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Amoxicillin and Augmentin Reduce Ethanol Intake and Increase GLT1 Expression as well as AKT Phosphorylation in Mesocorticolimbic Regions

By

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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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The University of Toledo.
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An Abstract of

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Pharmacological upregulation of glutamate transporter 1 (GLT1), a principal mediator of glutamate reuptake, in mesocorticolimbic regions of the brain is associated with reduced drug and ethanol self-administration. It has been shown that administration of the beta-lactam antibiotic, ceftriaxone (CEF) attenuated ethanol and cocaine seeking behaviors as well as prevented ethanol-induced downregulation of GLT1 levels in central reward brain regions. However, it is not known if these effects are compound-specific. Therefore, the present study examined the effects of two other beta-lactam antibiotics, amoxicillin (AMOX) and Amoxicillin/Clavulanate (Augmentin, AUG), on ethanol drinking, as well as GLT1 and phosphorylated-AKT (pAKT) expression levels in the nucleus accumbens (Acb) and prefrontal cortex (PFC) of alcohol-preferring (P) rats. P rats were exposed to free-choice of ethanol (15% and 30%) for five weeks and were given five consecutive daily i.p. injections of saline vehicle, 100 mg/kg AMOX or 100 mg/kg AUG. Both compounds significantly decreased ethanol intake and significantly increased GLT1 expression in the Acb. AUG also increased GLT1 expression in the PFC. Results for changes in pAKT levels matched those for GLT1, indicating that beta-lactam antibiotic-
induced reductions in ethanol intake are negatively associated with increases in GLT1 and pAKT levels within two critical brains regions mediating drug reward and reinforcement. These findings add to a growing literature that pharmacological increases in GLT1 expression are associated with decreases in ethanol, as well as drug, intake and suggest that one mechanism mediating this effect may be increased phosphorylation of AKT. Thus, GLT1 may serve as molecular targets for the treatment of alcohol and drug abuse.

Key words: GLT1, Amoxicilllin, Augmentin, pAKT
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List of Abbreviations

Acb .............................. Nucleus Accumbens
AMG ............................ Amygdala
AMOX .......................... Amoxicillin
ANOVA ........................ Analysis of Variance
ALDH2 .......................... Aldehyde Dehydrogenase 2
AUG ............................ Augmentin, Amoxicillin/Clavulanate
BBB ............................. Blood Brain Barrier
CEF .............................. Ceftriaxone
CNS ............................. Central Nervous System
CSF ............................. Cerebrospinal Fluid
GLM ............................. General Linear Model
GLT1 ............................ Glutamate Transporter 1
HPLC ............................ High-Performance Liquid Chromatography
NADH ........................... Nicotinamide Adenine Dinucleotide
NMTT ............................ N-Methylthiotetrazole
pAKT ............................ Phosphorylated-AKT
PFC ............................. Prefrontal Cortex
VTA …………………………..Ventral Tegmental Area
Chapter 1

Introduction

1.1 Overview

Glutamatergic neurotransmission in key mesocorticolimbic brain regions, such as the prefrontal cortex (PFC) (Goldstein and Volkow, 2002) and the nucleus accumbens (Acb) (Childress et al., 1999, Obara et al., 2009), plays a key role in the development of ethanol and drug dependence. For example, clinical neuroimaging studies performed during periods of craving for commonly abused drugs such as ethanol, heroin, methamphetamine, cocaine and nicotine revealed the importance of glutamatergic projections from the PFC to the Acb and the ventral-tegmental area (VTA) (Childress et al., 1999, Goldstein and Volkow, 2002). In preclinical studies, ethanol consumption increased extracellular glutamate levels in several mesocorticolimbic brain regions (Moghaddam and Bolinao, 1994, Dahchour et al., 2000, Melendez et al., 2005, Szuminski et al., 2007, Ding et al., 2012, Ding et al., 2013). Extracellular glutamate levels are regulated by several glutamate transporters (Gegelashvili and Schousboe, 1997, Seal and Amara, 1999, Anderson and Swanson, 2000, Danbolt, 2001). Of these,
glutamate transporter 1 (GLT1, a sodium dependent astro-glial protein) is responsible for 90% of glutamate reuptake from the synapse (Rothstein et al., 1996, Danbolt, 2001, Holmseth et al., 2012). It is noteworthy that a constituent GLT1 gene knockout reduced glutamate reuptake by approximately 90% (Tanaka et al., 1997, Otis and Kavanaugh, 2000, Kiryk et al., 2008). Impaired glutamate uptake or down-regulation of GLT1 expression has been implicated in the development of drug abuse using various animal models (Xu et al., 2003, Nakagawa and Satoh, 2004, Knackstedt et al., 2009, Sari et al., 2009, Knackstedt et al., 2010, Sari et al., 2011, Rao and Sari, 2012, Sari and Sreemantula, 2012, Sari et al., 2013a, Sari et al., 2013b).

Pharmacological upregulation of GLT1 levels in mesocorticolimbic brain regions can be induced by CEF, a beta-lactam antibiotic. For instance, CEF increased mesocorticolimbic GLT1 gene and/or protein expression and concomitantly attenuated (a) reinstatement to cocaine-seeking behavior (Sari et al., 2009, Knackstedt et al., 2010, Fischer et al., 2013), (b) relapse to methamphetamine self-administration (Abulseoud et al., 2012) as well as (c) ethanol drinking in alcohol-preferring (P) rats (Sari et al., 2011, Sari et al., 2013a, Sari et al., 2013b). Additionally, we and others have identified a compound GPI-1046 (3-(3-pyridyl)-1-propyl (2S)-1-(3,3-dimethyl-1,2-dioxopentyl)-2-pyrrolidinecarboxylate), a neuroimmunophilin, that increases GLT1 expression in the brain (Ganel et al., 2006, Sari and Sreemantula, 2012) and concomitantly decreases ethanol consumption in male P rats (Sari and Sreemantula, 2012). These findings suggest that other drugs upregulating central GLT1 expression may serve as alternative treatments for drug abuse and dependence. However, only CEF and GPI-1046 have undergone systematic evaluation thus far.
1.2 Aims and Objectives

Rothstein et al. (2005) screened 1,040 compounds and found that β-lactam antibiotics were the most potent stimulators of GLT1 expression. Interestingly, like CEF, amoxicillin was one of the compounds screened and found to be proficient in upregulating GLT1 expression (Rothstein et al., 2005). Therefore in the present study, we investigated the effect of amoxicillin (AMOX) (Figure 1-1) and amoxicillin/clavulanate (augmentin, AUG) (Figure 1-2) on ethanol intake in male P rats. The effect of AMOX and AUG on sucrose intake as an appetitive control for drinking-motivated behavior in male P rats was also examined. Furthermore, we determined the effects of treatment with these drugs on GLT1 expression in the PFC and Acb. We then determined the effects of AMOX and AUG on pAKT expression, as a signaling pathway involved in regulating GLT1 expression (Wu et al., 2010). Additionally, in order to determine whether AMOX or AUG has or does not have a Disulfiram-like actions, liver mitochondrial aldehyde dehydrogenase (ALDH2) activity was analyzed after treatment with each of these two compounds.

![Chemical Structure of Amoxicillin](image)

**Figure 1-1** Chemical Structure of Amoxicillin
Figure 1-2 Chemical Structure of Amoxicillin + Clavulanic Acid
Chapter 2

Materials and Methods

2.1. Chemicals

Amoxicillin and Augmentin were procured from GlaxoSmithKline, France.

2.2. Animals

Adult male P rats were obtained from Indiana University School of Medicine, Indianapolis, IN at the age of 21-30 days and housed in the Department of Laboratory Animal Resources at the University of Toledo. All animals were housed in standard plastic tubs with corn-cob bedding and had ad lib access to food and water throughout the experimental procedures. Animal vivaria were maintained at a temperature of 21°C with 50% relative humidity on a 12-hour light/dark cycle (0700h/1900h). All of the experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of Toledo 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). The animal housing and testing areas are fully
accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, International.

At the age of three months, P rats were single housed in bedded plastic cages, and randomly separated into four different groups (a) Ethanol naïve vehicle group (water control) received i.p. injections of saline vehicle solution (n=6); (b) Ethanol vehicle group (ethanol control) received i.p. injections of saline vehicle solution (n=12); (c) Ethanol amoxicillin group (AMOX group) which received 100 mg/kg, i.p. injections of amoxicillin (n=12); and (d) Ethanol augmentin group (AUG group) which received 100 mg/kg, i.p. injections of augmentin (n=12). Ethanol control, ethanol AMOX and ethanol AUG groups had free access to water, 15% and 30% ethanol and food throughout the experimental procedure. We also tested the effect of AUG (100 mg/kg, i.p.) on sucrose intake as compared to a vehicle treated group (saline vehicle, i.p.).

2.3 Ethanol Drinking Paradigm

At three months of age, male P rats were given free-choice access to ethanol (concurrently 15% and 30%, v/v), water and food ad lib for five weeks. Ethanol consumption for each animal was measured as grams of ethanol consumed per kilogram of body weight per day. Ethanol and water intakes as well as body weight were measured three times a week. The amount of ethanol and water consumed were determined to the nearest tenth of a gram by subtraction of the measured bottle weights from their initial weights containing water or ethanol. A densitometry formula was used to convert actual grams of ethanol consumed per kilogram of body weight of animals (i.e., corrected for
the specific gravity of ethanol). All the animals were required to meet the criterion of ≥4 g/kg/day ethanol consumption for at least 2 weeks before saline vehicle or drug treatment to be included in the study. This criterion of ethanol consumption of ≥ 4 g/kg/day was based on our previous studies reporting the development of dependence to ethanol (Sari and Sreemantula, 2012, Sari et al., 2013b). Figure 2-1 illustrates the timeline of chronic ethanol drinking paradigm with dosing regimen. The average of the ethanol consumption for the two weeks prior to drug or vehicle treatment was taken as baseline drinking.

During week 6, P rats were injected with AMOX (100 mg/kg, i.p.), AUG (100 mg/kg, i.p) or vehicle (0.9% saline, i.p.) daily for five consecutive days. During the sixth week, ethanol and water consumption were measured daily along with the body weight of the animals.

Figure 2-1 Timeline for Ethanol Drinking Paradigm.

2.4 Sucrose Drinking Paradigm

Augmentin’s effects on sucrose (10%) intake were also examined, as an appetitive control for drinking-motivated behavior. Figure 2-2 illustrates the sucrose drinking procedure with the treatment regimen. A continuous free-choice access to 10%
sucrose solution, water and food was given to male P rats throughout the study. The animals were divided into two groups: (a) Saline vehicle (control) group which was injected with saline, i.p. and (b) Augmentin group which was injected with 100mg/kg of AUG, i.p. Starting on day 13 both groups received their respective treatments for four consecutive days. Baseline sucrose intake was taken as average sucrose intake on days 11 and day 12. Body weight of the animals, sucrose and water intakes were measured daily starting 2 days prior to injections i.e. day 11 through the end of the study. After the last injection the animals were given a period of (drug) washout for 3 days post-treatment.

![Timeline for Sucrose Drinking Paradigm.](image)

**Figure 2-2** Timeline for Sucrose Drinking Paradigm.

### 2.5 Brain Tissue Harvesting

Ethanol-drinking animals were euthanized 24 hours after receiving their last injection by CO₂ inhalation and rapidly decapitated with a guillotine. The brains were dissected and flash-frozen and stored at -70°C. Stereotaxic microdissections were carried out using a cryostat to isolate the PFC and Acb as described recently (Sari and Sreemantula, 2012). Stereotaxic coordinates for the identification of PFC and Acb in the rat brain were obtained from Paxinos and Watson’s Stereotaxic Atlas (2007). The PFC (medial) and Acb were isolated at the same level, using surgical blades through
visualized landmarks. These extracted brain regions were then frozen at -70°C for western blot procedures for detecting GLT1, pAKT, Akt and β-tubulin.

2.6 Western Blot Protocol

Isolated PFC and Acb brain regions from the male P rats were analyzed for changes in GLT1 and pAKT relative to total-Akt and β-tubulin using the Western Blot assay. PFC and Acb samples were homogenized in sodium dodecyl sulfate lysis buffer for detecting GLT1 and lysis buffer containing phosphatase inhibitor for pAKT and total-Akt (Ericsson and Nister, 2011, Pisu et al., 2011). Proteins were extracted and quantified using Bio-Rad DC protein reagents (Bio-Rad, Hercules, CA). After mixing equal amounts of extracted protein with 5x Laemmli loading dye, the proteins were further separated by electrophoresis (at 200 V) on 10-20% tris-glycine gel (Life Technologies, Grand Island, NY, USA). The proteins were then transferred on a PVDF membrane (Bio-Rad, Hercules, CA) electrophoretically. The membranes containing protein were blocked using 3% milk in TBST (50 mM Tris HCl; 150 mM Acbl, pH7.4; 0.1% Tween20) for 30 minutes at room temperature. The membranes were then incubated overnight at 4°C with one of the following antibodies: guinea pig-anti GLT1 antibody (1:5000; Millipore), rabbit pAKT (1:5000; Cell Signaling Technology), and mouse anti-Akt (1:5000; Cell Signaling Technology). Mouse anti β-tubulin antibody was used to assess equivalent protein loading (loading control). Membranes were then washed and incubated with horseradish peroxidase (HRP) donkey-anti-Guinea pig IgG (H+L) secondary antibody (1:5000), anti-mouse IgG, HRP-linked secondary antibody (1:5000), or anti-rabbit IgG, HRP-linked secondary antibody (1:5000). After incubation with HRP Chemiluminescent
kit (SuperSignal West Pico, Pierce Inc.), membranes were exposed to Kodak BioMax MR film (Fisher Inc.), and the films were developed on a SRX-101A machine. Digitized images of immunoreactive proteins were quantified using the MCID system (GE Healthcare Niagara Inc., US). The data are presented as percentage ratios of GLT1/β-tubulin and pAKT/total-Akt, relative to ethanol vehicle control (100% control-value) levels.

2.7 Aldehyde Dehydrogenase 2 (ALDH2) Enzyme Assay

The pharmacological attenuation of ethanol consumption might also be attributed to the Disulfiram-like effect of the drug, which prevents the conversion of acetaldehyde to acetate through inhibition of aldehyde dehydrogenases. Type 2 aldehyde dehydrogenase (ALDH2) enzyme is accountable for 60% of metabolism of acetaldehyde (Weiner, 1987). In order to determine the effect of amoxicillin and augmentin on aldehyde dehydrogenases, ALDH2 activity was determined after treating P rats for 5 consecutive days with amoxicillin or augmentin compared to ethanol vehicle treated group.

2.8.A Tissue Preparation for Aldehyde Dehydrogenase 2 Assay

P rats were euthanized 24 hours after the last injection and their liver was dissected and stored at -80°C. Liver tissue (1.2 g-1.5 g) was homogenized in 3 volumes (w/v) of ice-cold 0.25 M sucrose solution using a mechanical homogenizer. The
homogenate was then subjected to centrifugation at 700 x g for 10 min. The pellet was discarded and the supernatant was further centrifuged at 7200 x g for 20 min. The pellet obtained after this step was washed with 1.5 ml of 0.25 M sucrose solution and subjected to centrifugation at 10000 x g for 15 min. The final pellet obtained, mitochondrial fraction, was resuspended in 0.5 ml of sucrose solution containing 1% sodium deoxycholate and stored at -80 ° C until further use.

2.8.B Mitochondrial Aldehyde Dehydrogenase (mALDH2) Assay

The ALDH2 activity was analyzed using acetaldehyde as the substrate, as described previously (Karamanakos et al., 2007). The assay mixture comprised of 50 µM acetaldehyde, 75 mM sodium pyrophosphate buffer (pH 8.0), 1 mM pyrazole, and 2 µM rotenone. The NADH formation was determined spectrophotometrically at 340 nm over a 5 min time period.

2.9 Analytical Determination of AMOX and AUG in Male P Rats’ Plasma and CSF

Approximately one hour after receiving a 100 mg/kg i.p. injection of AMOX or 100 mg/kg of AUG, P rats were euthanized and blood and cerebrospinal fluid were collected and immediately frozen at -80°C for further analysis. A high-performance liquid chromatography system (HPLC) (Waters Alliance 2695 separation module,
Milford, MA) equipped with a Kinetex C18 column (250 X 4.6 mm, Phenomenex) and UV/Visible detector was used for analyses. AMOX was analyzed isocratically with a mobile phase containing phosphate buffer, pH 5 and acetonitrile (98:2) pumped at a flow rate of 1 ml/min. The retention time of AMOX ($\lambda_{\text{max}}$ = 230 nm) was found to be 7.8 minutes. Different calibration standards of AMOX were prepared in the mobile phase. For the calibration curve, each standard was analyzed in triplicate and the average peak area was plotted against the concentration. The drug content was determined quantitatively by plotting a calibration curve. The assay method was found to be linear in the range of 0.78 - 50 µg/ml with a correlation coefficient of 0.9994. The limit of detection and limit of quantification of AMOX was found to be 0.060 µg/ml and 0.20 µg/ml, respectively. The intra- and interday reliability (measured by %RSD) exceeded 98%.

2.10 Statistical Analyses

General Linear Model (GLM) Repeated Measures statistical analyses were conducted to determine significant main effects of Day or Day x Treatment interactions (day by treatment) on the body weight and ethanol, water and sucrose intake of the ethanol vehicle, ethanol AMOX and ethanol AUG groups. Significant main effect or interaction terms were followed by one-way ANOVAs and Dunnett t-test planned comparisons to determine the effects of AMOX or AUG treatment for each test day. Effects of chronic ethanol on protein expression were determined by performing independent t-tests on immunoblots comparing the ethanol-naïve vehicle group with
ethanol vehicle group initially. Effects of beta-lactam treatments on protein expression were determined by one-way ANOVAs and Newman-Keuls multiple-comparisons on the immunoblots, with the ethanol-vehicle group serving as the control. All statistical analyses were based on a p<0.05 level of significance.
Chapter 3

Results

3.1 Concentration of Amoxicillin in Plasma and CSF after Amoxicillin and Augmentin Injection

The average plasma (n=3) and CSF (n=5) concentration of AMOX after AMOX administration (i.p., 100mg/kg) was found to be 29.82±4.1 µg/mL and 352.87±20.58 ng/mL, respectively. However, the average concentration of AMOX in plasma (n=3) and CSF (n=2) after AUG administration (i.p., 100mg/kg) in P rats was found to be 7.3±0.79 µg/mL and 270.99±3.75 ng/mL, respectively.

3.2 Effects of Amoxicillin and Augmentin on Ethanol Intake in Male P Rats

Figure 3-1 illustrates effects of AMOX and AUG treatment on daily average ethanol consumption (grams/kg/day) in male P rats. Daily average ethanol intake was measured consecutively over a period of 5 days (starting 24 hours after the first injection)
in male P rats treated with saline vehicle or AMOX (100 mg/kg, i.p.) or AUG (100 mg/kg, i.p.). The baseline ethanol intake was assessed as an average ethanol intake for the last two weeks prior to saline vehicle or AMOX or AUG injections.

Statistical analysis using GLM Repeated Measures revealed a significant main effect of Day \( [F(1,5)=31.537, p<0.001] \) and significant Treatment x Day interaction \( [F(2,10)=8.698, p<0.001] \). One-way ANOVA followed by two-sided Dunnett’s post-hoc test demonstrated a significant reduction in ethanol intake in all animals treated with AMOX (100 mg/kg, i.p.; \( p<0.001 \)) or AUG (100 mg/kg, i.p.; \( p<0.001 \)) compared to ethanol vehicle animals. The attenuation in the ethanol consumption was observed starting 24 hours after the first injection.
Figure 3-1 Effects of amoxicillin and augmentin on average daily ethanol intake (g/kg/day) in male P rats. One-way ANOVA analyses revealed significant differences among control and treatment groups. Dunnett's t-test revealed a significant reduction in ethanol intake in amoxicillin and augmentin treated group starting Day 1 through the end of the study as compared to ethanol vehicle group. All data are expressed as mean ± SEM. (# p<0.001); (Ethanol vehicle, n=12; Ethanol Amoxicillin, n=12; Ethanol Augmentin, n=12).

3.3 Effects of Amoxicillin and Augmentin on Water Intake in Male P Rats

The effects of AMOX and AUG on water intake are illustrated in Figure 3-2. Starting 24 hours after the first injection the daily water intake was measured consecutively for 5 days in male P rats treated with saline vehicle (i.p.) or AMOX (100
mg/kg, i.p.) or AUG (100 mg/kg, i.p., n=12). The baseline water intake was determined as an average water intake for the last two weeks prior to saline vehicle or AMOX or AUG injections.

GLM Repeated Measures exhibited a significant main effect of Day \( [F(1,5)=12.290, p<0.001] \) and significant Treatment x Day interaction \( [F(2,10)=5.791, p<0.001] \). Post-hoc Dunnett’s test following one-way ANOVA revealed a significant increase in water intake in all animals treated with AMOX (100 mg/kg; \( p<0.001 \)) or AUG (100 mg/kg; \( p<0.001 \)) compared to ethanol vehicle animals. The increase in water intake was observed throughout the study in AUG treated group (as compared to ethanol vehicle group), however a significant increase in water intake was found from day 2 to day 5 of the treatment period in AMOX treated group as compared to ethanol vehicle group.
Effects of amoxicillin and augmentin on average daily water intake (g/kg/day) in male P rats. One-way ANOVA analyses revealed significant differences among control and treatment groups. Dunnett's t-test revealed a significant increase in water intake in augmentin treated animals starting Day 1 through the end of the study as compared to ethanol vehicle group. However there was a significant increase in water intake with amoxicillin treated group as compared to ethanol vehicle group starting Day 2 through the end of the study. All data are expressed as mean ± SEM. (# p<0.001); (Ethanol vehicle, n=12; Ethanol Amoxicillin, n=12; Ethanol Augmentin, n=12).

3.4 Effects of Amoxicillin and Augmentin on Body Weight of Male P Rats

Figure 3-3 illustrates the effects of AMOX and AUG on body weight of male P rats. Body weights of the animals were monitored throughout the study. A GLM
repeated measures analysis revealed a significant main effect of Day \( [F(1,5)=92.985, \ p<0.001] \) and a significant Day x Treatment interaction effect \( [F(2,10)=2.179, \ p<0.05] \).

One-way ANOVA did not show any significant effect of AMOX treatment as compared to ethanol vehicle group \( (p>0.05) \). However, one-way ANOVA revealed a significant effect on the body weight of animals treated with AUG as compared to ethanol vehicle group on Day 5 \( (p<0.05) \). Nevertheless, there was no significant effect on the body weight from Day 1 through Day 4 of AUG treated group as compared ethanol vehicle group.

**Figure 3-3 Effects of amoxicillin and augmentin on body weight in male P rats.** One-way ANOVA followed by Dunnett’s t-test revealed no significant difference with amoxicillin treatment as compared to ethanol vehicle group. However, there was a significant difference in body weight of augmentin treated group as compared to ethanol vehicle group at the end of the study as revealed by Dunnett’s t-test. All data are expressed as mean ± SEM. \( (# \ p<0.001; *: \ p<0.05) \); (Ethanol vehicle, n=12; Ethanol Amoxicillin, n=12; Ethanol Augmentin, n=12)
3.5 Effect of Augmentin on Sucrose Intake in Male P Rats

Furthermore, effect of AUG on sucrose (10%) intake was examined. A GLM repeated measures analysis revealed a significant main effect of Day \( [F(1,7)=2.906, \ p\leq0.01] \) and a significant Day x Treatment interaction effect \( [F(1,7)=4.172, \ p\leq0.001] \). However, unpaired t-test did not reveal any significant change in sucrose intake after AUG treatment (compared to ethanol vehicle group) at the end of the study.

3.6 Effect of Chronic Ethanol on GLT1 Expression in Acb and PFC

We analyzed the effect of chronic ethanol consumption on GLT1 expression in Acb and PFC. Figure 4 illustrates the effect of chronic ethanol consumption on GLT1 expression in Acb and PFC as compared to ethanol-naïve vehicle group. The Western blots analyzed using independent t-test demonstrated significant downregulation of GLT1 in ethanol vehicle group as compared to ethanol-naïve vehicle group in Acb \( (p<0.01) \). However, there was no significant change in the GLT1 expression in ethanol vehicle group as compared to ethanol-naïve vehicle group in PFC.
Figure 4. **Effects of chronic ethanol on GLT1 expression in Acb and PFC.**
A) Representative Western Blots for GLT1 and β-tubulin loading control for Acb and PFC. B) Quantitative analysis of the immunoblots revealed significant downregulation in GLT1 levels in Acb but not in PFC following chronic ethanol consumption by male P rats as compared to ethanol naïve group (100%). Data are expressed as mean ± SEM. (**p<0.01). (Ethanol-naïve vehicle, n=6; Ethanol Vehicle (Acb), n=6; Ethanol Vehicle (PFC), n=6).

3.7 Effects of Amoxicillin and Augmentin on GLT1 Expression in Acb

Figure 5 illustrates the effects of AMOX and AUG on GLT1 expression in Acb. Western blot analysis of GLT1 demonstrated a significant change in GLT1 expression following AMOX and AUG treatment. One-way ANOVA analysis of the blots revealed
a significant main effect of both AMOX and AUG treatment in Acb [F(2, 15) = 6.364].
Furthermore, a Newman-Keuls multiple-comparison post-hoc test, revealed a significant increase in GLT1 expression in both AMOX (p<0.05) and AUG (p<0.01) treated groups compared to ethanol vehicle group in Acb.

**Figure 5. Effects of amoxicillin and augmentin on GLT1 expression in Acb.**
A) Representative Western Blots for GLT1 and β-tubulin loading control for Acb. B) Quantitative analysis of the immunoblots revealed significant upregulation in GLT1 levels in Acb following amoxicillin and augmentin treatments as compared to ethanol vehicle group (100%). Data are expressed as mean ± SEM. (*p<0.05; **p<0.01). (Ethanol-naïve vehicle, n=6; Ethanol Amoxicillin, n=6; Ethanol Augmentin, n=6).
3.8 Effects of Amoxicillin and Augmentin on GLT1 Expression in PFC

Figure 6 also illustrates the effects of AMOX and AUG on GLT1 expression in PFC. Interestingly, one-way ANOVA revealed a significant difference between the treatment and the ethanol vehicle group \([F(2,12=7.507)]\). A Newman-Keuls multiple-comparison post-hoc test exhibited a significant upregulation of GLT1 expression in AUG treated group \((p<0.01)\)(compared to ethanol vehicle animals) but not in AMOX treated group (compared to ethanol vehicle animals) in PFC. Additionally, there was a significant upregulation in GLT1 levels in AUG treated group as compared to AMOX treated group \((p<0.05)\).

Figure 6. Effects of amoxicillin and augmentin on GLT1 expression in PFC.
A) Representative Western Blots for GLT1 and \(\beta\)-tubulin loading control in PFC. B) Quantitative analysis of the immunoblots did not reveal any significant upregulation in GLT1 levels in PFC following amoxicillin treatment as compared to ethanol vehicle group (100%). However there was a significant upregulation in GLT1 levels following augmentin treatment as compared to amoxicillin treated group and ethanol vehicle group (100%). Data are expressed as mean ± SEM. \((^*p<0.05; ^{**}p<0.01)\). (Ethanol-naïve vehicle, n=5; Ethanol Amoxicillin, n=5; Ethanol Augmentin, n=5).
3.9 Effect of Chronic Ethanol on pAKT Expression in Acb and PFC

Furthermore, we investigated the effect of chronic ethanol consumption on phosphorylation of AKT in Acb and PFC. Figure 7 illustrates the effect of chronic ethanol consumption on pAKT expression in Acb and PFC as compared to ethanol-naïve vehicle group. The Western blots analyzed using independent t-test demonstrated significant downregulation of pAKT in ethanol vehicle group as compared to ethanol-naïve vehicle group in Acb (p<0.0001) and PFC (p<0.01).

![Western blots](image)

**Figure 7. Effects of chronic ethanol on pAKT expression in Acb and PFC.**
A) Representative Western Blots for pAKT, total-Akt and β-tubulin loading control for Acb and PFC. B) Quantitative analysis of the immunoblots revealed significant downregulation in pAKT levels in Acb and PFC following chronic ethanol consumption by male P rats as compared to ethanol naïve group (100%). Data are expressed as mean ± SEM. (*p<0.05; ****p<0.0001). (Ethanol-naïve vehicle, n=6; Ethanol Vehicle (Acb), n=6; Ethanol Vehicle (PFC), n=6).
3.10 Effects of Amoxicillin and Augmentin on pAKT Level in Acb

We further explored the effect of AMOX and AUG on pAKT, which is known to regulate GLT1 expression (Li et al., 2006), on Acb (Figure 8). One-way ANOVA analysis demonstrated a significant main effect of treatment on pAKT level in Acb [F(2,15)=4.638]. Newman-Keuls multiple comparison post-hoc test revealed a significant increase in pAKT level (p<0.05) with both AMOX and AUG treatment as compared to ethanol vehicle group in Acb.

![Figure 8](image)

Figure 8. Effects of amoxicillin and augmentin on pAKT expression in Acb. A) Representative Western Blot for pAKT, total-Akt and β-tubulin loading control for Acb. B) Quantitative analysis of the immunoblots revealed significant upregulation in pAKT levels in Acb following amoxicillin and augmentin treatment as compared to ethanol vehicle group (100%). Data are expressed as mean ± SEM. (*p<0.05). (Ethanol-naïve vehicle, n=6; Ethanol Amoxicillin, n=6; Ethanol Augmentin, n=6).
3.11 Effects of Amoxicillin and Augmentin on pAKT Level in PFC

One-way ANOVA analysis demonstrated a significant main effect of treatment on pAKT level in PFC [F(2,15)=4.828]. Newman-Keuls multiple comparison post-hoc test revealed a significant increase in pAKT level (p<0.05) with AUG treatment compared to ethanol vehicle group in PFC (Figure 9). However, there was no significant increase in pAKT in PFC following AMOX treatment as compared to ethanol vehicle group. Interestingly, there was a significant increase in pAKT level in AUG treated group as compared to AMOX treated group (p<0.05).

![Figure 9. Effects of amoxicillin and augmentin on pAKT expression in PFC. A) Representative Western Blot for pAKT, total-Akt and β-tubulin loading control for Acb. B) Quantitative analysis of the immunoblots revealed significant upregulation in pAKT levels in Acb following augmentin treatment as compared to ethanol vehicle group (100%). Data are expressed as mean ± SEM. (*p<0.05). (Ethanol-naïve vehicle, n=6; Ethanol Amoxicillin, n=6; Ethanol Augmentin, n=6).](image-url)
3.12 Effects of Amoxicillin and Augmentin on Hepatic ALDH2 Activity

Additionally, we examined the effects of AMOX and AUG on ALDH2 activity compared to ethanol vehicle group (Figure 10). One-way ANOVA followed by Newman-Keuls multiple comparison post-hoc test revealed no significant effect of AMOX and AUG on hepatic ALDH2 activity as compared to ethanol vehicle group [F(2,15)=1.432].

Figure 10. Effects of amoxicillin and augmentin on Hepatic ALDH2 Activity. P rats treated with amoxicillin or augmentin did not reveal any significant change in hepatic ALDH2 activity as compared to ethanol vehicle group. Enzyme activities are expressed as mean ± SEM. (Ethanol-naïve vehicle, n=6; Ethanol Amoxicillin, n=6; Ethanol Augmentin, n=6).
Chapter 4

Discussion

We report here that administration of AMOX and AUG attenuated ethanol intake in male P rats. Importantly, augmentin had no effect on sucrose intake, which was used as an appetitive control for drinking-motivated behavior, thus indicating that the effect was specific to ethanol associated reward. Notably, this reduction in ethanol intake was found along with upregulation of GLT1 expression in Acb and PFC after AUG treatment. However, AMOX upregulated GLT1 expression in Acb but not in PFC.

Interestingly, there was a significant increase in water consumption following both AMOX and AUG treatment as compared to ethanol vehicle group. We propose that this increase in water intake in AMOX and AUG treated groups was a compensatory behavioral mechanism associated with the decrease in ethanol intake. Studies from our lab tested other upregulator of GLT1 such as CEF and GPI-1046 showed similar effects in water intake (Sari et al., 2011, Sari and Sreemantula, 2012, Sari et al., 2013a, Sari et al., 2013b). Furthermore, AMOX treatment did not induce any significant effect in body weight of animals throughout the study; however there was significant difference in body
weight in AUG-treated group (as compared to vehicle-treated group) on the last day of treatment. It is noteworthy that studies demonstrated that administration of AUG was associated with mild diarrhea, which is a well-documented side-effect associated with the drug (Bucher et al., 2003, Henry et al., 2003). This might be one of the side effects of the drug in association with significant reduction in body weight as compared to ethanol vehicle group.

P rats are known to consume high doses of ethanol (5-8 g/kg/day) voluntarily, producing blood-alcohol concentrations as high as 50-200 mg%, following chronic free-choice alcohol drinking (McBride et al., 2013). Uninterrupted exposure to free-choice ethanol for at least 5 weeks has been associated with development of behavioral tolerance in P rats (Stewart et al., 1991). Thus, previous studies from our lab have also used P rats on 24-hour uninterrupted access to 15% and 30% ethanol, water for five weeks as animal models (Sari and Sreemantula, 2012, Sari et al., 2013a, Sari et al., 2013b). Therefore, P rats on concurrent five weeks of free-choice alcohol-drinking paradigm represented the relevant animal model for the present study.

The glutamate transmission in the mesolimbic pathway, encompassing the VTA, Acb and PFC, along with amygdala (AMG) has been implicated in the initiation and expression of the reinforcing and addictive effects associated with the drugs of abuse, including ethanol (Kalivas et al., 2009, Carrara-Nascimento et al., 2011, Gass et al., 2011). The elevated glutamatergic neurotransmission in PFC and Acb plays a critical role in addictive properties associated with ethanol dependence [for review see (Sari, 2013)]. There is an increase in the extracellular glutamate levels in key regions of the reward pathway, especially Acb, following ethanol consumption (Moghaddam and
Bolinao, 1994, Dahchour et al., 2000, Melendez et al., 2005, Szumlinski et al., 2007). The extracellular glutamate level is regulated by several glial glutamate transporters amongst which GLT1 eliminates more than 90% of the extracellular levels of glutamate. Downregulation of GLT1 expression has been reported following self-administration with several drugs of abuse such as cocaine (Knackstedt et al., 2010), nicotine (Knackstedt et al., 2009) in animal models. In accordance with these findings, we also found a downregulation of GLT1 expression in Acb but not in PFC of the ethanol vehicle group as compared to ethanol-naïve vehicle group following five weeks of chronic ethanol consumption. These findings were in agreement with recent studies from our lab (Sari and Sreemantula, 2012, Sari et al., 2013b). These evidences suggest the potential therapeutic role of GLT1 for the treatment of ethanol dependence.

Alternatively, we have shown that CEF and GPI-1046 were efficient in upregulating the GLT1 expression in Acb and PFC consequently attenuating the ethanol intake in male P rats in a five week ethanol drinking paradigm (Sari and Sreemantula, 2012, Sari et al., 2013b). These findings support the hypothesis that drugs restoring the glutamate homeostasis by upregulating the GLT1 expression in Acb and PFC may be a feasible therapeutic approach to attenuate ethanol intake in male P rats. Based on the structural similarities between AMOX with CEF i.e. the presence of β-lactam ring, we hypothesized that the observed attenuation in ethanol intake following AMOX and AUG treatment might be due to the potential role of AMOX in altering GLT1 protein expression. Interestingly, we found a significant upregulation in GLT1 expression in Acb after AMOX and AUG treatment. These findings are in correspondence with the study by Rothstein et al where amoxicillin was amongst 1,040 compounds found to be most
potent stimulators of GLT1 expression in organotypic spinal cord cultures (Rothstein et al., 2005).

It is noteworthy that in the present study, although there was a trend observed with GLT1 upregulation after AMOX treatment in PFC, the upregulation in GLT1 expression was not significant as compared to ethanol vehicle animal group. It is known that distribution of systemically administered compounds across the brain regions is not even (Alavijeh et al., 2005). Therefore, one of the possible explanations for this finding can be the differential or region-specific distribution of drug in the brain. However, not much has been known about the distribution of AMOX in the brain, further analysis needs to be conducted to determine the concentration of AMOX in different brain regions.

Intriguingly, the study revealed that AUG but not AMOX treatment upregulated the GLT1 levels in PFC. Thus, we rationalized that this observed upregulation in GLT1 expression following AUG treatment in PFC might be attributed to the presence of clavulanic acid in the formulation. Ample evidences support the role of clavulanic acid as a CNS-modulator. Clavulanic acid also has a β-lactam structure with negligible intrinsic antibacterial activity and it crosses the BBB very easily, achieving a CSF/plasma ratio of 0.25 approximately (Nakagawa et al., 1994). It is also documented to have anxiolytic effects in primate and rodents (Kim et al., 2009). Rawls et al reported that clavulanic acid demonstrated anti-seizure properties in invertebrates (Rawls et al., 2010), although it was unclear whether this anti-seizure effects of clavulanic acid were due to its ability to enhance glutamate uptake or not. However, more studies needs to be performed to
establish the plausible role of clavulanic acid in upregulating GLT1 expression and restoring glutamate homeostasis.

Studies have reported that pAKT is downregulated following chronic alcohol treatment in several in-vitro and/or in-vivo models (Antonelli et al., 2009, Cynkar et al., 2010). In agreement with these studies, we found a downregulation in pAKT/total-AKT expression after chronic ethanol consumption in ethanol-vehicle group as compared to ethanol-naïve group in Acb and PFC. In contrast, studies demonstrated that systemic administration of alcohol resulted in the activation of AKT signaling pathway in Acb (Cozzoli et al., 2009, Neasta et al., 2011). The basis of disparity in these findings can be explained by the difference in the ethanol drinking paradigms used in these studies i.e. the chronic five-weeks ethanol drinking paradigm. This is characterized by the uninterrupted access to ethanol for five-weeks (in the present study) versus the binge alcohol drinking paradigms involved limited access drinking with intermittent withdrawal periods (Cozzoli et al., 2009, Neasta et al., 2011).

Additionally, we examined the effect of AMOX and AUG on pAKT level in Acb and PFC. Following treatment with AUG there was an upregulation in the pAKT levels in both Acb and PFC, however AMOX treatment upregulated pAKT expression only in Acb but not in PFC. These findings correlate with the changes in GLT1 expression observed in Acb and PFC after AMOX and AUG treatments. These findings were in accordance with large body of evidences supporting the role of AKT signaling pathway in upregulating of GLT1 expression (Li et al., 2006, Wu et al., 2010). Interestingly, a recent study revealed that CEF upregulated GLT1 expression in hippocampal astrocytes in-vitro and in-vivo through PI3K/Akt/NF-κB pathway (Cigana et al., 2009). Therefore,
we conclude that AMOX and AUG induced upregulation in GLT1 expression is regulated by pAKT.

Although AMOX is known to easily penetrate the blood-brain barrier (BBB) in the presence of infections and inflammation, its ability to penetrate the healthy subjects remain debatable. In order to determine whether AMOX crossed the BBB, the concentration of AMOX in the CSF of male P rats after the drug treatment was determined using HPLC. The results from our findings suggested that AMOX penetrated the BBB in detectable amounts after administration of AMOX 100 mg/kg i.p. and augmentin 100 mg/kg i.p. These data were in conjunction with the previous studies demonstrating that AMOX was detected in the CSF without the presence of any infection (Clumeck et al., 1978, Mingrino et al., 1981). However further studies need to be performed to determine the CSF-to-plasma ratio of the drug by determining the change in concentration of the drug in CSF and plasma over a period of time after drug administration.

Evidence suggests that N-methylthiotetrazole (NMTT) moiety in the β-lactam antibiotics is crucial to produce the disulfiram-like effect on the hepatic alcohol metabolizing enzymes, mainly aldehyde dehydrogenases (Brien et al., 1985, Matsubara et al., 1987). Consistent with these findings, we did not observe any significant change in the hepatic ALDH2 activity after treating the rats with AMOX or AUG as compared saline vehicle treated group. This indicated that the treatment did not have any disulfiram-like effect and the attenuation in ethanol-intake following AMOX and AUG treatment was only due to the central effect that is, modulation of glutamatergic neurotransmission through GLT1 upregulation.
CEF, a β-lactam antibiotic, has been widely studied in drug-abuse models, including ethanol. CEF has been well established for its neuroprotective effects and has proceeded to Phase III clinical trials for Amyotrophic lateral sclerosis (ALS). However, CEF can only be administered parenterally which can be a shortcoming for its long-term use due to limited patient compliance. Alternatively, AMOX is an orally active FDA approved drug categorized as a β-lactam antibiotic like CEF. Considering these advantages and evidences based on our findings, further studies are warranted to determine the effect of AMOX on GLT1 expression and ethanol intake after oral administration.

Despite the fact that the present study provides compelling evidences about the efficacy of AMOX and AUG in attenuating ethanol intake, it bears a few limitations of its own. One of the limitations with the study was selection of single dose of each drug, which consequently did not aid in establishing a clear dose-response relationship of the drug. Further studies are required to establish a dose-response relationship of the drug.

In summary, this study demonstrates, for the first time, the effectiveness of AMOX and AUG treatment in reducing ethanol intake in male P rats. This attenuation in ethanol intake was associated in part with upregulation of GLT1 in Acb, but not in PFC, following AMOX treatment. Alternatively, following augmentin treatment GLT1 levels were found to be upregulated in both Acb and PFC. The changes in GLT1 expression observed in this study, following both treatments were associated with significant upregulation of phosphorylated Akt levels. These findings provide strong evidence about the efficacy of amoxicillin and augmentin in attenuating ethanol intake in male P rats by restoring glutamate homeostasis.
References


