snyder SH (1972) High affinity uptake systems for glycine, glutamic and aspaspartic acids in synaptosomes of rat central nervous tissues. Brain research 42:413-431.

Lumeng L, Hawkins T, Li T (1977) New strains of rats with alcohol preference and nonpreference. Alcohol and aldehyde metabolizing systems 3:537-544.

Makowske M, Christensen HN (1982) Contrasts in transport systems for anionic amino acids in hepatocytes and a hepatoma cell line HTC. Journal of Biological Chemistry 257:5663-5670.

McKenna MC (2007) The glutamate-glutamine cycle is not stoichiometric: fates of glutamate in brain. Journal of neuroscience research 85:3347-3358.

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

Miura K, Ishii T, Sugita Y, Bannai S (1992) Cystine uptake and glutathione level in endothelial cells exposed to oxidative stress. American Journal of Physiology-Cell Physiology 262:C50-C58.

Moran MM, McFarland K, Melendez RI, Kalivas PW, Seamans JK (2005) Cystine/glutamate exchange regulates metabotropic glutamate receptor presynaptic inhibition of excitatory transmission and vulnerability to cocaine seeking. The Journal of neuroscience 25:6389-6393.

MORAN MM, MELENDEZ R, BAKER D, KALIVAS PW, SEAMANS JK (2003) Cystine/glutamate antiporter regulation of vesicular glutamate release. Annals of the New York Academy of Sciences 1003:445-447.

Murphy T, Schnaar R, Coyle J (1990) Immature cortical neurons are uniquely sensitive to glutamate toxicity by inhibition of cystine uptake. The FASEB Journal 4:1624-1633.

Murphy TH, Miyamoto M, Sastre A, Schnaar RL, Coyle JT (1989) Glutamate toxicity in a neuronal cell line involves inhibition of cystine transport leading to oxidative stress. Neuron 2:1547-1558.

Nakagawa H, Yamada M, Tokiyoshi K, Miyawaki Y, Kanayama T (1994) [Penetration of potassium clavulanate/ticarcillin sodium into cerebrospinal fluid in neurosurgical patients]. The Japanese journal of antibiotics 47:93-101.

Nakagawa T, Fujio M, Ozawa T, Minami M, Satoh M (2005) Effect of MS-153, a glutamate transporter activator, on the conditioned rewarding effects of morphine, methamphetamine and cocaine in mice. Behavioural brain research 156:233-239.

Neu HC, Wilson A, Grüneberg R (1993) Amoxycillin/clavulanic acid: a review of its efficacy in over 38,500 patients from 1979 to 1992. Journal of chemotherapy (Florence, Italy) 5:67-93.

Ohtsuka Y, Kondo T, Kawakami Y (1988) Oxidative stresses induced the cystine transport activity in human erythrocytes. Biochemical and biophysical research communications 155:160-166.

Paxinos G, Watson C (1997) The rat brain in stereotaxic coordinates, Compact. San Diego: Academic Press Figure 1:25-30.

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

Rao PS, Sari Y (2012) Glutamate transporter 1: target for the treatment of alcohol dependence. Current medicinal chemistry 19:5148-5156.

Rao PS, Saternos H, Goodwani S, Sari Y (2015) Effects of ceftriaxone on GLT1 isoforms, xCT and associated signaling pathways in P rats exposed to ethanol. Psychopharmacology (Berl).

Rauen T, Rothstein JD, Wassle H (1996) Differential expression of three glutamate transporter subtypes in the rat retina. Cell and tissue research 286:325-336.

Rauen T, Wiessner M (2000) Fine tuning of glutamate uptake and degradation in glial cells: common transcriptional regulation of GLAST1 and GS. Neurochemistry international 37:179-189.

Robinson TE, Berridge KC (1993) The neural basis of drug craving: an incentive-sensitization theory of addiction. Brain research Brain research reviews 18:247-291.

Rosenberg PA, Aizenman E (1989) Hundred-fold increase in neuronal vulnerability to glutamate toxicity in astrocyte-poor cultures of rat cerebral cortex. Neuroscience letters 103:162-168.

Roskoski R, Jr. (1979) Net uptake of aspartate by a high-affinity rat cortical synaptosomal transport system. Brain research 160:85-93.

Rothstein J (1994) Excitotoxicity and neurodegeneration in amyotrophic lateral sclerosis. Clinical neuroscience (New York, NY) 3:348-359.

Rothstein JD, Martin L, Levey AI, Dykes-Hoberg M, Jin L, Wu D, Nash N, Kuncl RW (1994) Localization of neuronal and glial glutamate transporters. Neuron 13:713-725.

Rothstein JD, Martin LJ, Kuncl RW (1992) Decreased glutamate transport by the brain and spinal cord in amyotrophic lateral sclerosis. New England Journal of Medicine 326:1464-1468.

Rothstein JD, Patel S, Regan MR, Haenggeli C, Huang YH, Bergles DE, Jin L, Hoberg MD, Vidensky S, Chung DS (2005) β-Lactam antibiotics offer neuroprotection by increasing glutamate transporter expression. Nature 433:73-77.

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

Sari Y (2013) Potential therapeutic role of glutamate transporter 1 for the treatment of alcohol dependence. OA alcohol 1:6.

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

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

Sari Y, Smith KD, Ali PK, Rebec GV (2009) Upregulation of GLT1 attenuates cue-induced reinstatement of cocaine-seeking behavior in rats. The Journal of neuroscience : the official journal of the Society for Neuroscience 29:9239-9243.

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

Sari Y, Sreemantula SN, Lee MR, Choi D-S (2013b) Ceftriaxone treatment affects the levels of GLT1 and ENT1 as well as ethanol intake in alcohol-preferring rats. Journal of Molecular Neuroscience 51:779-787.

Sari Y, Sreemantula SN, Lee MR, Choi DS (2013c) Ceftriaxone treatment affects the levels of GLT1 and ENT1 as well as ethanol intake in alcohol-preferring rats. Journal of molecular neuroscience : MN 51:779-787.

Sato H, Tamba M, Okuno S, Sato K, Keino-Masu K, Masu M, Bannai S (2002) Distribution of cystine/glutamate exchange transporter, system xc−, in the mouse brain. The Journal of neuroscience 22:8028-8033.

Schroeder JA, Tolman NG, McKenna FF, Watkins KL, Passeri SM, Hsu AH, Shinn BR, Rawls SM (2014) Clavulanic acid reduces rewarding, hyperthermic and locomotor-sensitizing effects of morphine in rats: A new indication for an old drug? Drug and alcohol dependence 142:41-45.

Sheldon AL, Robinson MB (2007) The role of glutamate transporters in neurodegenerative diseases and potential opportunities for intervention. Neurochemistry international 51:333-355.

Sims K, Robinson M (1998) Expression patterns and regulation of glutamate transporters in the developing and adult nervous system. Critical reviews in neurobiology 13:169-197.

Sondheimer I, Knackstedt LA (2011) Ceftriaxone prevents the induction of cocaine sensitization and produces enduring attenuation of cue-and cocaine-primed reinstatement of cocaine-seeking. Behavioural brain research 225:252-258.

Steinhauser C, Gallo V (1996) News on glutamate receptors in glial cells. Trends in neurosciences 19:339-345.

Storck T, Schulte S, Hofmann K, Stoffel W (1992) Structure, expression, and functional analysis of a Na (+)-dependent glutamate/aspartate transporter from rat brain. Proceedings of the National Academy of Sciences 89:10955-10959.

Szatkowski M, Barbour B, Attwell D (1991) The potassium-dependence of excitatory amino acid transport: resolution of a paradox. Brain research 555:343-345.

Takumi Y, Matsubara A, Danbolt NC, Laake JH, Storm-Mathisen J, Usami S, Shinkawa H, Ottersen OP (1997) Discrete cellular and subcellular localization of glutamine synthetase and the glutamate transporter GLAST in the rat vestibular end organ. Neuroscience 79:1137-1144.

Tan S, Schubert D, Maher P (2001) Oxytosis: a novel form of programmed cell death. Current topics in medicinal chemistry 1:497-506.

Tanaka K, Watase K, Manabe T, Yamada K, Watanabe M, Takahashi K, Iwama H, Nishikawa T, Ichihara N, Kikuchi T (1997) Epilepsy and exacerbation of brain injury in mice lacking the glutamate transporter GLT-1. Science 276:1699-1702.

Tessler S, Danbolt N, Faull R, Storm-Mathisen J, Emson P (1999) Expression of the glutamate transporters in human temporal lobe epilepsy. Neuroscience 88:1083-1091.

Tzingounis AV, Wadiche JI (2007) Glutamate transporters: confining runaway excitation by shaping synaptic transmission. Nature Reviews Neuroscience 8:935-947.

Volterra A, Bezzi P, Rizzini BL, Trotti D, Ullensvang K, Danbolt NC, Racagni G (1996) The competitive transport inhibitor L-trans-pyrrolidine-2, 4-dicarboxylate triggers excitotoxicity in rat cortical neuron-astrocyte co-cultures via glutamate release rather than uptake inhibition. European Journal of Neuroscience 8:2019-2028.

Watanabe T, Morimoto K, Hirao T, Suwaki H, Watase K, Tanaka K (1999) Amygdala-kindled and pentylenetetrazole-induced seizures in glutamate transporter GLAST-deficient mice. Brain research 845:92-96.

Watase K, Hashimoto K, Kano M, Yamada K, Watanabe M, Inoue Y, Okuyama S, Sakagawa T, Ogawa S, Kawashima N, Hori S, Takimoto M, Wada K, Tanaka K (1998) Motor discoordination and increased susceptibility to cerebellar injury in GLAST mutant mice. The European journal of neuroscience 10:976-988.

White AR, Kaye C, Poupard J, Pypstra R, Woodnutt G, Wynne B (2004) Augmentin (amoxicillin/clavulanate) in the treatment of community-acquired respiratory tract infection: a review of the continuing development of an innovative antimicrobial agent. Journal of Antimicrobial Chemotherapy 53:i3-i20.

Wüst J, Wilkins TD (1978) Effect of clavulanic acid on anaerobic bacteria resistant to beta-lactam antibiotics. Antimicrobial agents and chemotherapy 13:130-133.