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Effects of Modulating Glutamate Homeostasis in Methamphetamine and Alcohol Co-Abuse: Potential Therapeutic Targets

by

Yusuf S. Althobaiti

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the

Doctor of Philosophy Degree in Experimental Therapeutics

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Dr. Youssef Sari, Committee Chair

_________________________________________

Dr. F. Scott Hall, Committee Member

_________________________________________

Dr. Zahoor Shah, Committee Member

_________________________________________

Dr. Wissam AbouAlaiwi, Committee Member

_________________________________________

Dr. Amanda Bryant-Friedrich, Dean
College of Graduate Studies

The University of Toledo

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An Abstract of

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Methamphetamine (METH) is one of the most commonly used psychostimulants that is highly co-abused with ethanol. The use of METH and ethanol can lead to deteriorating health problems and can alter the brain glutamate homeostasis. Glutamate homeostasis can be maintained by several glutamate transporters and receptors such as glutamate transporter type 1 (GLT-1), cystine-glutamate exchanger (xC), glutamate aspartate transporter (GLAST), and group II metabotropic glutamate receptors (mGluR2/3). This project aimed to investigate the role of the key proteins involved in glutamate homeostasis using METH and ethanol co-abuse rat model. I showed an unprecedented evidence that repeated high doses of METH decreased GLT-1 expression in the nucleus accumbens (NAc) and prefrontal cortex (PFC) and increased body temperature. Interestingly, ethanol and METH were found to have an additive effect on the downregulation of GLT-1 expression in the NAc but not in the PFC. Ceftriaxone (CEF), a β-lactam antibiotic known to upregulate GLT-1, significantly reversed METH-induced hyperthermia, restored GLT-1 expression, and increased xCT expression in the NAc and PFC. We then tested a non-antibiotic β-lactam compound, clavulanic acid (CA)
which can be administered orally, has better brain penetrability and is relatively safe. To test its activity on a METH and ethanol co-abuse rat model of drug dependence, we investigated the effect of CA on the reinstatement model of METH using conditioned place preference (CPP) paradigm as well as free choice ethanol drinking in alcohol preferring (P) rats. We further assessed the expression of GLT-1, xCT, GLAST, and mGluR2/3 in the NAc shell and core as well as dorsomedial PFC (dmPFC). We have found that the GLT-1 down-regulatory effect of METH and ethanol exposure in the NAc is specific to the shell subregion rather than the core. We showed for the first time that CA treatment blocked the reinstatement effect of METH, decreased ethanol intake, restored the expression of GLT-1 and xCT in the shell, and increased the expression of mGluR2/3 in the shell and dmPFC. We further investigated the mechanism of action of CA on METH induced reinstatement of CPP. The anti-reinstatement effect of seven-day CA treatment was completely blocked by pretreatment with the selective mGluR2/3 antagonist LY341495. Alternatively, a single dose pretreatment with CA did not block the reinstatement of METH CPP. Finally, we conducted an in vivo microdialysis study in freely moving P rats to assess glutamate release in the NAc shell or core following the reinstatement dose of METH. The reinstatement dose of METH increased glutamate release in the NAc shell, but not the NAc core, which was blocked by CA pre-treatment. Thus, restoring glutamate homeostasis following the development of METH dependence could be an effective therapeutic strategy to prevent relapse to drug seeking.
I dedicate this dissertation to my beautiful family, with my sincere gratitude for their unconditional support, patience and love.
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List of Abbreviations

aCSF.................. Artificial Cerebrospinal Fluid
AMPA.................. α-Amino-3-hydroxy-5-Methyl-4-isoxazolepropionic Acid
ANOVA................. Analysis of Variance
AP...................... Anterior Posterior

CA........................ Clavulanic acid
CEF........................ Ceftriaxone
CNS........................ Central Nervous System
CPP...................... Conditioned Place Preference

DHK..................... Dihydro Kainate
DM....................... Dorsal Medial
dmPFC................... Dorsomedial Prefrontal Cortex

EAAT.................... Excitatory Amino Acid Transporter
EDTA.................... Ethylene Diamine Tetra Acetic acid
EM....................... Ethanol-METH group
EMC..................... Ethanol-METH-CEF group
ES....................... Ethanol-Saline group

GLAST.................. Glutamate Aspartate Transporter
GLT-1................... Glutamate Transporter 1

HPLC.................... High Performance Liquid Chromatography
HRP..................... Horseradish Peroxidase

I.M...................... Intra Muscular
I.P....................... Intra Peritoneal

MDMA................... 3,4-methylenedioxymethamphetamine
METH.................... Methamphetamine
mGluR................... Metabotropic Glutamate Receptors
ML...................... Medio Lateral

NAc..................... Nucleus Accumbens
NMDA................... N-Methyl-D-Aspartate
OD..........................Optical Density
PCR.......................Polymerase Chain Reaction
PE 20........................Poly Ethylene 20
PFC..........................Prefrontal Cortex
PVDF.......................Polyvinylidene Fluoride
SEM........................Standard Error of the Mean
SDS........................Sodium Dodecyl Sulfate
VTA..........................Ventral Tegmental Area
WM..........................Water-METH group
WMC........................Water-METH-CEF group
WS..........................Water-Saline group
xCT.........................Cystine-glutamate Anti-porter
List of Symbols

mg ............................ Milligram
kg ............................. Kilogram

C ............................. Centigrade
cm ............................ centimeter

m ............................. Meter
mm ............................ Millimeter
µm ............................ Micrometer
nm ............................ Nanometer
G ............................. Gauge

M ............................. Molar
mM ........................... Milli Molar
µM ............................ Micro Molar

µl ............................ Microliter
v/v ........................... Volume/Volume

kDa .......................... Kilo Dalton
Introduction

Methamphetamine (METH) is one of the most commonly used psychostimulants worldwide. According to the available data from national surveys between the years of 2002 and 2004, more than 16 million Americans over the age of 12 have used METH (Colliver, 2006). METH abusers frequently use alcohol and have a higher risk of reaching alcohol intoxication level (Furr et al., 2000). The prevalence of alcohol use disorder was found to be more than 75% among amphetamine dependent patients (Stinson et al., 2005). The use of METH and ethanol can lead to deteriorating health problems and can alter the brain glutamate homeostasis. Glutamate is the major excitatory amino acid in the central nervous system (CNS), which is controlled by a group of glutamate transporters and receptors. Glutamate transporter type 1 (GLT-1; human homolog is excitatory amino acid transporter 2, EAAT2) plays a major role in clearing extracellular glutamate and minimizing the subsequent activation of synaptic and non-synaptic glutamate receptors (Ginsberg et al., 1995, Rothstein et al., 1995, Danbolt, 2001, Mitani and Tanaka, 2003). The other important transporter and regulator of glutamate homeostasis is the cystine-glutamate exchanger (xCT), which has a significant role in maintaining basal glutamate concentration (Baker et al., 2002). Although, it is known that repeated METH exposure can cause hyperthermia and increase extracellular glutamate concentration (Nash and Yamamoto, 1992, Stephans and Yamamoto, 1994),
little is known about its effect on glutamate transporters. Importantly, chronic ethanol exposure was found to reduce GLT-1 expression (Alhaddad et al., 2014b, Aal-Aaboda et al., 2015, Goodwani et al., 2015b) and increase extracellular glutamate concentration in the NAc (Ding et al., 2013, Das et al., 2015, Pati et al., 2016). Of note, ceftriaxone (CEF) is known to increase GLT-1 expression in several brain regions (Miller et al., 2008, Sari et al., 2009, Sari et al., 2013) and can normalize extracellular glutamate concentration in cocaine and ethanol-seeking rat models (Trantham-Davidson et al., 2012, Das et al., 2015). Importantly, CEF was also revealed to reduce morphine-induced hyperthermia (Rawls et al., 2007). Therefore, we investigated, in the first study, the effect of ethanol and METH exposure and CEF posttreatment on hyperthermia and the expression of glutamate transporters in the nucleus accumbens (NAc) and prefrontal cortex (PFC). The NAc is a brain region that is involved in the rewarding and reinforcing effects of drugs of abuse (Koob and Bloom, 1988, Wise and Rompré, 1989, Bardo, 1998, Koob et al., 1998). Glutamatergic neurotransmission in the NAc plays a critical role in the relapse behavior to different drugs of abuse. Importantly, deficits in glutamate clearance in the NAc have been found to be associated with chronic drug use and drug seeking behavior (Fujio et al., 2005, Melendez et al., 2005, Knackstedt et al., 2010, Das et al., 2015). The NAc receives glutamatergic inputs from the PFC as well as other brain regions (Kelley et al., 1982, Phillipson and Griffiths, 1985). Of note, it has been shown that blocking glutamatergic activation of mGluR1, mGluR5, and AMPA receptors, and inhibiting glutamate release or activating mGluR2/3 in the NAc can decrease the reinstatement to several drugs of
abuse (Cornish and Kalivas, 2000, Park et al., 2002, McFarland et al., 2004, Tessari et al., 2004, Lee et al., 2005, Bossert et al., 2006, Bäckström and Hyytiä, 2007, Dravolina et al., 2007). Moreover, decreased glutamatergic tone on mGluR2/3 can lead to increase in the glutamate release in the NAc during drug, cue, and/or stress induced-reinstatement (Dietrich et al., 2002, Moran et al., 2005, Madayag et al., 2007, LaLumiere and Kalivas, 2008). Thus, glutamate plays a significant role in the reinstatement of drug seeking and, therefore, minimizing glutamatergic effect on synaptic and extrasynaptic receptors might be an effective strategy for attenuating reinstatement of drugs of abuse. Importantly, CEF has been reported to prevent the reinstatement of METH, cocaine, ethanol, nicotine, and heroin (Sari et al., 2009, Knackstedt et al., 2010, Abulseoud et al., 2012, Alajaji et al., 2013, Qrunfleh et al., 2013, Shen et al., 2014). However, CEF cannot be administered orally, requires high doses to show efficacy with subsequent side effects, and has an antimicrobial activity. Therefore, it is very important to find a better alternative that overcomes these effects. Since CEF’s up-regulatory effect on GLT-1 expression is thought to be mainly attributed to the β-lactam ring found in CEF and other β-lactam antibiotics (Rothstein et al., 2005), other β-lactam ring containing compounds may show similar activity. Clavulanic acid (CA), a non-antibiotic β-lactam compound that is orally available, relatively safe, and can cross blood brain barrier (Münch et al., 1981, Nakagawa et al., 1994). Therefore, in the second study, we tested the effect of CA on METH induced reinstatement using conditioned place preference (CPP) paradigm, and effects of METH and CA on ethanol drinking and the expression of glial glutamate
transporters and mGluR2/3 in the NAc shell and core as well as the dorsomedial PFC (dmPFC). In the final study, we further investigated the mechanism of action of CA on METH-induced reinstatement. We have tested the anti-reinstatement effect of CA following blocking mGluR2/3 by the selective mGluR2/3 antagonist (LY341495). We have also tested the effect of a single dose of CA on METH reinstatement to examine any direct activation of glutamate transporters or receptors by CA. Finally, the effect of CA and METH reinstatement on glutamate release in the NAc shell and core have been assessed using in vivo microdialysis.
Chapter 1

Alcohol interactions with other drugs of abuse: an overview of animal and human studies

Running title: Alcohol interactions with other drugs of abuse

Yusuf S. Althobaiti¹, Youssef Sari¹*

¹ University of Toledo, College of Pharmacy and Pharmaceutical Sciences, Department of Pharmacology and Experimental Therapeutics, Toledo, OH.

*Corresponding author:
Dr. Youssef Sari
University of Toledo, College of Pharmacy and Pharmaceutical Sciences
Department of Pharmacology and Experimental Therapeutics
Health Science Campus,
3000 Arlington Avenue, HEB 282G
Toledo, OH 43614. USA
E-mail: youssef.sari@utoledo.edu
Tel: 419-383-1507 (Office)

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Abstract

Alcohol can contribute to a significant number of disabilities due to psychological, medical, injury, or other detrimental effects. These effects can be dramatically severe when alcohol is consumed with other drugs of abuse. Alcohol consumption with other drugs of abuse is very common among drug users. There is little known about the possible pharmacological interactions between alcohol and other drugs of abuse. In this review, we discussed the available human and animal studies of the most commonly co-abused stimulants with alcohol, including methamphetamine (METH), cocaine, 3,4-methylenedioxymethamphetamine (MDMA), and nicotine with a focus on prevalence, possible pharmacological mechanisms, and other health risks. Chronic use of alcohol with certain drugs of abuse can result in serious health issues, including cancer, hepatotoxicity, hypotension, cardiotoxicity, and neurobehavioral disturbance.
Introduction

Alcohol dependence is considered a major public health problem worldwide (Room et al., 2005, Volkow and Li, 2005, Heilig and Egli, 2006, Arasteh et al., 2008, Sullivan and Zahr, 2008, Harper, 2009). Alcohol can contribute to a significant number of disabilities due to psychological, medical, injury, or other detrimental effects [For review see ref. (Saitz, 2005)]. These effects can be dramatically severe when alcohol is consumed with other drugs of abuse. Alcohol consumption with other drugs of abuse is very common among drug users. There are different pharmacological mechanisms that can occur with interactions of alcohol and other drugs. It is noteworthy that drugs of abuse have been shown to alter central brain reward circuitry, which can lead addicts to increase their alcohol intake for reward effects (Wise, 1980, Koob and Le Moal, 2001). Alcohol use with other drugs of abuse has been reported to hinder decision making, thinking, and neurocognitive capabilities (Rasch et al., 2000, Tapert et al., 2004, George et al., 2005, Fisher et al., 2007, Arasteh et al., 2008, Van Tieu and Koblin, 2009). Moreover, recent studies confirmed that alcohol and other drugs of abuse are usually found in the blood of deceased or seriously injured drivers involved in traffic accidents caused by psychomotor function impairment (Walsh et al., 2004, Walsh et al., 2005, Legrand et al., 2012, Poulsen et al., 2012, Legrand et al., 2013). We discussed here several findings related to alcohol interactions with drugs of abuse. According to previous reports, alcohol is commonly abused with methamphetamine (METH), cocaine and marijuana (Caetano and Weisner, 1995). Men have higher prevalence of co-abuse of alcohol and other drugs compared to women (Falk et al., 2008). The prevalence of drugs of abuse has been shown to have a positive correlation with the level of alcohol consumption (Falk et al., 2008). In fact,
alcohol dependence has been associated with the highest prevalence of drugs of co-abuse (Falk et al., 2008). We reviewed here the available literature about alcohol interactions with different drugs of abuse, including METH, cocaine, nicotine, and 3,4-methylenedioxy-methamphetamine (MDMA), according to animal experimental and clinical studies.

1.1. Alcohol and METH co-abuse

METH abuse is an increasing health problem worldwide. According to the available data from national surveys between the years of 2002 and 2004, more than 16 million Americans over the age of 12 have used METH (Colliver, 2006). METH is a derivative of amphetamine with increased CNS activity and effects. METH can be abused by different routes such as inhalation, ingestion, or intravenous injection, with acute effects that can last for up to 24 hours (Domier et al., 2000, Cunningham et al., 2008). It is well known that METH can stimulate the release of monoamines such as dopamine and norepinephrine to produce euphoria and to increase alertness and libido (Anglin et al., 2000, Rothman et al., 2001, Gibson et al., 2002). METH abusers frequently use alcohol and have a higher risk of reaching alcohol intoxication level (Furr et al., 2000). The prevalence of alcohol use disorder was found to be more than 75% among amphetamine dependent patients (Stinson et al., 2005). For example, a study reported that more than 60% of METH users in New York City reported abusing METH in combination with Alcohol (Halkitis et al., 2005). A recent study conducted on regular METH users showed that alcohol drinking increased the chances of METH use in same day by more than 4 folds (Bujarski et al., 2014). Despite this evidence of high prevalence of METH and
alcohol co-abuse, very few studies have investigated the effects of their co-abuse. A summary of possible effects of alcohol and METH co-abuse are presented in Table 1.1.

Table 1.1  Aspects and effects of drugs of abuse and alcohol interactions

<table>
<thead>
<tr>
<th>Drug of Abuse</th>
<th>Aspect of interaction</th>
<th>Effects of interactions</th>
</tr>
</thead>
<tbody>
<tr>
<td>METH</td>
<td>METH metabolism</td>
<td>Alcohol decreased p-hydroxylated metabolites of METH in the urine of METH abusers (Shimosato, 1988) Alcohol increased the levels of METH and its active metabolite, amphetamine, in rats and rabbits (Liang et al., 2012, Li et al., 2014)</td>
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<tr>
<td></td>
<td>Performance and sleep</td>
<td>Lower detrimental effects on performance and sleep compared to each drug alone (Kirkpatrick et al., 2012)</td>
</tr>
<tr>
<td></td>
<td>Euphoria</td>
<td>Increased euphoria in alcohol and methamphetamine co-abuse (Kirkpatrick et al., 2012)</td>
</tr>
<tr>
<td></td>
<td>Cardiac effects</td>
<td>Increased myocardial oxygen consumption and cardiac rate (Mendelson et al., 1995)</td>
</tr>
<tr>
<td></td>
<td>Prenatal exposure</td>
<td>Damage to striatal region of the brain (Sowell et al., 2010)</td>
</tr>
<tr>
<td>Oxidative stress</td>
<td>Combination caused more impairment of antioxidant enzymes in rats hippocampus and oxidative stress than either drug alone (Vaghef et al., 2014)</td>
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<tr>
<td><strong>Cocaine</strong></td>
<td><strong>Cocaine metabolism</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Alcohol decreased metabolism of cocaine (Boyer and Petersen, 1992). Alcohol decreased benzoylcegonine renal excretion, and increased in cocaine and cocaethylene blood concentrations (Harris et al., 2003).</td>
<td></td>
</tr>
<tr>
<td><strong>Cardiovascular and endocrine systems</strong></td>
<td>Exposure to cocaine and alcohol increased heart rate, systolic blood pressure, cortisol, and prolactin levels (Farré et al., 1997, Harris et al., 2003)</td>
<td></td>
</tr>
<tr>
<td><strong>Cerebral blood perfusion</strong></td>
<td>Cerebral hypo-perfusion occurred more in individuals taking cocaine and alcohol than in individuals taking cocaine or alcohol alone (Robinson et al., 1999, Gottschalk and Kosten, 2002)</td>
<td></td>
</tr>
<tr>
<td>Neurobehavioral performances</td>
<td>Negatively affected by concurrent intake of cocaine and alcohol compared to either drug alone (Bolla et al., 2000, Verdejo-García and Pérez-García, 2007)</td>
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</tr>
<tr>
<td>Mesocorticolimbic dopamine system</td>
<td>Increased extracellular dopamine concentration than either drug alone in nucleus accumbens in rats (Lindholm et al., 2001)</td>
<td></td>
</tr>
<tr>
<td>Drug reinforcement</td>
<td>Sense of pleasure and euphoria were found to be improved (McCance-Katz et al., 1998)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rats have established self-administration and place preference to combination of alcohol and cocaine in concentrations that did not provoke reinforcement to either drug alone (Busse et al., 2004, Ding et al., 2012)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cocaine potentiated alcohol seeking (Knackstedt et al., 2006, Hauser et al., 2014)</td>
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</tr>
<tr>
<td><strong>Nicotine</strong> Mesocorticolimbic dopamine system</td>
<td>Increased in dopaminergic neuron firings and dopamine release in an additive mechanism (Zhou et al., 2001, Tizabi et al., 2002, Melendez et al., 2003, Larsson et al., 2005, Tizabi et al., 2007, Schier et al., 2013).</td>
<td></td>
</tr>
<tr>
<td>Pleasure and drug seeking</td>
<td>Increased in the pleasurable effects of each</td>
<td></td>
</tr>
<tr>
<td>Cardiovascular system</td>
<td>Additive effect on heart rate and blood pressure was found in healthy human volunteers (Benowitz et al., 1986, Perkins et al., 1995). Synergistic increase in left ventricular pressure in dogs (Mehta et al., 1998).</td>
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<tr>
<td>Cancer</td>
<td>Increase in the risk of developing esophageal cancer. (MLWIINSKI, 1976, Williams and Horm, 1977, Wynder et al., 1977, Mashberg et al., 1993) Showed a multiplicative effect in increasing the risk of head and neck cancer in human (Hashibe et al., 2009)</td>
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<tr>
<td>Prenatal exposure</td>
<td>Increased the risk of fetal growth restrictions in human (Haste et al., 1991, Olsen et al., 1991, Aliyu et al., 2009). Offspring developed rapid nicotine self-administration and at a higher level in rats (Matta and Elberger, 2007).</td>
<td></td>
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**MDMA**  
Cardiovascular system  
Exacerbated cardiac cellular stress and
<table>
<thead>
<tr>
<th>Category</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood level</td>
<td>MDMA plasma concentration increased following alcohol intake (Hernández-López et al., 2002)</td>
</tr>
<tr>
<td>Drug reinforcement</td>
<td>MDMA and alcohol induce a longer duration of euphoria (Hernández-López et al., 2002)</td>
</tr>
<tr>
<td></td>
<td>Exposure to alcohol during adolescent age in mice increased the reinforcing effects of MDMA</td>
</tr>
<tr>
<td></td>
<td>(Ribeiro Do Couto et al., 2012)</td>
</tr>
<tr>
<td>Sedation</td>
<td>MDMA reversed the sedation effect induced by alcohol consumption (Hernández-López et al., 2002)</td>
</tr>
<tr>
<td>Learning and memory</td>
<td>Administration of alcohol and MDMA exhibited learning and memory impairments</td>
</tr>
<tr>
<td></td>
<td>(Vidal-Infer et al., 2012)</td>
</tr>
<tr>
<td>Dopamine reward effect</td>
<td>MDMA impaired dopaminergic reward pathway, leading to increase alcohol consumption (Izco et al., 2007)</td>
</tr>
<tr>
<td>Psychopathological effect</td>
<td>Long term consumption of MDMA and alcohol serotonin depletion causing psychopathological changes (Cassel et al., 2005b)</td>
</tr>
</tbody>
</table>
Previous findings demonstrated that alcohol can decrease p-hydroxylated metabolites of METH in the urine of METH abusers, suggesting that alcohol may inhibit METH metabolism (Shimosato, 1988). This may lead to higher METH blood concentration, with an increase in its stimulating effects on brain and heart. Moreover, recent findings showed that alcohol increased the absorption and distribution of METH and its active metabolite, amphetamine, in several organs, including brain in rats and rabbits (Fig. 1-3) (Liang et al., 2012, Li et al., 2014). However, it is not likely that METH is affecting the metabolism of alcohol since it has been shown that breath test was not changed in volunteers following concurrent METH and alcohol consumption as compared to alcohol consumed alone; and alcohol concentrations were not affected when self-administered with METH in rats (Kirkpatrick et al., 2012, Liang et al., 2012). A recent study compared the acute effects of alcohol, METH, and their combination on mood, performance, and physiological behaviors of nine adult males (Kirkpatrick et al., 2012). This study showed that when alcohol and METH were co-self-administered, a greater increase in heart rate, euphoria, lower detrimental effects on sleep, and performance were observed compared to each drug self-administered alone. This may explain why METH abusers tend to consume high level of alcohol (Kirkpatrick et al., 2012). These findings raise an alarming concern of METH and alcohol co-abuse because METH might mask
the signs of alcohol intoxication, such as sedation and compensated performance, allowing abusers to consume more alcohol with risk of developing alcohol toxicity.

Fig 1-1: Effects of alcohol on the pharmacokinetics of other drugs of abuse. (↑: increase or enhancement; ↓ decrease or deterioration)

A double blind study was conducted on eight alcohol and METH abusers (Mendelson et al., 1995). The abusers were found to have high myocardial oxygen consumption and increased heart rate (Fig. 1-2). In this study, the pharmacokinetics of METH did not change significantly, which is possibly due to the limited number of subjects recruited in this study. However, further testing should be done on more subjects to reach more conclusive evidence of the effect of alcohol on METH pharmacokinetics (Mendelson et al., 1995). A recent clinical study conducted on nine volunteers showed an increase in heart rate (Kirkpatrick et al., 2012). Furthermore, findings showed that concurrent consumption of METH and alcohol disrupted learning and discriminating behavior.
compared to METH self-administered alone in rats (Yamamura et al., 1992). However, this study did not focus on the effects of alcohol alone which may hinder the conclusion that METH and alcohol co-abuse may disturb the performance compared to METH administered alone. A recent study conducted on rats revealed that concurrent intake of METH and alcohol can lead to synergistic effect in impairment of spatial memory compared to METH administered alone (Vaghef et al., 2014). Interestingly, alcohol administered alone did not cause any changes in spatial memory suggesting the synergistic effects of both drugs on memory. Moreover, this study showed that although alcohol or METH administered alone can induce oxidative stress and impairment in antioxidant enzymes in rats hippocampus, co-abuse of METH and alcohol can cause synergistic effect in impairment and oxidative stress compared to drug administered alone (Fig. 1-1) (Vaghef et al., 2014).

Fig 1-2: Effects of alcohol interaction with other drugs of abuse on cardiovascular system. (↑: increase or enhancement; ↓ decrease or deterioration)
Concurrent exposure to METH and alcohol has also been observed in pregnant women. Indeed, a study demonstrated that more than 40% of pregnant women who abused METH reported using alcohol during their pregnancy stages (Smith et al., 2006). Another study was conducted on 61 participants; 13 of them were exposed prenatally to alcohol, 21 were exposed prenatally to METH, and the remaining 27 control participants were not prenatally exposed to either alcohol or METH (Sowell et al., 2010). Of the 21 participants in METH group, 18 children were also exposed to alcohol during their fetal life. The results of this study suggest that prenatal exposure to METH and alcohol can cause synergistic striatal structural damage than prenatal exposure to alcohol alone. Damage to the striatal brain region hinders the overall intellectual competence of the affected children (Sowell et al., 2010). In addition, in a study that found common damage in the fronto-striatal circuit of the prenatally METH exposed group, 15 out of 19 children were exposed to alcohol and METH prenatally (Fig. 1-4) (Roussotte et al., 2011). It is important to note, however, that these studies could not precisely predict the dosage, frequency, and duration of METH or alcohol exposure during pregnancy, which may hinder our understanding of the pharmacological and neuropathological basis of drug exposure and interaction. Preclinical studies are warranted to show the risk of concurrent exposure of METH and alcohol during different stages of pregnancy, which may provide information about the deteriorating effects of prenatal exposure of METH and alcohol.
1.2. Alcohol and cocaine co-abuse

Cocaine can produce different effects on the human body; these effects can last from minutes to hours, based on the route through which cocaine was administered into the body (Volkow et al., 2000). In the brain, cocaine can affect the reward circuitry by modulating dopamine neurotransmission (Mcelvain and Schenk, 1992), and acts by preventing the reuptake of dopamine from the synaptic cleft, which leads to prolongation of the pleasurable effects of dopamine (Missale et al., 1985, Mcelvain and Schenk, 1992). Cocaine can produce euphoria, alertness, dependence and tolerance as well as cardiovascular changes (Fischman et al., 1985, Gawin, 1991, Frank et al., 1992, Kloner et al., 1992, Carroll et al., 1994). Tolerance makes cocaine users increase its doses each time to reach the same level of euphoria that was reached on the first instance of taking the drug. Increasing the doses of cocaine can lead its side effects and toxicity (Petersen, 1977, Ambre et al., 1988, Emmett-Oglesby and Lane, 1992, Abuse and America, 1999).
It is important to note that the prevalence of alcohol use was found to be 89% higher among cocaine dependents (Stinson et al., 2006). Different findings of the effects of alcohol and cocaine co-abuse are presented in Table 1.1. This might be due to higher increase of reward effects when alcohol and cocaine co-abused compared to either drug self-administered alone, which have been shown in preclinical studies (Moolten and Kornetsky, 1990, Lewis and June, 1994, Busse et al., 2004). In a study conducted on rats, intravenous injections of cocaine increased alcohol drinking suggesting that cocaine potentiated alcohol seeking (Knackstedt et al., 2006). Interestingly, a preclinical study showed a higher genetic susceptibility of the reinforcing effects of cocaine in selectively bred alcohol preferring (P) rats compared to its outbred Wistar rats, which suggests a higher sensitivity of alcoholics to the reinforcing effects of cocaine (Katner et al., 2011). Similarly, it has been revealed that genetically predisposed subjects for alcohol dependence have a higher rate to be cocaine dependents (Nurnberger et al., 2004).

![Diagram of effects of prenatal exposure to alcohol and other drugs of abuse](image)

Fig 1-4: Effects of prenatal exposure to alcohol and other drugs of abuse. (↑: increase or enhancement; ↓ decrease or deterioration)
Cocaine co-administered with alcohol can lead to production of cocaethylene, which is more lethal than cocaine itself (Hearn et al., 1991, Jatlow et al., 1991). Cocaethylene can also produce most of the effects that are associated with cocaine (Perez-Reyes et al., 1994, Farré et al., 1997). Concurrent exposure of alcohol and cocaine may cause more lethality in rats than either drug administered alone, which probably due to the formation of cocaethylene (Busse and Riley, 2003). Interestingly, cocaethylene detection in wastewater has been utilized in recent study as an evidence of co-abuse of cocaine and alcohol in different cities (Rodríguez-Álvarez et al., 2015). Cocaethylene levels were found to be significantly higher during weekends compared to weekdays suggesting a higher co-abuse of cocaine and alcohol during weekends (Rodríguez-Álvarez et al., 2015).

Alcohol has been shown to increase the plasma concentration of cocaine (Boyer and Petersen, 1992). This is probably mediated through a decrease in the metabolism of cocaine by carboxylesterases, which hydrolyze it to benzoylecgonine and ecgonine methyl ester metabolites (Boyer and Petersen, 1992). Furthermore, it has been demonstrated that alcohol administered with cocaine can lead to increase in cocaethylene concentration in plasma and decrease benzoylecgonine renal excretion (Fig. 1-3) (Harris et al., 2003). It is noteworthy that different routes of drug exposure may produce different peak levels of cocaethylene (Herbst et al., 2011). For example, oral administration is considered the highest in raising cocaethylene concentration in blood as compared to intravenous route (Herbst et al., 2011). The inhalation route (i.e. smoking) showed the lowest effect on cocaethylene blood concentration compared to oral and IV
routes (Herbst et al., 2011). Furthermore, cocaine and cocaethylene blood concentrations were obtained following concurrent use of cocaine and alcohol (McCance-Katz et al., 1998). This study revealed that the concentration of cocaine in plasma was found to be increased by 15% after cocaine and alcohol exposure. Moreover, 22% of the absorbed cocaine was converted to cocaethylene. Although, cocaine half-life was not altered significantly by ingestion of alcohol, cocaethylene’s half-life increased in comparison to cocaine’s (McCance-Katz et al., 1998). Increasing the half-life of cocaethylene might impose serious health problem due to increasing body exposure to its deteriorating and associated toxic effects.

Concurrent exposure to cocaine and alcohol has deleterious effects on cardiovascular and endocrine systems. Co-abuse of cocaine and alcohol was found to increase heart rate, systolic blood pressure, cortisol, and prolactin concentrations (Fig. 1-2) (Farré et al., 1997, Harris et al., 2003). In addition, cerebral blood perfusion was found to be affected by co-exposure to cocaine and alcohol (Robinson et al., 1999, Gottschalk and Kosten, 2002). It has been shown that cerebral hypo-perfusion was more common among individuals taking cocaine and alcohol compared to individuals taking cocaine or alcohol alone (Robinson et al., 1999, Gottschalk and Kosten, 2002). This shows the significant deleterious effects of the co-abuse of alcohol and cocaine on cardiovascular system that might result in debilitating conditions.

Several tests were performed on intelligence, memory, verbal learning and other aspects of neuropsychological performances to explore the effects of co-abuse of alcohol and
cocaine (Bolla et al., 2000, Verdejo-García and Pérez-García, 2007). The resulting neuropsychological performances were found to be negatively affected by the concurrent intake of cocaine and alcohol compared to either drug administered alone (Fig.1-1) (Bolla et al., 2000, Verdejo-García and Pérez-García, 2007). It has been shown that the sense of pleasure and euphoria increased dramatically, which contributed to an increase in co-abuse of alcohol and cocaine and consequently elevated the risk of dependence and toxicity (McCance-Katz et al., 1998, Dackis and O'Brien, 2001). In addition, alcohol was found to significantly potentiate the effect of cocaine in conditioned place preference in rats and invertebrate animal model (Busse et al., 2004, Tallarida et al., 2014). Moreover, study showed that there is a synergistic effect in self-administration of both alcohol and cocaine in concentrations that did not provoke self-administration to either drug alone (Ding et al., 2012). Similarly, a recent study has shown the potentiating effect of cocaine on alcohol seeking and relapse-like alcohol intake in P rats (Hauser et al., 2014). This might indicate a cross reactivity between alcohol and cocaine on common drug seeking behavior.

Several studies have shown the involvement of mesolimbic dopaminergic system in reinforcing effects of cocaine (de Wit and Wise, 1977, Roberts and Koob, 1982, Pettit et al., 1984, Rodd et al., 2005a) and alcohol (Weiss et al., 1993, Rodd et al., 2005b). In fact, alcohol and cocaine co-exposure increased extracellular dopamine concentration in the nucleus accumbens, well known brain region involved in the rewarding and reinforcing effects of drugs of abuse in rats (Koob and Bloom, 1988, Wise and Rompré, 1989, Bardo, 1998, Koob et al., 1998), than either drug administered alone in rats (Lindholm et al.,
Furthermore, recent findings have demonstrated the critical role of glutamate and its uptake in central brain reward regions in the seeking and reinforcing effects of cocaine (Sari et al., 2009, Reissner et al., 2014, Shen et al., 2014) and alcohol (Ding et al., 2013, Alhaddad et al., 2014, Das et al., 2015, Das et al., 2016). Further studies are needed for investigating the role of glutamatergic system in alcohol and cocaine co-abuse in brain regions involved in rewarding and reinforcing effects of these drugs.

Alternatively, studies have shown the detrimental effects of prenatal exposure to cocaine such as low birth weight, preterm delivery, decrease in head circumference (Chouteau et al., 1988, Bateman et al., 1993, Singer et al., 2001, Singer et al., 2002). However, prenatal co-exposure to cocaine and alcohol has not been well studied despite the findings that more than 85% of women who reported using cocaine during pregnancy, also reported concurrent alcohol use (Singer et al., 2002). One recent study, however, has demonstrated a significant interaction in prenatal co-exposure of cocaine and alcohol on cortical thickness in youths prenatally exposed to these drugs (Gautam et al., 2015). Furthermore, it has been shown that prenatal exposure to alcohol increased the rewarding and reinforcing effects of cocaine in rats (Fig. 1-4) (Barbier et al., 2009).

1.3. Alcohol and nicotine co-abuse

Alcohol and nicotine have serious global health problems. Table 1.1 summarizes different studies of the effects of alcohol and nicotine co-abuse. Nicotine dependents may have high tendency to be alcohol dependents (Grant et al., 2004). It has been reported that more than 80% of chronic alcohol users are also smokers (Burling and Ziff, 1988, DiFranza and Guerrera, 1990, Batel et al., 1995). In a preclinical study, rats chronically
exposed to alcohol and nicotine showed increased nicotine self-administration as compared to drug administered alone (Deehan Jr et al., 2015). Although, it has been suggested that nicotine or alcohol consumed alone may have some beneficial effect at low doses, it is clear that co-abuse of these drugs may have negative effects in human health [For review see ref. (Hurley et al., 2012)].

Nicotine and alcohol activate the mesocorticolimbic dopaminergic system; there is potential synergistic effect in the increase of dopamine release when the drugs are consumed concurrently [For review see ref. (Doyon et al., 2013)]. Furthermore, studies showed that alcohol and nicotine co-abuse can lead to increase dopaminergic neuronal firings and dopamine release (Zhou et al., 2001, Tizabi et al., 2002, Melendez et al., 2003, Larsson et al., 2005, Tizabi et al., 2007, Schier et al., 2013). It is suggested that the synergistic effect of these drugs may influence drug reinforcement to each other and may predispose smokers to become alcoholics and vice versa (Collins et al., 1996, Doyon et al., 2013). Interestingly, an additive effect on dopamine release in the nucleus accumbens shell was found between alcohol and nicotine in rats (Tizabi et al., 2007). This additive effect on dopamine release was inhibited by mecamylamine pretreatment, a nicotinic receptor antagonist, suggesting the involvement of nicotinic receptors in the reinforcing effects of alcohol. Importantly, alcohol-induced dopaminergic neurons firing in ventral tegmental area was inhibited in mice lacking nicotinic acetylcholine receptors that contain α6 subunit (Liu et al., 2013). Moreover, it has been shown that alcohol and nicotine co-abuse can increase the pleasurable effects of each drug (Rose et al., 2004). This may explain some of the pharmacological mechanisms of action involving the co-abuse of nicotine and alcohol in the modulation of dopamine release (Fig. 1-1).
The risk of developing cancer in general is higher in heavy tobacco smokers and alcohol drinkers (MLWIINSKI, 1976, Williams and Horm, 1977, Wynder et al., 1977, Mashberg et al., 1993). In a case-controlled clinical study conducted on European and American subjects, alcohol and tobacco smoke revealed increasing risk of head and neck cancer in patients addicted to both drugs (Hashibe et al., 2009). The exact mechanism of alcohol and nicotine interaction that results in the development of cancer is not well known and remains controversial. Studies have suggested that alcohol and nicotine co-abuse may produce toxic metabolites such as acetaldehyde, which may contribute to cancer development (Homann et al., 2000, Salaspuro and Salaspuro, 2004). Other studies have suggested that alcohol and nicotine co-abuse promotes the formation of premalignant lesions (Fig. 1-3) (Lee et al., 2003, Chang et al., 2004, Maserejian et al., 2006).

The effects of alcohol and nicotine co-abuse on cardiovascular system have been also investigated. Synergistic effect on heart rate and blood pressure was found in healthy human volunteers following alcohol and nicotine exposure (Benowitz et al., 1986, Perkins et al., 1995). Interestingly, the order of self-administering alcohol and nicotine plays a role in their negative interactive effect on cardiovascular system. When self-administration of alcohol was followed by nicotine, a synergistic effect in the increase in left ventricular pressure was revealed, which was alleviated when self-administration of nicotine was followed by alcohol in dogs (Fig. 1-2) (Mehta et al., 1998).

In a study investigating the link between alcohol and nicotine use during pregnancy in more than 14000 previous pregnant mothers, it was found that more than 55% of
pregnant alcohol users reported smoking (Aliyu et al., 2009). Alcohol and smoking exposure during gestational period increased the risk of fetal growth abnormalities more than the exposure to alcohol alone (Haste et al., 1991, Olsen et al., 1991, Aliyu et al., 2009). Interestingly, in a study conducted on rats, alcohol and nicotine were co-administered to pregnant rats throughout the gestational period (Matta and Elberger, 2007). This study showed that offsprings prenatally exposed to nicotine and alcohol developed rapid increase in nicotine self-administration as compared to controls (Fig. 1-4) (Matta and Elberger, 2007).

1.4. Alcohol and MDMA co-abuse

According to 2001-2002 national epidemiologic survey in the United States, the prevalence of alcohol use in MDMA users is more than 95% (Keyes et al., 2008). MDMA and alcohol exposure in adolescent mice induced physiological and behavioral alteration than either drug administered alone (Ros-Simó et al., 2012). In fact, a recent study has shown that the co-abuse of MDMA and alcohol exacerbated cardiac cellular stress and toxicity through augmented activation of cardiac sympathetic system in adolescent mice (Fig. 1-2) (Navarro-Zaragoza et al., 2015). MDMA can induce a rapid release of dopamine and serotonin (Gudelsky and Nash, 1996). High consumption of MDMA may result in the depletion of serotonin in the brain, resulting in serious psychological consequences (Battaglia et al., 1987, Ricaurte et al., 1988, Gudelsky et al., 1994, STEELE et al., 1994, Chu et al., 1996, Gurtman et al., 2002, Cole and Sumnall, 2003, Green et al., 2003). MDMA is usually consumed with many drugs such as amphetamine, cocaine, cannabis, and alcohol. Alcohol and MDMA co-abuse is
considered the most popular form of MDMA co-abuse (Gamella et al., 1997, Topp et al., 1999, Hernández-López et al., 2002, Lora-Tamayo et al., 2004, Schifano, 2004). Different aspects and effects of interaction between alcohol and MDMA are presented in Table 1.1. Importantly, mice pretreated with MDMA were found to consume high amount of alcohol compared to control mice (Izco et al., 2007). Therefore, mice consumed higher amounts of alcohol in order to reach the same reward effect that was normally reached at lower doses of alcohol. This study also found that MDMA impaired the dopaminergic pathway. Furthermore, findings revealed that presynaptic modulation of serotonin release in the hippocampus is affected by exposure to both MDMA and alcohol (Cassel et al., 2005a). This study also showed that long term consumption of MDMA and alcohol caused serotonin depletion. The alteration in the serotonergic system might be associated with the psychopathological disturbances observed in MDMA and alcohol co-abusers (Cassel et al., 2005a).

It has been found, in a double blind study conducted on nine healthy human volunteers, that MDMA and alcohol co-abuse induced a longer duration of euphoria and feeling well as compared to drug use alone (Hernández-López et al., 2002). Therefore, MDMA and alcohol use together can increase the abuse potential more than abusing alcohol or MDMA alone. In a preclinical study, exposure to alcohol during adolescent age in mice increased the reinforcing effects of MDMA (Ribeiro Do Couto et al., 2012). Moreover, exposure to MDMA and alcohol during adolescence potentiated anxiety measures, impaired learning and memory, and decreased striatal dopamine contents during adult life in mice (Fig. 1-1) (Rodríguez-Arias et al., 2011, Vidal-Infer et al., 2012). Studies have
demonstrated an increase in the MDMA plasma concentration by 13% following alcohol intake and a decrease in blood alcohol concentration of about 12% compared to either drug administered alone (Fig. 1-3) (Hernández-López et al., 2002). In addition, these studies found that MDMA reversed the subjective sedation effect induced by alcohol consumption.

A recent study aimed to find the effect of MDMA and alcohol co-abuse on learning and memory (Vidal-Infer et al., 2012). In this study, alcohol and MDMA were administered either together or alone to measure their effects on learning and memory in adult mice. Both drugs caused impairment of learning and memory impairments, as the affected mice displayed an imbalance in the interaction of dopamine and serotonin. These findings suggest that the brain in adulthood is very sensitive to MDMA and alcohol damage (Vidal-Infer et al., 2012). However, other study did not demonstrate any additive effect of combining alcohol and MDMA on declarative memory in mice (Ros-Simó et al., 2013). This might be due to several factors, including the doses used for alcohol and MDMA.

Prenatal exposure to alcohol and MDMA is understudied topic, although pregnant women who reported MDMA use during pregnancy also reported higher alcohol use compared to non MDMA users (Singer et al., 2012a, Singer et al., 2012b). Importantly, a preclinical study found that prenatal exposure to both alcohol and MDMA impaired working memory, exploratory activity, and neurogenesis in rats offspring (Fig. 1-4) (Canales and Ferrer-Donato, 2014).
1.5. Conclusion

Alcohol interaction with drugs of abuse is currently not well understood, however, there are studies that demonstrated numerous pathological and side effects, which have occurred with drugs co-abuse. The prevalence of concurrent abuse of alcohol with METH, cocaine, nicotine, or MDMA is extremely high. This increase in prevalence of co-abuse of alcohol with other drugs is most likely due to potentiated euphoria and pleasure effects as well as decrease detrimental subjective effects of either alcohol or other drugs of abuse. Co-abuse of alcohol with other drugs can lead to serious negative consequences on the brain such as decreasing antioxidant enzymes, disrupting learning and memory processes, cerebral hypo-perfusion, neurotransmitters depletion as well as potentiating drug seeking behavior. Moreover, co-abuse of alcohol and other drugs can lead to increase in heart rate, blood pressure, myocardial oxygen consumption and cellular stress as well as increase in the risk of developing different types of cancer. Alcohol has been shown to increase the blood concentration of different drugs of abuse and its active metabolites. It is suggested that the pharmacokinetics of drugs of abuse, including METH, MDMA, cocaine, and nicotine, might be altered when alcohol is consumed concurrently with these drugs. We suggest here that there might be an association with alcohol metabolism and its metabolites, which may increase the blood concentration of different drugs of abuse, and consequently elevate the risk of toxicity. Importantly, alcohol co-abuse with other drugs during pregnancy can impose critical structural and functional damages in the fetal brain as well as it can promote drug
addiction in the affected offspring later in life. Further studies are needed to investigate possible pharmacodynamics and pharmacokinetics aspects of interactions of alcohol with other drugs of abuse as well as the neurochemical basis of their interactions and possible therapeutic interventions of drugs co-abuse.
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Chapter 2

Effects of ceftriaxone on glial glutamate transporters in Wistar rats administered sequential ethanol and methamphetamine

Running title: Effects of ethanol and methamphetamine on GLT-1 and xCT

Yusuf S. Althobaiti¹, Fahad S. Alshehri¹, Atiah H. Almalki², Youssef Sari¹,²*

¹University of Toledo, College of Pharmacy and Pharmaceutical Sciences, Department of Pharmacology and Experimental Therapeutics, Toledo, OH.
²University of Toledo, College of Pharmacy and Pharmaceutical Sciences, Department of Medicinal Chemistry, Toledo, OH.

*Send correspondence to:
Dr. Youssef Sari
University of Toledo, College of Pharmacy and Pharmaceutical Sciences
Department of Pharmacology and Experimental Therapeutics
Department of Medicinal Chemistry
Health Science Campus,
3000 Arlington Avenue, HEB 282G
Toledo, OH 43614. USA
E-mail: youssef.sari@utoledo.edu
Tel: 419-383-1507 (Office)

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Abstract:

Methamphetamine (METH) is one of the psychostimulants that is co-abused with ethanol. Repeated high doses of METH have been shown to cause increases in extracellular glutamate concentration. We have recently reported that ethanol exposure can also increase the extracellular glutamate concentration and downregulate glutamate transporter subtype 1 (GLT-1). GLT-1 is a glial transporter that regulates the majority of extracellular glutamate. A Wistar rat model of METH and ethanol co-abuse was used to examine the expression of GLT-1 as well as other glutamate transporters (xCT and GLAST). We also examined the body temperature in rats administered METH, ethanol or both drugs. We further investigated the effects of ceftriaxone (CEF), a β-lactam antibiotic known to upregulate GLT-1, in this METH/ethanol co-abuse rat model. After seven days of either ethanol (6 g/kg) or water oral gavage, Wistar rats received either saline or METH (10 mg/kg i.p. every 2 hrs x 4), followed by either saline or CEF (200 mg/kg) posttreatment. METH administered alone decreased GLT-1 expression in the NAc and PFC and increased body temperature, but did not reduce either xCT or GLAST expression in ethanol and water-pretreated rats. Interestingly, ethanol and METH were found to have an additive effect on the downregulation of GLT-1 expression in the NAc, but not in the PFC. Moreover, ethanol alone caused GLT-1 downregulation in the NAc and elevated body temperature compared to control. Finally, CEF posttreatment significantly reversed METH-induced hyperthermia, restored GLT-1 expression, and increased xCT expression. These findings suggest the potential therapeutic role of CEF against METH- or ethanol/METH-induced hyperglutamatergic state and hyperthermia.

**Keywords:** Methamphetamine, GLT-1, hyperthermia, ethanol gavage, xCT, GLAST
Introduction

Methamphetamine (METH) abusers frequently use alcohol with a higher risk of reaching alcohol intoxication (Furr et al., 2000). The prevalence of alcohol use disorder was found to be more than 75% among amphetamine-dependent subjects (Stinson et al., 2005). Exposure to a high dose of METH induces depletion of dopamine and serotonin at the nerve terminals (Ricaurte et al., 1980, Ricaurte et al., 1982, Seiden et al., 1988, Hirata et al., 1995, Cass et al., 2006) and increases extracellular glutamate concentration in rat striatum (Nash and Yamamoto, 1992, Stephans and Yamamoto, 1994). Repeated exposure to higher doses of amphetamine has also been shown to increase extracellular glutamate concentration in the nucleus accumbens (NAc) and the ventral tegmental area (VTA) in rats (Xue et al., 1996). Although, it is known that repeated METH exposure can increase extracellular glutamate concentration, there is less known about its effect on glutamate transporters. In general, these transporters are responsible for clearing extracellular glutamate to maintain glutamate homeostasis. Among these transporters, glutamate transporter 1 (GLT-1; human homolog is excitatory amino acid transporter 2, EAAT2) plays a major role in clearing the majority of the extracellular glutamate concentration (Ginsberg et al., 1995, Rothstein et al., 1995, Danbolt, 2001, Mitani and Tanaka, 2003). Importantly, chronic ethanol exposure was found to reduce GLT-1 expression (Alhaddad et al., 2014b, Aal-Aaboda et al., 2015, Goodwani et al., 2015b) and increase extracellular glutamate concentration in the NAc (Ding et al., 2013, Das et al., 2015, Pati et al., 2016). Since repeated high dose of METH exposure can increase extracellular glutamate concentration (Halpin et al., 2014), we investigated in this study for any potential additive effect of ethanol and METH exposure on GLT-1 expression as
well as other glial glutamate transporters such as cystine/glutamate transporter (xCT) and glutamate aspartate transporter (GLAST) in the NAc and PFC. The NAc is a brain region that is involved in the rewarding and reinforcing effects of drugs of abuse (Koob and Bloom, 1988, Wise and Rompré, 1989, Bardo, 1998, Koob et al., 1998). The NAc receives glutamatergic inputs from the PFC as well as other brain regions (Kelley et al., 1982, Phillipson and Griffiths, 1985). In this study, we examined the effect of ceftriaxone (CEF) posttreatment on GLT-1, xCT, and GLAST expression in the NAc and PFC in rats that were exposed to repeated high-dose METH. CEF is known to increase GLT-1 expression in several brain regions (Miller et al., 2008, Sari et al., 2009, Sari et al., 2013) and can normalize extracellular glutamate concentration in the NAc in cocaine and ethanol-seeking rat models (Trantham-Davidson et al., 2012, Das et al., 2015). CEF was also shown to reduce ethanol intake and cocaine seeking, in part, through upregulation of GLT-1 and xCT expression in the NAc and PFC (Sari et al., 2009, Knackstedt et al., 2010, Sari et al., 2011, Fischer et al., 2013, Alhaddad et al., 2014a, Rao and Sari, 2014). It is noteworthy that repeated exposure to high dose METH was found to cause hyperthermia (Chan et al., 1994, Lan et al., 1998, Ishigami et al., 2003). Importantly, CEF was also revealed to reduce morphine-induced hyperthermia (Rawls et al., 2007). Thus, we have also investigated the effects of CEF on METH-induced hyperthermia. We administered CEF after ethanol and METH exposure for clinical relevance.
2.1. Materials and methods

2.1.1 Subjects

Male Wistar rats, weighing 200-300 g at the beginning of the study, were obtained from Harlan, Inc. (Indianapolis, IN). Rats were single-housed in standard plastic cages with controlled temperature (21°C) and humidity (30%) on 12:12 light-dark cycle and were allowed to habituate to these conditions before the experiments. Rats had ad libitum food and water throughout the experimental procedure, except two hours fasting before each oral gavage administration. Animal experimental procedures were approved by the Institutional Animal Care and Use Committee of The University of Toledo in accordance with the guidelines of the Institutional Animal Care and Use Committee of the National Institutes of Health and the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, Commission on Life Sciences, 1996).

2.1.2 Drugs

(+)-METH hydrochloride was purchased from Sigma-Aldrich (St. Louis, MO). CEF (Sandoz Inc., Princeton, NJ) was purchased from The University of Toledo’s pharmacy. Saline solution (0.9% NaCl) was used to dissolve either (+) METH or CEF. Ethanol (95%; Decon Labs, Inc.) was diluted in water.
2.1.3. Experimental design

An experimental schedule is illustrated in Figure 1. Rats were administered oral gavage of either water or ethanol (6 g/kg) for seven days, followed by either METH (10 mg/kg, i.p.) or saline vehicle (i.p.). We first orally gavaged the rats with ethanol in order to initially induce a reduction in GLT-1 expression and glutamate uptake, as was performed in recent study from our laboratory (Das et al., 2015); we then followed with METH i.p. injections to further reduce glutamate uptake. After completion of the four METH i.p. injections, rats were randomly assigned to receive either CEF (200 mg/kg i.p.) or saline vehicle (i.p.) for two days; control and experimental groups have been summarized in Table 2.1. The rationale for testing repeated high doses of METH (10 mg/kg i.p. every 2 hrs x 4) exposure was chosen based on previous studies that showed neurotoxicity and elevation of extracellular glutamate concentration in rat brains (Bowyer et al., 1994, Hirata et al., 1995, Yamamoto and Zhu, 1998, Mark et al., 2004, Mark et al., 2007). The rationale for testing the ethanol binge gavage paradigm was based on recent studies from our laboratory and others (Faingold, 2008, Abulseoud et al., 2014, Das et al., 2016). Control and treated rats were then quickly euthanized by CO₂ inhalation and rapidly decapitated. Brains were then extracted and immediately frozen in dry ice and stored at -80 °C. The PFC and NAc were micropunched using a cryostat apparatus as described in a previous study from our laboratory (Sari and Sreemantula, 2012). Rat Brain Stereotaxic Atlas was used to identify the selected structures (PFC and NAc) (Paxinos, 2007).
Figure 2-1. Experimental schedule for METH and ethanol administration. Rats were administered oral gavage of either water or ethanol (6 g/kg) for 7 days followed by either METH (10 mg/kg i.p., every 2 hrs for 4 times) or saline vehicle (i.p.). After completion of the four METH injections, rats were randomly assigned to receive either CEF (200 mg/kg i.p.) or saline vehicle (i.p.) for two days. Control and treated rats were then quickly euthanized (72 and 48 hrs following last water/ethanol and saline/METH administration, respectively) by CO2 inhalation and rapidly decapitated.

Table 2.1. Experimental groups according to the administration of water or ethanol oral gavage, METH or saline, as well as CEF or saline.

<table>
<thead>
<tr>
<th>Group</th>
<th>Day 1-7</th>
<th>Day 8</th>
<th>Day 8-10</th>
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<tbody>
<tr>
<td>1- Water-Saline-Saline</td>
<td>Water</td>
<td>Saline</td>
<td>Saline</td>
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<tr>
<td></td>
<td>Drug</td>
<td>Dose</td>
<td>Drug</td>
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<tr>
<td></td>
<td>Meth</td>
<td>(1 ml/kg, i.p. every 2 hrs x 4)</td>
<td>(1 ml/kg, i.p. every day x 3)</td>
</tr>
<tr>
<td>2- Water-METH-Saline</td>
<td>Water</td>
<td>METH</td>
<td>Saline</td>
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<tr>
<td></td>
<td>Drug</td>
<td>Dose</td>
<td>Drug</td>
</tr>
<tr>
<td></td>
<td>Meth</td>
<td>(10 mg/kg, i.p. every 2 hrs x 4)</td>
<td>(1 ml/kg, i.p. every day x 3)</td>
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<tr>
<td>3- Water-METH-CEF</td>
<td>Water</td>
<td>METH</td>
<td>CEF</td>
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<tr>
<td></td>
<td>Drug</td>
<td>Dose</td>
<td>Drug</td>
</tr>
<tr>
<td></td>
<td>Meth</td>
<td>(10 mg/kg, i.p. every 2 hrs x 4)</td>
<td>(200 mg/kg, i.p. every day x 3)</td>
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<tr>
<td>4- Ethanol-Saline-Saline</td>
<td>Ethanol</td>
<td>Saline</td>
<td>Saline</td>
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<td></td>
<td>Drug</td>
<td>Dose</td>
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<tr>
<td></td>
<td>Meth</td>
<td>(1 ml/kg, i.p. every 2 hrs x 4)</td>
<td>(1 ml/kg, i.p. every day x 3)</td>
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<tr>
<td>5- Ethanol-METH-Saline</td>
<td>Ethanol</td>
<td>METH</td>
<td>Saline</td>
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<td></td>
<td>Drug</td>
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<td></td>
<td>Meth</td>
<td>(10 mg/kg, i.p. every 2 hrs x 4)</td>
<td>(1 ml/kg, i.p. every day x 3)</td>
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<tr>
<td>6- Ethanol-METH-CEF</td>
<td>Ethanol</td>
<td>METH</td>
<td>CEF</td>
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<td></td>
<td>Drug</td>
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<tr>
<td></td>
<td>Meth</td>
<td>(10 mg/kg, i.p. every 2 hrs x 4)</td>
<td>(200 mg/kg, i.p. every day x 3)</td>
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2.1.4. Western blot

The Western blot procedure was performed as previously described (Sari et al., 2009). Briefly, brain tissue was lysed in lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 0.5% NP-40, 1% Triton, 0.1% SDS) containing a protease inhibitor cocktail. A Bio-Rad protein assay method was used to determine total protein content in the tissue extracts (Bio-Rad, Hercules, CA, USA). The extracted proteins were loaded onto 10-20% tris-glycine gel. After separation, proteins were transferred electrophoretically from the gel onto the PVDF membranes. The membranes were then blocked using 3% milk in Tris-buffered saline Tween 20 for 30 minutes. Guinea pig anti-GLT1 (1:5000 dilution; Millipore Bioscience Research Reagents), rabbit anti-xCT antibody (1:1000 dilution: Novus), rabbit anti-GLAST (1:5000 dilution; Abcam), or mouse anti β-tubulin antibody (1:5000 dilution; Covance) was then added to the blocking buffer, and the membrane was incubated overnight at 4°C. The membrane was then washed and incubated with horseradish peroxidase-labeled (HRP) anti-Guinea pig, anti-rabbit, or anti-mouse secondary antibody (1:5000). A chemiluminescent kit (SuperSignal West Pico) was used to incubate the membrane for protein detection. Subsequently, the membrane was exposed to Kodak BioMax MR films (Thermo Fisher Scientific). The films were then developed using an SRX-101A machine by Konica Minolta Medical & Graphic, Inc. The blots for each protein were digitized, and densitometric analysis was obtained using an MCID software (Imaging Research, Inc.). Data were calculated as ratios of GLT-1/β-tubulin, xCT/ β-tubulin and GLAST/ β-tubulin. The control group (Water-Saline-Saline) was included with the experimental groups each time the 10-well gel was run. The
control group was then set arbitrary as 100% and the changes in protein expression of the remaining five groups was obtained relative to the control in that particular gel. Expression of proteins was consistent each time we run samples. This calculation method has been used in several previous publications from our lab and others (Li et al., 2003, Raval et al., 2003, Miller et al., 2008, Zhang and Tan, 2011, Simões et al., 2012, Devoto et al., 2013, Goodwani et al., 2015a, Hakami et al., 2016).

2.1.5. Body temperature measurement

The body temperature was measured rectally using digital thermometer (Thermalert TH-5, Physitemp, NJ, USA) at three time points to minimize handling following METH exposure: at baseline, after the last METH injection (Time 0) when the rats were randomly assigned to receive either saline or CEF, and finally 12 hrs after last METH injection.

2.1.6. Statistical analysis

Two-way ANOVA (Pretreatment x Posttreatment) was used to analyze immunoblot data. Newman-Keuls multiple comparisons test was used when significant interaction or significant main effect was revealed using GraphPad Prism. Mixed-model factorial ANOVA [Time x Pretreatment x Posttreatment, with repeated measures on the time factor (Baseline, 0, 12 hrs), with Pretreatment and Posttreatment as the between-subjects factor] was used to analyze body temperature data using SPSS software. All statistical tests were based on p<0.05 level of significance.
2.2. Results

2.2.1. Effects of METH administered alone or with ethanol as well as effects of CEF posttreatment on GLT-1 expression in the NAc and PFC

This study investigated the effect of METH on GLT-1 expression in the NAc and PFC 48 hrs following the last METH i.p. injection in Wistar rats. Two-way ANOVA revealed a significant effect of posttreatment in the NAc \([F (2, 30) = 39.09, p < 0.0001]\) and PFC \([F (2, 30) = 14.10, p < 0.0001]\), significant effect of oral gavage pretreatment in the NAc \([F (1, 30) = 11.69, p < 0.0018]\) but not PFC \([F (1, 30) = 0.05634, p = 0.8140]\), and significant interaction between posttreatment and oral gavage pretreatment in the NAc \([F (2, 30) = 3.949, p = 0.0300]\) but not in the PFC \([F (2, 30) = 0.009251, p = 0.9908]\). Newman-Keuls multiple comparisons test showed a significant increase in GLT-1 expression in METH-CEF-treated rats compared to METH-Saline-treated rats in the NAc [water group \((p< 0.001)\) and ethanol group \((p< 0.0001; \text{Fig. } 2-2A,B)\)] and PFC [water group \((p< 0.01)\) and ethanol group \((p< 0.01; \text{Fig. } 2-2C,D)\)]. Moreover, statistical analyses showed a significant downregulation of GLT-1 expression in the NAc [water group \((p< 0.05)\) and ethanol group \((p< 0.01; \text{Fig. } 2-2B)\)] and in the PFC [water group \((p< 0.05)\) and ethanol group \((p< 0.05; \text{Fig. } 2-2D)\)] of the METH-Saline group compared to the corresponding saline control group. Alternatively, post hoc analyses showed a significant decrease in GLT-1 expression in ethanol-Saline-Saline compared to water-Saline-Saline in the NAc \((p < 0.01; \text{Fig. } 2-2B)\). Interestingly, GLT-1 expression was significantly decreased in ethanol-METH-Saline-treated rats compared to water-METH-Saline-treated rats in the NAc \((p< 0.01; \text{Fig. } 2-2B)\).
Figure 2-2. Effects of METH (10 mg/kg i.p. every 2 hrs x 4), ethanol and CEF (200 mg/kg) on GLT-1 expression in the NAc and PFC. (A,C) Immunoblots for GLT-1 as well as β-tubulin, which was used as a control loading protein, in the NAc and PFC, respectively, as compared to water-pretreated groups and ethanol-pretreated groups. (B,D) Quantitative analysis revealed a significant increase in the ratio of GLT-1/β-tubulin in METH-CEF-treated rats compared to the METH-Saline-treated rats in the water and the ethanol groups, in the NAc and PFC, respectively. Significant downregulation of GLT-1 expression was revealed in the METH-Saline-treated groups compared to control in water- and ethanol-treated groups in the NAc and PFC. Significant downregulation of GLT-1 expression was revealed in ethanol-Saline-Saline and ethanol-METH-Saline groups compared to its corresponding water control groups in the NAc, but not in the PFC. No significant difference in GLT-1 expression was revealed in water-METH-CEF-treated rats compared to water control groups. However, a significant increase in GLT-1
expression was found in the ethanol-METH-CEF group compared to ethanol control group in the NAc, but not in the PFC. * p<0.05, ** p<0.01 (or &&, for comparison between ethanol and its corresponding water control groups), *** p<0.001, and **** p<0.0001. Values shown as means ± S.E.M. n = 6 for each group.

2.2.2. Effects of CEF treatment on xCT expression in the NAc and PFC of groups administered METH alone or METH and ethanol

We further investigated the effect of METH on xCT expression in the NAc and PFC 48 hrs following the last METH i.p. injection in Wistar rats. Two-way ANOVA revealed a significant effect of posttreatment in the NAc [F (2, 30) = 12.92, p < 0.0001] and PFC [F (2, 30) = 11.01, p < 0.001], no significant effect of oral gavage pretreatment in the NAc [F (1, 30) = 0.04864, p = 0.8269] or PFC [F (1, 30) = 0.3730, p = 0.5460], and no significant interaction between posttreatment and oral gavage pretreatment in the NAc [F (2, 30) = 0.05490, p = 0.9467] or PFC [F (2, 30) = 0.1945, p = 0.8243]. Newman-Keuls multiple comparisons test showed a significant increase in xCT expression in METH-CEF-treated rats compared to METH-Saline and Saline-Saline treated rats in the NAc [water group (p< 0.05) and ethanol group (p< 0.05; Fig. 2-3A,B)] and PFC [water group (p< 0.05) and ethanol group (p< 0.05; Fig. 2-3C,D)]. However, statistical analyses did not show any significant change in xCT expression in the NAc [in water group (p> 0.05) or ethanol group (p>0.05; Fig. 2-3B)] and PFC [water group (p> 0.05) or ethanol group (p> 0.05; Fig. 2-3D)] of the METH-Saline group compared to the corresponding saline control group.
Figure 2-3. Effects of METH (10 mg/kg i.p. every 2 hrs x 4), ethanol and CEF (200 mg/kg) on xCT expression in the NAc and PFC. (A,C) Immunoblots for xCT as well as β-tubulin, which was used as a control loading protein, in the NAc and PFC, respectively, as compared to water-pretreated groups and ethanol-pretreated groups. (B,D) Quantitative analysis revealed a significant increase in the ratio of xCT /β-tubulin in METH-CEF-treated rats compared to the METH-Saline and Saline-Saline treated rats in the water and ethanol groups in the NAc and PFC, respectively. No significant change in xCT expression was revealed in the METH-Saline-treated groups compared to control in water- and ethanol-treated groups in either the NAc or PFC. * p<0.05. Values shown as means ± S.E.M. n = 6 for each group.
2.2.3. Effects of CEF treatment in GLAST expression in the NAc and PFC in groups administered METH alone or with ethanol

We further investigated the effect of METH in GLAST expression in the NAc and PFC. Two-way ANOVA did not reveal any significant effect of posttreatment in the NAc [F (2, 30) = 0.4872, p = 0.6191] or PFC [F (2, 30) = 0.02371, p = 0.9766], no significant effect of oral gavage pretreatment in the NAc [F (1, 30) = 2.810, p = 0.1041] and PFC [F (1, 30) = 0.0008578, p = 0.9768], and no significant interaction between posttreatment and oral gavage pretreatment in the NAc [F (2, 30) = 0.4643, p = 0.6330] (Fig 2-4A,B) and PFC [F (2, 30) = 0.003179, p = 0.9968] (Fig 2-4C,D).

**Figure 2-4.** Effects of METH (10 mg/kg i.p. every 2 hrs x 4), ethanol and CEF (200 mg/kg) on GLAST expression in the NAc and PFC. (A,C) Immunoblots for GLAST as well as β-tubulin, which was used as a control loading protein, in the NAc and PFC,
respectively, as compared to water-pretreated groups and ethanol-pretreated groups. (B,D) Quantitative analysis did not reveal any significant differences in the ratio of GLAST/β-tubulin among all groups in the NAc and PFC, respectively. Values shown as means ± S.E.M. n = 6 for each group.

2.2.4. Effect of CEF on METH-induced hyperthermia

A mixed-model factorial ANOVA conducted on body temperature revealed a significant effect of time \[ F (2, 39) = 240.305, p < 0.0001 \], a significant interaction between time and pretreatment \[ F (2,39) = 7.848, p = 0.001 \], a significant interaction between time and posttreatment \[ F (4, 80) = 33.22, p < 0.0001 \], and a significant interaction between time, pretreatment and posttreatment \[ F (4, 80) = 4.335, p = 0.003 \]. Contrasts revealed that ethanol pretreatment significantly elevated body temperature compared to water control at baseline \( p < 0.05 \) (Fig. 2-5). Similarly, contrast analyses showed that following the last dose of METH (at time 0), METH significantly elevated body temperature compared to saline in water and ethanol pretreated groups as well as in comparison to the baseline point \( p < 0.0001 \). In addition, body temperature was significantly higher in the ethanol-Saline-Saline group as compared to the water-Saline-Saline group at this time point \( p < 0.001 \). CEF posttreatment (at time 12 hrs) restored body temperature compared to saline in the water \( p < 0.0001 \) and ethanol \( p < 0.001 \) pretreated groups. Similarly, body temperature was significantly higher in the water-METH-Saline \( p < 0.0001 \), ethanol-Saline-Saline \( p < 0.05 \), ethanol-METH-Saline \( p < 0.0001 \), and ethanol-METH-CEF groups \( p < 0.01 \) as compared to the water-Saline-Saline (Fig. 2-5) group. Significant increase in body temperature was revealed in METH-Saline treated groups in water and
ethanol pretreatment groups as compared to its baseline point. No significant difference was found between water-METH-CEF and water-Saline-Saline groups or between ethanol-METH-CEF and ethanol-Saline-Saline groups (p > 0.05) (Fig. 2-5).

**Figure 2-5.** Effects of METH (10 mg/kg i.p. every 2 hrs x 4), ethanol, and CEF posttreatment (200 mg/kg) on body temperature compared to water-Saline-Saline control group. Ethanol pretreatment significantly elevated body temperature at baseline compared to water pretreatment. METH significantly elevated body temperature in all groups after the last METH injection (time 0) compared to water-Saline-Saline group. CEF posttreatment restored body temperature compared to saline posttreatment in water and ethanol-METH treated rats. * p<0.05, ** p<0.01, # p<0.001, and @ p<0.0001 (& p<0.01, $ p<0.001 compared to baseline point) (mixed-model repeated measure factorial
ANOVA). Values are represented as mean ± SEM (Error bars were deleted for clarity). n = 7-9 for each group.

2.3. Discussion

The present study revealed for the first time that repeated high doses of METH significantly decreased GLT-1 expression in the NAc and PFC in the co-abuse METH and ethanol group as well as in the METH alone group. Our findings contradict a previous report in which METH induced an upregulation of GLT-1 in the PFC (Qi et al., 2012). However, this contradictory result could be due to different experimental designs and dosing regimens. A single low dose of METH (2 mg/kg), as compared to 10 mg/kg every 2 hrs for 4 times in this study, was used in the previous report by Qi et al. Mice were then euthanized at different time points following a single METH injection (0.5, 1, 2, and 4hrs) compared to rats that were euthanized 48 hrs after the last METH injection in our report. The GLT-1 expression was not changed in the PFC at the first two time points tested. However, GLT-1 expression was then increased after 2 hrs of METH injection (~ 250% of control) and then decreased dramatically to reach 140% of control after 4 hrs of METH injection. This previous report did not further investigate GLT-1 expression beyond 4 hrs of METH administration. The pattern of changes in GLT-1 expression presented in this previous report suggests a transient increase of GLT-1 that might be followed by a reduction in GLT-1 expression. By contrast, in our study, we have used repeated high dose METH, which is well known to produce neurotoxicity and hyperthermia (Sonsalla et al., 1989, Bowyer et al., 1994, Halpin and Yamamoto, 2012). This neurotoxic dosing paradigm of METH produced neurotoxicity comparable to the
one produced in other studies that have used a very high dose of METH 50 mg/kg 2-3 times per day for 4 days (Bittner et al., 1981, Ricaurte et al., 1982). However, previous study has investigated a single dose of METH in order to produce neurotoxicity and hyperthermia comparable to the dosing paradigm used in this study (Fukumura et al., 1998). The least effective dose that produced neurotoxicity and hyperthermia was 10 times higher than the dose used by Qi et al. (i.e., 20 mg/kg), while doses that produced a comparable neurotoxicity and hyperthermia are 30 and 40 mg/kg (Fukumura et al., 1998, Qi et al., 2012).

The GLT-1 is a glial glutamate transporter that plays a critical role in clearing the majority of extracellular glutamate to maintain glutamate homeostasis (Ginsberg et al., 1995, Rothstein et al., 1995, Rothstein et al., 1996, Danbolt, 2001, Mitani and Tanaka, 2003). The METH-induced downregulation of GLT-1 expression could be due to the fact that binge METH exposure induced hepatotoxicity in rats, with subsequent elevation in plasma and brain ammonia (Halpin and Yamamoto, 2012). Chronic ammonia exposure for at least 48 hrs was shown to decrease glutamate uptake in cultured astrocytes due to a possible decrease in the expression of glutamate transporters (Bender and Norenberg, 1996). It has also been shown that ammonia is responsible for GLT-1 downregulation in brains of a rat model with acute liver failure (Knecht et al., 1997, Chan and Butterworth, 1999). Moreover, rats treated with ammonium acetate develop a significant downregulation in GLT-1 expression compared to control rats (Norenberg et al., 1997). Therefore, downregulation of GLT-1 expression found in this study could be due to elevation in plasma and brain ammonia caused by METH exposure. Further studies are
warranted to determine the concentration of ammonia in the plasma and brain of rats exposed to repeated doses of METH.

A METH-induced decrease in GLT-1 expression in the NAc and PFC was restored by CEF posttreatment in both ethanol and water-pretreated rats. CEF is known to upregulate GLT-1 expression in disease and naïve animal models (Rothstein et al., 2005, Miller et al., 2008, Ramos et al., 2010). Since METH caused GLT-1 downregulation, we further investigated GLT-1 expression in ethanol and water pretreated rats to explore whether there is any additive effect of ethanol and METH in this protein. The present data revealed that METH exacerbates the reduction in GLT-1 expression in ethanol-pretreated rats compared to water-pretreated rats in the NAc, but not in the PFC. This indicates that there is no additive effect of ethanol on GLT-1 expression in the PFC, which is consistent with our recent findings, which demonstrated that free choice exposure to ethanol does not reduce GLT-1 expression in the PFC (Sari et al., 2013, Alhaddad et al., 2014b). Moreover, GLT-1 expression was downregulated in the NAc following saline treatment in the ethanol group compared to the water group. This is in accordance with recent findings that demonstrated that chronic ethanol exposure decreases GLT-1 expression and increases extracellular glutamate concentration in the NAc (Das et al., 2015).

Although the mechanism of ethanol-induced downregulation of GLT-1 is not known, studies from our laboratory showed that ethanol decreases phosphorylation of Akt (Alhaddad et al., 2014b, Goodwani et al., 2015a). Certain studies, however, have reported contradicting findings regarding the effects of ethanol exposure on GLT-1 expression. For example, GLT-1 expression was not altered following intermittent
ethanol exposure (Pati et al., 2016) or continuous ethanol exposure for eight weeks in female P rats (Ding et al., 2013). These contradictory results could be due to the differences in ethanol exposure paradigm and study design. The report by Pati et al. (2016) used intermittent ethanol exposure, while in our present study, we have used repeated daily ethanol exposure, which has been shown to increase extracellular glutamate concentration shortly after the last ethanol exposure in different brain regions such as the VTA, hippocampus, NAc, PFC, and striatum (Rossetti and Carboni, 1995, Dahchour and Witte, 1999, Dahchour and De Witte, 2000, Melendez et al., 2005, Kapasova and Szumlinski, 2008, Ding et al., 2012, Hermann et al., 2012) and decrease GLT-1 expression and/or glutamate clearance (Melendez et al., 2005, Ding et al., 2012, Aal-Aaboda et al., 2015, Das et al., 2015, Goodwani et al., 2015b). Alternatively, the report by Ding et al. (2013) used free choice continuous ethanol exposure for eight weeks (compared to oral gavage of ethanol for seven days in this current study). Ding et al. noted a trend of decrease in GLT-1 expression that was suggested to be masked by high variations in samples. Moreover, this previous report used female rats, as opposed to male rats that showed a decrease in GLT-1 expression following ethanol exposure (Alhaddad et al., 2014b, Goodwani et al., 2015b). Further studies are needed to investigate different gender responses to ethanol exposure and consequent changes in GLT-1 expression.

We also tested xCT, a glial protein that exchanges intracellular glutamate for extracellular cystine to maintain glutamate homeostasis (Bannai and Kitamura, 1980, Baker et al., 2002). However, we did not find any downregulation of xCT expression.
following METH treatment in either ethanol or water-pretreated rats. Importantly, CEF upregulated xCT expression in the NAc and PFC of ethanol and water pretreated rats, which is consistent with studies from our laboratory and others (Lewerenz et al., 2009, Knackstedt et al., 2010, Alhaddad et al., 2014a, Rao and Sari, 2014). CEF-induced upregulation of xCT expression might be another mechanism that modulates glutamate homeostasis to alleviate METH effects. In addition, xCT has been shown to facilitate cystine uptake with the subsequent synthesis of glutathione (Sato et al., 1999, Lewerenz et al., 2006). An in vitro study showed that CEF-induced upregulation of xCT expression was associated in part with increased glutathione concentration, which is independent of GLT-1 upregulation (Lewerenz et al., 2009). It is noteworthy that several studies indicated that METH can cause oxidative stress in different brain regions (Cubells et al., 1994, Açikgöz et al., 1998, Yamamoto and Zhu, 1998, Gluck et al., 2001, Ramirez et al., 2009). Furthermore, glutathione was found to be reduced in the striatum following repeated high doses of METH (Moszczyska et al., 1998). As a result, the CEF-induced increase in xCT expression may eventually improve glutathione synthesis.

Furthermore, we did not find any changes in GLAST expression in the NAc and PFC in either ethanol or water-pretreated rats. In accordance, ethanol exposure and/or CEF treatment did not significantly reduce GLAST expression (Alhaddad et al., 2014b, Hakami et al., 2016). Studies suggested that GLAST is highly expressed in the cerebellum and predominantly regulates glutamate uptake as compared to forebrain regions, including the PFC and NAc. However, GLT-1 is predominant in the forebrain. The differential predominance of GLAST versus GLT-1 in the PFC and NAc might be a
key factor involving the effects of ethanol and METH co-abuse in the expression of these transporters. Studies are warranted to investigate the differential effects of these glial transporters in an ethanol and METH co-abuse model.

METH significantly elevated body temperature in both ethanol and water-pretreated rats compared to saline, which is consistent with previous reports (Cass et al., 2006, Shioda et al., 2010, Halpin and Yamamoto, 2012). The present data showed that CEF posttreatment significantly reversed the increase in body temperature compared to saline when measured 12 hrs following the last METH dose. Although the rapid onset of action of CEF on body temperature is unclear, studies demonstrated that a single dose of CEF can increase the activity of GLT-1 and improve the survival of neurons (Thöne-Reineke et al., 2008). In fact, acute administration of CEF has many effects, including an anti-inflammatory response and analgesic action (Wei et al., 2012, Macaluso et al., 2013). Further studies are warranted to investigate the acute effects of CEF on the glutamatergic system and body temperature. The mechanism of action of CEF in reversing hyperthermia is unknown, but is most likely through its ability to upregulate GLT-1 and improve glutamate uptake. This is in line with a previous report by Rawls and colleagues in which CEF reversed morphine-induced hyperthermia (Rawls et al., 2007). This latter study demonstrates that CEF’s inhibition of hyperthermia was prevented by administering glutamate uptake blocker (TBOA), which suggests that upregulation of GLT-1 expression may be critical in the attenuation of hyperthermia. It is unclear whether the normalizing effect of CEF on body temperature might be associated with upregulation of GLT-1 and reduced extracellular glutamate concentration in central
reward brain regions such as the NAc and PFC. However, it is suggested that glutamate might be implicated in thermoregulation, since treatment with glutamate receptor antagonists attenuates the increase in body temperature in animal models (Madden and Morrison, 2003, Nakamura et al., 2004, Cao and Morrison, 2006, Nakamura and Morrison, 2008). Importantly, the NAc and PFC were found to be implicated in thermoregulation (Tseng et al., 1980, Hori et al., 1984, Shibata et al., 1988). Changes in body temperature and heat production were also found when functional ablation of PFC was applied (Shibata et al., 1981, Shibata et al., 1985). In addition, a recent study has shown that microinjections of METH into the PFC evoked measures of non-shivering thermogenesis (Hassan et al., 2015). Further studies are warranted to explore any possible associative effects between thermoregulation and glutamate homeostasis in an ethanol and METH co-abuse animal model and to investigate the key brain regions involved in this mechanism.

In summary, our findings provide evidence of the important role of GLT-1 using high doses of METH, well known to cause a hyperglutamatergic state and hyperthermia. Importantly, we found for the first time additive effects of ethanol and METH on GLT-1 downregulation in the NAc as compared to drug administered alone. This study also showed for the first time that CEF, a β-lactam antibiotic, was effective in restoring GLT-1 expression and reversing hyperthermia in the ethanol and METH co-abuse rat model. These findings suggest that CEF might be used as a potential drug for treatment against METH- or ethanol/METH-induced downregulation of GLT-1 expression and hyperthermia.
Disclosure

The authors declare no conflict of interest.

Acknowledgments

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Chapter 3

Effects of clavulanic acid treatment on GLT-1, xCT and mGluR2/3 expression on co-abuse of methamphetamine and ethanol in alcohol-preferring rat model

Yusuf S. Althobaiti1, Fahad S. Alshehri1, Alqassem Y. Hakami1, Alaa M. Hammad1, Youssef Sari1*

1University of Toledo, College of Pharmacy and Pharmaceutical Sciences, Department of Pharmacology and Experimental Therapeutics, Toledo, OH.

*Send correspondence to:
Dr. Youssef Sari
University of Toledo, College of Pharmacy and Pharmaceutical Sciences
Department of Pharmacology and Experimental Therapeutics
Health Science Campus,
3000 Arlington Avenue, HEB 282G
Toledo, OH 43614. USA
E-mail: youssef.sari@utoledo.edu
Tel: 419-383-1507 (Office)
Abstract

Relapse to different drugs of abuse, including methamphetamine (METH) is a major challenge in treating addiction. Studies show that there is a high rate of METH use in combination with ethanol. In this study, we investigated METH reinstatement using conditioned place preference (CPP) in alcohol-preferring (P) rats as an animal model of alcoholism. Among other neurotransmitters, glutamate has been shown to be implicated in mediating relapse to several drugs of abuse, including METH and ethanol. Glutamate homeostasis is maintained by number of glutamate transporters, such as glutamate transporter type 1 (GLT-1), cystine/glutamate transporter (xCT), and glutamate aspartate transporter (GLAST). In addition, group II metabotropic glutamate receptors (mGluR2/3) were found to be implicated in relapse to different drugs of abuse. Here, we tested the effect of clavulanic acid (CA), a non-antibiotic β-lactam compound, on METH reinstatement, ethanol drinking, the expression of glial glutamate transporters, and mGluR2/3 in the nucleus accumbens (NAc) shell and core as well as the dorsomedial prefrontal cortex (dmPFC). CPP for METH was induced in P rats, followed by extinction training. A priming injection of METH reinstated preference in the METH-paired chamber following extinction. Chronic exposure to ethanol decreased the expression of GLT-1 and xCT in the NAc shell, but not the NAc core or dmPFC. CA treatment blocked the reinstatement of METH, decreased ethanol intake and restored the expression of GLT-1 and xCT in the NAc shell. Moreover, the expression of mGluR2/3 was increased by CA in the NAc shell and dmPFC. These findings suggest that CA has the potential to modulate the expression of glial glutamate transporters and receptors, which consequently attenuate dependence to METH and ethanol.
Introduction

Methamphetamine (METH) is a derivative of amphetamine with greater addictive property potential. Relapse to METH and other drugs of abuse is considered a serious challenge in treating addiction. One of the main brain regions involved in the rewarding and reinforcing effects of drugs of abuse is the nucleus accumbens (NAc) (Koob and Bloom, 1988, Wise and Rompré, 1989, Bardo, 1998). Glutamatergic neurotransmission in the NAc plays a critical role in the relapse behavior to different drugs of abuse. Importantly, deficits in glutamate clearance in the NAc have been found to be associated with chronic drug use and drug seeking behavior (Fujio et al., 2005, Melendez et al., 2005, Knackstedt et al., 2010, Das et al., 2015). The NAc receives glutamatergic inputs from the prefrontal cortex (PFC) as well as other brain regions (Kelley et al., 1982, Phillipson and Griffiths, 1985). Glutamate release from dorsal PFC projections to the NAc core has been found to mediate cocaine reinstatement (McFarland et al., 2003, McFarland et al., 2004). Dorsomedial PFC (dmPFC), in particular, has been implicated in mediating the reinstatement of cocaine (McLaughlin and See, 2003, Berglind et al., 2009). Of note, it has been shown that blocking glutamatergic activation of mGluR1, mGluR5, activating mGluR2/3, and blocking AMPA receptors and glutamate release in the NAc can decrease the reinstatement of several drugs of abuse (Cornish and Kalivas, 2000, Park et al., 2002, McFarland et al., 2004, Tessari et al., 2004, Lee et al., 2005, Bossert et al., 2006, Bäckström and Hyytiä, 2007, Dravolina et al., 2007). Thus, glutamate plays a significant role in the reinstatement of drug seeking and, therefore, minimizing glutamatergic effects on synaptic and extrasynaptic receptors might be an effective strategy for attenuating reinstatement of drugs of abuse. Glutamate homeostasis...
is maintained by several glutamate transporters such as the glutamate transporter type 1 (GLT-1), the cystine/glutamate transporter (xCT), and the glutamate aspartate transporter (GLAST). GLT-1 clears about 90% of extracellular glutamate and it is frequently reported to be down-regulated in the NAc following exposure to drugs of abuse (Rothstein et al., 1995, Knackstedt et al., 2009, Sari and Sreemantula, 2012, Fischer et al., 2013, Shen et al., 2014). Furthermore, xCT was found to be down-regulated in the NAc following cocaine withdrawal (Baker et al., 2003, Knackstedt et al., 2010). In fact, decreased xCT expression in the NAc, which has a significant role in regulating glutamate homeostasis (Baker et al., 2002), is associated with decreased basal glutamate concentrations and subsequent impairment of the activation of mGluR2/3. Decreased glutamatergic tone on mGluR2/3 can lead to an increase in glutamate release in the NAc during drug, cue, and/or stress induced-reinstatement (Dietrich et al., 2002, Moran et al., 2005, Madayag et al., 2007, LaLumiere and Kalivas, 2008).

Importantly, several studies suggest that alcohol use disorder is extremely high in amphetamine-dependent subjects (Stinson et al., 2005). METH users frequently use ethanol (Furr et al., 2000, Bujarski et al., 2014), which may result in several deteriorating effects [for review see ref. (Althobaiti and Sari, 2016)]. Despite the evidence of the high rate of the co-abuse of METH and ethanol, little research has been focused on this area. Little is known about the effect of METH on ethanol drinking in rodent models. Moreover, little is known about the effects of exposure to METH and ethanol on the expression of GLT-1 in the NAc and the dmPFC. However, treatment with ceftriaxone (CEF), a known GLT-1 up-regulator, has been reported to prevent the reinstatement of
METH, cocaine, ethanol, nicotine, and heroin (Sari et al., 2009, Knackstedt et al., 2010, Abulseoud et al., 2012, Alajaji et al., 2013, Qrunfleh et al., 2013, Shen et al., 2014). Moreover, our lab recently showed that there is a significant decrease in ethanol intake in alcohol preferring (P) rats following CEF treatment, which was associated with significant up-regulation of GLT-1 in the NAc and the PFC (Sari et al., 2011, Sari et al., 2013b, Alhaddad et al., 2014a). Although CEF is a powerful up-regulator of GLT-1, it cannot be administered orally, requires high doses to show efficacy with subsequent side effects, and has antimicrobial activity. Therefore, it is very important to find a better alternative that overcomes these limitations. Since CEF’s up-regulatory effect on GLT-1 expression is thought to be mainly attributed to the β-lactam ring found in CEF and other β-lactam antibiotics (Rothstein et al., 2005), other β-lactam ring containing compounds may show similar activity. CA is a non-antibiotic β-lactam compound that is orally available, relatively safe, and can cross the blood brain barrier (Münch et al., 1981, Nakagawa et al., 1994). These advantages suggest that CA may have a beneficial effect in METH and ethanol exposed rats. We hypothesize that CA would prevent reinstatement of METH CPP and decrease ethanol intake. To explore the possible role of glutamatergic systems in these effects, we investigated the expression of different glutamate transporters (GLT-1, xCT, and GLAST) and mGluR2/3 in the NAc shell and core, as well as dmPFC. We also investigated the effect of METH on ethanol drinking and the effect of this co-abuse rat model on locomotor activity.
3.1. Methods and materials

3.1.1. Subjects

Alcohol preferring (P) rats were obtained from the Indiana University Medical Center (Indianapolis, IN) Indiana Alcohol Research Center breeding colonies. Animals were single-housed in standard plastic cages with a controlled temperature (21°C) and humidity (30%) on 12:12 light-dark cycle and had free access to food, water and two ethanol concentrations (15% and 30% v/v). Rats had ad libitum food, water, and ethanol throughout the experimental procedure, except the control group which did not have access to ethanol. Animal experimental procedures were approved by the Institutional Animal Care and Use Committee of The University of Toledo in accordance with the guidelines of the Institutional Animal Care and Use Committee of the National Institutes of Health and the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, Commission on Life Sciences, 1996).

3.1.2. Drugs

(+)METH hydrochloride and CA were purchased from Sigma-Aldrich (St. Louis, MO). Saline solution (0.9% NaCl) was used to dissolve either drug. Ethanol (95%; Decon Labs, Inc.) was diluted in water.

3.1.3. Conditioned place preference paradigm

3.1.3.1. Apparatus

A three-chambered CPP apparatus made of Plexiglas was used in this study. Two equal-sized conditioning chambers were distinguished by both visual and tactile cues (40 cm x
40 cm x 40 cm) and one smaller middle chamber (30 cm x 40 cm x 40 cm). The inner walls of the first conditioning chamber are white with horizontal black stripes and textured black floor (chamber 1). The inner walls of the other conditioning chamber (chamber 2) are black with vertical white stripes and smooth white floor. The middle chamber is featureless and separated from the two conditioning chambers by two guillotine doors. The CPP experiment was conducted in four distinct phases.

3.1.3.2. The habituation phase

The first day of habituation is considered as Day 1. On Days 1, 2, and 3 each rat was placed in the middle chamber with both doors closed for 3 minutes. Then, both doors were opened and the rat had a free access to explore the entire apparatus for 20 minutes. On Day 3, the test was recorded by a digital camera fixed above the apparatus (the CPP Test). The time spent in each chamber was measured by a blinded observer and locomotor activity was analyzed using ANY-maze video tracking system. Rats showing strong initial preference to any chamber, more than 67% of total time, were excluded from the study as suggested previously (Fujio et al., 2005). One way repeated measures ANOVA did not reveal any significant main effect preference for Chamber 1, Chamber 2, or the middle chamber [F (1.851, 66.62) = 0.7889, P = 0.4497].

3.1.3.3. The conditioning phase

The conditioning phase (Days 4-10) consisted of fourteen conditioning sessions conducted on 7 consecutive days (morning and afternoon sessions). That is, each rat, in the morning session, received i.p. injections of either 2.5 mg/kg METH or saline, and was
placed in the corresponding chamber with the door closed for 20 minutes. In the afternoon session (at least 5 hours after the morning session), each rat received saline or 2.5 mg/kg METH i.p. and was placed in the opposite chamber with the door closed for 20 minutes. Since initial time spent in Chamber 1, Chamber 2, and the middle chamber was not significantly different, an unbiased design was utilized. That is, random assignment of rats was utilized in which half of the animals received METH in Chamber 1, and the other half received it in Chamber 2. Control rats in both morning and afternoon sessions received saline and were placed in either chamber (i.e. in chamber 1 in the morning and chamber 2 in the afternoon session) with doors closed for 20 minutes. The order of administering METH and saline was counterbalanced. That is, on one day, METH was administered in the morning for half of rats and saline in the afternoon. On the following day, saline was administered to same rats in the morning and METH was given in the afternoon, and the pattern was reversed for the other half. Similarly, the orientation of the chambers was alternated (i.e. chamber 1 was in the left side on one day, then it was moved to be in the right side the following day) as described in a previous study (Cunningham et al., 2006). On Day 11, the CPP Test was performed again as described previously.

3.1.3.4. The extinction phase

This phase is similar to the conditioning phase except the rats that received METH in the conditioning phase were randomly assigned to receive either CA (5 mg/kg, i.p.) or saline (1 ml/kg) instead of 2.5 mg/kg METH (Day 12-18). Control rats in both morning and afternoon sessions received saline as described in the conditioning phase. On Day 19
another CPP Test was performed. If time spent in the METH-paired chamber following conditioning training was not decreased by 25% following extinction training, the rat was not considered to be extinguished and excluded from the study as described in previous work (Abulseoud et al., 2012).

3.1.3.5. Reinstatement phase

On the morning of Day 20, each rat was administered a single dose of either METH (2.5 mg/kg) or saline and placed in the corresponding chamber with the door closed for 20 minutes. In the afternoon, saline or METH injection was administered and the rat was placed in the opposite chamber. On Day 21, each rat was tested for CPP as described before.

3.1.4. Experimental design

Experimental groups and schedule are illustrated in Table 3.1. A separate group of rats was utilized to investigate whether CA has any effect on CPP by itself. These rats were administered CA (5 mg/kg) instead of METH and underwent the same procedure as described before in conditioning phase. After completion of the seven conditioning days, rats were tested for CPP.

Rats had a free access to two ethanol concentrations (15% and 30% v/v), water and food for five weeks before starting the CPP experiment. Ethanol and water intake as well as body weight were measured three times a week during week 4 and 5 and the average values of these two weeks were considered as the baseline. The measurements of fluid intake and body weight were then recorded daily throughout the experiment. During
week 6, the CPP experiment was started, in which each group received either saline or METH (2.5 mg/kg, i.p.) injections for 7 days (Conditioning phase), followed by 7 days treatment with either saline or CA (5 mg/kg, i.p.) (Extinction phase). Finally, rats were tested for reinstatement produced by an i.p. injection of METH (2.5 mg/kg). There were four groups: a) Control group, this group had free access to water and food throughout the whole experiment and received saline injections in conditioning, extinction and reinstatement phases; b) Saline-Saline, this group had free access to ethanol (15% and 30% v/v), as well as ad libitum food and water throughout the experiment. Rats in this group received saline injections in conditioning, extinction and reinstatement phases; c) METH-Saline, this group had free access to ethanol (15% and 30% v/v), as well as ad libitum food and water throughout the experiment. Rats in this group received METH injections in conditioning, saline in extinction and METH in the reinstatement phase; d) METH-CA, this group had free access to ethanol (15% and 30% v/v), as well as ad libitum food and water throughout the experiment. Rats in this group received METH in conditioning, CA in extinction and METH in the reinstatement phase.
Table 3.1. Experimental groups and schedule of CPP and drinking paradigms. METH (2.5 mg/kg) was administered during conditioning and reinstatement phases, while CA (5 mg/kg) was administered during the extinction phase.

<table>
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<th>Group</th>
<th>Drinking</th>
<th>Phase</th>
<th>Days</th>
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<td>Habituation</td>
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<td>Conditioning</td>
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<td>Extinction</td>
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<td>Reinstatement</td>
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<td>1- Control</td>
<td>Water</td>
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<td>2- Saline-Saline</td>
<td>Ethanol (1% and 3%)</td>
<td>AM (Day 3)</td>
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<tr>
<td>3- METH-Saline</td>
<td>Water</td>
<td>PM</td>
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<td>4- METH-CA</td>
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<td>CPP Test</td>
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<td>CPP Test + Animal euthanasia</td>
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3.1.5. Brain tissue harvesting

Rats were rapidly euthanized following reinstatement CPP test by CO₂ inhalation and immediately decapitated. Brains were then removed and rapidly frozen on dry ice and stored at -80 °C. Brain sectioning and micro-punch procedure were performed as
described previously (McBride et al., 2009). The NAc shell and core, as well as dmPFC, were identified using Rat Brain Stereotaxic Atlas (Fig. 9) (Paxinos, 2007).

3.1.6. Western blot
Western blot procedure was performed as previously described (Sari et al., 2009). Briefly, brain tissue was lysed in lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 0.5% NP-40, 1% Triton, 0.1% SDS) containing protease inhibitor cocktail. A Bio-Rad protein assay method was used to determine protein amount in the tissue extracts (Bio-Rad, Hercules, CA, USA). Extracted proteins were loaded onto 10-20% tris-glycine gel. After separation, proteins were transferred from the gel onto PVDF membrane. Consequently, membrane was blocked using 3% milk in Tris-buffered saline Tween 20 for 30 minutes. Guinea pig anti-GLT1 (Millipore Bioscience Research Reagents; 1:5000 dilution), rabbit anti-xCT antibody (Abcam; 1:1000 dilution), rabbit anti-mGluR2/3 antibody (Upstate Biotechnology; 1:1000 dilution), rabbit anti-GLAST (Abcam; 1:5000 dilution), or mouse anti β-tubulin antibody (Covance; 1:5000 dilution) was then added to the blocking buffer, and the membrane was incubated overnight at 4 °C. Membrane was then washed and incubated with horseradish peroxidase-labeled (HRP) anti-Guinea pig, anti-rabbit, or anti-mouse secondary antibody (1:5000). Chemiluminescent kit (SuperSignal West Pico) was used to incubate membrane for protein detection. Subsequently, membrane was exposed to Kodak BioMax MR films (Thermo Fisher Scientific). Films were then developed using an SRX-101A machine. Blots for each detected protein were digitized and quantified using an MCID system.
Data calculated as ratios of GLT-1/β-tubulin, xCT/β-tubulin, GLAST/β-tubulin, and mGluR2/3/β-tubulin.

3.1.7. Statistical analysis
Time spent in conditioning chambers and locomotor activity were analyzed using two-way repeated measures ANOVA (Time x Chamber), and (Time x Treatment), respectively. One-way ANOVA was used to analyze immunoblot data. Newman-Keuls multiple comparisons were used for immunoblot data as well as time spent and locomotor activity. Average ethanol intake, ethanol preference, water intake, and body weight were analyzed using two-way repeated measures ANOVA (Time x Treatment) followed by Bonferroni’s multiple comparisons test. Post hoc multiple comparisons were performed when a significant interaction or a significant main effect was revealed. GraphPad Prism was used to statistically analyze all data in this study that were based on p<0.05 level of significance.

3.2. Results
3.2.1. Conditioned place preference
3.2.1.1. Effect of CA alone on CPP
Two-way repeated measures ANOVA revealed a non-significant effect of time [F (1, 6) = 0.02707, p = 0.8747], a non-significant effect of chamber [F (1, 6) = 0.04274, p = 0.8431], and a non-significant interaction between time and chamber [F (1, 6) = 1.397, p = 0.2820] (Fig. 3-1A).
3.2.1.2. Effect of CA on METH-induced reinstatement using CPP paradigm

3.2.1.2.1. METH-Saline treated group

Two-way repeated measures ANOVA revealed a non-significant effect of time \([F(3, 24) = 1.005, p = 0.4077]\), a non-significant effect of chamber \([F(1, 8) = 1.514, p = 0.2534]\), and a significant interaction between time and chamber \([F(3, 24) = 10.39, p = 0.0001]\). Newman-Keuls multiple comparisons test showed a significant increase in time spent following conditioning in the METH-paired chamber as compared to pre-conditioning \((p< 0.05)\) and the saline-paired chamber in the post-conditioning test \((p< 0.01; \text{Fig. 3-1B})\). A significant decrease in time spent in the METH-paired chamber was revealed following extinction training as compared to post-conditioning \((p< 0.01; \text{Fig. 3-1B})\). The reinstatement dose of METH significantly increased time spent in METH-paired chamber as compared to the extinction phase \((p<0.05)\). The reinstatement dose of METH significantly increased time spent in METH-paired chamber as compared to the saline-paired chamber \((p<0.05)\) in the reinstatement phase.

3.2.1.2.2. METH-CA treated group

Two-way repeated measures ANOVA revealed a significant effect of time \([F(3, 33) = 2.066, p = 0.1236]\), a non-significant effect of chamber \([F(1, 11) = 2.401, p = 0.1495]\), and a significant interaction between time and chamber \([F(3, 33) = 10.56, p < 0.0001]\). Newman-Keuls multiple comparisons showed a significant increase in time spent following conditioning training in the METH-paired chamber as compared to pre-conditioning \((p<0.05)\) and the saline-paired chamber in post-conditioning \((p< 0.001; \text{Fig. 3-1C})\). A significant decrease in time spent in the METH-paired chamber was revealed
following extinction training and reinstatement as compared to post-conditioning (p<0.001 and p<0.01, respectively; Fig. 3-1C). Time spent in the METH-paired chamber was not significantly changed following reinstatement as compared to extinction or the saline-paired chamber (p>0.05).

3.2.2. Locomotor activity

Two-way repeated measures ANOVA revealed a non-significant effect of time [F (1, 32) = 1.223, p = 0.2771], a significant effect of treatment [F (2, 32) = 15.73, p < 0.0001], and a significant interaction between time and treatment [F (2, 32) = 8.657, p = 0.0010]. A significant decrease in locomotor activity was revealed at baseline in Ethanol-Saline and Ethanol-METH groups as compared to the control group (p<0.0001; Fig. 3-1D). Newman-Keuls multiple comparisons showed a significant increase in locomotor activity following seven days conditioning treatment with METH as compared to saline treatment in ethanol exposed rats (p<0.001; Fig. 3-1D). A significant decrease in locomotor activity was revealed between the Ethanol-Saline group as compared to the water control group (p<0.0001; Fig. 3-1D). No significant change in locomotor activity was revealed between the METH-Saline group and the control group following conditioning. Importantly, CA by itself did not change locomotor activity following seven days treatment with this drug; mean distance traveled ± SEM in post-conditioning = 56.16 ± 4.57 m as compared to baseline = 50.46 ± 5.39 m [Paired t-test, t(6) = 1.662, p = 0.1477; data not shown].
Fig 3-1: Time spent and locomotor activity. Time spent in each conditioning chamber during different CPP phases in CA alone A), METH-Saline group (B) and METH-CA group (C). Time spent in CA-paired chamber was not significantly changed following 7 days treatment with CA (A). Time spent in the METH-paired chamber was significantly increased in reinstatement as compared to extinction training in METH-Saline treated rats (B). CA blocked the reinstatement effect of METH (C). Distance traveled for rats during baseline and after conditioning with METH (D). Locomotor activity was lower in ethanol groups during baseline as compared to the control group. Following the conditioning with
METH, the locomotor activity was increased as compared to saline. No significant
difference in distance traveled between the control group and Ethanol-METH group
following conditioning (n = 9-12 for each group). Values shown as means ± S.E.M.
*p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001. [(@p<0.05) as compared to pre-
conditioning, (#p<0.05, ##p<0.01 and ###p<0.001) as compared to post-conditioning,
while (&p<0.05) as compared to extinction phase].

3.2.3. Ethanol drinking and preference, water intake and body weight

3.2.3.1. Effect of METH during the conditioning phase

We tested the effect of METH during the conditioning phase (conditioning 1-7) on
ethanol intake and preference as well as water intake and body weight. Statistical analysis
using two-way repeated measures ANOVA revealed a significant effect of time [F (7,
182) = 11.43, p < 0.0001], treatment [F (1, 26) = 11.01, p = 0.0027], and a significant
interaction between time and treatment [F (7, 182) = 5.372, p < 0.0001]. Bonferroni’s
multiple comparisons revealed that METH significantly decreased average ethanol intake
during Day 1, 3, 4, and 5 of conditioning as compared to saline treatment (p < 0.05,
0.0001, 0.05, and 0.05, respectively; Fig. 3-2A). No significant change in ethanol intake
between METH and saline treatment was seen during Day 2, 6, and 7 of conditioning
phase.

We further investigated the effect of METH on ethanol preference. Two-way repeated
measures ANOVA revealed a significant effect of time [F (7, 182) = 7.597, p < 0.0001],
treatment [F (1, 26) = 11.29, p = 0.0024], and a significant interaction between time and
treatment [F (7, 182) = 3.956, p < 0.0005]. Bonferroni’s multiple comparisons revealed
that METH significantly decreased ethanol preference during Day 3 through 6 of conditioning as compared to saline treatment (p < 0.01 for Day 3 and 5, p < 0.05 for Day 4 and 6; Fig. 3-2B). No significant change in ethanol preference between METH and saline treatment was seen during Day 1, 2, and 7 of the conditioning phase.

Two-way repeated measures ANOVA conducted on water intake revealed a significant effect of time [F (7, 182) = 2.171, p = 0.0387], treatment [F (1, 26) = 13.34, p = 0.0011], and a non-significant interaction between time and treatment [F (7, 182) = 1.416, p = 0.2014]. Bonferroni’s multiple comparisons revealed that METH significantly increased average water intake during Day 1, 2, and 4 of conditioning as compared to saline treatment (p < 0.05 for Day 1 and 4, p < 0.01 for Day 2; Fig. 3-3A). No significant change in ethanol intake between METH and saline treatment was observed during Day 3, 5, 6, and 7 of the conditioning phase.

A significant effect of time [F (7, 182) = 24.27, p < 0.0001], non-significant effect of treatment [F (1, 26) = 3.676, p = 0.0662], and a significant interaction between time and treatment [F (7, 182) = 10.89, p < 0.0001] was revealed on body weight using two-way repeated measures ANOVA. Bonferroni’s multiple comparisons test did not reveal any significant change in body weight following METH treatment in all conditioning days as compared to saline (p > 0.05; Fig. 3-3B).
Fig 3-2: Effect of METH on ethanol intake and preference during the conditioning phase.

Rats had a free access to three bottles containing water, 15% ethanol and 30% ethanol for five weeks. Ethanol intake and preference were measured during the last two weeks and served as a baseline. Rats were then administered METH (2.5 mg/kg/day) or saline for 7
days during the conditioning phase and ethanol intake (A) and preference (B) were assessed daily. METH significantly decreased ethanol intake as compared to saline during Day 1, 3-5 of conditioning. Ethanol preference was decreased following METH in Day 3-6 of conditioning. *p<0.05, **p<0.01, and ****p<0.0001. (n = 7-21).
Fig 3-3: Effect of METH on water intake and body weight during the conditioning phase. Rats had a free access to three bottles containing water, 15% ethanol and 30% ethanol for five weeks. Water intake and body weight were measured during the last two weeks and served as baseline. Rats were then administered METH (2.5 mg/kg/day) or saline for 7 days during the conditioning phase and water intake (A) and body weight (B) were assessed daily. METH significantly increased water intake as compared to saline during Day 1, 2 and 4 of conditioning. Body weight was not significantly changed following METH during the conditioning phase. *p<0.05 and **p<0.01 (n = 7-21).

3.2.3.2. Effect of CA during the extinction phase

We tested the effect of CA during the extinction phase (extinction 1-7) on ethanol intake and preference as well as water intake and body weight. Statistical analysis using two-way repeated measures ANOVA revealed a non-significant effect of time [F (7, 175) = 1.027, p = 0.4141], a significant effect of treatment [F (2, 25) = 18.14, p < 0.0001], and a significant interaction between time and treatment [F (14, 175) = 4.333, p < 0.0001]. Bonferroni’s multiple comparisons test revealed that average ethanol intake was significantly decreased in the METH-CA group during Day 1 of extinction as compared to the Saline-Saline group (p < 0.05; Fig. 3-4A) and decreased ethanol intake was observed as compared to METH-Saline and Saline-Saline groups during Day 2 through 7 of the extinction phase. No significant change in ethanol intake was observed between METH-Saline and Saline-Saline groups in all days of extinction.
We further investigated the effect of CA on ethanol preference. Two-way repeated measures ANOVA revealed a significant effect of time \([F (7, 175) = 2.832, p = 0.0081]\), treatment \([F (2, 25) = 10.64, p = 0.0005]\), and a non-significant interaction between time and treatment \([F (14, 175) = 1.476, p = 0.1244]\). Bonferroni’s multiple comparisons revealed that ethanol preference was significantly decreased in the METH-CA group as compared to the METH-Saline group during Day 1 through 7 of extinction \((p < 0.01-0.05; \text{Fig. 3-4B})\). Statistical analysis revealed that ethanol preference was significantly decreased in the METH-CA group as compared to Saline-Saline group during Day 1, 2 and 5 of extinction \((p < 0.05; \text{Fig. 3-4B})\). No significant change in ethanol preference was observed between the METH-Saline and Saline-Saline groups in all days of extinction.

A significant effect of time \([F (7, 175) = 4.13, p = 0.0003]\), a non-significant effect of treatment \([F (2, 25) = 1.255, p = 0.3025]\), and a non-significant interaction between time and treatment \([F (14, 175) = 1.066, p = 0.3920]\) was revealed using two-way repeated measures ANOVA conducted on water intake. Bonferroni’s multiple comparisons test did not reveal any significant change in water intake among all groups during the extinction phase \((p > 0.05; \text{Fig. 3-5A})\).

Two-way repeated measures ANOVA conducted on body weight revealed a significant effect of time \([F (7, 175) = 5.486, p < 0.0001]\), non-significant effect of treatment \([F (2, 25) = 1.722, p = 0.1992]\), and a non-significant interaction between time and treatment \([F (14, 175) = 1.352, p = 0.1815]\). Bonferroni’s multiple comparisons did not reveal any
significant change in body weight among any groups during the extinction phase (p > 0.05; Fig. 3-5B).

Fig 3-4: Effect of CA on ethanol intake and preference during the extinction phase. Rats continued to have a free access to three bottles containing water, 15% ethanol and 30% ethanol. Rats received either CA (5 mg/kg/day) or saline for 7 days during the extinction
phase. Ethanol intake (A) and preference (B) were measured daily during the extinction phase. CA significantly decreased ethanol intake and preference during Day 1-7 of the extinction phase. *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001. (n = 7-12).

Fig 3-5: Effect of CA on water intake and body weight during the extinction phase. Rats continued to have a free access to three bottles containing water, 15% ethanol and 30%
ethanol. Rats were administered either CA (5 mg/kg/day) or saline for 7 days during the extinction phase. Water intake (A) and body weight (B) were measured daily during the extinction phase. CA did not significantly alter either water intake or body weight during the extinction phase. (n = 7-12 for each final groups).

3.2.4. The expression of GLT-1, xCT, GLAST, and mGluR2/3 in the NAc shell and core as well as the dmPFC

3.2.4.1. GLT-1 expression

We investigated the effect of ethanol, METH and CA on GLT-1 expression level in the dmPFC and the NAc shell and core following the reinstatement test. One-way ANOVA revealed a significant main effect of treatment among Control, Saline-Saline, METH-Saline, and METH-CA groups in the NAc shell [F (3, 24) = 7.866, P = 0.0008; Fig. 3-6A], but not in the core [F (3, 24) = 0.5286, P < 0.6669; Fig. 3-7A] or dmPFC [F (3, 24) = 0.8643, P = 0.4731; Fig. 3-8A]. Newman-Keuls multiple comparisons showed a significant increase in the expression of GLT-1 in METH-CA treated rats compared to METH-Saline treated rats in the NAc shell (p<0.05; Fig. 3-6A). By contrast, statistical analyses showed a significant downregulation in GLT-1 expression in the Saline-Saline and METH-Saline group compared to the control group in the NAc shell (p<0.05 and p<0.001, respectively; Fig. 3-6A). No significant difference in GLT-1 expression level was revealed between the METH-CA group and the control group.
3.2.4.2. xCT expression

We further tested the effect of ethanol, METH and CA on another glutamate transporter, xCT, which plays a key role in maintaining glutamate homeostasis. Therefore, we investigated xCT expression level in the NAc shell and core, as well as dmPFC, following the reinstatement test. One-way ANOVA revealed a significant main effect of treatment among Control, Saline-Saline, METH-Saline, and METH-CA groups in the NAc shell \([F (3, 20) = 4.831, P = 0.0109]\), but not in the NAc core \([F (3, 24) = 0.2322, P < 0.8730; \text{Fig. 3-7B}]\) or dmPFC \([F (3, 24) = 1.15, P = 0.3491; \text{Fig. 3-8B}]\). Newman-Keuls multiple comparisons showed a significant increase in the expression level of xCT in METH-CA treated rats compared to the Saline-Saline treated rats in the NAc shell (p<0.05; Fig. 3-6B). By contrast, statistical analyses showed a significant downregulation in xCT expression in the Saline-Saline group compared to the control group in the NAc shell (p<0.05; Fig. 3-6B). No significant difference in xCT expression level was revealed between the METH-CA or METH-Saline groups as compared to the control group.

3.2.4.3. GLAST expression

We further tested the effect of ethanol, METH and CA on expression levels of GLAST, another glutamate transporter, in the NAc shell and core, as well as the dmPFC, following the reinstatement test. One-way ANOVA revealed a non-significant main effect of treatment among Control, Saline-Saline, METH-Saline, and METH-CA groups in the NAc shell \([F (3, 24) = 0.7276, P = 0.5456; \text{Fig. 3-6C}]\), NAc core \([F (3, 20) = 1.096, P = 0.3740; \text{Fig. 3-7C}]\) and dmPFC \([F (3, 20) = 0.2637, P = 0.8507; \text{Fig. 3-8C}]\).
3.2.4.4. mGluR2/3 expression

We investigated the effect of ethanol, METH and CA on mGluR2/3 expression in the dmPFC, and NAc shell and core, following the reinstatement test. One-way ANOVA revealed a significant main effect of treatment among Saline-Saline, METH-Saline, and METH-CA groups in the NAc shell [F (3, 24) = 3.996, P = 0.0193; Fig. 3-6D], dmPFC [F (3, 20) = 3.116, P = 0.0492; Fig. 3-8D], but not in the NAc core [F (3, 24) = 0.294, P = 0.8293; Fig. 3-7D]. Newman-Keuls multiple comparisons showed a significant increase in the expression level of mGluR2/3 in METH-CA treated rats compared to the control group treated rats in the NAc shell (p<0.05; Fig. 3-6D), dmPFC (p<0.05; Fig. 3-8D). Moreover, statistical analyses showed a significant increase in mGluR2/3 expression level in the METH-CA group compared to Saline-Saline and METH-Saline group in the NAc shell (p<0.05; Fig. 3-6D), but not in the dmPFC (p>0.05; Fig. 3-8D).
Fig 3-6: Effects of ethanol, METH (2.5 mg/kg) and CA (5 mg/kg) on the expression of GLT-1 (A), xCT (B), GLAST (C), and mGluR2/3 (D) in the NAc shell. Quantitative analysis revealed a significant increase in the ratio of GLT-1/β-tubulin in METH-CA treated rats compared to the METH-Saline treated rats. A significant downregulation of GLT-1 expression was revealed in the METH-Saline and Saline-Saline groups compared to the control water group. Significant increase in the ratio of xCT/β-tubulin was revealed in the METH-CA group compared to the Saline-Saline group. Significant downregulation of xCT expression was revealed in Saline-Saline treated group compared to the control water group. Significant increase in the ratio of mGluR2/3/β-tubulin was revealed in METH-CA compared to METH-Saline, Saline-Saline, and the control groups. No significant difference in GLAST expression was revealed among all tested groups in the NAc shell. * p<0.05. Values shown as means ± S.E.M. n = 6-7 for each group.
Fig 3-7: Effects of METH (2.5 mg/kg) and CA (5 mg/kg) on the expression of GLT-1 (A), xCT (B), GLAST (C), and mGluR2/3 (D) in the NAc core. Quantitative analysis did not reveal any significant change in the expression of either GLT-1, xCT, GLAST, or mGluR2/3 among all tested groups in the NAc core. Values shown as means ± S.E.M. n = 6-7 for each group.

Fig 3-8: Effects of METH (2.5 mg/kg) and CA (5 mg/kg) on the expression of GLT-1 (A), xCT (B), GLAST (C), and mGluR2/3 (D) in dmPFC. Quantitative analysis revealed a significant increase in the ratio of mGluR2/3/β-tubulin in the METH-CA group compared to the control group. No significant difference in GLT-1, xCT or GLAST
expression was revealed among all tested groups in dmPFC. * p<0.05. Values shown as means ± S.E.M. n = 6-7 for each group.

Fig 3-9: Representative micro-punch samples in the NAc (A) shell (open circles) and core (closed circles), as well as dmPFC (B). Samples were taken from both sides of the NAc and dmPFC; however, they are indicated in one side of the brain for clarity in the diagram. Numbers indicate anteroposterior distance from bregma according to the atlas of Paxinos and Watson (1998).

3.3. Discussion

In this study, we showed for the first time that CA blocked the reinstatement of METH CPP and decreased ethanol drinking. We have also shown that the down-regulatory effect of chronic ethanol drinking on the expression of GLT-1 and xCT in the NAc is specific to the NAc shell as compared to the NAc core. CA treatment restored the expression of
GLT-1 and xCT in the NAc shell as well as increased the expression of mGluR2/3 in the NAc shell and dmPFC. Importantly, the blocking effect of CA on METH induced reinstatement is unlikely to be caused by CA-induced changes in CPP or locomotor activity. In fact, CA failed to affect CPP by itself, suggesting a lack of CPP or aversion due to CA treatment, and we confirmed that CA did not affect locomotor activity. Moreover, CA did not affect water intake and its decreasing effect on drinking was specific to ethanol. Together, the anti-reinstatement effect of CA is unlikely to be affected by its direct effect on CPP or due to a negative effect on locomotor activity. Thus, the CA effects on reinstatement and ethanol drinking are most likely due to its improvement of glutamatergic tone in brain regions known to mediate the rewarding and reinforcing effects of drugs of abuse. Chronic ethanol exposure caused reduction in the locomotor activity of rats as compared to control group, which is consistent with the sedative effect of ethanol. Interestingly, METH reversed the negative effect of ethanol drinking on locomotor activity which corroborates a recent clinical study (Kirkpatrick et al., 2012). METH stimulant effects and the resulting improvement on the detrimental effect of ethanol on locomotor activity might indicate that the high prevalence of METH and ethanol co-abuse found in clinical studies may involve some degree of compensation or self-medication [for review see ref. (Althobaiti and Sari, 2016)].

Ethanol exposure caused a significant downregulation of GLT-1 and xCT in the NAc shell but not the NAc core or dmPFC. This new finding adds to previous reports that show the important role of the shell subregion of the NAc in mediating the rewarding and reinforcing effects of ethanol. Ethanol exposure has been shown to increase the release of
dopamine in the NAc shell of rats selectively bred to prefer ethanol more than rats bred to avoid ethanol (Bustamante et al., 2008). Moreover, a recent report showed that blocking the dopaminergic D1 receptor in the NAc shell but not the core decreases ethanol seeking behavior in P rats (Hauser et al., 2015). Similarly, ethanol has been shown to be self-infused in the NAc shell, but not the NAc core, in both Wistar and P rats, with increased sensitivity of the NAc shell to the reinforcing effect of ethanol in P rats (Engleman et al., 2009). Moreover, the reinforcing effect of ethanol has been shown to be mediated through the activation of dopaminergic receptors in the NAc shell, but not the NAc core (Ding et al., 2015). Moreover, studies showed that chronic ethanol drinking can decrease the expression of GLT-1 and xCT in the NAc and other brain regions, and restoring their expression can decrease ethanol drinking (Sari and Sreemantula, 2012, Alhaddad et al., 2014a, Alhaddad et al., 2014b, Aal-Aaboda et al., 2015, Das et al., 2015). Here, we revealed that the reduction in the expression of GLT-1 and xCT following chronic ethanol exposure is specific to the shell rather than the core subregion of the NAc. However, we have previously shown that ethanol drinking for six weeks caused downregulation of GLT-1 expression in both the NAc shell and the core (Sari et al., 2013b). In this study, the rats were exposed to chronic ethanol consumption for a longer period of time (eight weeks). This might indicate possible neuroadaptation that might have occurred in the core, but not the NAc shell, to the effect of ethanol on GLT-1 expression. We suggest that GLT-1 is more sensitive to the effect of ethanol in the shell subregion of the NAc than the core. The expression of GLAST, however, was not altered in any brain regions tested, which is in line with previous reports (Alhaddad et al., 2014b, Hakami et al., 2016). Studies suggested that GLAST is highly expressed in cerebellum
and retina, while GLT-1 and xCT predominantly regulate glutamate homeostasis as compared to GLAST in forebrain regions, including the NAc (Rothstein et al., 1994, Rothstein et al., 1995, Lehre et al., 1997, Danbolt, 2001, Baker et al., 2002). This might be a key factor mediating the effects of ethanol on the expression of these transporters. However, the expression of the glutamate transporters as measured by Western blot does not reflect functional activity. Further studies are needed to investigate any changes in the activity of GLT-1 and the other glutamate transporters following METH and ethanol exposure.

In this study, we showed that METH initially caused reduction, followed by gradual restoration of ethanol intake. The cause of this effect is unclear, however, it might be due to possible interactions between METH and ethanol on dopaminergic pathways in the NAc. *In vivo* microdialysis studies have reported increases in dopamine release in the NAc following oral ethanol as well as METH self-administration (Weiss et al., 1993, Lominac et al., 2012, Jang et al., 2016). Dopamine in the NAc is known to regulate motivated behavior and reward to drugs of abuse [for review see ref. (Ikemoto and Panksepp, 1999)]. Tolerance to the effects of METH on dopamine release in the NAc has been reported (Le Cozannet et al., 2013), which might help to explain the pattern shown here. It is possible that METH initially increases dopamine release in the NAc with the associated motivational states, which in turn decreases ethanol seeking behavior. This initial effect might be followed by gradual decreases in dopamine release following METH exposure with the associated increases in ethanol drinking to reach to the same
dopamine release and hedonic effects. Further studies are needed to investigate the neurochemical basis of this effect.

METH and ethanol co-exposure showed a trend toward more downregulation of GLT-1 in the NAc shell, but not the NAc core. This adds to the previous body of research that the NAc shell, rather than the NAc core, has been implicated in the rewarding and reinforcing effects of amphetamine and other drugs of abuse (Pontieri et al., 1995, Carlezon and Wise, 1996, Ikemoto et al., 2005). In fact, increasing the expression of the GLT-1 by viral-mediated gene transfer in the NAc shell or treatment with GLT-1 activator, MS-153, has been shown to block the acquisition of morphine, METH, and cocaine CPP in rats and mice (Fujio et al., 2005, Nakagawa et al., 2005). METH exposure did not cause any added effect to ethanol on the expression of xCT or GLAST. This suggests a specific effect of METH on GLT-1 rather than the other transporters. Further studies are needed to investigate the role of METH by itself on GLT-1 expression as well as other proteins involved in regulating extracellular glutamate in mesocorticolimbic brain regions.

Interestingly, the expression of GLT-1 and xCT was restored following CA treatment in the NAc shell which might be, in part, the underlying mechanism in blocking the reinstatement of METH and reducing ethanol drinking. One recent report has shown that CA is capable of increasing the expression of GLT-1 in the NAc (Kim et al., 2016). Here we showed for the first time that CA can also increase the expression of xCT and restore GLT-1 expression in the shell subregion of the NAc but not the core. Moreover, novel
evidence has been shown in this study that CA increased the expression of mGluR2/3 in both the NAc shell and dmPFC. Increasing the expression of mGluR2/3 in the PFC has been shown to decrease ethanol seeking, making it a potential target for treating ethanol dependence (Meinhardt et al., 2013). Moreover, activation of mGluR2/3 in the NAc shell but not the NAc core blocked context induced reinstatement of heroin (Bossert et al., 2006). Interestingly, activating mGluR2/3 was shown to block amphetamine induced release of dopamine in the NAc (Pehrson and Moghaddam, 2010). Together, restoring the expression of GLT-1 and xCT in the NAc shell as well as increasing the expression of mGluR2/3 in the NAc shell and dmPFC can result in improving the overall glutamatergic tone and prevent ethanol drinking as well as relapse to METH CPP. Further studies are needed to investigate whether the anti-reinstatement effect of CA is mediated through mGluR2/3 or other glutamate receptors or transporters.

Finally, treatment with CEF, a well-known β-lactam antibiotic and GLT-1 upregulator, has been proven to be effective in several models of drug addiction and relapse (Rawls et al., 2007, Knackstedt et al., 2010, Abulseoud et al., 2012, Trantham-Davidson et al., 2012, Alajaji et al., 2013, Sari et al., 2013a, Abulseoud et al., 2014, Rao and Sari, 2014, Das et al., 2015). However, CEF has a limited brain penetrability which requires higher parenteral doses (100-200 mg/kg) with the associated side effects. Here, we showed that CA (5 mg/kg), in a dose that is lower than CEF by 20-40 fold, blocked the reinstatement of METH CPP, decreased ethanol drinking, restored the expression of GLT-1 and xCT and increased mGluR2/3 expression. Moreover, recent studies by Rawls and colleagues have shown that CA blocked the reinforcing effects of cocaine, morphine CPP, and
increased GLT-1 expression in the NAc (Schroeder et al., 2014, Kim et al., 2016).
Together, CA could be a potential therapeutic agent for treating drug addiction since it has more advantages compared to CEF. CA can be given orally, is relatively safe, and does not have any intrinsic antibiotic activity.
In conclusion, our findings show for the first time that CA prevented the reinstatement of METH and decreased ethanol drinking. These behavioral effects of CA are most likely due to restoration of GLT-1 and xCT expression, and increased mGluR2/3 expression, in brain regions that are involved in the reinforcing effects of drugs of abuse. These findings suggest that CA could be a potential safe therapeutic agent for management of drug addiction and relapse.

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References


Chapter 4

Regulating GLT-1 and mGluR2/3 for the attenuation of methamphetamine reinstatement

Yusuf Althobaiti\textsuperscript{1}, Alqassem Y. Hakami\textsuperscript{1}, Fahad S. Alshehri\textsuperscript{1}, Alaa M. Hammad\textsuperscript{1}, Sujan C. Das\textsuperscript{2}, Youssef Sari\textsuperscript{1,2}\textsuperscript{*}

\textsuperscript{1}University of Toledo, College of Pharmacy and Pharmaceutical Sciences, Department of Pharmacology and Experimental Therapeutics, Toledo, OH.

\textsuperscript{2}University of Toledo, College of Pharmacy and Pharmaceutical Sciences, Department of Medicinal Chemistry, Toledo, OH

*Send correspondence to:
Dr. Youssef Sari
University of Toledo, College of Pharmacy and Pharmaceutical Sciences
Department of Pharmacology and Experimental Therapeutics
Health Science Campus,
3000 Arlington Avenue, HEB 282G
Toledo, OH 43614. USA
E-mail: youssef.sari@utoledo.edu
Tel: 419-383-1507 (Office)
Abstract

Methamphetamine (METH) is highly addictive psychostimulant with high rates of relapse. Relapse to several drugs of abuse has been suggested to be mediated by glutamatergic mechanisms. We previously showed the anti-reinstatement effect of clavulanic acid (CA), a non-antibiotic β-lactam compound known to upregulate glutamate transporter type 1 (GLT-1), on the reinstatement of METH conditioned place preference (CPP). Thus, the effect of blocking mGluR2/3 on the anti-reinstatement effect of CA expression of GLT-1 was tested in this study. The expression levels of GLT-1, cystine/glutamate antiporter (xCT), glutamate aspartate transporter (GLAST), and group II metabotropic glutamate receptors (mGluR2/3) in the nucleus accumbens (NAc) shell and core, as well as the dorsomedial prefrontal cortex (dmPFC) were also investigated. Moreover, the effect of CA and METH reinstatement on glutamate release in the NAc shell and core have been investigated by in vivo microdialysis. A priming injection of METH reinstated CPP and increased the glutamate release in the NAc shell, but not the core. CA treatment for seven days blocked the reinstatement of METH CPP and the release of glutamate in the NAc shell. In contrast, a single dose pretreatment with CA did not affect the reinstatement of METH CPP. The effect of treatment with CA for seven days on the reinstatement was blocked by pretreatment with the selective mGluR2/3 antagonist LY341495. GLT-1 protein expression was found to be downregulated only in the NAc shell in METH-treated rats, and this effect was restored by CA treatment. CA also increased the protein expression of mGluR2/3 and mRNA expression of mGluR3 in the NAc shell and dmPFC. These findings reveal GLT-1 and mGluR2/3 as potential therapeutic targets for the treatment of relapse to METH that is mediating glutamatergic system.
Introduction

METH addiction is an increasing health problem worldwide. According to the available data from national surveys between the years of 2002 and 2004, more than 16 million Americans over the age of 12 have used METH (Colliver, 2006). In fact, relapse to drug use is a major challenge in the treatment of drug addiction. There are several reasons for relapse to drug use. The main suggested reasons for relapse to drug use are re-exposure to a single dose of the drug, re-exposure to environmental stimuli associated with previous drug use, and stress [for review see (Shaham et al., 2003, Ghitza et al., 2006)]. The nucleus accumbens (NAc) is well-known to be involved in the rewarding and reinforcing effects of drugs of abuse (Koob and Bloom, 1988, Wise and Rompré, 1989, Bardo, 1998). Deficits in glutamate clearance in the NAc have been found to be associated with chronic drug use and drug seeking behavior (Fujio et al., 2005, Melendez et al., 2005, Knackstedt et al., 2010, Das et al., 2015). Glutamate release from prefrontal cortex (PFC) projections to the NAc have been found to mediate the reinstatement of cocaine seeking (McFarland et al., 2003, McFarland et al., 2004). Of note, blocking glutamatergic activation of mGlu1, mGlu5, AMPA receptors, and blocking glutamate release in the NAc have been shown to decrease the reinstatement of self-administration for several drugs of abuse (Cornish and Kalivas, 2000, Park et al., 2002, McFarland et al., 2004, Tessari et al., 2004, Lee et al., 2005, Bäckström and Hyytiä, 2007, Dravolina et al., 2007). Thus, glutamate plays a significant role in the reinstatement of drug seeking and, therefore, minimizing its release during reinstatement and/or increasing its clearance should be an effective strategy for managing this disorder.
Normal extracellular glutamate concentrations are maintained by a group of glutamate transporters including the glutamate transporter type 1 (GLT-1), the cystine/glutamate exchanger (xCT), and the glutamate aspartate transporter (GLAST). GLT-1 is responsible for transporting the majority of extracellular glutamate into the astrocytes in several forebrain regions that are implicated in drug dependence, while GLAST is the predominant glutamate transporter in cerebellum and retina (Rothstein et al., 1994, Rothstein et al., 1995, Lehre et al., 1997, Danbolt, 2001). Moreover, GLT-1 was frequently reported to be down-regulated in the NAc following exposure to various drugs of abuse (Knackstedt et al., 2009, Sari and Sreemantula, 2012, Fischer et al., 2013, Shen et al., 2014). Importantly, treatment with ceftriaxone (CEF), a GLT-1 up-regulator, has been reported to prevent reinstatement of METH seeking, as well as cocaine, ethanol, nicotine, and heroin seeking (Sari et al., 2009, Knackstedt et al., 2010, Abulseoud et al., 2012, Alajaji et al., 2013, Qrunfleh et al., 2013, Shen et al., 2014). Moreover, our lab recently showed that there is a significant decrease in ethanol intake in P rats following CEF treatment, associated with a significant up-regulation of GLT-1 in the NAc and PFC (Sari et al., 2011, Sari et al., 2013, Alhaddad et al., 2014).

Furthermore, xCT was found to be downregulated in the NAc following withdrawal from cocaine (Baker et al., 2003). Decreased NAc expression of xCT, which has a significant role in regulating glutamate homeostasis (Baker et al., 2002), is associated with decreased basal glutamate concentrations and subsequent lower activation of presynaptic group II metabotropic glutamate receptors (mGluR2/3). Decreased glutamatergic tone on mGluR2/3 receptors promotes reinstatement due to increased glutamate release in the
NAc during drug, cue, and/or stress induced-reinstatement of drug seeking (Dietrich et al., 2002, Moran et al., 2005, Madayag et al., 2007, LaLumiere and Kalivas, 2008). In fact, systemic treatment with an mGluR2/3 agonist (LY379268) blocked context-induced relapse to heroin, stress- and cue-induce relapse to ethanol seeking, and cue- and drug-induced reinstatement to METH and cocaine seeking (Baptista et al., 2004, Bossert et al., 2004, Zhao et al., 2006, Kufahl et al., 2013). Therefore, increasing glutamate transport would increase activation of mGluR2/3, increase inhibition of glutamate release, and reduce reinstatement of drug seeking.

Although CEF has been shown to increase the expression of GLT-1 and xCT and influence glutamate homeostasis, it still has some drawbacks as a potential anti-addiction medication. CEF cannot be administered orally, requires high doses to show efficacy, has substantial side effects, and is an antibiotic. Therefore, it is very important to find an alternative that overcomes such drawbacks. Since CEF’s up-regulatory effect of GLT-1 is thought to be mainly attributed to the β-lactam ring found in CEF and other β-lactam antibiotics (Rothstein et al., 2005). Thus, other β-lactam ring containing compounds may show similar activity. Clavulanic acid (CA) is a non-antibiotic β-lactam compound, which is orally available, relatively safe, and can cross blood brain barrier (Münch et al., 1981, Nakagawa et al., 1994). These advantages suggest that CA may be a potentially effective compound that should be tested in animal model of METH relapse. Recent studies have shown that CA blocked the reinforcing effects of cocaine and morphine, and increased GLT-1 expression in the NAc (Schroeder et al., 2014, Kim et al., 2016). We hypothesize that CA would prevent the reinstatement of METH conditioned place
preference (CPP) in rats by improving glutamatergic tone on mGluR2/3 receptors. In this study, we tested the effect of CA on METH-induced reinstatement of CPP. We also investigated the involvement of mGluR2/3 receptors in the effect of CA on METH-induced reinstatement. Since the NAc has two subregions, the shell and the core, that have been implicated in drug addiction (Groenewegen et al., 1987, Heimer et al., 1991, Hutcheson et al., 2001, Di Chiara et al., 2004), we examined the expression of GLT-1, xCT, GLAST, and mGluR2/3 receptors in these subregions, as well as the dmPFC, following METH and CA treatment. We also assessed extracellular glutamate release in the NAc shell and core following extinction of METH CPP, and during reinstatement of METH CPP, using in vivo microdialysis.

4.1. Methods and materials

4.1.1. Subjects

Male alcohol preferring (P) rats were obtained from the Indiana University Medical Center (Indianapolis, IN) Indiana Alcohol Research Center breeding colonies. Animals were single-housed in standard plastic cages with a controlled temperature (21°C) and humidity (30%) on 12:12 light-dark cycle and had free access to food and water. Animal experimental procedures were approved by the Institutional Animal Care and Use Committee of The University of Toledo in accordance with all NIH guidelines, including those adopted from the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, Commission on Life Sciences, 1996).
4.1.2. Drugs

(+) METH hydrochloride and CA were purchased from Sigma-Aldrich (St. Louis, MO). The selective mGluR2/3 antagonist LY341495 (disodium salt) was purchased from Tocris (Bristol, UK). All drugs were dissolved in sterile saline solution (0.9% NaCl).

4.1.3. Conditioned place preference paradigm

The CPP apparatus is made of Plexiglas and consists of two equal-sized conditioning chambers (40cm x 40cm x 40cm: length x width x height), and one smaller middle chamber (30cm x 40cm x 40cm: length x width x height). The two conditioning chambers are distinguished by both visual and tactile cues. The inner walls of the first chamber are white with horizontal black stripes and textured black floor (chamber 1). The inner walls of the other chamber (chamber 2) are black with vertical white stripes and smooth white floor. The middle chamber is featureless and separated from the two conditioning chambers by two guillotine doors.

4.1.3.1. The habituation phase

The experimental schedule is illustrated in Figure 1. On Days 1, 2, and 3, each rat was placed in the middle chamber with both doors closed for 3 minutes. Then, both doors were opened and each rat had free access to explore the entire apparatus for 20 minutes. On Day 3, the test was recorded by a digital camera fixed above the apparatus and time spent in each chamber was measured by a blinded observer (preference test). Rats showing strong initial preference to any chamber, more than 67% of total time, were excluded from the study as suggested previously (Fujio et al., 2005, Nakagawa et al.,...
There was no significant difference in time spent in chambers during the habituation phase, (mean time spent ± SEM in Chamber 1 = 417.70 ± 13.47 sec, Chamber 2 = 372.67 ± 11.88 sec, and middle chamber = 409.63 ± 13.85 sec; one way repeated measures ANOVA, F (2, 66) = 2.242, p = 0.1143; data not shown). Since initial time spent in Chamber 1, Chamber 2, and the middle chamber was not significantly different, an unbiased design was utilized. That is, random assignment of rats was utilized in which half of the animals received METH in Chamber 1, and the other half received it in Chamber 2.

4.1.3.2. The conditioning phase
The conditioning phase (Days 4-10) included fourteen 20-min conditioning sessions conducted over 7 consecutive days (morning and afternoon sessions). That is, in the morning session, each rat received either 2.5 mg/kg METH or saline, and was placed in the pre-assigned chamber with the door closed for 20 minutes. In the afternoon session, each rat received saline or 2.5 mg/kg METH and was placed in the opposite chamber with the door closed for 20 minutes. Control rats received saline in both morning and afternoon sessions. The order of administering METH and saline was counterbalanced. That is, on one day, METH was administered in the morning for half of the rats and saline in the afternoon. On the following day, saline was administered in the morning and METH was given in the afternoon, and the pattern was reversed for the other half of the rats. Similarly, the orientation of the chambers was alternated (i.e. chamber 1 was in the left side on one day, then it was moved to be in the right side the following day) as
described in a previous study (Cunningham et al., 2006). On Day 11, a preference test was performed again, as described previously.

4.1.3.3. The extinction phase
On Day 12-18, rats were randomly assigned to receive either CA (5 mg/kg, i.p.) or saline instead of 2.5 mg/kg METH and the procedure proceeded as described above for the conditioning phase. On Day 19, another CPP Test was performed. If time spent in the METH-paired chamber compared to the post-conditioning test was not decreased by 25% following extinction training, rats were not considered to be extinguished and were excluded from the study as described in previous work (Abulseoud et al., 2012).

4.1.3.4. Reinstatement phase
On Day 20, each rat was administered a single dose of METH (2.5 mg/kg) or saline and placed in the assigned chamber with the door closed for 20 minutes. In the afternoon, saline or METH (2.5 mg/kg, i.p.) was administered and rat was placed in the opposite chamber. On Day 21, each rat did not receive any injection and was tested for CPP.

![Figure 4-1. Experimental schedule of the CPP and in vivo microdialysis experiments.](image-url)
4.1.4. Experimental design

Experimental groups and schedule are illustrated in Table 4.1. In Experiment 1, CA was tested to investigate whether this drug has any effect on CPP by itself. Rats were administered CA (5 mg/kg) in one session and were placed in one chamber and were administered saline in the next session and placed in the opposite chamber as described before. After seven days, rats were tested for CPP as described above. In Experiment 2, the effect of CA on METH-induced reinstatement of CPP was investigated. Following conditioning with METH, rats were randomly assigned to receive either CA or saline instead of METH during extinction training. The effect of CA on reinstatement was tested by exposing the rats to a dose of METH and a CPP test was performed. In Experiment 3, the possible involvement of mGluR2/3 receptors in the effect of CA on METH-induced reinstatement of CPP was investigated. This was performed by administering the selective mGluR2/3 antagonist LY341495 (2 mg/kg, i.p.) 30 minutes before the injection of METH. In Experiment 4, we tested whether the effect of CA is mediated through direct effects on mGluR2/3 receptors. Therefore, CA was administered 30 minutes before the reinstatement dose of METH.

There were five groups of subjects in Experiment 1-4, assigned as follow: a) a Saline-Saline control group, in which rats received saline injections in sessions in the acquisition, extinction and reinstatement phases; b) a METH-Saline group, in which rats received METH/saline in the acquisition phase, saline was administered in both sessions in the extinction phase, and METH/saline in the reinstatement phase; c) a METH-CA group in which METH/saline was administered in the acquisition phase, CA/saline was
administered in the extinction phase, and METH/saline was administered in the reinstatement phase; d) a METH-CA + mGluR2/3 antagonist group in which METH/saline was administered in acquisition phase, CA/saline was administered in the extinction phase, and the mGluR2/3 antagonist was administered 30 minutes before METH in the reinstatement phase; e) a METH-Saline-CA group, in which rats received METH/saline in the acquisition phase, saline was administered in both sessions in the extinction phase, and CA was administered 30 minutes before the reinstatement dose of METH in the reinstatement phase. In Experiment 5, we measured extracellular glutamate concentrations following the extinction phase and during the reinstatement dose of METH in the NAc shell and core using *in vivo* microdialysis in freely moving rats. Similar experimental groups to those described above (a, b and c) were utilized except that following the extinction test, rats underwent surgery and insertion of microdialysis probes. On the following day, *in vivo* microdialysis was conducted and rats received a dose of METH to induce reinstatement.
Table 4.1. Experimental groups and the schedule of the CPP experiment. METH (2.5 mg/kg) was administered during conditioning and reinstatement phases, while CA (5 mg/kg) was administered during the extinction phase. The mGluR2/3 antagonist LY341495 (2 mg/kg) or CA (5 mg/kg) was administered 30 min before METH.

<table>
<thead>
<tr>
<th>Group</th>
<th>Phase</th>
<th>Days</th>
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<tr>
<td></td>
<td>Habituation</td>
<td>1-3</td>
<td>3</td>
<td>4-10</td>
<td>11</td>
<td>12-18</td>
<td>19</td>
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<tr>
<td>1- Saline-Saline</td>
<td>AM</td>
<td>Saline</td>
<td>PM</td>
<td>Saline</td>
<td>Saline</td>
<td>Saline</td>
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<tr>
<td>2- METH-Saline</td>
<td>AM</td>
<td>Saline</td>
<td>PM</td>
<td>Saline</td>
<td>Saline</td>
<td>Saline</td>
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</tr>
<tr>
<td>3- METH-CA</td>
<td>AM</td>
<td>Saline</td>
<td>PM</td>
<td>Saline</td>
<td>Saline</td>
<td>Saline</td>
<td>Saline</td>
</tr>
<tr>
<td>4- METH-CA+ mGluR2/3 antagonist</td>
<td>CPP Test</td>
<td>Alternating METH or Saline</td>
<td></td>
<td>Alternating CA or Saline</td>
<td></td>
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<tr>
<td>5- METH-Saline-CA</td>
<td>AM</td>
<td>Saline</td>
<td>PM</td>
<td>Saline</td>
<td>Saline</td>
<td>Saline</td>
<td>Saline</td>
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</table>

* Half of the rats received the reinstatement dose of METH in the morning, while the other half received it in the afternoon session.
4.1.5. *In vivo* microdialysis

In Experiment 5, we further investigated the hypothesis that the anti-reinstatement effect of CA is mediated through improving glutamatergic tone and blocking glutamate release in the NAc following METH in the reinstatement phase. We measured extracellular glutamate concentrations using *in vivo* microdialysis. Once rats met the extinction criteria, surgeries were immediately performed after anesthetizing rats with ketamine (75 mg/kg) and xylazine (5 mg/kg). Consequently, microdialysis probes were bilaterally inserted using stereotaxic coordinates targeting the NAc shell (AP +1.6, ML -1.0, DV +7.5mm) and the NAc core (AP +1.6, ML +2.0, DV +7.5mm). The microdialysis probes were constructed as previously described (Halpin et al., 2014) using silica tubes (OD of 150 µm, Polymicro Technologies), PE 20 tubes (Becton Dickinson), 26-ga stainless steel hypodermic tubing (Small Parts), hollow fiber Microdialysis Membrane (1.7 mm of active membrane, MWCO 13000, 216 µm, Spectrum Labs), 2-ton waterproof epoxy, and tygon microbore tubes. On the following day, after a 1 hr equilibration period, baseline samples were collected every 20 min for two hr, at a flow rate of 2 µl/min of the artificial cerebrospinal fluid (aCSF). Rats were then injected with either saline, in the case of the Saline-Saline group, or METH (2.5 mg/kg), in the case of the METH-Saline or METH-CA groups. Samples were then collected every 20 minutes for three hours. Only rats with correct probe placement, as verified histologically, were included in the study. *In vivo* microdialysis experiments were not performed in the same CPP apparatus to eliminate any possible effects of METH paired cues on extracellular glutamate concentration.

4.1.6. HPLC quantification of glutamate
Glutamate contents in the microdialysis samples (20 µl) were analyzed using an HPLC system (ESA, Inc). The samples were derivatized for electrochemical detection with O-phthalaldehyde (OPA) and sodium sulfite with an ESA model 540 autosampler before injecting onto a C18 column (3.0 × 50 mm, 2.5 µm particle size, Waters). The mobile phase used for elution contained 0.1 M Na2HPO4, 0.1 mM EDTA, and 7.5% Methanol (pH 3.0). After detection of glutamate by a CoulArray coulometric detector (model 5600A, ESA, Inc.), the data were recorded using CoulArray software. The glutamate concentration in each sample was determined by peak height compared with an external standard. Glutamate standards were run every day for recalibration of electrochemical potentials.

4.1.7. Brain tissue harvesting

All Rats were rapidly euthanized following the CPP test by CO2 inhalation and immediately decapitated. Brains were then removed and rapidly frozen in dry ice and stored at -80 ºC. Brain sectioning and micro-punch procedure were performed as described previously (McBride et al., 2009). The NAc shell and core as well as the dmPFC, were identified using a rat brain stereotaxic atlas (Paxinos, 2007). The isolated brain regions were utilized for western blot and quantitative reverse transcription PCR experiments.

4.1.8. Western blot

Western blot was performed on tissue samples as previously described (Sari et al., 2009). Briefly, brain tissue was lysed in lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 1 mM
EDTA, 0.5% NP-40, 1% Triton, 0.1% SDS) containing a protease inhibitor cocktail. A Bio-Rad protein assay method was used to determine total protein content in the tissue extracts (Bio-Rad, Hercules, CA, USA). Extracted proteins were loaded onto 10-20% tris-glycine gel. After separation, proteins were transferred from the gel onto a PVDF membrane. Consequently, the membrane was blocked using 3% milk in Tris-buffered saline Tween 20 for 30 minutes. Guinea pig anti-GLT1 (Millipore Bioscience Research Reagents; 1:5000 dilution), rabbit anti-xCT antibody (Abcam; 1:1000 dilution), rabbit anti-mGluR2/3 antibody (Upstate Biotechnology; 1:1000 dilution), rabbit anti-GLAST (Abcam; 1:5000 dilution), or mouse anti β-tubulin antibody (Covance; 1:5000 dilution) was then added to the blocking buffer, and the membrane was incubated overnight at 4 °C. The membrane was then washed and incubated with horseradish peroxidase-labeled (HRP) anti-guinea pig, anti-rabbit, or anti-mouse secondary antibody (1:5000). A chemiluminescent kit (SuperSignal West Pico) was used to incubate the membrane for protein detection. Subsequently, the membrane was exposed to Kodak BioMax MR films (Thermo Fisher Scientific). Films were then developed using an SRX-101A machine. Blots for each detected protein were digitized and quantified using an MCID system. Data was calculated as the following ratios: GLT-1/β-tubulin, xCT/β-tubulin, GLAST/β-tubulin, and mGluR2/3/β-tubulin.

4.1.9. RNA extraction and quantitative reverse transcription PCR (qRT-PCR)

Total RNAs from dmPFC and NAc shell were isolated using TRizol reagent according to the manufacturer’s instructions (Invitrogen). RNA was then utilized for cDNA synthesis
by reverse transcription using a Verso cDNA synthesis kit (Thermo Scientific) according to the manufacturer’s protocol. Samples were tested in triplicate in a total volume of 20 µl of reaction mixture using SYBR Green as fluorescent dye (BIO-RAD) on an RT-PCR system using an iCycler (Bio-Rad laboratories, München, Germany) (3 x 39 cycles of 95°C for 15 sec, 55°C for 30 sec, and 72°C for 30 sec). The primers for the genes of interest are shown in Table 4.2. The selection of mGluR2 and mGluR3 forward and reverse primers were based on a previous report (Chen et al., 2002), while the GAPDH forward and reverse primers were from previous work (Tawfik et al., 2006). The obtained threshold and fluorescence values (CT) for each sample were used to compare the relative amount of target mRNA in treated groups to control group using the 2^{-\Delta\Delta CT} method (Livak and Schmittgen, 2001). The mean CT value for control gene (GAPDH) was subtracted from the mean CT value of the gene of interest to get ΔCT. The ΔCT values for the control group was then averaged and subtracted from the ΔCT for the treated groups to obtain the ΔΔCT. The relative fold change from control was then expressed by calculation of 2^{-\Delta\Delta CT} for each sample.

Table 4.2. List of forward and reverse primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<tr>
<td>mGluR 2</td>
<td>5’-GTGGTGACATTGCGCTGAA-3’</td>
<td>5’-GGATGAGGAGGACATTGTA-3’</td>
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<tr>
<td>mGluR 3</td>
<td>5’-CTGGTGATCCCTAGCATGTG-3’</td>
<td>5’-GAGGAATGCACACCCAGATGA-3’</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5’-CCCCCAATGTATCCGTTG-3’</td>
<td>5’-TAGCCAGGATGCCCTTTAGT-3’</td>
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</table>
4.1.10. Statistical analysis

Time spent in conditioning chambers and brain extracellular glutamate concentrations were analyzed using two-way repeated measure ANOVA, (Time x Chamber) and (Time x Treatment), respectively. Newman-Keuls multiple comparisons were used when significant interactions or significant main effects were revealed. One-way ANOVA was used to analyze immunoblot and qRT-PCR data followed by Newman-Keuls post hoc tests. GraphPad Prism was used to statistically analyze all data in this study, using a 0.05 level of significance.

4.2. Results

4.2.1. Effect of CA on METH-induced reinstatement of CPP

In experiment 1, CA did not cause any preference or aversion in the CA-paired chamber (mean time spent ± SEM in post-conditioning = 405.39 ± 25.30 sec as compared to mean time spent in habituation = 413.08 ± 40.11 sec; Paired t-test, t(5) = 0.2610, p = 0.8045; data not shown). In Experiment 2, animals were conditioned with METH for seven days followed by extinction training in which rats received saline in the METH-Saline group or CA in the METH-CA group instead of METH. Reinstatement of METH CPP was then tested using a priming injection of METH following extinction training: A) METH-Saline treated group: two-way repeated measures ANOVA revealed no significant effect
of time $[F(2, 18) = 3.505, p = 0.0518]$, a significant effect of chamber $[F(1, 9) = 26.95, p = 0.0006]$, and a significant interaction between time and chamber $[F(2, 18) = 26.98, p < 0.0001]$. Newman-Keuls multiple comparisons showed a significant increase in time spent following conditioning training in the METH-paired chamber as compared to saline-paired chamber ($p < 0.0001$; Fig. 4-2A). A significant decrease in time spent in the METH-paired chamber was revealed following extinction training as compared to post-conditioning ($p < 0.0001$; Fig. 4-2A). METH before the reinstatement test significantly increased time spent in the METH-paired chamber as compared to the saline-paired chamber ($p < 0.01$), and as compared to time spent in the METH-paired chamber in the extinction ($p < 0.01$).

B) METH-CA treated group: two-way repeated measures ANOVA revealed a significant effect of time $[F(2, 16) = 5.397, p = 0.0162]$, no significant effect of chamber $[F(1, 8) = 1.685, p = 0.2305]$, and a significant interaction between time and chamber $[F(2, 16) = 8.83, p = 0.0026]$. Newman-Keuls multiple comparisons test showed a significant increase in time spent following conditioning training in METH-paired chamber as compared to the saline-paired chamber ($p < 0.0001$; Fig. 4-2B). A significant decrease in time spent in the METH-paired chamber was revealed following extinction training as compared to post-conditioning ($p < 0.05$; Fig. 4-2B). METH administration prior to the reinstatement test did not change the time spent in the METH-paired chamber as compared to extinction or the saline-paired chamber ($p > 0.05$).

In Experiment 3, we tested the effect of blocking mGluR2/3 receptors on the anti-reinstatement effect of CA, in order to investigate the possible involvement of
glutamatergic neurotransmission in this effect. Two-way repeated measures ANOVA revealed no significant effect of time \([F (2, 16) = 3.265, p = 0.0647]\), a significant effect of chamber \([F (1, 8) = 12.7, p = 0.0074]\), and a significant interaction between time and chamber \([F (2, 16) = 14.61, p = 0.0002]\). Newman-Keuls multiple comparisons showed a significant increase in time spent following conditioning training in the METH-paired chamber as compared to the saline-paired chamber \((p < 0.001; \text{Fig. } 4-2C)\). A significant decrease in time spent in the METH-paired chamber was revealed following extinction training as compared to post-conditioning \((p < 0.001; \text{Fig. } 4-2C)\). The anti-reinstatement effect of CA was prevented by administering the selective mGluR2/3 receptors antagonist LY341495 (2 mg/kg, i.p.) 30 minutes prior to the injection of METH for the reinstatement test. METH significantly increased time spent in the METH-paired chamber as compared to the saline-paired chamber \((p < 0.01)\), and as compared to time spent in the METH-paired chamber in the extinction test \((p < 0.05)\).

In Experiment 4, animals were conditioned with METH followed by extinction training by administering saline instead of METH. CA was administered 30 minutes prior to METH in the reinstatement test to investigate any possible direct effect of CA on glutamate transporters or receptors. Two-way repeated measures ANOVA revealed no significant effect of time \([F (2, 10) = 0.7022, p = 0.5183]\), a significant effect of chamber \([F (1, 5) = 9.331, p = 0.0283]\), and a significant interaction between time and chamber \([F (2, 10) = 11.59, p = 0.0025]\). Newman-Keuls multiple comparisons showed a significant increase in time spent following conditioning training in the METH-paired chamber as compared to the saline-paired chamber \((p < 0.01; \text{Fig. } 4-2D)\). A significant decrease in time spent in the METH-paired chamber was revealed following extinction training as compared to post-
conditioning (p< 0.05; Fig. 4-2C). CA pretreatment did not affect the reinstatement effect produced by METH. METH significantly increased time spent in the METH-paired chamber as compared to the saline-paired chamber (p<0.01), and as compared to time spent in METH-paired chamber during the extinction test (p<0.05).

A

B

C

D
Figure 4-2. Time spent in each conditioning chamber during post-conditioning, extinction, and reinstatement phases in METH-Saline (A), METH-CA (B), METH-CA+LY341495 (C), and METH-Saline+CA treated (D) groups. Time spent in the METH-paired chamber was significantly increased in the reinstatement test as compared to the extinction test in METH-Saline treated rats. CA blocked reinstatement by METH administration. In contrast, the mGluR2/3 antagonist prevented the effect of CA on reinstatement, restoring the effects of METH. Pretreatment with CA did not block reinstatement by METH. n = 6-10 for each group. Values shown as means ± S.E.M. *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001. (#p<0.05, ##p<0.01, and ####p<0.0001 as compared to post-conditioning, while &p<0.05, &&p<0.01 as compared to the extinction test).

4.2.2. Effects of METH and CA treatment on the protein expression of glutamate transporters and mGluR2/3 in dmPFC and NAc shell and core

4.2.2.1. GLT-1 expression

We investigated the effect of METH and CA on GLT-1 expression levels in dmPFC and NAc shell and core following the reinstatement test. One-way ANOVA analyses revealed
a significant main effect between Saline-Saline, METH-Saline, and METH-CA groups in the NAc shell \[F (2, 15) = 9.998, P = 0.0017; \text{Fig. 4-3A}\], but not in the NAc core \[F (2, 18) = 1.404, P < 0.2712; \text{Fig. 4-4A}\] or dmPFC \[F (2, 18) = 1.865, P = 0.1836; \text{Fig. 4-5A}\]. Newman-Keuls multiple comparisons showed a significant increase in the expression of GLT-1 in METH-CA treated rats compared to the METH-Saline treated rats in the NAc shell \(p<0.01; \text{Fig. 4-3A}\). By contrast, statistical analyses showed a significant downregulation in GLT-1 expression in the METH-Saline group compared to the control group in the NAc shell \(p<0.01; \text{Fig. 4-3A}\). No significant difference in GLT-1 expression level was revealed between the METH-CA group and the control group.

4.2.2.2. xCT expression

We further tested the effect of METH and CA on another glutamate transporter, xCT, which plays a key role in maintaining glutamate homeostasis. We investigated xCT expression levels in NAc shell and core, as well as dmPFC, following the reinstatement test. One-way ANOVA revealed no significant main effect between Saline-Saline, METH-Saline, and METH-CA groups in the NAc shell \[F (2, 15) = 2.972, P = 0.0818; \text{Fig. 4-3B}\], NAc core \[F (2, 18) = 0.5099, P < 0.6090; \text{Fig. 4-4B}\], or dmPFC \[F (2, 18) = 0.3327, P = 0.7213; \text{Fig. 4-5B}\].

4.2.2.3. GLAST expression

We further tested the effect of METH and CA on GLAST expression levels, another glutamate transporter, in NAc shell and core, as well as the dmPFC, following the reinstatement test. One-way ANOVA revealed no significant main effect between Saline-
Saline, METH-Saline, and METH-CA groups in the NAc shell \( [F (2, 18) = 1.713, P = 0.2084; \text{Fig. 4-3C}] \), NAc core \( [F (2, 15) = 0.317, P = 0.7331; \text{Fig. 4-4C}] \), or dmPFC \( [F (2, 18) = 1.122, P = 0.3474; \text{Fig. 4-5C}] \).

4.2.2.4. mGluR2/3 expression

We investigated the effect of METH and CA on mGluR2/3 receptor expression levels in dmPFC, NAc shell, and NAc core following the reinstatement test. One-way ANOVA revealed a significant main effect between Saline-Saline, METH-Saline, and METH-CA groups in the NAc shell \( [F (2, 18) = 13.63, P = 0.0002; \text{Fig. 4-3D}] \), and dmPFC \( [F (2, 15) = 5.281, P = 0.0184; \text{Fig. 4-5D}] \), but not in the NAc core \( [F (2, 18) = 0.09878, P < 0.9064; \text{Fig. 4-4D}] \). Newman-Keuls multiple comparisons showed a significant increase in the expression level of mGluR2/3 receptors in METH-CA treated rats compared to the METH-Saline treated rats in the NAc shell \( (p < 0.001; \text{Fig. 4-3D}) \) and the dmPFC \( (p < 0.05; \text{Fig. 4-5D}) \). Alternatively, statistical analyses showed a significant increase in mGluR2/3 receptors expression levels in the METH-CA group compared to the control group in the NAc shell \( (p < 0.001; \text{Fig. 4-3D}) \) and dmPFC \( (p < 0.05; \text{Fig. 4-5D}) \).
Figure 4-3. Effects of METH and CA on the expression of GLT-1/β-tubulin (A), xCT/β-tubulin (B), GLAST/β-tubulin (C), and mGluR2/3/β-tubulin (D) in the NAc shell. Quantitative analysis revealed a significant increase in the ratio of GLT-1/β-tubulin in METH-CA treated rats compared to METH-Saline treated rats. Significant downregulation of GLT-1 expression was revealed in the METH-Saline treated groups compared to the Saline-Saline control group. A significant increase in the mGluR2/3/β-tubulin ratio was revealed in METH-CA as compared to METH-Saline and Saline-Saline groups. No significant difference in xCT or GLAST expression were revealed. ** p<0.01 and *** p<0.001. Values shown as means ± S.E.M. n = 6-7 for each group.
Figure 4-4. Effects of METH and CA on the expression of GLT-1/β-tubulin (A), xCT/β-tubulin (B), GLAST/β-tubulin (C), and mGluR2/3/β-tubulin (D) in the NAc core. Quantitative analysis did not reveal any significant change in the expression of GLT-1, xCT, GLAST, or mGluR2/3 in the NAc core. Values shown as means ± S.E.M. n = 6-7 for each group.
Figure 4-5. Effects of METH and CA on the expression of GLT-1/β-tubulin (A), xCT/β-tubulin (B), GLAST/β-tubulin (C), and mGluR2/3/β-tubulin (D) in dmPFC. Quantitative analysis revealed a significant increase in the mGluR2/3/β-tubulin ratio in METH-CA compared to METH-Saline and Saline-Saline groups. No significant differences in GLT-1, xCT or GLAST expression were revealed. * p<0.05. Values shown as means ± S.E.M. n = 6-7 for each group.

4.2.3. Effects of METH and CA treatment on the mRNA expression of mGluR2 and mGluR3 in dmPFC and NAc shell

Since CA treatment increased the protein expression of mGluR2/3 in the dmPFC and NAc shell, we further investigated the effect of METH and CA treatment on mGluR2 and mGluR3 mRNA expression level in these brain regions.
4.2.3.1. \textit{mGluR2} mRNA expression

One-way ANOVA revealed a non-significant main effect among Saline-Saline, METH-Saline, and METH-CA groups in the NAc shell \( [F (2, 13) = 0.01798, P = 0.9822; \text{Fig. 4-6A}] \) and dmPFC \( [F (2, 12) = 2.71, P = 0.1069; \text{Fig. 4-6C}] \).

4.2.3.2. \textit{mGluR3} mRNA expression

One-way ANOVA revealed a significant main effect between Saline-Saline, METH-Saline, and METH-CA groups in the NAc shell \( [F (2, 13) = 6.025, P = 0.0141; \text{Fig. 4-6B}] \) and dmPFC \( [F (2, 12) = 5.511, P = 0.0201; \text{Fig. 4-6D}] \). Newman-Keuls multiple comparisons showed a significant increase in the mRNA expression level of \textit{mGluR3} in METH-CA treated rats compared to METH-Saline treated rats in the NAc shell (\( p<0.05; \text{Fig. 4-6B} \)) and dmPFC (\( p<0.05; \text{Fig. 4-6D} \)). Additionally, statistical analyses showed a significant increase in \textit{mGluR3} mRNA expression level in the METH-CA group compared to the control group in the NAc shell (\( p<0.05; \text{Fig. 4-6B} \)) and dmPFC (\( p<0.05; \text{Fig. 4-6D} \)).
Effects of METH and CA on the mRNA expression of mGluR2 (A,C) and mGluR3 (B,D) in the NAc shell and dmPFC, respectively. Quantitative analysis revealed a significant increase in the mRNA expression of mGluR3 in METH-CA compared to METH-Saline and Saline-Saline groups in both the NAc shell and dmPFC. No significant differences in the mRNA expression of mGluR2 were revealed in either the NAc shell or dmPFC. * p<0.05. Values shown as means ± S.E.M. n = 5-6 for each group.

4.2.4. Extracellular glutamate concentration

In Experiment 5, we investigated the effect of METH reinstatement on glutamate release in the NAc shell and core following extinction training in Saline-Saline, METH-Saline, and METH-CA treated groups.

4.2.4.1. The NAc shell
Two-way repeated measure ANOVA revealed a significant effect of time [$F(15, 165) = 2.104$, $p = 0.0120$], a significant effect of treatment [$F(2, 11) = 18.28$, $p = 0.0003$], and a significant interaction between time and treatment [$F(30, 165) = 4.24$, $p < 0.0001$].

Newman-Keuls multiple comparisons showed a significant increase in extracellular glutamate concentration following METH in the METH-Saline group as compared to the Saline-Saline and METH-CA groups at all time points (Fig. 4-7C). No significant change in extracellular glutamate concentration was revealed following METH in the METH-CA group as compared to the Saline-Saline group at all time points (Fig. 4-7C).

4.2.4.2. The NAc core

Two-way repeated measure ANOVA revealed a significant effect of time [$F(15, 210) = 2.092$, $p = 0.0115$], no significant effect of treatment [$F(2, 14) = 0.09012$, $p = 0.9144$], and no significant interaction between time and treatment [$F(30, 210) = 1.021$, $p = 0.4424$]. Newman-Keuls multiple comparisons did not show any significant changes in extracellular glutamate concentration following METH between treatment groups at any time points (Fig. 4-7D).
Figure 4-7. Effects of METH on extracellular glutamate release during the reinstatement phase. (A,B) locations of microdialysis probes in the NAc shell and core, respectively. Numbers indicate anteroposterior distance from bregma according to the atlas of Paxinos and Watson (1998). (C,D) extracellular glutamate concentrations in the NAc shell and core, respectively. Percentage of baseline was obtained from the average of six baseline samples (0-120 min) collected prior to administering METH in the METH-Saline and METH-CA groups, or saline, in the Saline-Saline group, as indicated by the arrow. Ten samples were then collected following the injection of METH or saline (120-320 min). METH significantly increased glutamate efflux in the NAc shell, which was blocked by CA treatment. No significant change in glutamate efflux was found following METH i.p. injection in the NAc core. n = 4-7 for each group. Values shown as means ± S.E.M. **p<0.01, ***p<0.001 and ****p<0.0001 as compared to the Saline-Saline control group. (#p<0.05, ##p<0.01, ###p<0.001, and ####p<0.0001 as compared to the METH-CA treated group.

4.3. Discussion
In this study, we showed that CA treatment for seven days during the extinction phase inhibited the reinstatement of METH CPP in an animal model of METH relapse. We have also shown for the first time that this anti-reinstatement effect of CA is mediated through the activation of mGluR2/3 receptors. However, it is unlikely that CA caused a direct activation of glutamate transporters or mGluR2/3 receptors, since we found that CA administered 30 minutes before METH injection did not block the ability of METH to reinstate METH CPP. METH caused a downregulation of GLT-1 expression in the NAc shell only. This glutamate transporter is responsible for transporting the majority of extracellular glutamate into astrocyte (Rothstein et al., 1995, Danbolt, 2001). Astrocytic glutamate can be exchanged for extracellular cystine, allowing glutamate to be released in the extra-synaptic space. Non-synaptic glutamate can then activate mGluR2/3 receptors, resulting in feedback inhibition of presynaptic release of glutamate (Baker et al., 2002). The presynaptic release of glutamate during reinstatement to different drugs of abuse has been found to be critical in mediating relapse to drug seeking (McFarland et al., 2003, Moran et al., 2005, LaLumiere and Kalivas, 2008). In this study, CA restored GLT-1 expression in the NAc shell and increased the expression of mGluR2/3 receptors in the NAc shell and dmPFC. Moreover, CA did not affect CPP by itself when it was administered alone (without exposure to METH) for the same period of post-treatment. In fact, the anti-reinstatement effect of CA was completely blocked by the selective mGluR2/3 receptor antagonist LY341495. Importantly, the METH increased the release of glutamate in the NAc shell, but not the NAc core, during the reinstatement phase, which was blocked by CA treatment. Therefore, the CA effect on the reinstatement of METH CPP is most likely to be mediated by increasing glutamatergic tone on mGluR2/3 receptors.
receptors. This is in line with previous reports showing that systemic pretreatment with the potent mGluR2/3 agonist LY379268 blocked reinstatement of drug seeking to heroin, cocaine, and ethanol (Baptista et al., 2004, Bossert et al., 2005, Kufahl et al., 2011). Furthermore, several studies have shown that glutamate release in the NAc originating from PFC afferents is elevated in the reinstatement of cocaine seeking in rats (McFarland et al., 2003, Madayag et al., 2007). Thus, blocking glutamate release in the NAc during the reinstatement of drug seeking should be an effective strategy in treating this drug relapse.

These findings suggest the important role of the NAc shell rather than the NAc core in METH reinstatement. This is in line with previous reports that investigated the differential role of the NAc shell and core in drug addiction. For example, cocaine and other dopaminergic compounds have been reported to be self-administered in the NAc shell rather than the core (Ikemoto et al., 1997, Ikemoto, 2003). Moreover, other in vivo microdialysis studies showed that the elevation of dopamine concentrations are more pronounced in the shell than the core compartment of the NAc following systemic administration of morphine, cocaine or amphetamine in rats (Pontieri et al., 1995). Moreover, a recent report showed that blocking the dopaminergic D1 receptor in the NAc shell but not the NAc core decreases ethanol seeking behavior in P rats (Hauser et al., 2015). Similarly, ethanol has been shown to be self-infused in the NAc shell, but not the core, in both Wistar and P rats, with increased sensitivity of the NAc shell to the reinforcing effect of ethanol in P rats (Engleman et al., 2009). Moreover, the reinforcing effect of ethanol has been shown to be mediated through the activation of dopaminergic
receptors in the NAc shell, but not the NAc core (Ding et al., 2015). Furthermore, GLT-1 overexpression in the NAc shell by viral mediated gene transfer has been reported to attenuate the reinforcing effect of METH and morphine in the CPP model in rats (Fujio et al., 2005). In fact, 6-hydroxydopamine lesions of the NAc shell, but not the NAc core blocked amphetamine CPP (Sellings and Clarke, 2003). Interestingly, activation of mGluR2/3 receptors in the NAc shell but not the core blocked context induced reinstatement of heroin (Bossert et al., 2006).

However, the NAc core has been reported to play a significant role in the rewarding and reinforcing effects of different drugs of abuse (Hutcheson et al., 2001, McFarland et al., 2003, LaLumiere and Kalivas, 2008, Fischer et al., 2013). Interestingly, cue-induced relapse has been shown to be mediated by the NAc core while context induced relapse is mediated by the NAc shell in an ethanol relapse animal model (Chaudhri et al., 2010). Of note, relapse to several drugs of abuse has been suggested to be mediated by re-exposure to either drug exposure, cues associated with drug use, or stressors [for review see ref. (Shaham et al., 2003, Ghitza et al., 2006)]. We have investigated the effect of drug-induced reinstatement in CPP model. Further studies are warranted to investigate the differential role of the NAc shell and core in stress- or cue-induced reinstatement since previous studies suggest a distinct pathway to be involved in each trigger of relapse[for review see ref. (Shaham et al., 2003, Bossert et al., 2005)].

Prevention of the drug relapse in addiction to METH, and other drugs, is the primary goal in the management of addictive disorders (O'Brien, 1997, Brecht et al., 2000, Newton et
al., 2009). Unfortunately, there is no approved pharmacotherapy for treating METH dependence, and most strategies for its management have failed in clinical studies (Piasecki et al., 2002, Fechtner et al., 2006, Heinzerling et al., 2006, Shoptaw et al., 2006, Stoops, 2006). However, clinical trials investigating the effect of modafinil, a wake promoting drug, on METH dependence have produced promising results (McGregor et al., 2008, McElhiney et al., 2009). In preclinical studies, modafinil has shown efficacy in blocking reinstatement of morphine CPP in rats (Tahsili-Fahadan et al., 2010).

Interestingly, the anti-reinstatement effect of modafinil on morphine CPP was blocked by a specific mGluR2/3 antagonist, suggesting the involvement of the glutamatergic system in the activity of modafinil (Tahsili-Fahadan et al., 2010). Of note, modafinil treatment also blocked the reinstatement of METH self-administration in rats (Reichel and See, 2010). Moreover, the drug- and cue-primed reinstatement of METH seeking has been shown to be associated with glutamate release in the NAc and dmPFC in rats (Parsegian and See, 2014). Interestingly, this later study found decreases in basal glutamate concentrations following METH self-administration in the NAc and dmPFC, which might support the hypothesis in decreases of glutamatergic tone on mGluR2/3, resulting in augmented release of glutamate during primed reinstatement of drug seeking (Dietrich et al., 2002, Moran et al., 2005, Madayag et al., 2007, LaLumiere and Kalivas, 2008, Parsegian and See, 2014). Furthermore, improving the clearance of extracellular glutamate by upregulating the expression of GLT-1 using ceftriaxone, has been shown to block METH-induced reinstatement of CPP (Abulseoud et al., 2012). Together, both ceftriaxone and modafinil have shown efficacy in preventing the reinstatement of METH
responses in animal models that are most likely to be mediated through glutamatergic systems, however, both drugs have limitations.

Ceftriaxone has antibiotic activity, is unavailable for oral delivery, and has limited brain penetrability, which requires higher doses with associated toxicity and side effects. Alternatively, modafinil is a controlled substance due to its abuse potential. Importantly, besides its multiple side effects on the cardiovascular system, modafinil has been shown to have a higher abuse potential and drug reinforcement property in the case of a pre-existing history of stimulant abuse or exposure, in preclinical and clinical studies (Gold and Balster, 1996, Jasinski and Kovacevic-Ristanovic, 2000, Deroche-Gamonet et al., 2002, Rush et al., 2002, Stoops et al., 2005, Bernardi et al., 2009). Therefore, CA could be a better therapeutic option as compared to modafinil in preventing relapse to METH. Moreover, CA, as compared to ceftriaxone, can be given orally, is devoid of antimicrobial activity, has a good brain penetrability, and has been in clinical use for more than three decades with a very good safety and tolerability profile.

In conclusion, a METH priming injection induced reinstatement of METH CPP in extinguished rats and caused increases in glutamate release in the NAc shell but not the NAc core. CA treatment for seven days during extinction blocked the reinstatement of METH CPP and the release of glutamate in the NAc shell. In contrast, a single dose pretreatment with CA did not affect the reinstatement of METH CPP. Selective mGluR2/3 receptor antagonist pretreatment, when administered 30 min before the priming injection of METH, completely blocked the effect of seven days CA treatment on reinstatement.
Moreover, GLT-1 expression was only downregulated in the NAc shell in METH treated rats, which was restored by CA treatment. CA also increased the protein expression of mGluR2/3 and mRNA expression of \textit{mGluR3} in the NAc shell and dmPFC. Together, the effect of CA on the reinstatement of METH CPP is most likely to be mediated by improving glutamatergic tone on mGluR2/3 receptors. These findings reveal a new therapeutic avenue for managing METH relapse that is mediated by glutamatergic mechanism.

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The authors declare no conflict of interest.

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Chapter 5

Summary

The effects of alcohol and METH co-abuse on glutamate homeostasis in the NAc and PFC were investigated in this project. We also tested the effect of CEF, a β-lactam antibiotic known to upregulate GLT-1 expression, posttreatment on glutamate transporters and METH-induced hyperthermia. Our findings provide evidence of the important role of GLT-1 using high doses of METH, well known to cause a hyperglutamatergic state and hyperthermia. METH administered alone decreased GLT-1 expression in the NAc and PFC and increased body temperature, but did not reduce either xCT or GLAST expression in ethanol and water-pretreated rats. Importantly, we found for the first time additive effects of ethanol and METH on GLT-1 downregulation in the NAc as compared to either drug administered alone. In addition, we showed for the first time that CEF was effective in restoring GLT-1 expression and reversing hyperthermia in the ethanol and METH co-abuse rat model. These findings suggest that β-lactams might be potential treatments against METH and ethanol/METH co-abuse.

Since the NAc has two main subregions, the shell and core, we further investigated the effect of METH and alcohol co-abuse on glutamate transporters in these subregions as
well as the dmPFC. We have also investigated another non-antibiotic β-lactam compound on METH reinstatement, ethanol drinking, the expression of glial glutamate transporters, and mGluR2/3 in the NAc shell and core, as well as the dmPFC. A priming injection of METH reinstated preference in a METH-paired chamber, which was blocked by CA treatment. Additionally, chronic exposure to ethanol decreased the expression of GLT-1 and xCT in the NAc shell, but not the NAc core or dmPFC. METH and ethanol downregulated GLT-1 more in the NAc shell. CA decreased ethanol intake and restored the expression of GLT-1 and xCT in the NAc shell. Moreover, CA increased the expression of mGluR2/3 in the NAc shell and dmPFC. These findings suggest that CA blocked the reinstatement of METH CPP by modulating the expression of glial glutamate transporters and receptors.

To further test this hypothesis, we have tested the effects of METH alone and seven days CA posttreatment, administered during the extinction phase, on the reinstatement of METH CPP and the expression of the proteins involved in glutamate homeostasis in the NAc shell and core, as well as the dmPFC. CA treatment for seven days blocked the reinstatement of METH CPP. Moreover, GLT-1 was downregulated in the NAc shell in METH treated rats, which was restored by CA treatment. CA also increased the protein expression of mGluR2/3 and mRNA expression of mGluR3 in the NAc shell and dmPFC. In addition, pretreatment with the selective mGluR2/3 antagonist LY341495 blocked the effect of seven days CA treatment on the reinstatement of METH. However, it is unlikely that CA caused a direct activation of glutamate transporters or mGluR2/3, since we found that CA, when administered 30 minutes before METH injection, did not block the
reinstatement to METH CPP. Therefore, the effect of CA on the reinstatement of METH CPP is most likely mediated by increasing the expression of proteins involved in glutamate homeostasis and the altering glutamatergic tone on mGluR2/3 receptors. Increasing glutamatergic tone on mGluR2/3 receptors results in reduced release of glutamate in the NAc during drug primed reinstatement. Finally, to test this hypothesis, we assessed extracellular glutamate release following reinstatement of METH seeking in Saline-Saline, METH-Saline, and METH-CA treated rats. METH increased the release of glutamate in the NAc shell, but not the NAc core of METH-Saline treated rats, which was blocked by CA treatment during the extinction phase. Therefore, the effect of CA on the reinstatement of METH CPP is most likely mediated by increasing glutamatergic tone on mGluR2/3 receptors and blocking the release of glutamate during METH-primed reinstatement. Together, these findings reveal a new therapeutic approach for the potential management of METH relapse via a glutamatergic mechanism.
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Appendix A

List of Articles Published based on this Dissertation

