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Role of glutamate transporters in alcohol and methamphetamine co-abuse

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Entitled

Role of Glutamate Transporters in Alcohol and Methamphetamine Co-Abuse

By

Fahad Alshehri

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

Master of Science Degree in Pharmaceutical Science

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August-2015

An Abstract of

Role of Glutamate Transporters in Alcohol and Methamphetamine Co-Abuse

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Alcohol and methamphetamine are one of the most widespread drugs of abuse. Recently, studies are focusing on the association between drugs of abuse and glutamatergic system. It has been found that both alcohol and methamphetamine can disturb glutamate transport, release, and clearance. Alternatively, ceftriaxone, known to upregulate one of the major glutamate transporters glutamate transporter 1 (GLT-1), showed a promising effect against cocaine seeking, methamphetamine reinstatement in conditioned place preference, and alcohol consumption. These effects are suggested to be associated in part with upregulation of GLT-1 expression levels in central reward brain regions leading to normalization of extracellular glutamate concentrations in these regions. However, there is little known about co-abuse of alcohol and methamphetamine. Therefore, this study examined the effects of ceftriaxone on alcohol and methamphetamine co-abuse in both striatum and hippocampus of Wistar rats. Wistar rats were gavaged with ethanol 6 g/kg for seven days and injected intraperitoneally with

methamphetamine 10 mg/kg, four times with two hours apart. Furthermore, studies have shown the hyperglutamatergic state with drug uses, including methamphetamine and alcohol. We suggest here that this hyperglutamatergic state might be associated with a deficit in glutamate transport. Thus, we tested ceftriaxone to prevent downregulation of GLT-1 induced by administration of both alcohol and methamphetamine. Three ceftriaxone injections were given after the administration of high doses of methamphetamine. As a result, administration of alcohol and methamphetamine caused a significant downregulation of GLT-1 expression levels as compared to control naïve groups in both striatum and hippocampus. Conversely, both xCT and GLAST expression levels remained unaffected as compared to naïve control group. Ceftriaxone treatment normalized the level of GLT-1 expression levels in both striatum and hippocampus. These findings show the important role of GLT-1 in alcohol and methamphetamine co-abuse. Therefore, GLT-1 could be considered as a target for future treatment against neurotoxicity induced by administration of alcohol and methamphetamine.

Keywords: GLT-1, Methamphetamine, Alcohol, glutamate.

Acknowledgments

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List of Abbreviations

ALS............................... Amyotrophic Lateral Sclerosis

AMPA.......................... α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor.

GLAST.......................... Glutamate Aspartate Transporter.

GLT-1........................... Glutamate Transporter 1.

KA................................ Kainate receptor.

MND............................. Motor Neuron Disease.

NMDA.......................... N-methyl-D-aspartate receptor.

xCT.............................. Cystine/glutamate transporter.

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Chapter 1

Introduction

1.1. Overview

Addiction is disorder characterize by repetitive consumption of the same chemical substance which leads to destructive behavioral and severe consequences. According to the National Institute on Drug Abuse, addiction is increasing among the young population in recent years with drugs of abuse such as marijuana, amphetamines, cocaine, heroin, and alcohol. One of the most widespread substances of abuse is alcohol (Alcohol & Drug Information, 2015). Chronic consumption of alcohol leads to a disease called Alcoholism which includes dependence on the substance and withdrawal symptoms in case of sudden cessation. Similarly, methamphetamine is a psychostimulant substance with high rate of dependence and tolerance, and it is considered as the second illegal drug in most parts of the world with prevalence around 0.4% (Petit et al., 2012). Concomitant use of methamphetamine and alcohol is becoming a growing problem in the young generation, recent studies showed an increase in alcohol and methamphetamine users in United States (Windle, 2003, Iritani et al., 2007). According to a recent study examining

the effects of administration of alcohol and methamphetamine, it has been revealed an increase in alcohol drinking pattern with methamphetamine administration as a result of greater reward effects as compared to each drug administered alone (Kirkpatrick et al., 2012, Bujarski et al., 2014a). Overall, concomitant consumption of alcohol and methamphetamine increases their harmful effects and the risk of more complications. Thus, co-abuse increases the clinical challenges for treatment of substance of abuse (Sepehrmanesh et al., 2014).

The human brain is considered the main target of drug addiction, mostly the mesolimbic system (Adinoff, 2004). The pleasurable effects associated with long term uses of drug of abuse lead to addiction, tolerance, and dependence (Smith, 1969, Hyman, 2005). Dopamine has been studied extensively in the neuroscience field. Studies have found that dopamine play a crucial role in reward mechanisms in the mesolimbic system (Björklund and Dunnett, 2007), and also in the treatment of addiction. However, glutamate has been now the focus as a target for the treatment of drug abuse. Glutamate is an excitatory neurotransmitter essential for the normal physiological functions of the brain, and acts on different receptors such as N-methyl-D-aspartate (NMDA), Kainate receptor (KA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA), and mGluRs (metabotropic glutamate receptors) (Schoepp and Conn, 1993, Gao et al., 2000). Glutamate transmission is regulated by a number of transporters, including glutamate transporter -1 (GLT-1/ its human homolog known as excitatory amino acid transporter 2, EAAT2), cysteine glutamate antiporter (xCT), and glutamate aspartate transporter (GLAST/ its human homolog, EAAT1) (Danbolt, 2001, Baker et al., 2002, Kanai and Hediger, 2004). It has been found in animal models that, glutamatergic

neurotransmission is altered with drug abuse (Kalivas, 2009). GLT-1 and xCT have been identified in literatures as a potential target for targeting drug (Fujio et al., 2005, Nakagawa et al., 2005, Gass and Olive, 2008, Rasmussen et al., 2011). As a matter of fact, some of drugs of abuse have been found to downregulate GLT-1 and xCT such as alcohol, cocaine and morphine (Mao et al., 2002, Gass and Olive, 2008, Knackstedt et al., 2010, Sari and Sreemantula, 2012b, Sari, 2013, Alhaddad et al., 2014b). On the other hand, it has been found that some compounds can upregulate glutamate transporters such as β-lactam antibiotics, MS153, and GPI-1046 (Nakagawa et al., 2005, Sari and Sreemantula, 2012a, Alhaddad et al., 2014a, Rao and Sari, 2014a). Although, it is clear that several drugs of abuse downregulated GLT1 and xCT, the effects of administration of alcohol and methamphetamine on GLT-1 and xCT are not fully investigated and further studies are needed.

1.2. Alcohol and Methamphetamine Co-Abuse

Alcohol abuse in United States is considered as one of the nation problems, according to National Institute on Alcohol Abuse and Alcoholism; in 2012 around 87% of people (18 and older) stated that during their lifetime they have had alcohol. Moreover, alcohol-related death is increasing; approximately 88,000 people die yearly because of diseases linked to alcohol, such as cardiovascular, liver, and central nervous system diseases.

Concomitant use of alcohol and drugs of abuse can lead to several health risks. The use of alcohol and cocaine aggravated the loss of judgments, mortality and morbidity, difficulties in diagnosis emergency cases, and potential of becoming addicted to both drugs (Grant and Harford, 1990). Alternatively, methamphetamine is one of the widest spread illegal drugs in United States, according to National Survey on Drug Use and Health (NSDUH). It is estimated that, more than 12 million people (4.7%) have used methamphetamine at some point in their lifetimes. Methamphetamine can cause depletion of the neurotransmitters such as dopamine, serotonin, and norepinephrine (Hanson et al., 2009, Thomas et al., 2010). Concomitant use of alcohol and Methamphetamine is popular between young age due to the rewarding effects (Bujarski et al., 2014a). It has been found that concomitant use of amphetamines and alcohol can lead to complicated cardiovascular problems, slow in the metabolism of both drugs, neural injuries in several parts of the brain, and reduction in cortical thickness (Todzy et al., 1978, Mendelson et al., 1995, Lawyer et al., 2010, Li et al., 2014). Moreover, recent studies found that chronic alcohol drinking can downregulate GLT-1 in many areas in the brain such as accumbens, prefrontal cortex, and hippocampus (Alhaddad et al., 2014b, Aal-Aaboda et al., 2015, Rao et al., 2015). In addition, methamphetamine has found to cause increase of glutamate level after administration (Abekawa et al., 1994). Studies from our lab have demonstrated that chronic alcohol consumption downregulated GLT-1 expression levels in several brain reward regions (Sari and Sreemantula, 2012b, Sari, 2013, Alhaddad et al., 2014b, Rao and Sari, 2014a, Aal-Aaboda et al., 2015), which may have an effect in increasing extracellular glutamate concentrations. Thus, these findings suggest a potential relationship between alcohol and methamphetamine in glutamatergic system. We

hypothesized here that methamphetamine administered with alcohol may exacerbate glutamatergic excitotoxicity. Thus, in this study, we investigated the role of administration of alcohol and methamphetamine on key glutamatergic transporters such as GLT-1, xCT and GLAST in striatum and hippocampus.

1.3.   Methamphetamine abuse

Methamphetamine use has been growing epidemically in the last decade and linked to many neuropsychological complications. Methamphetamine stimulates the release of dopamine, norepinephrine, serotonin, and glutamate (Nash and Yamamoto, 1992, Bowyer et al., 1994, Thomas et al., 2004). It is known that chronic use of methamphetamine can lead to severe and dangerous neurological changes in the brain (Chang et al., 2007). Chronic use of methamphetamine can lead to dependence and tolerance, which can result from the repetitive administration of the drug in order to have the same reward effect. Consumption of high amount of methamphetamine leads to a condition called "methamphetamine neurotoxicity" (Cubells et al., 1994, Halpin and Yamamoto, 2012)). Acute doses of methamphetamine characterized by depletion of dopamine, lethal hyperthermia, psychosis, and dopaminergic neural damage (Kita et al., 2003). The depletion of dopamine has been associated with behavioral and psychological changes, some are transitory and others are permanent.

It has been suggested that dopaminergic neurons play an important role in the neurotoxicity associated with chronic use of methamphetamine (Meredith et al., 2005).

The exact mechanism is not fully understood; however, methamphetamine was shown to trigger the release of dopamine from the presynaptic vesicles and facilitate its release to the synaptic space, which leads to neural damage via dopamine auto-oxidation, oxidative stress, decrease density of dopamine transporter, free radical formation, and calcium efflux (Davidson et al., 2001, Kita et al., 2003, Riddle et al., 2006). Acute use of methamphetamine leads to a persistent release of dopamine and as a consequence, depletion of presynaptic dopaminergic vesicles and deficiency in the amount of dopamine stored in the vesicles (Wagner et al., 1980). Thus, symptoms of neurodegenerations appear like Parkinson's disease, autism, and schizophrenia-like symptoms which can persist for years after abstinence due to neural damage (Davidson et al., 2001, Kita et al., 2003). Similarly, glutamate released after the administration of methamphetamine increases calcium influx, resulting in mitochondrial damage and cell death (Stephans and Yamamoto, 1994, Schinder et al., 1996). Another important factor with methamphetamine is the production of nitric oxide after the glutamatergic activation of the NMDA (N-Methyl-D-aspartate) receptor, which may facilitate the release of calcium and its binding to calmodulin, and in turn lead to nitric oxides release and cGMP accumulation (Garthwaite and Boulton, 1995, Kita et al., 2003). It has also been found that metabotropic receptors 5 (mGluR5) receptor play a role in neurotoxicity and cell death by a similar mechanism. Norepinephrine and serotonin have been examined in term of acute methamphetamine use, but their implications after methamphetamine use are minor (Cook and Schanberg, 1970, Kuczenski et al., 1995). Furthermore, several studies are focusing on the relationship between methamphetamine-induced hyperthermia and dopamine neurotoxicity (Halladay et al., 2003, Numachi et al., 2007, Ito et al., 2008).

Lethal hyperthermia has been associated with repetitive methamphetamine uses at high doses due to increase extracellular catecholamines (Bowyer et al., 1994, Imam and Ali, 2001, Kita et al., 2003). Other studies have also connected methamphetamine neurotoxicity with an increase in the production of peripheral ammonia, liver damage, and the possible role of ammonia (Kamijo et al., 2002, Halpin and Yamamoto, 2012).

1.4. Targeted Areas in Alcohol and Methamphetamine Co-Abuse

1.4.1 Striatum

Striatum is located in the subcortical part of the forebrain; it has an important role in learning pattern in the limbic structure, which contains both dopamine receptor and glutamate receptors (Robbins and Everitt, 2002). It has been found that striatum is a potential target for examining the effect of drugs of abuse on the release of dopamine and other neurotransmitters (Abekawa et al., 1994, Cass, 1997, McCann et al., 1998). In addition, glutamate in striatum plays a crucial role through activation of glutamate receptors, which lead to inhibition of mitochondrial succinate dehydrogenase and impairment of ATP synthesis and mitochondrial damage (Meldrum, 2000). Methamphetamine affects striatum by activating both dopaminergic and glutamatergic systems. A study done recently found that current methamphetamine users and recent

abstinent suffered from enlargement in striatum compared to normal individuals due to the inflammations and release of free radicals (Chang et al., 2007). Another study examined the effects of alcohol drinking on the dopamine receptors (D1 and D2) and found that D1 receptors have been reduced in striatum compared to control group (Martinez et al., 2005). Other areas are also important in alcohol and methamphetamine co-abuse and further studies are needed.

1.4.2 Hippocampus

Hippocampus plays an important role in drug addiction, hippocampus has a projection connecting several regions in the limbic system, such as drug sensitization, fear conditioning, and stress (Belujon and Grace, 2011). Studies has shown neurodegenerations induced by methamphetamine use in hippocampus similarly to striatum, and this effect was associated behavioral changes (Kuczenski et al., 1995). Furthermore, it was revealed that methamphetamine can lead intensification of the inflammation process and the migration of the phagocytes in the hippocampus area associated with alteration of the blood brain barrier (BBB) (Bowyer and Ali, 2006). Alternatively, alcohol can lead to behavioral changes, memory formation, and c-fos gene in hippocampus (Ryabinin, 1998, Weitemier and Ryabinin, 2003). These facts support the importance of hippocampus as a key area in alcohol and methamphetamine co-abuse.

1.5. Glutamate and Glutamate Transporters

Glutamate is an excitatory neurotransmitter in the nervous system. Glutamate has a key role in neural development and growth (Bar-Peled et al., 1997). Alternatively, glutamate plays an essential role in neurodegeneration through activation of the glutamate receptors and facilitation of cell death. Certain disorders have been connected to glutamate excitotoxicity such as amyotrophic lateral sclerosis (ALS), motor neuron disease (MND), Parkinson's disease, and Alzheimer's disease (Maragos et al., 1987, Fray et al., 1998, Howland et al., 2002, Schaeffer and Duplantier, 2010). Recently, glutamate clearance found to be decreased in chronic alcohol drinking with downregulation of GLT-1 and xCT expression levels (Sari, 2013, Alhaddad et al., 2014b, Rao et al., 2015). Glutamate activates different types of receptors, inotropic receptors such as NMDA and AMPA and metabotropic receptors such as Group I, II, and III. On the other hand, five types of glutamate transporters have been found in the nervous system(excitatory amino acid transporters 1-5), some are located on the astrocytes and the other on the glutamatergic neurons (Vezzani et al., Lehre et al., 1995, Meldrum, 2000). GLT-1 is responsible for the majority of glutamate clearance after its release in the synaptic cleft (Haugeto et al., 1996, Bergles et al., 1999, Danbolt, 2001). GLAST has a minor role in transporting glutamate with predominance in the cerebellum. xCT controls the homeostasis of glutamate in the synaptic cleft in exchange with cystine for glutamate in the processes of glutathione biosynthesis. xCT has been associated with

neurodegeneration diseases such as multiple sclerosis (Pampliega et al., 2011). Also, xCT has been identified to play a potential role in alcohol intake (Rao and Sari, 2014a). Together, these suggest that GLT-1 and xCT might be target proteins for the treatment of alcohol dependence.

1.6. Ceftriaxone and glutamatergic system

Cephalosporins are semisynthetic antibiotics which share the beta-lactam moiety. These compounds are derived initially from a fungus called "cephalosporium acremonium". Cephalosporins are classified to more than one generation; each generation contains antibiotics that share the similar spectrum of action against bacteria. Ceftriaxone is a third-generation antibiotic, with a broad spectrum of activity against many gram-positive and gram-negative bacteria (Hall et al., 1981). Ceftriaxone has better activity than the first and second generation of cephalosporin. These compounds affect the bacterial cell wall synthesis by disturbing the peptidoglycan layer and weakening the cell wall which leads to the death of the bacteria. Ceftriaxone has more resistance against beta lactamase enzymes produced by resistant gram-negative or gram-positive bacteria. Beta-lactam antibiotics recently showed a neuroprotective effect against glutamate neurotoxicity (Rothstein et al., 2005, Lipski et al., 2007a). Most beta-lactam antibiotics show upregulation of GLT 1 (Rothstein et al., 2005). Further studies have been conducted on the effects of ceftriaxone with conditions such as stroke, dementia associated human

immunodeficiency virus protein, cerebral ischemia, and spinal muscle atrophy (Lipski et al., 2007b, Rumbaugh et al., 2007, Verma et al., 2010, Nizzardo et al., 2011). Ceftriaxone neuroprotective properties can be associated in part through upregulating the GLT-1 (Rothstein et al., 2005, Lipski et al., 2007a, Verma et al., 2010). It is suggested that ceftriaxone may have neuroprotective effects through reduction of extracellular glutamate concentrations and subsequent attenuation of neurotoxicity.

1.7. Aims and Objective

Several studies are focusing on the role of glutamatergic system in drugs of abuse such as methamphetamine (Abekawa et al., 1994, Bowyer et al., 1994, Cubells et al., 1994, Davidson et al., 2001). Methamphetamine stimulates the release of glutamate and increases the level of catecholamines in the synaptic cleft in different part of the brain. Chronic alcohol consumption may lead to increase in extracellular glutamate concentration (Dahchour and De Witte, 2003, Melendez et al., 2005). Furthermore, GLT-1 and xCT expression levels are downregulated after chronic alcohol use (Schreiber and Freund, 2000, Gass and Olive, 2008, Sari and Sreemantula, 2012b, Alhaddad et al., 2014b, Aal-Aaboda et al., 2015). Thus, the decrease in the clearance of glutamate extracellular concentrations could be associated with a deficiency in its clearance. The present study is aimed to examine the effect of administration alcohol and methamphetamine at higher doses on GLT-1, xCT, and GLAST. We suggest here that the

hyperglutamatergic state might be associated with deficit in glutamate transport. We hypothesize that ceftriaxone would normalize the expression levels of these glutamate transporters in methamphetamine and alcohol co abuse model in both striatum and hippocampus. Normalizing the expression levels of GLT-1and xCT may lead to the attenuation of glutamate-induced neurotoxicity.

Chapter 2

Materials and Methods

2.7.   Ceftriaxone

Ceftriaxone was purchased from Apotex Corporation and reconstituted with saline. Animals were given intraperitoneal injections based on their weight (200 mg / kg).
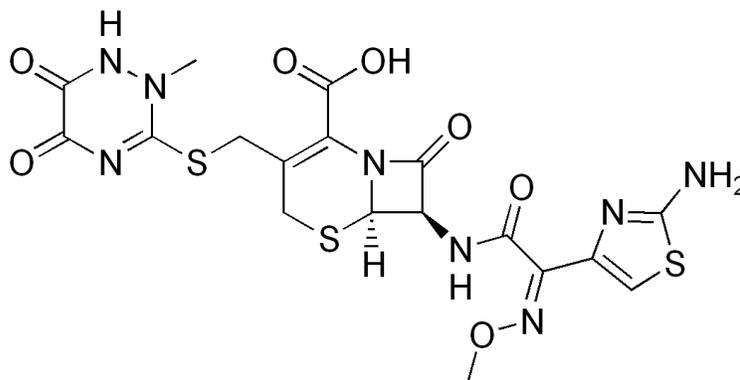
Figure 2-1 Chemical structure of ceftriaxone

2.2.　Methamphetamine

Methamphetamine was dissolved in saline. Intraperitoneal injections were given according to the body weight of the animals (10 mg / kg).
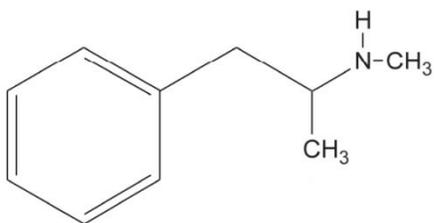
Figure 2-2 Chemical structure of methamphetamine

2.3.　Animals

Male Wistar rats were used in this study to examine the effect methamphetamine on glutamate transporters. Wistar rats were received from Harlan Laboratories at age of 40 – 45 days. Rats were separated in standard plastic tube with free access to food and water during the experiment. Animals kept in 25°C with relative humidity of 50% on a regular 12 hours light cycle. The experiments and housing procedures were approved by the Institutional Animal Care and Use committee at The University of Toledo with guidelines followed by the Institutional Animal Care and Use Committee of the National Institutes of Health and the Guide for the Care and Use of Laboratory.

2.6. Administration of Alcohol and Methamphetamine

At age of 60 days, male Wistar rats were separated in single housing and divided randomly into six groups: (a) water gavaged group received i.p saline vehicle; (b) water gavaged group received i.p methamphetamine; (c) water gavaged group received i.p methamphetamine then ceftriaxone; (d) ethanol gavaged group received i.p saline vehicle; (e) ethanol gavaged group received i.p methamphetamine; (f) ethanol gavaged group received i.p methamphetamine then ceftriaxone. Animals received oral gavage of water or ethanol (6 g/kg) for 7 days and received 4 injections of saline or (methamphetamine 10 mg/kg) with 2 hours apart at day 8. Ceftriaxone (200 mg/kg) injections were i.p administered given 30 minutes after the last methamphetamine injection, 24 hour, and 72 hours. At day 10, animals were euthanized one hour after the last ceftriaxone injection.
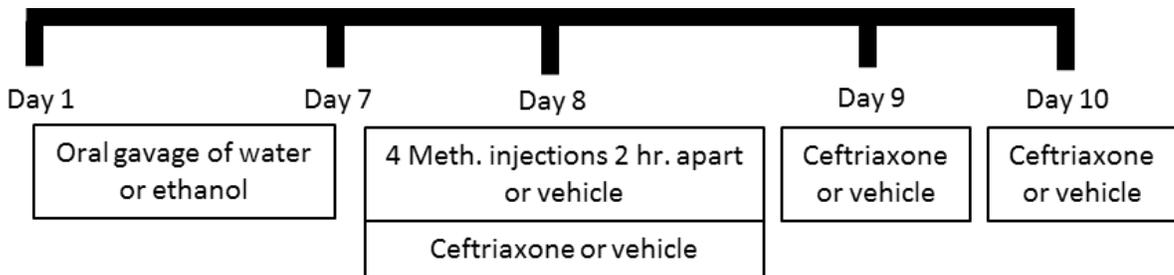
Figure 2-3 Methamphetamine injections schedule

2.5. Brain Tissue Collection

At the end of the experiment, all animals were euthanized with carbon dioxide and decapitated with the guillotine. Brains were extracted and kept frozen in $-70°C$ for immunoblotting. Dorsal striatum and Hippocampus were isolated using a cryostat machine (Leica CM1950) with guidance of rat brain atlas (The Rat Brain in Stereotaxic Coordinates, 6th edition). Brain regions were kept at -70ºC for immunoblotting and protein detection.

2.6. Immunoblotting procedures

Striatum and hippocampus were lysed in lysis buffer (5 ml 1M Tris HCl, 5 ml 3M NaCl, 0.2 ml .05 EDTA , 5 ml 10% NP-40, 10 ml 10% Triton, and 1 ml 10% SDS, protease inhibitor and phosphatase inhibitors). Then, samples were vortexed and centrifuged for 15 minutes at 13,200 rpm at $4°C$, the supernatant of each sample aliquoted in eppendorf tubes and frozen in -70$°C$. Protein quantification assays were performed to determine the total amount of protein using BioRad kit. Moreover, proteins were mixed with laemmli dye, heated and then centrifuged. The supernatants were separated through 10% polyacrylamide gels at 100-150 volt in 1X laemmli buffer (1X prepared from 10X laemmli buffer 30.2 g Tris base,144 g Glycine, and 10 SDS, and 1000 ml). Proteins were then transferred on PVDF membrane (Bio-Rad Laboratories) on 24 Volt for a period of 3-4 hours. After the transfer, membranes were blocked with 3 %

blocking buffer (fat free milk, LabScientific) in 100 ml 1X TBST (1 X prepared from 10X TBST 242.88g Tris base, 350.64g NaCl, 7.4 pH, and 4 L). The membranes were then incubated with primary antibodies (GLT-1, xCT or GLAST) over night at 4°C. β-tubulin was used as a loading control. Membranes were incubated with chemiluminescent substrate (Super Signal West Pico, Thermo Scientific) are exposed to autoradiography films (Denvilla Scientific Inc.). The membranes were then developed using X-Ray film processor (Konica SRX101A – Tabletop). Images of immunoblots were quantified using MCID Digital Imaging Software.

2.7.   Statistical Analysis

Effects of methamphetamine, ethanol, and ceftriaxone administrations on protein expression levels (immunoblots) for GLT-1, xCT, and GLAST were performed using one-way analysis of variance (ANOVA) followed by Newman-Keuls multiple comparison tests (Prism GraphPad). Effects of ethanol gavage on GLT-1 expression levels as compared to water gavage were done by using independent t-test (Prism GraphPad). All statistical analysis were done using P value less than 0.05 (P<0.05) as level of significance.

Chapter 3

Results

3.1. Effects of Methamphetamine and Ceftriaxone on GLT-1 Expression Levels in Striatum.

Figure 3-1 demonstrates the effect of ceftriaxone on GLT-1 expression levels in striatum in the group receiving water through gavage procedure and challenged with high dose of methamphetamine. One-way ANOVA followed by Newman-Keuls multiple comparison post-hoc test, demonstrates a significant increase in the expression levels of GLT-1 in ceftriaxone-treated group ($P<0.05$). Alternatively, quantitative analysis of western blot showed a significant downregulation of GLT-1expression levels in methamphetamine-treated group as compared to naïve control- and ceftriaxone-treated groups ($P<0.05$). Statistical analysis revealed no significant effect between naïve control- and ceftriaxone-treated groups.

WS   WM   WMC

A.   GLT-1

β-tubulin

B.
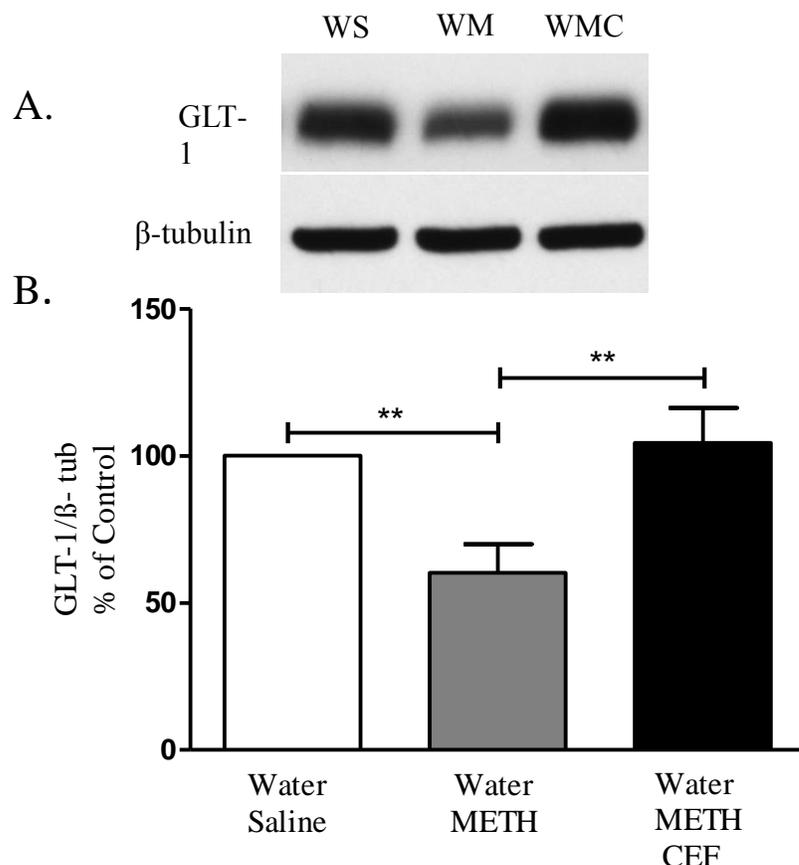
Figure 3-1 Effects of methamphetamine (10mg/kg i.p. every 2 hours x4) and ceftriaxone (200mg/kg) on GLT-1 expression levels in striatum. A) Immunoblots of GLT-1 and β-tubulin in striatum. B) Quantitative analysis revealed a significant increase in the ratio of GLT-1/β-tubulin in water-methamphetamine-ceftriaxone (WMC) group compared to the water-methamphetamine (WM). Significant downregulation of GLT-1 expression levels was revealed in the water-methamphetamine groups compared to the control naïve water-saline (WS) group. No significant difference in GLT-1 expression levels was revealed in methamphetamine-ceftriaxone group compared to the water-saline control group. Values shown as means $\pm$ S.E.M ($*p < 0.05$) (n=6 for each group).(

3.2. Effects of Methamphetamine, Ethanol, and Ceftriaxone on GLT-1 Expression Levels in Striatum.

Figure 3-2 demonstrates the effect of ceftriaxone on GLT-1 expression levels in striatum in the group receiving ethanol through gavage procedure and challenged with high dose of methamphetamine. One-way ANOVA followed by Newman-Keuls multiple comparison post-hoc test, revealed a significant increase in the expression levels of GLT-1 in ceftriaxone-treated group ($P<0.05$). However, quantitative analysis of western blot showed a significant downregulation of GLT-1 expression levels in ethanol-methamphetamine- treated group as compared to ethanol-saline- and ethanol-methamphetamine-ceftriaxone treated groups ($P<0.05$). Statistical analysis did not reveal any significant effect between ethanol-saline- and ethanol-methamphetamine-ceftriaxone treated groups.

Figure 3-2 <u>**Effects of methamphetamine (10mg/kg i.p. every 2 hours x4), and ethanol**</u>
<u>**(6g/kg), and ceftriaxone (200mg/kg) on GLT-1 expression levels in striatum.**</u>
A) Immunoblots of GLT-1 and β-tubulin in striatum. B) Quantitative analysis revealed a
significant increase in the ratio of GLT-1/β-tubulin in ethanol-methamphetamine-
ceftriaxone group compared to the ethanol-methamphetamine (EMC) group. Significant
downregulation of GLT-1 expression levels was revealed in the Ethanol-METH (EM)
group compared to the ethanol-saline control (ES) group. No significant difference in
GLT-1 expression levels was revealed in ethanol-methamphetamine-ceftriaxone group
compared to the ethanol-saline control group. Values shown as means ± S.E.M (*p <
0.05) (n=6 for each group).

3.3 Effects of Ethanol on GLT-1 Expression Levels in Striatum

Figure 3-3 shows the effect of ethanol on GLT-1 expression levels in striatum. Quantitative analysis of western blot showed a significant downregulation of GLT-1 expression levels in ethanol saline- treated group as compared to water-saline. An Independent t-test indicated a significant decrease in GLT-1 expression levels in striatum in ethanol saline- treated groups ($P<0.05$).

Figure 3-3 Effects of ethanol (6g/kg) on GLT-1 expression levels in striatum. A) Immunoblots of water and ethanol groups for GLT-1 and β-tubulin in the striatum. B) Quantitative analysis revealed a significant downregulation of GLT-1 expression levels in the ethanol-saline group compared to control naïve water-saline group. Values shown as means ± S.E.M ($*p < 0.05$) (n = 6 for each group).

3.4 Effect of Ethanol and Methamphetamine Co-abuse on GLT-1 Expression Levels in Striatum

Figure 3-4 shows the effect of ethanol on GLT-1 expression levels in striatum. Quantitative analysis of western blot revealed a significant downregulation of GLT-1expression levels in ethanol-methamphetamine-treated group as compared to water-methamphetamine-treated group. An Independent t-test indicated a significant decrease of GLT-1 expression levels in striatum after ethanol treatment in methamphetamine-treated group ($P<0.05$).

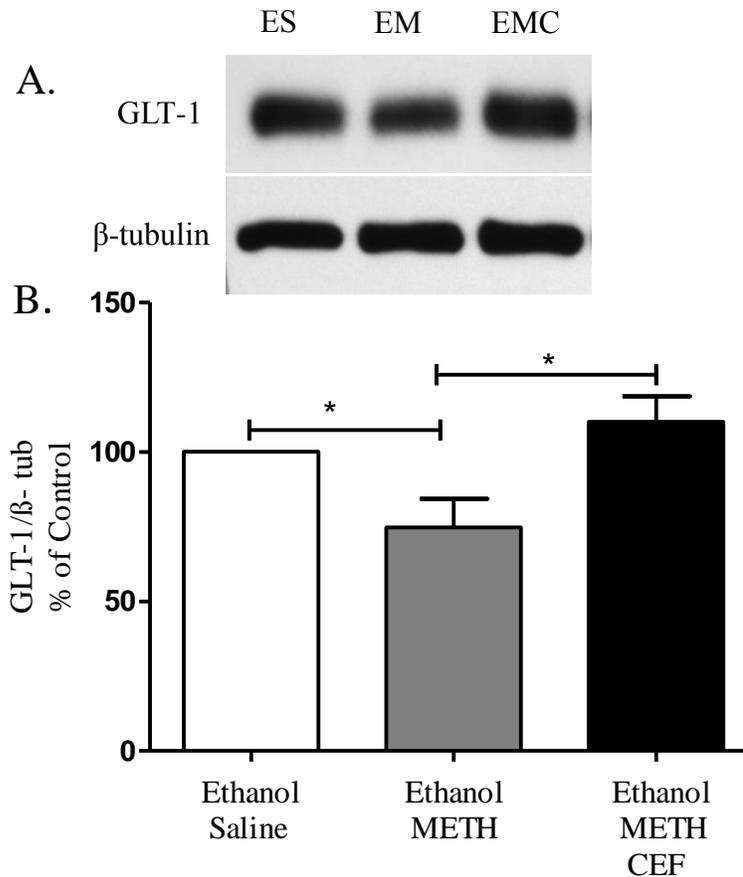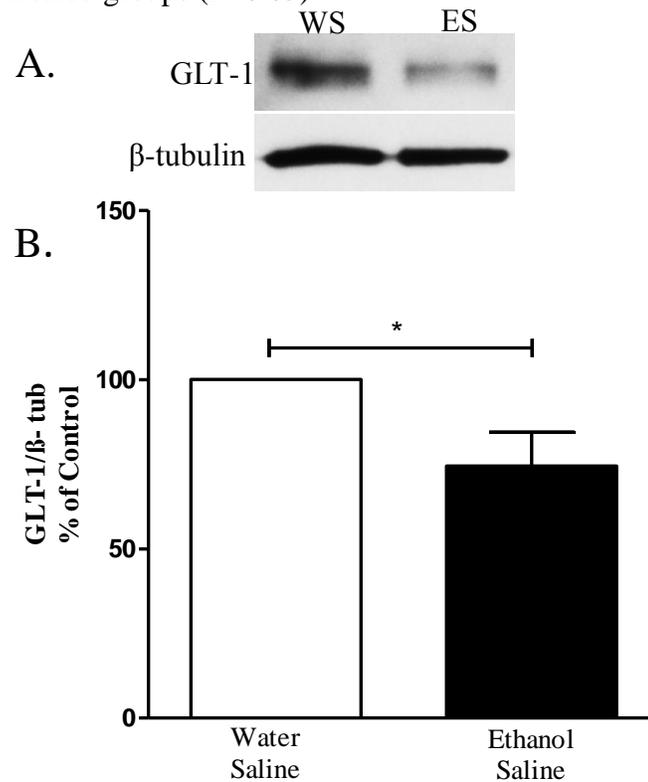**Figure 3-4 <u>Effects of methamphetamine (10mg/kg i.p. every 2 hours x4) and ethanol (6g/kg) co-abuse on GLT-1 expression levels in striatum.</u>** A) Immunoblots of water and ethanol groups for GLT-1 and β-tubulin in the striatum. B) Quantitative analysis revealed a significant downregulation in the ethanol-methamphetamine (EM) group compared to the water-methamphetamine (WM) control group. Values shown as means ± S.E.M (∗$p < 0.05$) (n = 6 for each group).

3.5. Effect of Methamphetamine and Ceftriaxone on xCT Expression Levels in Striatum

Figure 3-5 reveals the effect of ceftriaxone on xCT expression levels in striatum in the group receiving water through gavage procedure and challenged with high dose of methamphetamine. Quantitative analysis of western blot did not reveal any significant changes in xCT expression levels among all groups. One-way ANOVA followed by Newman-Keuls multiple comparison post-hoc test showed no significant changes in the expression levels of xCT between all groups ($P<0.05$).
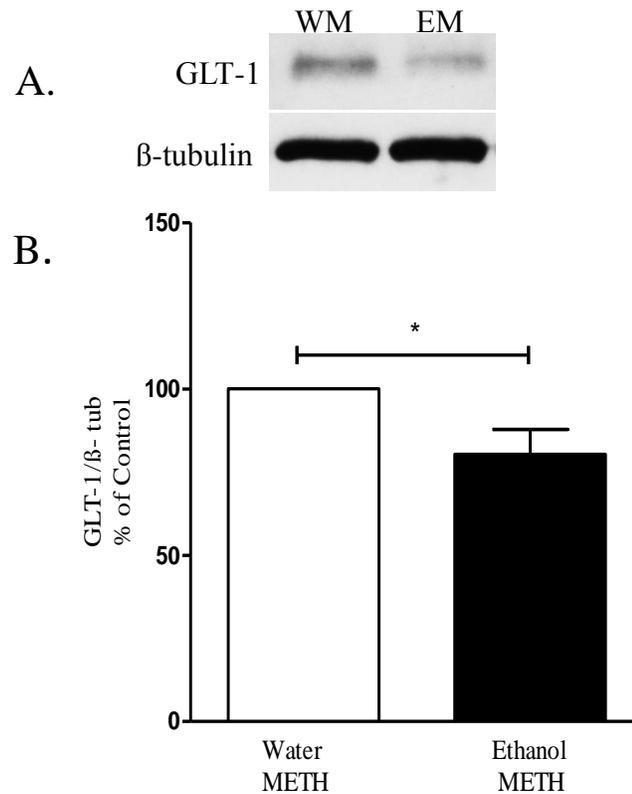

Figure 3-5 <u>**Effects of methamphetamine (10mg/kg i.p. every 2 hours x4) and ceftriaxone (200mg/kg) on xCT expression levels in striatum.**</u> A) Immunoblots of xCT and β-tubulin in striatum.   B) Quantitative analysis revealed no significant difference in the ratio of xCT/β-tubulin between all water groups. Values shown as means ± S.E.M ($*p < 0.05$) (n=6 for each group).

3.6. Effect of Methamphetamine, Ethanol, and Ceftriaxone on xCT Expression Levels in Striatum.

Figure 3-6 reveals the effect of ceftriaxone on xCT expression levels in striatum in the group receiving ethanol through gavage procedure and challenged with high dose of methamphetamine. Quantitative analysis of western blot did not reveal any significant changes of xCT expression levels among all groups. One-way ANOVA followed by Newman-Keuls multiple comparison post-hoc test demonstrated no significant changes in the expression levels of xCT between all treated groups ($P<0.05$).

Figure 3-6 **Effects of methamphetamine (10 mg/kg i.p. every 2 hours x 4), ethanol (6g/kg), and ceftriaxone (200mg/kg) on xCT expression levels in striatum.** A) Immunoblots of xCT and β-tubulin in striatum. B) Quantitative analysis revealed no significant difference in the ratio of xCT/β-tubulin between all Ethanol groups. Values shown as means ± S.E.M ($*p < 0.05$) (n=6 for each group).

3.7. Effect of Methamphetamine and Ceftriaxone on GLAST Expression Levels in Striatum.

Figure 3-7 reveals the effect of ceftriaxone on GLAST expression levels in striatum in the group receiving water through gavage procedure and challenged with high dose of methamphetamine. Quantitative analysis of western blot did not show any significant changes of GLAST expression levels among all groups. One-way ANOVA followed by Newman-Keuls multiple comparison post-hoc test revealed no significant changes in the expression levels of GLAST in all treated groups ($P<0.05$).

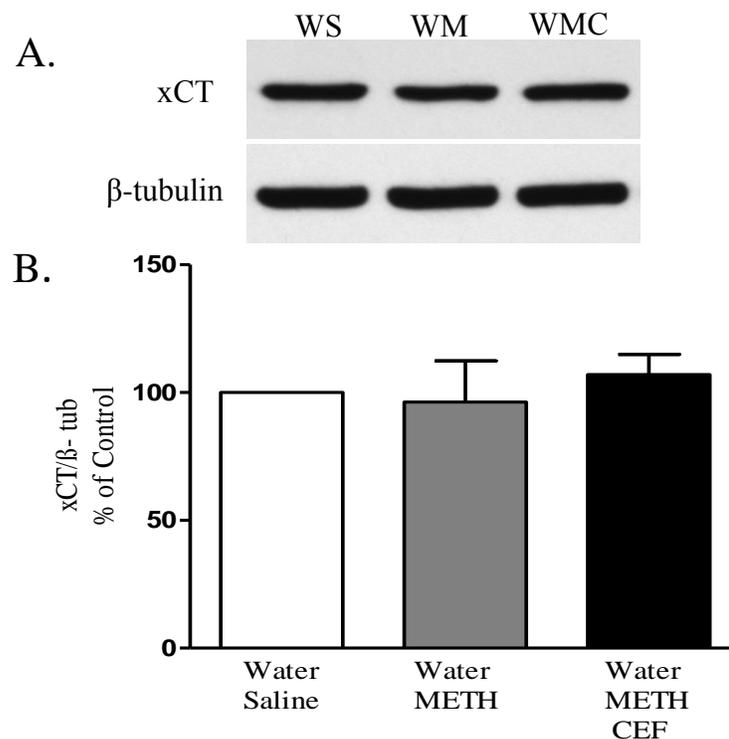**Figure 3-7** <u>**Effects of methamphetamine (10 mg/kg i.p. every 2 hours x 4) and ceftriaxone (200mg/kg) on GLAST expression levels in striatum.**</u> A) Immunoblots of GLAST and β-tubulin in striatum. B) Quantitative analysis revealed no significant difference in the ratio of GLAST/β-tubulin between all Water groups. Values shown as means ± S.E.M ($*p < 0.05$) (n=6 for each group).

3.8. Effect of Methamphetamine, Ethanol, and Ceftriaxone on GLAST Expression Levels in Striatum

Figure 3-8 demonstrates the effect of ceftriaxone on GLAST expression levels in striatum in the group receiving ethanol through gavage procedure and challenged with high dose of methamphetamine. Quantitative analysis of western blot did not reveal any significant changes of GLAST expression levels among all groups. One-way ANOVA followed by Newman-Keuls multiple comparison post-hoc test revealed no significant changes in the expression levels of GLAST between all treated groups ($P<0.05$).

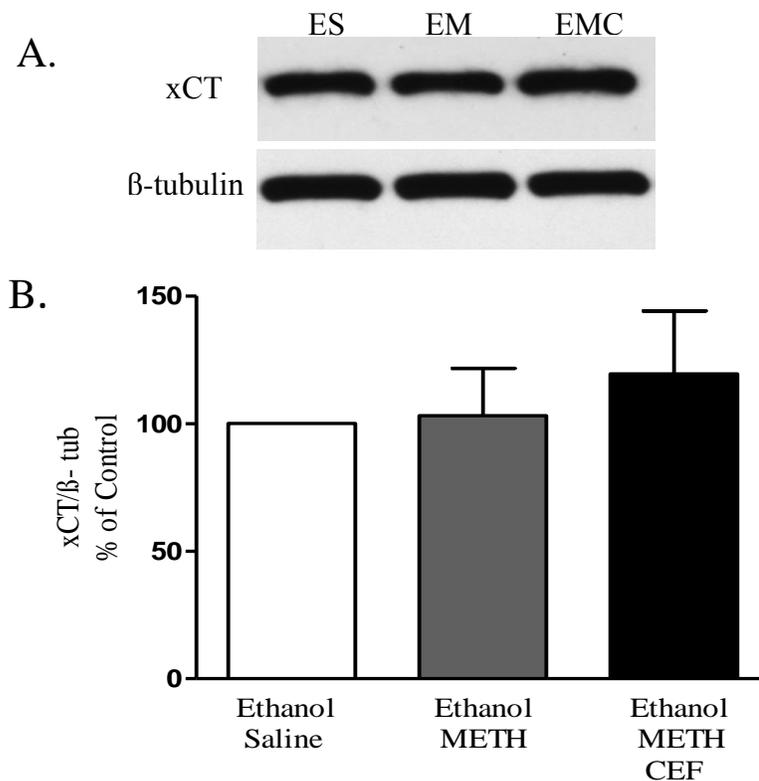**Figure 3-8 Effects of methamphetamine (10mg/kg i.p. every 2 hours x 4), ethanol (6 g/ kg), and ceftriaxone (200mg/kg) on GLAST expression levels in striatum.** A) Immunoblots of GLAST and β-tubulin in striatum. B) Quantitative analysis revealed no significant difference in the ratio of GLAST/β-tubulin between all ethanol groups. Values shown as means ± S.E.M ($*p < 0.05$) (n=6 for each group).

3.9. Effect of Methamphetamine and Ceftriaxone on GLT-1 Expression Levels in Hippocampus.

Figure 3-9 demonstrates the effect of ceftriaxone on GLT-1 expression levels in hippocampus in the group receiving water through gavage procedure and challenged with high dose of methamphetamine. One-way ANOVA followed by Newman-Keuls multiple comparison post-hoc test, demonstrate a significant increase in the expression levels of GLT-1 in ceftriaxone treated group ($P<0.05$). Alternatively, quantitative analysis of western blot showed a significant downregulation of GLT-1 expression levels in methamphetamine-treated group as compared to naïve control- and ceftriaxone-treated groups ($P<0.05$). Statistical analysis revealed no significant effect between naïve control- and ceftriaxone-treated groups.

A.

WS   WM   WMC

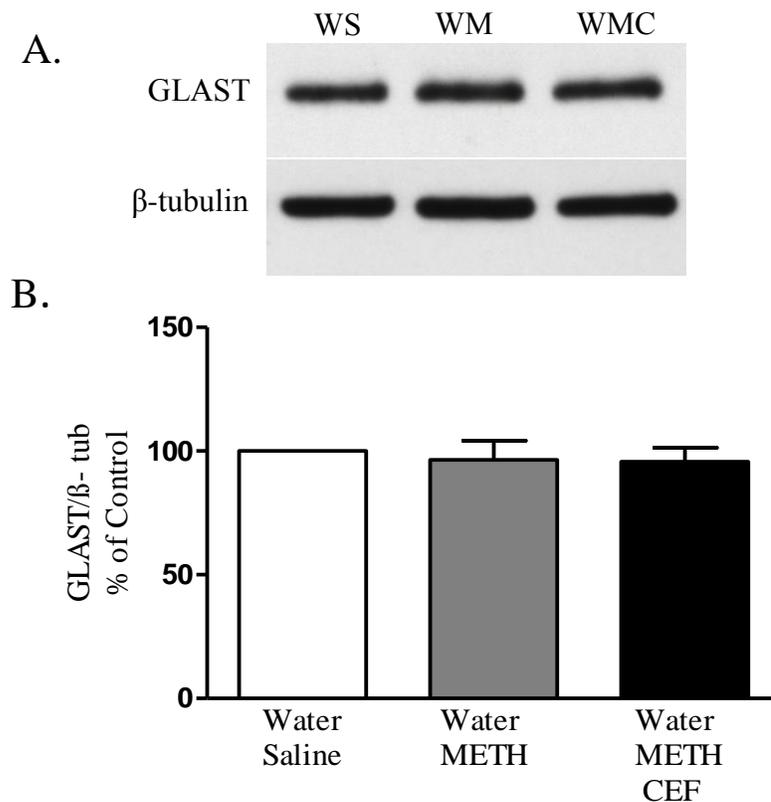GLT-1

β-tubulin

B.

GLT-1/ß- tub
% of Control
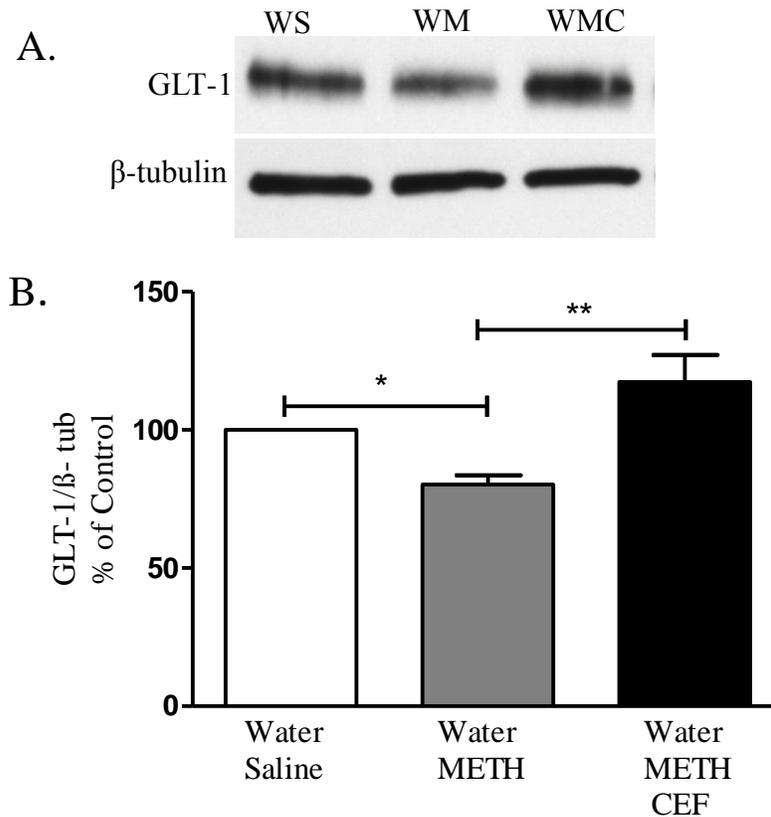
Figure 3-9 <u>Effects of methamphetamine (10mg/kg i.p. every 2 hours x4) and ceftriaxone (200mg/kg) on GLT-1 expression levels in hippocampus.</u> A) Immunoblots of GLT-1 and β-tubulin in hippocampus. B) Quantitative analysis revealed a significant increase in the ratio of GLT-1/β-tubulin in water-methamphetamine-ceftriaxone group compared to the water-methamphetamine. Significant downregulation of GLT-1 expression levels was revealed in the water-methamphetamine groups compared to the control naïve water-saline group. No significant difference in GLT-1 expression levels was revealed in methamphetamine-ceftriaxone group compared to the water-saline control group. Values shown as means ± S.E.M ($*p < 0.05$) (n=6 for each group).

3.10. Effect of Methamphetamine, Ethanol, and Ceftriaxone on GLT-1 Expression levels in Hippocampus.

Figure 3-10 shows the effect of ceftriaxone on GLT-1 expression levels in hippocampus in the group receiving ethanol through gavage procedure and challenged with high dose of methamphetamine. One-way ANOVA followed by Newman-Keuls multiple comparison post-hoc test, revealed a significant increase in the expression levels of GLT-1 with ceftriaxone- treated group ($P<0.05$). However, quantitative analysis of western blot showed a significant downregulation in GLT-1 expression levels in ethanol-methamphetamine-treated group as compared to ethanol-saline- and ethanol-methamphetamine-ceftriaxone-treated groups ($P<0.05$). Statistical analysis did not reveal any significant effect between ethanol-saline and ethanol-methamphetamine-ceftriaxone-treated group.
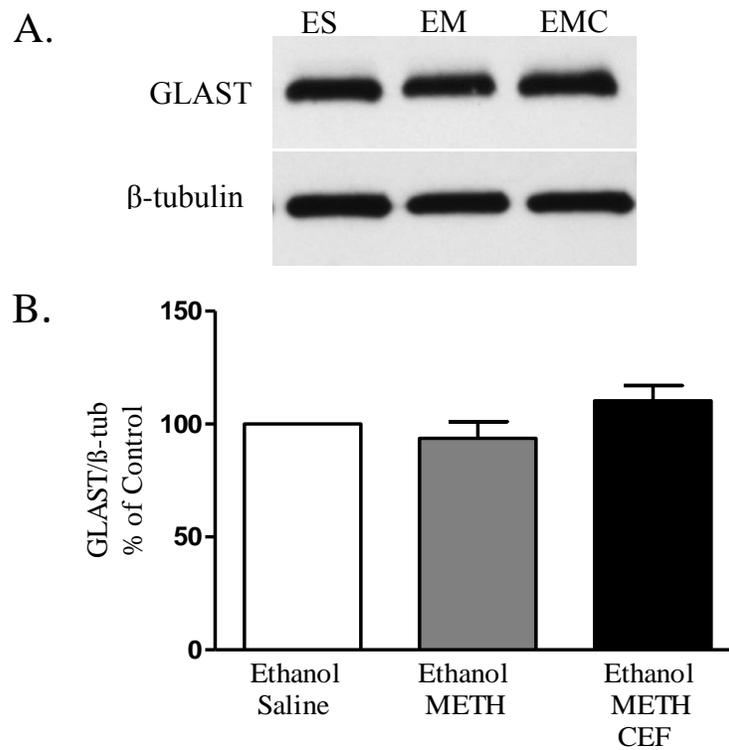
A.

ES EM EMC

GLT-1

β-tubulin

B.

Figure 3-10 <u>Effects of methamphetamine (10 mg/kg i.p. every 2 hours x4), and ethanol (6g/kg), and ceftriaxone (200mg/kg) on GLT-1 expression levels in hippocampus.</u> A) Immunoblots of GLT-1 and β-tubulin in hippocampus. B) Quantitative analysis revealed a significant increase in the ratio of GLT-1/β-tubulin in ethanol-methamphetamine-ceftriaxone group compared to the ethanol-methamphetamine group. Significant downregulation of GLT-1 expression levels was revealed in the ethanol-methamphetamine group compared to the ethanol-saline control group. No significant difference in GLT-1 expression levels was revealed in methamphetamine-ceftriaxone group compared to the ethanol-saline control group. Values shown as means ± S.E.M (*p < 0.05) (n=6 for each group).

3.11. Effect of Ethanol on GLT-1 Expression Levels in Hippocampus

Figure 3-11 shows the effect of ethanol on GLT-1 expression levels in hippocampus. Quantitative analysis of western blot showed a significant downregulation of GLT-1 expression levels in ethanol-saline-treated group as compared to water-saline-treated group. An Independent t-test indicated a significant decrease of GLT-1 expression levels in HPC after ethanol treatment in saline-treated groups (P<0.05).
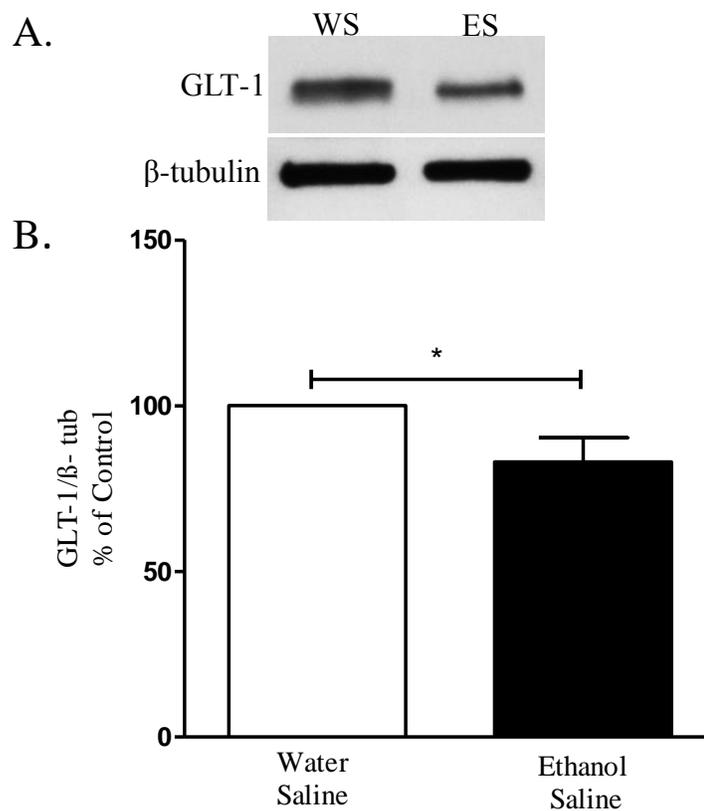
Figure 3-11 <u>Effects ethanol (6g/kg) on GLT-1 expression levels in hippocampus.</u> A) Immunoblots of water and ethanol groups for GLT-1 and β-tubulin in the hippocampus. B) Quantitative analysis revealed a significant downregulation of GLT-1 expression levels in the ethanol-saline group compared to control naïve water-saline group. Values shown as means ± S.E.M (∗p < 0.05) (n = 6 for each group).

3.12. Effect of Ethanol and Methamphetamine Co-abuse on GLT-1 Expression Levels in Hippocampus

Figure 3-12 shows the effect of ethanol on GLT-1 expression levels in hippocampus. Quantitative analysis of western blot revealed a significant downregulation of GLT-1 expression levels in ethanol-methamphetamine-treated group as compared to water-methamphetamine-treated group. An Independent t-test indicated a significant decrease of GLT-1 expression levels in HPC after ethanol treatment in both saline- and methamphetamine-treated groups (P<0.05).

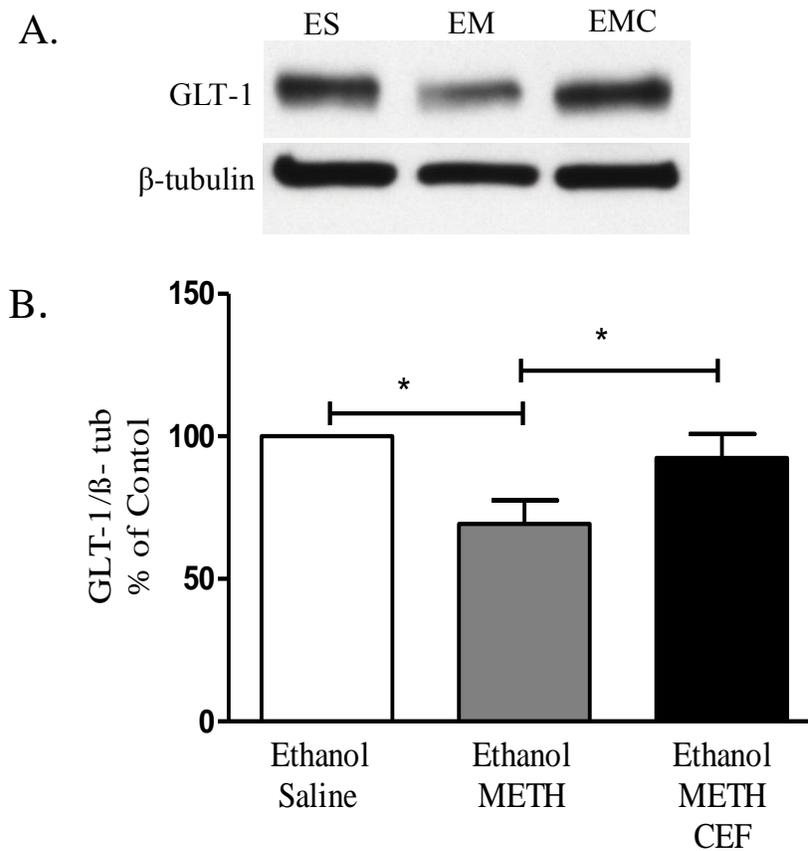**Figure 3-12 <u>Effects of methamphetamine (10mg/kg i.p. every 2 hours x4) and ethanol (6g/kg) co-abuse on GLT-1 expression levels in hippocampus.</u>** A) Immunoblots of water and ethanol groups for GLT-1 and β-tubulin in the hippocampus. B) Quantitative analysis revealed a significant downregulation in ethanol-methamphetamine group compared to water-methamphetamine control group. Values shown as means ± S.E.M ($*p < 0.05$)   (n = 6 for each group)

3.13. Effect of Methamphetamine and Ceftriaxone on xCT Expression Levels in Hippocampus

Figure 3-13 shows the effect of ceftriaxone on xCT expression levels in hippocampus in the group receiving water through gavage procedure and challenged with high dose of methamphetamine. Quantitative analysis of western blot did not reveal any significant changes of xCT expression levels among all groups. One-way ANOVA followed by Newman-Keuls multiple comparison post-hoc test showed no significant changes in the expression levels of xCT in all treated groups ($P<0.05$).

Figure 3-13 Effects of methamphetamine (10mg/kg i.p. every 2 hours x4) and ceftriaxone (200mg/kg) on xCT expression levels in hippocampus. A) Immunoblots of xCT and β-tubulin in hippocampus. B) Quantitative analysis revealed no significant difference in the ratio of xCT/β-tubulin between all water groups. Values shown as means ± S.E.M (∗$p < 0.05$) (n=6 for each group).

3.14. Effect of Methamphetamine, Ethanol, and Ceftriaxone on xCT Expression Levels in Hippocampus

Figure 3-14 shows the effect of ceftriaxone on xCT expression levels in hippocampus in the group receiving ethanol through gavage procedure and challenged with high dose of methamphetamine. Quantitative analysis of western blot did not reveal any significant changes of xCT expression levels among all groups. One-way ANOVA followed by Newman-Keuls multiple comparison post-hoc test demonstrated no significant changes in the expression levels of xCT between all treated groups ($P<0.05$).

Figure 3-14 <u>Effects of methamphetamine (10mg/kg i.p. every 2 hours x4), ethanol (6g/kg), and ceftriaxone (200mg/kg) on xCT expression levels in hippocampus</u>. A) Immunoblots of xCT and β-tubulin in hippocampus. B) Quantitative analysis revealed no significant difference in the ratio of xCT/β-tubulin between all Ethanol groups. Values shown as means ± S.E.M ($*p < 0.05$) (n=6 for each group).

3.15. Effect of Methamphetamine and Ceftriaxone on GLAST Expression Levels in Hippocampus

Figure 3-15 reveals the effect of ceftriaxone on GLAST expression levels in hippocampus in the group receiving water through gavage procedure and challenged with high dose of methamphetamine. Quantitative analysis of western blot did not reveal any significant changes in GLAST expression levels among all groups. One-way ANOVA followed by Newman-Keuls multiple comparison post-hoc test showed no significant changes in the expression levels of GLAST in all treated groups ($P<0.05$).
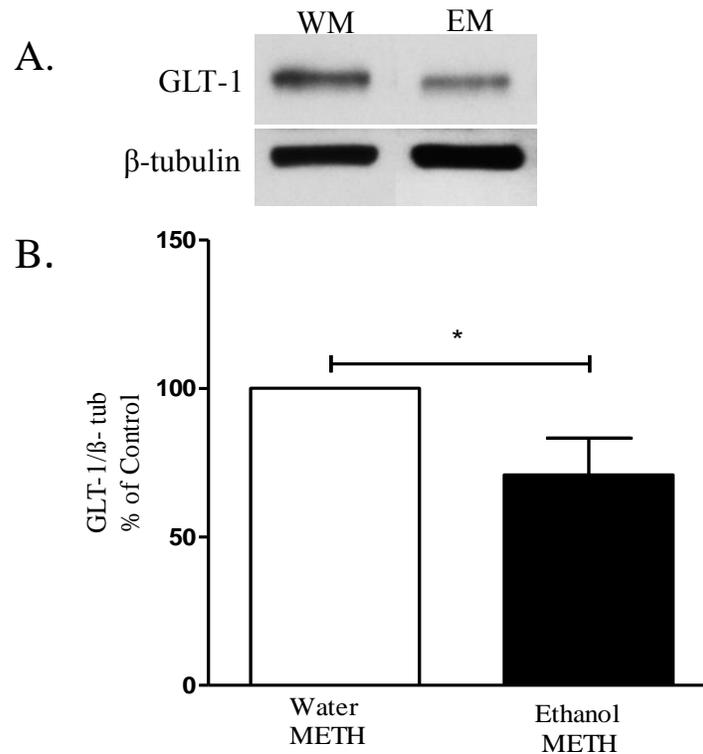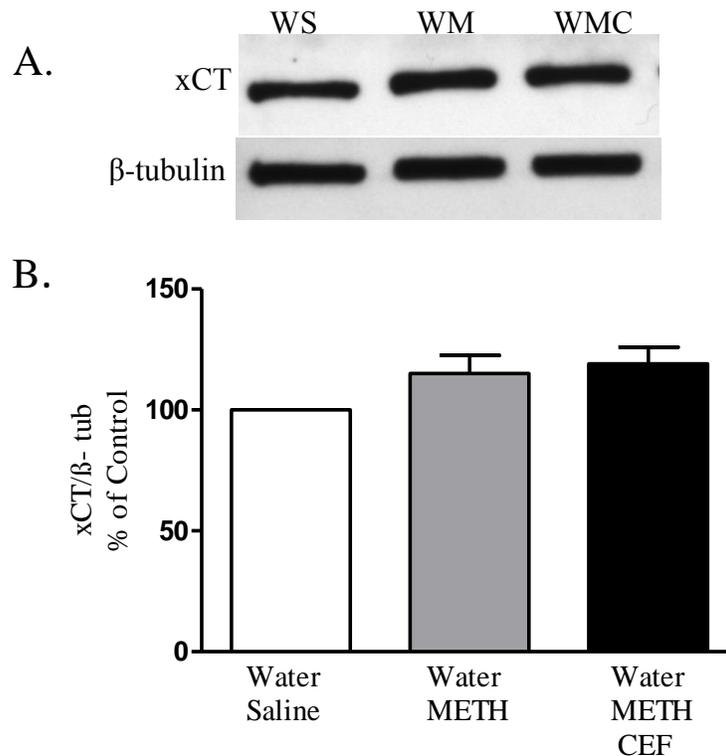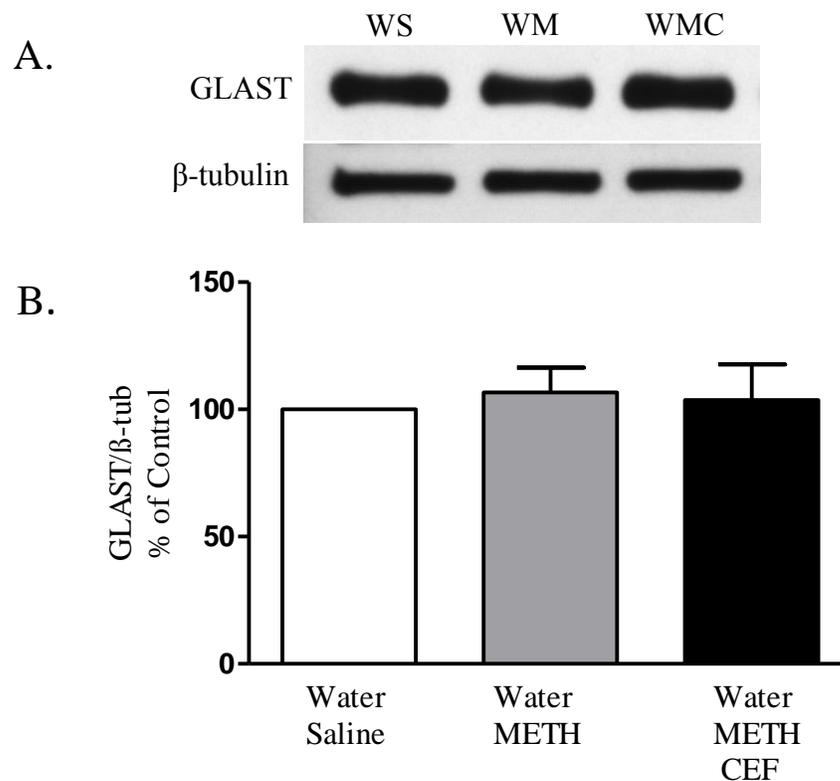

Figure 3-15 Effects of methamphetamine (10mg/kg i.p. every 2 hours x4) and ceftriaxone (200mg/kg) on GLAST expression levels in hippocampus. A) Immunoblots of GLAST and β-tubulin in hippocampus.   B) Quantitative analysis revealed no significant difference in the ratio of GLAST/β-tubulin between all Water groups. Values shown as means ± S.E.M (∗p < 0.05) (n=6 for each group).

3.16. Effect of Methamphetamine, Ethanol, and Ceftriaxone on GLAST Expression Levels in Hippocampus

Figure 3-16 demonstrates the effect of ceftriaxone on GLAST expression levels in hippocampus in group receiving ethanol through gavage procedure and challenged with high dose of methamphetamine. Quantitative analysis of western blot did not reveal any significant changes in GLAST expression levels among all groups. One-way ANOVA followed by Newman-Keuls multiple comparison post-hoc test revealed no significant changes in the expression levels of GLAST between all treated groups ($P<0.05$).

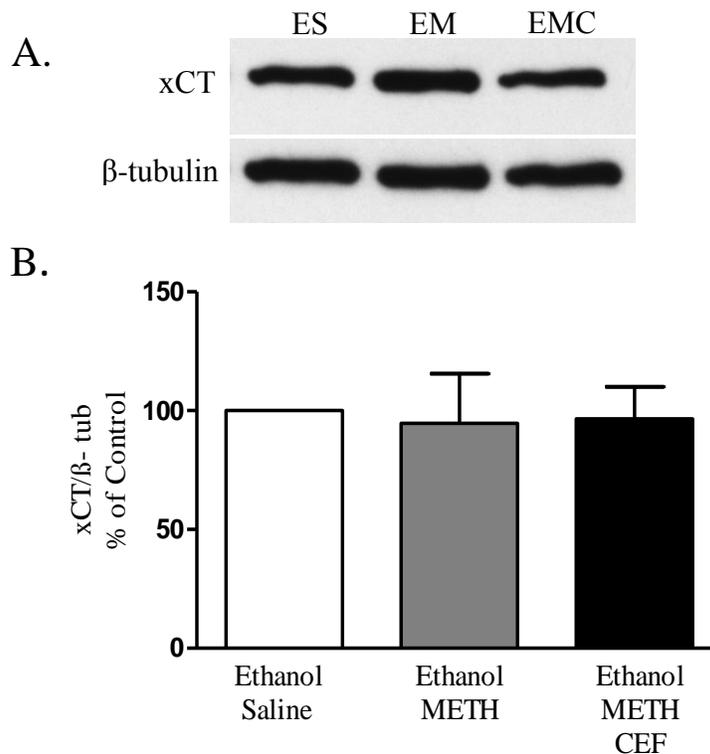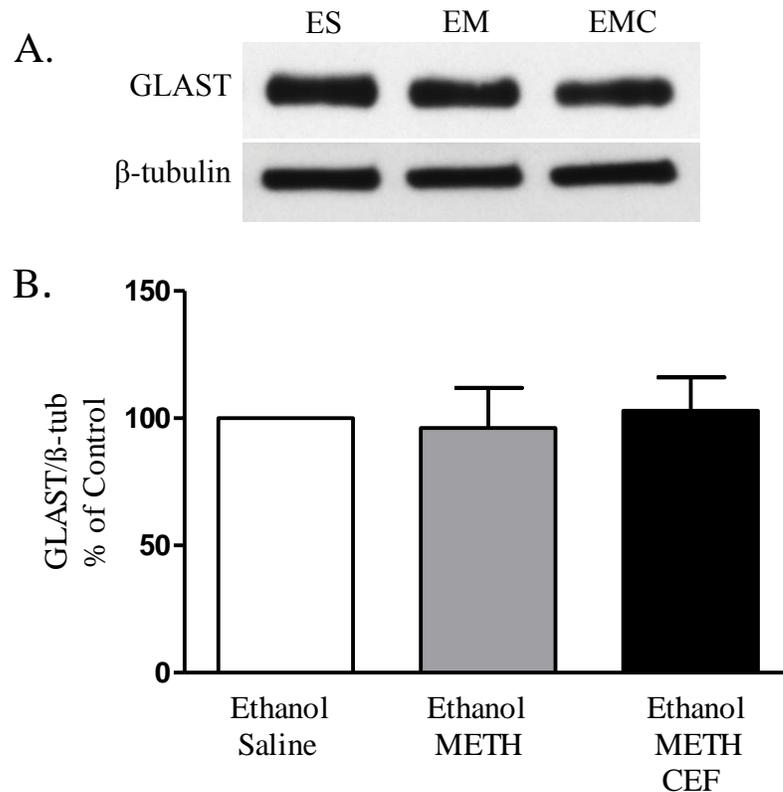**Figure 3-16** <u>**Effects of methamphetamine (10mg/kg i.p. every 2 hours x 4), ethanol (6 g/ kg), and ceftriaxone (200mg/kg) on GLAST expression levels in hippocampus.**</u> A) Immunoblots of GLAST and β-tubulin in hippocampus. B) Quantitative analysis revealed no significant difference in the ratio of GLAST/β-tubulin between all ethanol groups. Values shown as means ± S.E.M ($*p < 0.05$) (n=6 for each group).

Chapter 4

Discussion

Several studies have reported the harmful effects of co-abuse of alcohol and methamphetamine (Lawyer et al., 2010, Kirkpatrick et al., 2012, Bujarski et al., 2014b). The combination of the two drugs is lethal, as methamphetamine can produce stimulating effects that counteract the depressing effects of alcohol where users tend to consume more alcohol (Bujarski et al., 2014b). We reveal in this study the effects of repeated high doses of methamphetamine alone in comparison to alcohol and methamphetamine co-abuse on glutamate transporters: GLT-1, xCT, and GLAST in Wister rats. Ethanol was administrated through gavage and water was chosen as a control for the gavage procedure. In this study, we showed that GLT-1 was downregulated in both water and ethanol groups after administrating methamphetamine. However, xCT and GLAST were not affected by administration of methamphetamine in water and ethanol groups as compared to the control groups. Remarkably, ceftriaxone was able to normalize the levels of GLT-1 in both water and ethanol groups.

Interestingly, GLT-1 levels in alcohol and methamphetamine administered group were downregulated significantly compared to methamphetamine group, which suggests that co-abuse of alcohol and methamphetamine could exacerbate the effects on GLT-1 expression levels. Studies found that methamphetamine increased the rewards effects and reduced central alcohol depression effects (Kirkpatrick et al., 2012). Studies from our laboratory reported that chronic alcohol drinking in alcohol preferring rats (P-rats) downregulated GLT-1 (Sari and Sreemantula, 2012b, Sari et al., 2013, Alhaddad et al., 2014a, Alhaddad et al., 2014b, Rao and Sari, 2014b, Aal-Aaboda et al., 2015). In addition, studies have shown that methamphetamine indirectly increased the release of glutamate (Nash and Yamamoto, 1992, Mark et al., 2004), which suggest that administration of both alcohol and methamphetamine may dramatically increase this release.

The results of this study are the first to show that GLT-1 is downregulated after acute repeated methamphetamine administrations. Studies have found that excess glutamate can cause excitotoxicity and cell death (Choi, 1992, Sattler and Tymianski, 2001). In this study, we have investigated the expression levels of xCT and GLAST, which did not show any changes as compared the control naïve groups. These results indicated that the effects of administration of acute high doses of methamphetamine are more specific on GLT-1 but not on xCT or GLAST expression levels. In order to normalize the level of GLT-1, ceftriaxone were administered i.p. after methamphetamine injections, as ceftriaxone is known to upregulate GLT-1 expression levels in different brain regions, including both striatum and hippocampus (Aal-Aaboda et al., 2015). Importantly, ceftriaxone was able to restore the level of GLT-1 in both striatum and hippocampus,

which may have effects in reducing the excess of extracellular glutamate and eliminate the effects of glutamate excitotoxicity. We investigated the effects of oral gavage of ethanol alone (6 g/kg) in binge dosing pattern (Faingold, 2008) in the expression levels of GLT-1, xCT, and GLAST. As a result, we found a reduction in GLT-1 expression levels as compared to control naïve group. It is noteworthy, that xCT did not show any changes compared to control naïve groups. Similarly, we did not observe any effects in the expression levels of GLAST. These findings demonstrated the specific action in GLT-1.

Alcohol and methamphetamine co-abuse may enhance the possibility of glutamate mediating excitotoxicity and neural death, which may involve glutamate receptors such as NMDA, AMPA, and KA receptors (Choi, 1992, Meldrum, 2000, Sattler and Tymianski, 2001). Studies have found a strong association between excessive overstimulation of glutamate receptors and neurodegenerative diseases (Maragos et al., 1987, Gao et al., 2000, Howland et al., 2002, Scott et al., 2002). Though, the exact mechanism is still not fully understood, it is believed that calcium plays an important role in the mechanism of glutamate excitotoxicity and neural death (Choi, 1985). In addition, alcohol and methamphetamine administration was shown to disrupt glutamate homeostasis via facilitating the glutamate release and decrease the expression levels of GLT-1, which may increase the level of synaptic glutamate and overstimulate glutamate receptors and increase calcium influx (Choi, 1985). Studies suggested that glutamate facilitates excitotoxicity through an increase in the calcium influx due to the overstimulation of glutamate receptors, which can lead to mitochondrial loss and neuronal death (Stephans and Yamamoto, 1994, Schinder et al., 1996). Other studies suggest the involvement of nitric oxide after NMDA receptor activation, which can

facilitate the calcium binding to calmodulin, release of nitric oxides and increase cGMP accumulation (Garthwaite and Boulton, 1995, Kita et al., 2003).

Striatum considers one of densest dopaminergic parts of the brain; it has been found that striatum is an important target in drugs of abuse. In addition, hippocampus is also important in addiction and drug of abuse. Hippocampus connects different parts the limbic system and plays an important role in drug sensitization, fear conditioning, and stress. In this study, we tested the effects of alcohol and methamphetamine on GLT-1, xCT, and GLAST in both striatum and hippocampus. We found that GLT-1 expression levels were reduced in both of these regions after administrating alcohol and methamphetamine together. We also found a reduction in the level of GLT-1 expression levels after administering repeated high doses of methamphetamine in both areas. Similarly, GLT-1 expression levels were also reduced after alcohol administration alone in both areas. In contrast, both xCT and GLAST expression levels remained unaffected in both areas after administering alcohol alone, methamphetamine alone, or methamphetamine and alcohol administered together. Importantly, ceftriaxone normalized the levels of GLT-1 expression levels in both areas.

It is noteworthy that studies have shown that several β-lactam antibiotics can upregulate GLT-1 leading to normalization of extracellular glutamate as well as neuroprotection (Rothstein et al., 2005). Ceftriaxone have been used in several studies due to its effectiveness in upregulating GLT-1. A daily dose of 200 mg/kg for five to seven days is usually chosen for animal models of drug abuse and neurodegenerative diseases (for review see ref. Rao and Sari, 2012). For example, ceftriaxone was able to prevent cocaine seeking through normalizing GLT-1 and restoring glutamate homeostasis

(Knackstedt et al., 2010). In addition, studies from our laboratory showed similar effects using cocaine seeking animal model (Sari et al., 2009). Another study found that ceftriaxone could attenuate hyperactivity and behavioral changes associated with amphetamine (Rasmussen et al., 2011). Furthermore, studies from our laboratory have confirmed the ability of ceftriaxone on attenuating alcohol consumption in P-rats (Sari et al., 2013, Alhaddad et al., 2014a, Rao and Sari, 2014b). Thus, we choose to investigate the effects of ceftriaxone on alcohol and methamphetamine co-abuse animal model.

In this study, we used acute repeated high doses of methamphetamine (10mg/kg). This paradigm is well established to produce methamphetamine neurotoxicity. This model provides the best example of repeated high doses of methamphetamine that produces dopaminergic nerve ending damage and neurotoxicity (Thomas et al., 2004). Studies have shown an increase in the extracellular concentrations of all monoamine neurotransmitters, including dopamine and glutamate particularly in the striatum (Nash and Yamamoto, 1992) and hippocampus (Rocher and Gardier, 2001). Moreover, this regimen of methamphetamine administration shows greater loss of dopamine transporter (DAT) and increased of mortality rate (Fumagalli et al., 1998). We found here that GLT-1 is also downregulated, which suggests that glutamatergic system is also affected after repeated high doses of methamphetamine.

In conclusion, this study revealed for the first time the effects of methamphetamine in GLT-1, xCT, and GLAST expression levels in both striatum and hippocampus. In addition, we showed that administration of both alcohol and methamphetamine could lead to dramatic decrease in the expression levels of GLT-1 in striatum and hippocampus

without any effects in the expression levels of xCT and GLAST in these brain regions. Importantly ceftriaxone was able to normalize GLT-1 expression levels in all groups that received methamphetamine or alcohol and methamphetamine. These findings suggest that GLT-1 might be a target in a model of alcohol and methamphetamine co-abuse and that ceftriaxone could have beneficial effects in upregulating GLT-1 expression levels and consequently reducing alcohol and methamphetamine glutamate excitotoxicity.

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