A Dissertation

entitled

Glutamate Transporter 1 and Cystine-glutamate Anti-porter: Therapeutic Targets for Alcohol Dependence

by

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Alcohol dependence leads to long-lasting changes in corticostriatal brain circuits, which make addicts unable to stop alcohol-taking and -seeking. Alteration in glutamate homeostasis within nucleus accumbens is one of the neuroadaptive changes caused by alcohol exposure, which promotes further alcohol dependence. Little is known about the involvement of glutamate transporters in altered glutamate homeostasis in alcohol dependence. This research project aimed to investigate the role of glutamate transporters using alcohol preferring rats in three different models, which mimic different stages of alcohol dependence. In chronic alcohol drinking paradigm, five weeks of voluntary alcohol drinking caused two-fold increase in extracellular glutamate concentration in nucleus accumbens with downregulation of both GLT-1 and xCT expression. Ceftriaxone, a β-lactam antibiotic, attenuated chronic alcohol consumption with normalization of extracellular glutamate concentration and the expression of both GLT-1 and xCT. Reverse in vivo microdialysis of dihydrokainic acid (potent GLT-1 blocker) blocked the ceftriaxone-induced normalization of extracellular glutamate concentration, which suggests that ceftriaxone normalizes extracellular glutamate concentration in GLT-1 and xCT expression.
mediated pathways. In contrast, relapse-like alcohol drinking did not alter ex-vivo glutamate uptake and relative gene expression of GLT-1 and xCT in nucleus accumbens. However, relapse-like alcohol drinking increased relative gene expression of mGlu5 in nucleus accumbens. Ceftriaxone prevented relapse-like alcohol drinking in similar way of upregulation of both GLT-1 and xCT. Suppression of expression of GLT-1 or xCT prevented the action of ceftriaxone in attenuation of alcohol drinking suggesting that upregulation of both GLT-1 and xCT are the key in mechanism of this drug. Surprisingly, relapse-like alcohol drinking after binge-withdrawal downregulated expression of GLT-1 without affecting xCT. Taken together, it is evident that different stages of alcohol dependence alter glutamate homeostasis within nucleus accumbens, which may involve different pharmacological mechanisms. Affecting GLT-1 and xCT were one common factor contributing to different stages of alcohol dependence. Thus, GLT-1 and xCT might be potential therapeutic targets for alcohol dependence.
To Lord Krishna, the supreme personality of Godhead, I dedicate this dissertation.
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List of Abbreviations

aCSF..........................Artificial Cerebrospinal Fluid
ADE ..........................Alcohol Deprivation Effect
ADP ..........................Adenosine Diphosphate
AMPA .......................α-Amino-3-hydroxy-5-Methyl-4-isoxazolepropionic Acid
Amy ..........................Amygdala
ANOVA ......................Analysis of Variance
AP ............................Anterior Posterior
AUD ..........................Alcohol Use Disorder
AWS ..........................Alcohol Withdrawal Syndrome

BAC ..........................Blood Alcohol Concentration
BBB ..........................Blood Brain Barrier
BLA ..........................Basolateral Amygdala

cAMP ........................Cyclic Adenosine Monophosphate
CEF ..........................Ceftriaxone
CNS ..........................Central Nervous System

DA ..........................Dopamine
DHK ..........................Dihydro Kainate
DPM ..........................Disintegrations Per Minute
DV ..........................Dorsal Ventral

EAAT ........................Excitatory Amino Acid Transporter
EDTA ........................Ethylene Diamine Tetra Acetic acid
EtOH ........................Ethanol
EW ..........................Ethanol Withdrawal

GABA ........................Gamma Amino Butyric Acid
GLAST ........................Glutamate Aspartate Exchanger
GLT-1 ........................Glutamate Transporter 1
GPCR ........................G Protein-Coupled Receptor
GS ..........................Glutamine Synthetase

HEPES ........................Hydroxy Ethyl Piperazine Ethane Sulfonate
Hipp ..........................Hippocampus
HPLC ..........................High Performance Liquid Chromatography
HRP ..........................Horse Reddish Peroxidase

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

KRP............................Kreb’s-Ringer’s Phosphate buffer

mGluR..........................Metabotropic Glutamate Receptors
ML...............................Medio Lateral
mPFC..............................Medial Prefrontal Cortex
MSN...............................Medium Spiny Neurons

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

SDS-PAGE .................Sodium Dodecyl Sulfate-Poly Acrylamide Gel Electrophoresis

VTA............................Ventral Tegmental Area

WD...............................Withdrawal

xCT...............................Cystine-glutamate Anti-porter
List of Symbols

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

C .......................Centigrade

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

M .......................Molar
mM .....................Milli Molar
µM .....................Micro Molar
N .......................Normal
Pmol ....................Pico Mole

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

kDa .....................Kilo Dalton
Chapter 1

Glutamate Homeostasis within Cortico-striatal Brain Circuits in Alcohol Dependence

**Running title:** Glutamatergic neurotransmission in alcoholism

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Abstract

Alcohol dependence is a very complex neuropsychiatric disorder. Alcohol affects multiple body organs, including brain, making it a leading cause of death throughout the world. In brain, alcohol affects multiple neurotransmission pathways which make addicts unable to stop alcohol drinking even though unfavorable outcomes appear. As alcohol abuses progress to dependence, glutamatergic neurotransmission within cortico-striatal circuits appears to play critical roles for making addicts prone to further alcohol drinking. Alteration in glutamate homeostasis, a process of maintaining non-toxic glutamate concentration, within cortico-striatal pathways has been shown to shape alcohol-seeking and -taking behaviors. In this chapter, we will discuss the glutamate homeostasis within nucleus accumbens, a gateway of connecting limbic and motor sub-circuits, with its processing of addiction formation in lights of alcohol dependence. Besides, we will give an overview of current findings of altered glutamate homeostasis within nucleus accumbens imparted by alcohol dependence. Finally, we will discuss the various alcohol drinking paradigms with rodent model that best mimic the human alcohol dependence scenarios.
Introduction

Alcohol dependence is a chronic psychological disorder. In 2014, 87.6 percent of adults (18 or older) drank alcohol at some points of their life and 6.8 % of them had alcohol use disorder (AUD) ((SAMHSA), 2014). Alcohol-related deaths are approximately 88,000 per year making it fourth leading cause of death in United States (US) (Stahre et al., 2014). Alcohol consumption affects multiple organs of the body such as brain, liver, heart and pancreas. In this chapter, we will confine our discussion on how drugs of abuse, including alcohol can affect the reward circuitry of the central nervous system (CNS). In physiological condition, excitatory and inhibitory neurotransmissions exist in equilibrium. Short-term (acute) alcohol consumption increases inhibitory neurotransmission and decreases excitatory neurotransmission resulting in net depressive effects on brain (Valenzuela, 1997). In contrary, long-term alcohol (chronic) consumption may counteract the depressive effects of alcohol resulting in neuroexcitation (Tabakoff and Hoffman, 1996). Reduction or cessation of alcohol consumption results in excessive excitation of brain due to the absence of compensatory effects of alcohol. Besides, abrupt cessation/reduction of binge alcohol intake results in alcohol withdrawal syndrome (AWS).

Extensive pre-clinical and clinical studies have been conducted to decipher the reward processing (natural/drugs of abuse) by the CNS. Unlike the natural rewards, drugs of abuse cause long-lasting neuro-adaptive changes in reward circuit resulting in compulsive addictive behavior. Dysfunction of the cortico-striatal glutamatergic neurotransmission has been found to play a critical role in long-lasting drug-seeking behavior.
It is well established that alcohol dependence increases glutamate neurotransmission in cortico-striatal pathways. But little is known about the exact neuro-pharmacological mechanism(s) through which glutamatergic neurotransmission increases(s) following alcohol consumption or withdrawal. In this chapter, we will focus our discussion on the reward circuitry in brain, glutamate homeostasis in cortico-striatal circuit with its pathology incurred by drugs of abuse, including alcohol, and animal model to study alcohol dependence.

1.1. Brain circuitry in alcohol dependence

The positive reinforcements (rewarding stimulus or experience) are the critical motivating factors for early stage of alcohol uses and abuses. Alternatively, negative reinforcing effects of alcohol play a critical role during transition stage of alcohol abuse to alcohol dependence (Gilpin and Koob, 2008). Thus, alcohol dependence (drug addiction, in general) is considered as a motivation disorder characterized by excessive control over behavior imparted by drugs (Di Chiara, 1999). Dopamine release from the ventral tegmental area (VTA) into nucleus accumbens (NAc) is an acute rewarding effect of alcohol and repeated alcohol use abnormally strengthen the alcohol-seeking behavior (Kalivas, 2009). Relapse to alcohol-seeking is mediated through long-lasting neuroadaptive changes in cortico-striatal circuitry, which make the addicts vulnerable to relapse many years after abstinence of alcohol.
The cortico-striatal circuitry is responsible for initiating learnt behavior (e.g. drug-taking) and generating new adaptive behaviors in response to change in environmental contingencies (Yin and Knowlton, 2006, Kalivas, 2009). Hence, dysfunction of the cortico-striatal projections impairs the ability of addicts to stop drug-taking even though there is change in environmental contingencies. The cortico-striatal circuits can be divided into two sub-circuits: limbic sub-circuit and motor sub-circuit (Kalivas, 2009) (Fig 1-1).

Fig 1-1: Cortico-striatal circuitry with connections of various regions.

The limbic sub-circuit is involved in processing /attending the newly/previousy learned information. If the established behavior gives the adaptive outcome then the influence of limbic sub-circuit diminishes and motor sub-circuit becomes well organized around task performance. But if the environmental contingency changes, then the motor sub-circuit becomes more disorganized while limbic sub-circuit becomes strongly engaged to establish a response with adaptive outcomes (Barnes et al., 2005, Kalivas, 2009).
The inability of the limbic sub-circuit to process the negative environmental contingencies effectively results in relapse to drug seeking. This means the pathology in limbic sub-circuit dominate the adaptive behaviors. Since prefrontal cortex (PFC) is responsible for executing goal directed behavior and NAc serves as a gateway for communicating limbic and motor sub-circuits, our research will focus on NAc and PFC as brain regions modulating alcohol dependence. We will investigate the neuropharmacological basis of alcohol consumption in NAc and PFC of alcohol preferring (P) rats.

1.1.1. Nucleus accumbens:

The NAc is the ventral part of the striatum. The primary neurons (>90%) in NAc are GABAergic medium spiny neurons (MSN) receiving dopaminergic inputs from VTA and glutamatergic inputs from PFC, hippocampus, thalamus and amygdala (Kalivas and Volkow, 2011, van Huijstee and Mansvelder, 2014). Natural rewards or drugs of abuse increase release of dopamine from VTA into NAc in tonic or phasic (high frequency) firing manner. DA released into NAc binds with low-affinity D1 and high-affinity D2 receptors (Volkow and Morales, 2015). Activation of D1-receptors works through direct striatal pathway and mediates rewards. On the other hand, activation of D2-receptors works in striatal indirect pathways and opposes aversive responses. Thus, activation of both D-1 and D2- receptors exert maximum rewards. Conditioned stimuli for natural rewards (e.g food, sex) increase phasic firing of DA into NAc and acquisition of reward attenuates DA firing (Volkow and Morales, 2015).
In contrast, most of the drugs of abuse increase phasic DA firing and continue to increase DA release even during acquisition resulting in compulsive acquisition (Volkow and Morales, 2015). Thus, DA acts for reinforcement of acquisition of drug-seeking behaviors.

While DA plays a central role for acute rewards and initiation of associative learnings, considerable evidences suggest the involvement of cortico-striatal glutamatergic neurotransmission for chronic and long-lasting drug-seeking behavior (Kalivas and Volkow, 2005). Reinstatement of drug-seeking behavior in operant chamber paradigm was associated with increased glutamate release from PFC to NAc suggesting the role of glutamatergic neurotransmission within NAc for drug-seeking behavior (Reissner and Kalivas, 2010). The blockade of AMPA glutamate receptors within NAc has been shown to attenuate drug-seeking behaviors in rodents. The reinstatement of drug-seeking has been shown to change the synaptic strength and dendritic spine diameter in NAc of rodents (Scofield and Kalivas, 2014). Human neuroimaging studies showed that representation of drug-paired cues resulted in metabolic activation of PFC and NAc (Goldstein and Volkow, 2002). Together, these evidences suggest a crucial role of glutamate homeostasis in NAc.
1.1.2. Pre-frontal cortex:

Pyramidal neurons are the main neuronal population in PFC. PFC receives dopaminergic inputs from ventral tegmental area (VTA) and sends out glutamatergic projections into NAc, VTA and basolateral amygdala (BLA) (van Huijstee and Mansvelder, 2014). PFC is involved in integrating limbic information and process goal-directed behavior through projections into NAc (Del Arco and Mora, 2009). PFC can control the function of NAc in both direct (glutamatergic) and indirect (dopamine/acetylcholine) pathways (Del Arco and Mora, 2009). Alcohol drinking has been shown to increase neuronal firing rate in PFC of alcohol preferring rats (Linsenbardt and Lapish, 2015). Withdrawal from alcohol was associated with increased tonic glutamate in PFC of rodents (Hwa et al., 2015). Thus, PFC has drawn a substantial attention for finding potential therapeutic targets for alcohol dependence.

1.2. Glutamate homeostasis

Glutamate is a major excitatory neurotransmitter (~70% of synaptic transmission) in the central nervous system (CNS) (Danbolt, 2001). The action of glutamate propagates through fast-acting ligand-gated glutamate receptors and slow-acting metabotropic glutamate receptors. The outcome of glutamate neurotransmission has two avenues. In one side, normal glutamate neurotransmission is essential for cognitive functions (Rahn et al., 2012). On the other side, excessive glutamate or altered glutamate neurotransmission is a cause of neuro-toxicity, neurodegenerative diseases, cognitive
deficit, and propensity to drugs of abuse (Reissner and Kalivas, 2010, Merritt et al., 2013, Scofield and Kalivas, 2014). Thus, excessive glutamate is removed from the extra-synaptic space to maintain non-toxic level of glutamate.

The glutamate homeostasis is maintained through equilibrium between glutamate release and removal into or out of extra-synaptic space. Glutamate release into synapse and extra-synaptic space is regulated through metabotropic glutamate receptors (mGluRs) and cystine-glutamate anti-porter (xCT), respectively. On the other hand, excess glutamate is removed from extra-synaptic space though glutamate transporters (Fig 1-2).
1.2.1. Glutamate transporters:

Glutamate is the major excitatory neurotransmitter in the CNS. There are five sub-types of excitatory amino acid transports: EAAT 1 (GLAST), EAAT 2 (GLT-1), EAAT 3, EAAT 4 and EAAT 5. Among these glutamate transporters, EAAT 2 (GLT-1) transports the majority (~94%) of extracellular glutamate within the astrocytes in sodium-dependent manner (Tanaka et al., 1997).

Within the astrocytes, glutamine synthetase (GS) enzyme converts glutamate into glutamine to be reused by presynaptic neurons. This mechanism is termed as glutamate-glutamine cycle. xCT, catalytic subunit of xCT system, release glutamate from inside of astrocytes into extrasynaptic space in exchange of cystine (dimer of cysteine) in sodium-independent manner. xCT accounts for majority of the extracellular glutamate (Baker et al., 2002). Glutamate uptake from extrasynaptic space through GLT-1 and glutamate release into extrasynaptic space through xCT results in tight regulation of glutamate concentration termed as glutamate homeostasis.

1.2.2. Metabotropic glutamate receptors:

Metabotropic glutamate (mGlu) receptors are a family of G protein-coupled receptors (GPCR), which consists of an extracellular N-terminal domain, a heptahelical transmembrane domain and an intracellular C-terminal domain. The seven hydrophobic segments of heptahelical transmembrane domain are separated by three extracellular loops and three intracellular loops (Masu et al., 1991). The cysteine-rich N-terminus is involved in glutamate binding, the extracellular loops interact with positive and negative
allosteric modulators, and intracellular C-terminus is involved in signal transduction (Kew and Kemp, 2005). mGlu receptors are classified into three subgroups due to their distinct sequence pattern, pharmacology and signal transduction mechanism (Pin and Duvoisin, 1995, Conn and Pin, 1997, Ferraguti and Shigemoto, 2006, Pomierny-Chamiolo et al., 2013, Pitsikas, 2014). Group I mGlu receptors include mGlu1 and mGlu5 receptors and their splice variants, group II mGlu receptors include mGlu2 and mGlu3 receptors and group III mGlu receptors include mGlu4, mGlu6, mGlu7 and mGlu8 receptors (Fig 1-3).

Group I mGlu receptors are positively coupled to phosphoinositide hydrolysis through G\textsubscript{q} protein whereas group II and group III mGlu receptors are negatively coupled to adenylyl cyclase through G\textsubscript{i} protein. Activation of group I mGlu receptors (mGlu\textsubscript{1} and mGlu\textsubscript{5} receptors) lead to increased level of phosphoinositide, intracellular Ca\textsuperscript{2+} and cAMP, and thus result in increased neurotransmission through activation of NMDA receptors (Ferraguti and Shigemoto, 2006, Lesage and Steckler, 2010, Hu and Gereau, 2011, Caraci et al., 2012, Cleva and Olive, 2012). Alternatively, activation of group II (mGlu\textsubscript{2} and mGlu\textsubscript{3} receptors) and group III mGlu (mGlu\textsubscript{4}, mGlu\textsubscript{6}, mGlu\textsubscript{7} and mGlu\textsubscript{8} receptors) receptors lead to decrease intracellular cAMP and activation of various K\textsuperscript{+} channels, and thus results in decrease in glutamate release (Nicoletti et al., 2011, Caraci et al., 2012, Pomierny-Chamiolo et al., 2013).
Group I mGlu (mGlu₁ and mGlu₅) receptors are predominantly expressed on post-synaptic terminals near the synaptic cleft. Group II mGlu (mGlu₂ and mGlu₃) and group III mGlu (mGlu₄, mGlu₇ and mGlu₈, not mGlu₆) receptors are primarily expressed on presynaptic neurons (Cleva and Olive, 2012, Pomierny-Chamiolo et al., 2013). mGlu₁,₃,₅,₇ receptors are enormously found throughout the brain, however, mGlu₂,₄,₆ receptors are confined to particular brain areas and mGlu₆ receptors are found on retina (Ferraguti and Shigemoto, 2006) (Fig 1-3).

Fig 1-3: mGlu receptors with their signaling pathways.
1.3. Effects of alcoholism on glutamate transporters/anti-porters:

Dopamine release is an acute rewarding effect of alcohol. Although DA release is a significant phenomenon following alcohol consumption, it is suggested this might not be essential for alcohol reinforcement (Gilpin and Koob, 2008). It is noteworthy that glutamate neurotransmission in central reward brain region may play a role in this mechanism. It is now well established that alcohol dependence increases glutamatergic neurotransmission in cortico-striatal circuits. There are differences in the effects of alcohol exposure in the expression of glutamate transporters and these might be due to different experimental drinking paradigms. Thus, non-contingent alcohol administration has been shown to decrease glutamate uptake without affecting GLT-1 expression in NAc of rats (Melendez et al., 2005). Another study found that 20 months of voluntary alcohol drinking decreased glutamate uptake in cortex of alcohol preferring (P) rats (Schreiber and Freund, 2000). Voluntary alcohol drinking for five weeks has been shown to decrease GLT-1 expression in NAc, without affecting GLT-1 expression in PFC, of P rats (Sari and Sreemantula, 2012). Ceftriaxone (CEF), a beta lactam antibiotic, has been shown to attenuate voluntary alcohol drinking associated with increase in GLT-1 expression (Sari et al., 2011, Qrunfleh et al., 2013, Rao and Sari, 2014).
1.4. Effects of alcohol consumption on metabotropic glutamate receptors

Metabotropic glutamate receptors drew immense interest, compared to inotropic glutamate receptors, due to their modulatory effects for maintaining glutamate homeostasis. As mentioned earlier, activation of group-I (mGlu$_{1/5}$) mGlu receptors results in increase glutamate neurotransmission, and activation of group-II and III mGlu receptors results in decrease glutamate neurotransmission. Thus, antagonism of mGlu$_{1/5}$ receptors has been shown to decrease alcohol consumption (or self-administration) and relapse-like behaviors in rodents (Backstrom et al., 2004, Besheer et al., 2010, Lum et al., 2014). In addition, activation of mGlu$_{2/3}$ receptors decreases alcohol seeking and reinstatement (Rodd et al., 2006, Kufahl et al., 2011). Suppression of mGlu$_7$ receptors in NAc of rats has been shown to increase alcohol consumption, which indicates the crucial role of this receptor in alcohol dependence (Bahi, 2013).

1.5. Animal model for alcohol dependence

One difficulty in pre-clinical study of alcohol dependence is that most of the rodent lines do not drink alcohol voluntarily without experimental manipulations (McBride et al., 2014). Several rat lines, including alcohol preferring (P) rats, have been developed through selective breeding, which drink significant amount of alcohol voluntarily and mimic human condition of alcoholism (Li et al., 1991, Li et al., 1993). Our current research used P rats exposed to two alcohol concentrations (15% and 30%, concurrently) as a model of alcohol dependence. The use of multiple concentrations concurrently has
been shown to increase alcohol intake and blood alcohol concentration (BAC) (Rodd-Henricks et al., 2001, McBride et al., 2013).

P rat model meet the criteria as alcohol dependence model. First, P rats drink high amount of alcohol (5-8 g/kg/day) voluntarily with pharmacologically relevant BAC (50-200 mg %) (Li et al., 1993, McBride and Li, 1998, Murphy et al., 2002). Second, P rats drink alcohol to achieve the pharmacological effects of alcohol, not due to its taste, smell or calorie value (Bice and Kiefer, 1990, Lankford et al., 1991, Toalston et al., 2008). Third, P rats self-administer alcohol due to its reinforcing properties evident from the fact that P rats have a high breakpoint (maximum responses for a stimulus) for alcohol (Czachowski and Samson, 2002, Rodd et al., 2003). Finally, P rats develop metabolic and pharmacological tolerance of alcohol and show withdrawal symptoms upon reduction or cessation of alcohol (Lumeng and Li, 1986, Kampov-Polevoy et al., 2000).

1.6. Alcohol drinking paradigms

We will use three alcohol drinking paradigms which mimic various alcohol drinking scenarios in human. In chronic alcohol drinking paradigm, p rats will have continuous access to alcohol and/or water for five weeks. This continuous alcohol drinking paradigm mimics the chronic alcohol drinking in human. P rats will be treated with ceftriaxone for five consecutive days on week six. In relapse-like alcohol drinking, P rats will have five weeks of alcohol access followed by two weeks of alcohol abstinence. After two-weeks of alcohol abstinence, P rats will be re-exposed to alcohol consumption for one more
week. Finally, we will use a binge alcohol exposure model where P rats will be re-exposed to alcohol after 48 hours of withdrawal from binge alcohol administration.

These three models have been summarized in the following figure.

A) Chronic voluntary alcohol consumption model:

B) Relapse-like voluntary alcohol consumption following withdrawal period model:

C) Relapse-like voluntary alcohol consumption following binge alcohol withdrawal model:
1.7. Conclusion

It is evident from existing pre-clinical and clinical studies that glutamatergic neurotransmission from PFC into NAc underlies the addictive behaviors. The alteration of glutamate homeostasis within PFC and NAc warrants extensive study to find out novel therapeutic targets for alcohol dependence. Our research aimed at determining how glutamate transporters were affected by alcohol dependence. The subsequent chapters will discuss in detail the effects of alcohol drinking on GLT-1 and xCT in chronic, relapse-like and binge alcohol drinking paradigms.
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Chapter 2

Ceftriaxone attenuates ethanol drinking and restores extracellular glutamate level through normalization of GLT-1 in nucleus accumbens of male alcohol-preferring rats

Running title: Ceftriaxone on glutamate

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Abstract:

Alteration of glutamatergic-neurotransmission is a hallmark of alcohol abuse. We have previously reported that chronic ethanol-drinking downregulated glutamate transporter 1 (GLT-1) in nucleus accumbens (NAc) in male P rats in a manner that was reversed by ceftriaxone treatment. However, the effect of ceftriaxone on extracellular glutamate concentrations in NAc after chronic ethanol-drinking has not yet been studied. In the present study, male P rats were treated with ceftriaxone (100 mg/kg/day, i.p.) for five consecutive days following five-weeks of free choice ethanol (15% and 30%) drinking. In vivo microdialysis was performed to measure the extracellular glutamate concentrations in NAc and the effect of blockade of GLT-1 with dihydrokainic acid (DHK) on extracellular glutamate in NAc of ceftriaxone-treated rats was determined. Ceftriaxone treatment attenuated ethanol intake as well as ethanol preference. Extracellular glutamate was significantly higher in NAc after five-weeks of ethanol drinking in saline-treated compared to water control rats. Ceftriaxone treatment blocked the increase extracellular glutamate produced by ethanol intake. Blockade of GLT-1 by DHK reversed the effects of ceftriaxone on glutamate and implicated the role of GLT-1 in the normalization of extracellular glutamate by ceftriaxone. In addition, GLT-1 protein was decreased in ethanol exposed animals and ceftriaxone treatment reversed this deficit. Ceftriaxone treatment also increased glutamine synthetase activity in NAc as compared to ethanol drinking saline-treated rats. Our present study demonstrates that ceftriaxone treatment prevents ethanol drinking in part through normalization of extracellular glutamate concentrations in NAc of male P rats via GLT-1. **Keywords:** Alcohol abuse; GLT-1; non-net-flux microdialysis; ceftriaxone; dihydrokainic acid.
Introduction

Alcoholism is a common glutamate-related neuropsychiatric disorder (Tsai et al., 1995). An alteration of cortico-striatal glutamatergic neurotransmission is a hallmark of alcohol abuse. Furthermore, the changes in cortico-striatal glutamatergic-neurotransmission produced by ethanol include: 1) decreased levels of glutamate transporter 1 (GLT-1, its human homolog termed excitatory amino acid transporter 2, EAAT2) (Sari et al., 2013b) and cystine/glutamate exchanger (xCT) (Alhaddad et al., 2014) in NAc following chronic ethanol drinking, 2) increased extracellular glutamate concentrations in the NAc following acute ethanol administration (Dahchour et al., 2000, Melendez et al., 2005), 3) decreased glutamate uptake in the NAc following repeated ethanol exposure (Melendez et al., 2005), and 4) increased extracellular glutamate concentrations in NAc shell following chronic ethanol drinking (Ding et al., 2013).

Extracellular glutamate is regulated by several glutamate transporters (Gegelashvili and Schousboe, 1997, Seal and Amara, 1999, Anderson and Swanson, 2000), however GLT-1 regulates the majority of extracellular glutamate (Rothstein, 1995, Danbolt, 2001, Mitani and Tanaka, 2003). Ceftriaxone (CEF), a β-lactam antibiotic, is known to cross the blood-brain barrier (BBB) (Lucht et al., 1990, Prasil et al., 2010) and has been shown to upregulate GLT-1 (Rothstein et al., 2005, Lee et al., 2008, Ramos et al., 2010, Sari et al., 2010), xCT levels (Lewerenz et al., 2009, Knackstedt et al., 2010, Alhaddad et al., 2014) and produce sustained reductions of extracellular glutamate in the NAc of rats (Rasmussen et al., 2011b). Furthermore, CEF is known to have neuroprotective effects (Mimura et al., 2011) and attenuated cocaine-seeking (Sari et al., 2009, Knackstedt et al., 2010, 2011).
Sondheimer and Knackstedt, 2011), cannabinoid tolerance (Gunduz et al., 2011), amphetamine-induced hyperactivity and behavioral sensitization (Rasmussen et al., 2011a), and morphine-evoked hyperthermia (Rawls et al., 2007). CEF has also been reported to attenuate both chronic and relapse-like ethanol drinking (Sari et al., 2011, Qrunfleh et al., 2013, Sari et al., 2013a, Sari et al., 2013b, Alhaddad et al., 2014, Rao and Sari, 2014), and ethanol-withdrawal manifestations (Abulseoud et al., 2014).

Drugs of abuse, including ethanol, activate dopaminergic projections from ventral tegmental area (VTA) to PFC and NAc. Chronic drug abuse induces transition of dopaminergic projections to glutamatergic projections. Nucleus accumbens (NAc) receives glutamatergic inputs from PFC, hippocampus (Hipp) and amygdala (Amy) (Kalivas and O'Brien, 2008). The PFC→NAc glutamate projection commences adaptive behavior and Hipp/Amy→NAc glutamate projection helps to retrieve previously experienced emotional and circumstantial information (Moussawi and Kalivas, 2010). Thus, NAc becomes a key brain region modulating addiction and has been focused in this study. Less is known about the role of extracellular glutamate in NAc following chronic ethanol consumption. Although CEF treatment produced sustained reductions of basal extracellular glutamate in NAc (Rasmussen et al., 2011b), it is unknown whether CEF modulates extracellular glutamate in NAc following chronic ethanol drinking. In this study, P rats voluntarily drank ethanol using a three-bottle choice paradigm for five weeks as a model of chronic alcoholism. We tested the hypothesis that chronic voluntary ethanol-drinking would increase the extracellular glutamate concentrations in NAc of P rats and CEF treatment would reverse the increases in extracellular glutamate after chronic
voluntary ethanol drinking. We used in-vivo microdialysis with no-net-flux to measure extracellular concentrations of glutamate in NAc of P rats. Western blot analysis was used to examine the relative expression level of GLT-1. Furthermore, dihydrokainic acid, a GLT-1 blocker, was locally perfused into the NAc of CEF-treated P rats to investigate the contribution of GLT-1 to the modulation of extracellular glutamate by CEF. Since glutamatergic neurotransmission is regulated by the glutamate-glutamine cycle and glutamine synthetase (GS) (Tani et al., 2014), we also investigated the effect of chronic ethanol-drinking and CEF treatment on GS activity in PFC and NAc of P rats.

2.1. Materials and methods

2.1.1 Subjects

Male P rats were shipped at their age of 3-4 weeks from Indiana University School of Medicine, Indianapolis; IN. At the age of 80-85 days, P rats were single housed with 21°C temperature and 50% humidity in a 12h light/dark cycle. Food and water were available unrestricted throughout the experiments; and all experimental procedures were conducted in light cycle. All housing and experimental procedures are 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 of Life sciences, 1996).
2.1.2. *Ethanol drinking procedures*

At the age of 90 days, male P rats had continuous and free access to ethanol (15% and 30%, v/v, concurrently), and/or water for five weeks. Ethanol and water intake were measured (as g/kg/day) three times per week during the last two weeks and served as baseline intake. During week six of ethanol drinking, rats were randomly divided into two groups: ethanol-drinking saline-treated (EtOH-Sal) rats and ethanol-drinking CEF-treated (EtOH-CEF) rats. The Water-drinking saline-treated (WD-Sal) group had free access to water and food only throughout the experiment. The EtOH-CEF group received CEF at a dose of 100 mg/kg (i.p.) once daily for five consecutive days during week six. Both WD-Sal and EtOH-Sal groups received saline (i.p.) once daily for five consecutive days during week six. The stereotaxic surgery for implantation of microdialysis probes was performed on day six and in-vivo reverse microdialysis with no-net-flux was performed on day seven of week six. Another group of CEF-treated rats was perfused with DHK (500 µM) during the microdialysis procedure and termed as EtOH-CEF+DHK group.

CEF 100 mg/kg/day was used because it was reported to significantly increase the level of GLT-1 in both PFC and NAc of P rats (Sari et al., 2011, Sari et al., 2013b). This dose also significantly reduced the baseline extracellular glutamate concentrations in the NAc of rats (Rasmussen et al., 2011b).
2.1.3. *In-vivo reverse microdialysis (no-net-flux microdialysis)*

Probes for reverse microdialysis were constructed as previously described (Halpin et al., 2014). Briefly, probes were made of PE 20 tubing (Becton Dickinson), silica tubing (OD, 150 μm; Polymicro Technologies), 26 ga stainless steel hypodermic tubing (Small parts), Hollow Fiber Microdialysis Membrane (active membrane, 1.7 mm; 13,000 molecular cutoff; 216 μm outer diameter; Spectrum Laboratories), 2 ton waterproof epoxy and tygon microbore tubing. Surgeries for the probe placement were performed a day before the microdialysis experiment. The microdialysis procedure on next day of probe placement has been shown to provide stable baseline glutamate level (Halpin et al., 2014). The probes were stereotaxically placed into the NAc (AP+1.8, ML+1.5, DV-6.5/-7mm). Xylazine (5 mg/kg) and ketamine (75 mg/kg) were used as anesthetics.

Artificial cerebrospinal fluid (aCSF) was perfused at a flow rate of 1.5 μl/min through the probes during next day. The flow rate was fixed at 1.5 μl/min throughout the microdialysis procedure. Following a 1 h equilibration period, baseline samples were collected every 30 min for 3 h after which the aCSF was switched to aCSF containing 2.5, 5, and 10 μM glutamate. Two samples were collected at 30 min interval for each concentration. For EtOH-CEF+DHK group, DHK (500 μM) was perfused for 30 min at flow rate of 1.5 μl/min prior to baseline samples and each concentration switch. Only rats with probe placement in NAc (core or shell) were included in the study. A concentration of 500 μM DHK was used based on previous literature showing effective blockade of GLT-1 (Fletcher and Johnston, 1991, Fischer et al., 2013).
2.1.4. **Quantification of glutamate**

Glutamate content of the dialysates was analyzed using an HPLC system (ESA, Inc) with electrochemical detection as previously described (Breier et al., 2006). Dialysate samples were derivatized with O-phthalaldehyde (OPA) and sodium sulfite with an ESA Model 540 autosampler before injecting onto a C18 column (3.0x50 mm, 2.5 µm particle size, Waters). The elution was performed with a mobile phase containing 0.1M Na$_2$HPO$_4$, 0.1mM EDTA, and 7.5% Methanol (pH 3.0). Glutamate was detected by CoulArray coulometric detector (model 5600A, ESA, Inc.), and the data were recorded using CoulArray software. Glutamate concentration in each dialysate was analyzed by peak height and compared with an external standard.

2.1.5. **Brain tissue harvesting**

After the microdialysis procedure, P rats were euthanized with CO$_2$ inhalation and rapidly decapitated with a guillotine. Brains were removed and instantly frozen with dry ice and stored at -70°C.

2.1.6. **Western blot**

After probe placement verification, the whole NAc in the hemisphere contralateral to the probe placement was dissected from cryostat sections through the NAc (Paxinos and Watson, 1998). The whole NAc was dissected since CEF increased the levels of GLT-1 in both the core and shell of NAc (Sari et al., 2013b). The cytoplasmic fraction of the
dissected NAc was used to determine the level of GLT-1. Cytoplasmic and nuclear fractions were separated as described previously (Ahmed et al., 2006). Equal amounts of protein were loaded and separated by 10-20% tris-glycine gel (Life Technologies, Grand Island, NY). Proteins were then transferred onto PVDF membrane and incubated at room temperature with blocking buffer for 30 min. The membranes were incubated overnight at 4°C with guinea pig-anti GLT-1 (1: 5000, Millipore, 60 kDa). The membranes were then washed with TBST, incubated with HRP-linked secondary antibody (1:5000) at room temperature for 90 minutes, and developed using HRP chemiluminescent kit (SuperSignal West Pico, Pierce Inc.). Furthermore, membranes were exposed to Kodak BioMax MR films (Fisher Inc.), and the films were developed by SRX-101A machine. The bands were quantified using MCID software and normalized to a β-tubulin (50 kDa) internal loading control.

2.1.7. Glutamine synthetase (GS) activity assay

Cytoplasmic fractions of PFC and NAc were used to determine glutamine synthetase (GS) activity. GS activity was measured by γ-glutamyl transfer assay as previously described (Miller et al., 1978, van der Vos et al., 2012). Briefly, equal volume of cytoplasmic fractions of PFC and NAc were mixed with assay mixture (50 mM imidazole chloride (pH 7.4), 50 mM L-glutamine, 25 mM hydroxylamine, 25 mM Na-arsenate, 2 mM MnCl₂ and 0.16 mM ADP) and incubated in a 96-well plate at 37°C for 30 min. The reaction was terminated by the addition of GS stop solution (2.42% FeCl₃, 1.45% trichloroacetic acid and 1.82 N HCl). The absorbance of the formed γ-glutamyl
hydroxamate was measured at 570 nm and converted into nanomoles by a calibration curve using authentic \( \gamma \)-glutamyl hydroxamate. Parallel incubations of cytoplasmic fractions with assay mixture lacking Na-arsenate and ADP served as blank.

2.1.8. Statistical analysis

Differences in ethanol intake, ethanol preference (amount of ethanol consumed/total amount of fluid consumed \( \times 100 \)) and body weight between EtOH-Sal and EtOH-CEF groups were analyzed with two-way (repeated measure) ANOVA followed by Bonferroni’s multiple comparison test. No-net-flux microdialysis data were analyzed with linear regression analysis where the x-intercept and slope represented extracellular glutamate concentrations and probe recovery, respectively. Differences in extracellular glutamate concentration and probe recovery were further analyzed with one-way ANOVA followed by Tukey’s post hoc test. Western blot and GS activity assay data were analyzed with one-way ANOVA followed by Tukey’s post hoc test. All statistical analyses were performed using GraphPad Prism. All data are presented as mean ± SEM.

2.1.9. Drugs

CEF was purchased from Apotex Corp (USA) and dissolved in saline solution (0.9% NaCl). Dihydrokainic acid (DHK) was purchased from Tocris Bioscience (Ellisville, MO, USA) and dissolved in dialysis buffer.
2.2. Results

2.2.1. Attenuation of ethanol intake and ethanol preference by ceftriaxone treatment

We determined the effects of CEF treatment on ethanol intake and ethanol preference as well as body weights. Two-way (repeated measure) ANOVA showed a significant effect of day on ethanol intake [F (5, 35) = 9.84, p<0.0001], a significant effect of treatment [F (1, 7) = 83.77, p<0.0001], and a significant day × treatment interaction [F (5, 35) = 14.25, p<0.0001]. Bonferroni’s multiple comparison test revealed a significant reduction in ethanol intake in EtOH-CEF group compared to EtOH-Sal group (p<0.0001 for day 1 through day 5) (Fig. 2-1A).

A two-way (repeated measure) ANOVA conducted on ethanol preference revealed a significant effect of day [F (5, 35) = 3.33, p=0.01], a significant effect of treatment [F (1, 7) = 18.66, p=0.003] and a significant interaction between day and treatment [F (5, 35) = 6.04, p=0.0004]. Bonferroni’s multiple comparison test revealed that CEF treatment significantly decreased ethanol preference in EtOH-CEF group compared to EtOH-Sal group (p<0.001 for day 1 and p<0.0001 for day 2 through day 5). Baseline ethanol preference between EtOH-Sal and EtOH-CEF groups was not significantly different (p>0.99) (Fig. 2-1B).
Figure 2-1. Effect of CEF treatment on ethanol intake and ethanol preference in male P rats. P rats had free access to ethanol (15% and 30%, concurrently) and/or water for five weeks. P rats were treated with CEF (100 mg/kg/day, i.p.) or saline (i.p.) for five consecutive days during week six. Ethanol and water intake were measured during the last two weeks and served as baseline intake. A. CEF treatment significantly reduced ethanol intake in EtOH-CEF group during treatment period (day 1 through day 5) compared to the EtOH-Sal group (#, p<0.0001). B. CEF treatment significantly decreased ethanol preference in EtOH-CEF group during treatment period (day 1 through day 5) compared to the EtOH-Sal group (&, p<0.001; #, p<0.0001). All data are expressed as mean ± SEM. n=8/ group. EtOH, ethanol; Sal, Saline; CEF, ceftriaxone.

2.2.2. Ceftriaxone treatment normalizes extracellular glutamate concentration in NAc

Figure 2 shows microdialysis probe placements in NAc. We conducted a one-way ANOVA on basal glutamate concentrations (x-intercept of linear regression curve when y=0) and on the slopes of the linear regression analysis. A one-way ANOVA conducted on basal glutamate data revealed a significant main effect among WD-Sal, EtOH-Sal,
EtOH-CEF and EtOH-CEF+DHK groups \([F (3, 24) =6.48, p=0.002]\) (Fig 2-3A). Tukey’s multiple comparison test revealed that the extracellular glutamate level in NAc is significantly higher in EtOH-Sal group compared to WD-Sal group \((p=0.03)\) (Fig. 2-3B). CEF treatment for five consecutive days significantly reduced extracellular glutamate level in NAc in EtOH-CEF group compared to EtOH-Sal group \((p=0.002)\). Extracellular glutamate concentrations in NAc in EtOH-CEF group were not significantly different from WD-Sal group \((p=0.71)\). Importantly, blockade of GLT-1 by perfusion of DHK (500 µM) significantly increased extracellular glutamate concentrations in NAc in EtOH-CEF+DHK group compared to EtOH-CEF group \((p=0.04)\). Extracellular glutamate in NAc in EtOH-CEF+DHK group was not significantly different from EtOH-Sal group \((p=0.76)\) (Fig. 2-3B).

A one-way ANOVA conducted on slope of regression analysis data revealed a significant main effect among groups \([F (3, 24) =7.37, p=0.001]\). The slope of linear regression line (probe recovery) was significantly lower in EtOH-Sal group compared to WD-Sal group \((p=0.03)\). CEF treatment normalized the slope of linear regression line in EtOH-CEF group compared to the EtOH-Sal group \((p=0.0008)\). In addition, the blockade of GLT-1 significantly lowered the slope of regression line in EtOH-CEF+DHK group compared to EtOH-CEF group \((p=0.03)\). The slope of regression line in EtOH-Sal group was not significantly different from EtOH-CEF+DHK group \((p=0.57)\) (Fig. 2-3C).
Figure 2-2. Coronal slices of rat brains showing microdialysis probe tips in NAc. The dots in bottom pictures [adapted from Paxinos G and Watson C. (1998)] represent the microdialysis probe tips of rats included in the study.
Figure 2-3. Effect of chronic ethanol-drinking and CEF treatment on extracellular glutamate concentration in NAc of P rats. A. CEF (100 mg/kg, i.p., every 24h×5) treatment restored extracellular glutamate concentration in NAc, and the effect of CEF was reversed by intra-NAc infusion of DHK (500 µM). B. Extracellular glutamate concentration in EtOH-Sal was significantly increased in NAc following five-week of voluntary ethanol drinking as compared to the water drinking saline-treated (WD-Sal) group. CEF treatment restored glutamate concentration in NAc. In addition, intra-NAc infusion of DHK (500 µM) significantly increased extracellular glutamate concentration in EtOH-CEF group. C. EtOH-Sal group displayed reduced slope of the line of regression compared to WD-Sal animals, and CEF-treated animals increased slope of the line of regression compared to the EtOH-Sal group. Intra-NAc infusion of DHK reversed slope of the line of regression in EtOH-CEF animals. *p<0.05; **p<0.01; and ***p<0.001. n= 6-8/group. WD, water drinking; Sal, saline; EtOH, ethanol; CEF, ceftriaxone; DHK, dihydrokainate.

2.2.3. Chronic ethanol consumption caused downregulation of GLT-1 level and ceftriaxone treatment normalized GLT-1 level

We used western blot analysis to test the effect of voluntary ethanol-drinking and CEF treatment on expression level of GLT-1 in NAc (Fig. 2-4A). One-way ANOVA, followed by Tukey’s post hoc, revealed a significant main effect of ethanol-drinking and CEF treatment on GLT-1 level [F (2, 21) = 6.85, p= 0.005]. Tukey’s multiple comparison test revealed a significantly lower level of GLT-1 in EtOH-Sal group
compared to WD-Sal group (p=0.04), and significantly higher level of GLT-1 in EtOH-CEF group compared to EtOH-Sal group (p=0.004). There was no significant difference in GLT-1 level between WD-Sal and EtOH-CEF groups (Fig. 2-4B).

**Figure 2-4.** Effect of voluntary ethanol drinking and CEF treatment on GLT-1 expression level in NAc.  **A.** Representative Western blots for GLT-1 and β-tubulin as loading control.  **B.** GLT-1 level was significantly downregulated in NAc of EtOH-Sal group compared to WD-Sal group.  CEF treatment restored GLT-1 level in NAc of EtOH-CEF group compared to EtOH-Sal group.  *p<0.05, **p<0.01 (n=8/group). WD, water drinking; Sal, saline; EtOH, ethanol; CEF, ceftriaxone.
2.2.4. Increase in GS activity by CEF treatment in NAc of P rats

We further tested the effect of voluntary ethanol-drinking on GS activity in PFC and NAc of P rats. In PFC, one-way ANOVA revealed no significant main effect among WD-Sal, EtOH-Sal and EtOH-CEF groups [F (2, 18) = 0.34, p= 0.71] (Fig. 2-5A). Tukey’s multiple comparison test revealed no significant difference among WD-Sal, EtOH-Sal and EtOH-CEF groups. In NAc, One-way ANOVA, followed by Tukey’s post hoc, revealed a significant main effect among WD-Sal, EtOH-Sal and EtOH-CEF groups [F (2, 20) = 3.623, p= 0.04]. Tukey’s multiple comparison tests revealed that GS activity was not significantly different between EtOH-Sal and WD-Sal groups following five-week of voluntary ethanol drinking (p= 0.34) (Fig. 2-5B). We further tested whether CEF treatment had any effect on GS activity in NAc. CEF treatment indeed significantly increased GS activity of NAc in EtOH-CEF group compared to EtOH-Sal group (p=0.03). GS activity in NAc was not significantly different between WD-Sal and EtOH-CEF groups (p=0.40) (Fig. 2-5B).
Figure 2-5. Effect of chronic ethanol-drinking and CEF treatment on glutamine synthetase (GS) activity. **A.** GS activity was not significantly different among WD-Sal, EtOH-Sal and EtOH-CEF groups in PFC. **B.** GS activity was not significantly altered following five weeks of voluntary ethanol drinking in NAc of EtOH-Sal group compared to WD-Sal group. However, CEF increased GS activity in NAc of EtOH-CEF group compared to the EtOH-Sal group. *p<0.05 (n=7-8/group). WD, water drinking; Sal, saline; EtOH, ethanol; CEF, ceftriaxone.
2.3. Discussion

Our current study is unique in several aspects. First, we used reverse microdialysis (no-net-flux microdialysis) to evaluate the effect of CEF treatment on extracellular glutamate level in NAc of P rats following chronic ethanol drinking. Second, we blocked GLT-1 through perfusion of DHK to investigate the involvement of GLT-1 in CEF-modulated glutamate level in NAc of P rats. Third, we also investigated the effect of CEF treatment on GS activity in NAc of P rats.

The present findings showed that CEF treatment significantly attenuated voluntary ethanol drinking in male P rats using three-bottle choice (15% ethanol, 30% ethanol and water) drinking paradigm. The attenuated ethanol-drinking was associated with a significant decrease in ethanol preference during the five days of CEF treatment. However, CEF treatment didn’t cause any significant changes on the body weight of male P rats (data not shown). These results are in accordance with previous findings that showed an attenuation of both chronic (Sari et al., 2011, Alhaddad et al., 2014), and relapse-like (Qrunfleh et al., 2013) ethanol drinking in P rats, and decreased ethanol preference in mice (Lee et al., 2013) treated with CEF. Here, we used a three-bottle choice drinking model because P rats were found to drink more than 4 g/kg/day with this paradigm (Sari et al., 2011, Sari et al., 2013b, Alhaddad et al., 2014). The voluntary ethanol drinking paradigm is also known to produce physical dependence on ethanol (Kampov-Polevoy et al., 2000) and produce pharmacologically relevant blood alcohol concentrations (BACs) of 50-200 mg% (Murphy et al., 2002, McBride et al., 2013).
In the present study, in-vivo microdialysis revealed that a high amount of voluntary ethanol drinking (≥4 g/kg/day) was associated with significantly higher extracellular glutamate level in NAc of ethanol-drinking saline (EtOH-Sal) rats compared to water-drinking saline-treated (WD-Sal) rats. These are in accordance with previous findings, which reported increased glutamate levels and decreased glutamate uptake in NAc following ethanol exposure (Melendez et al., 2005, Ding et al., 2013, Griffin III et al., 2014). The increased glutamate has also been reported during the ethanol-withdrawal period (Rossetti et al., 1999). It is very important to note that our P rats were not in withdrawal during the microdialysis procedure since P rats have been reported to show onset of withdrawal symptoms only after 20 hours of cessation of severe ethanol-intoxication only (Chefer et al., 2011, Abulseoud et al., 2014).

Importantly, we revealed that CEF normalized the extracellular glutamate concentrations in NAc as compared to EtOH-Sal rats. This effect is in accordance with previous reports showing normalization of accumbal glutamatergic neurotransmission by CEF treatment (Rasmussen et al., 2011b, Trantham-Davidson et al., 2012). To investigate the mechanism by which CEF normalizes glutamate in NAc, we blocked GLT-1 though perfusion of DHK in NAc of CEF-treated P rats. Interestingly, blockade of GLT-1 in CEF-treated P rats by perfusion of DHK significantly increased the glutamate concentrations in NAc. The reversal of CEF effect by DHK suggests that CEF normalized extracellular glutamate through modulation of GLT-1. Since DHK was reverse perfused through microdialysis probe, there is possibility of presence of DHK in dialysate samples. Hence, we verified that DHK was not co-eluted with glutamate in
HPLC system. This confirms that the increased glutamate concentration in DHK-perfused rats was due to the pharmacological action of DHK, not for co-elution of DHK with glutamate in HPLC system.

The observed changes in glutamate in EtOH-Sal rats indicate decreased clearance of glutamate (Bungay et al., 2003, Trantham-Davidson et al., 2012). The decreased glutamate clearance might arise from dysregulation of Na\(^+\)-dependent glutamate transporters such as EAATs. Among all five subtypes of EAATs, GLAST (EAAT1) and GLT-1 (EAAT2) account for the majority of glutamate transport in CNS. GLT-1 is the major glutamate transporter in forebrain and striatum (Rothstein, 1995, Danbolt, 2001, Mitani and Tanaka, 2003, Abulseoud et al., 2014) and consequently plays a key role in maintaining striatal glutamate homeostasis (Rothstein, 1995, Danbolt, 2001, Mitani and Tanaka, 2003, Melendez et al., 2005). Thus, the decrease in GLT-1 following chronic voluntary ethanol drinking may be related to increased extracellular glutamate concentrations in NAc. This finding is consistent with previous reports showing downregulation of GLT-1 in NAc following chronic voluntary ethanol drinking (Sari and Sreemantula, 2012, Sari et al., 2013b).

The decreased GLT-1 immunoreactive protein found in our present study differs from other studies showing no change of GLT-1 level following ethanol administration (Melendez et al., 2005, Ding et al., 2013). This disparity might be due to five-week of voluntary ethanol drinking in contrast to acute systemic ethanol administration or differential chronic ethanol exposure used in previous studies. It remains to be examined
if the change in extracellular glutamate after ethanol exposure is mediated primarily by the downregulation of GLT-1 protein or the impaired transport function of GLT-1. Regardless, CEF treatment normalized GLT-1 protein levels in NAc and is consistent with earlier reports showing GLT-1 upregulation in NAc by CEF (Rothstein et al., 2005, Sari et al., 2010, Sari et al., 2011, Qrunfleh et al., 2013, Sari et al., 2013a, Sari et al., 2013b, Abulseoud et al., 2014, Rao and Sari, 2014).

Furthermore, we report here that CEF treatment did not show any significant effect on GS activity in PFC of P rats. But CEF treatment significantly increased GS activity in NAc of EtOH-CEF treated rats as compared to ethanol exposed rats. GS is present in astrocytes and converts imported glutamate (via EAATs) into glutamine (Miguel-Hidalgo, 2006). Although, GS activity is decreased in astrocytes by ethanol treatment (Davies and Vernadakis, 1984), we did not observe any significant difference in GS activity in NAc between WD-Sal and EtOH-Sal P rats. This discrepancy might be due to neuroadaptive changes which occurred during the five-week of voluntary ethanol drinking. Our finding is consistent with another report that showed no significant difference in packing density of GS-immunoreactive astrocytes between water drinking and ethanol drinking P rats for 2 months (Miguel-Hidalgo, 2006). It has been shown that activation of EAATs stimulates the release of astrocytic glutamine, a converted product of glutamate by glutamine synthetase (GS) (Uwechue et al., 2012). Therefore, the increased GS activity in NAc with CEF treatment might be due to normalization of glutamate inflow through GLT-1 within astrocytes. We previously showed that GLT-1 level was not significantly altered in PFC of P rats following five weeks of ethanol.
drinking (Sari and Sreemantula, 2012). Thus the unaltered GS activity in PFC might be correlated with unaltered GLT-1 level in PFC. Further studies are required to investigate the underlying direct mechanisms of action of CEF on GS activity in NAc of P rats.

In summary, consecutive five-day CEF treatment attenuated voluntary ethanol drinking as well as ethanol preference in male P rats. Five-week voluntary ethanol drinking was associated with the increase in extracellular glutamate concentrations in NAc that was reversed by CEF treatment. Blockade of GLT-1 by DHK reversed the effect of CEF treatment in extracellular glutamate suggesting the involvement of GLT-1 in the mechanism of CEF. Furthermore, ethanol-induced higher extracellular glutamate was associated, in part, with decreased expression of GLT-1 in NAc in a manner reversed by CEF treatment. In addition, CEF treatment increased GS activity in NAc of EtOH-CEF rats compared to EtOH-Sal rats. Overall, our present study suggests that CEF prevented ethanol drinking through normalization of extracellular glutamate concentrations in NAc via restoration of GLT-1 levels.

**Disclosure statements**

The authors declare no conflict of interest.
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References


GLT-1 and xCT are both critical to attenuate relapse-like alcohol drinking in alcohol preferring rats model

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Abstract

Relapse to alcohol drinking is a common phenomenon among alcohol addicts. Alteration in glutamate homeostasis within nucleus accumbens is one of the neuroadaptive changes promoting relapse to alcohol drinking. One of the mechanisms to prevent relapse-like alcohol drinking is upregulation of GLT-1 and xCT through ceftriaxone (a β-lactam antibiotic) in nucleus accumbens. Little is known about the comparative contribution of GLT-1, xCT and metabotropic receptors to the alteration of glutamate homeostasis caused by relapse-like alcohol drinking. It is also unknown whether upregulation of both GLT-1 and xCT are required for ceftriaxone-induced attenuation of relapse-like alcohol drinking. We used P rats in relapse-like alcohol drinking paradigm. We reveal here that relapse-like alcohol drinking did not affect Na\(^+\)-dependent and Na\(^+\)-independent glutamate uptake as well as relative gene expression of GLT-1 and xCT in nucleus accumbens of P rats. The relapse-like alcohol drinking increased the relative gene expression of mGlu\(_5\) without affecting relative gene expression of mGlu\(_{1/2/3}\) in nucleus accumbens. Suppression of GLT-1 or xCT through vivo-morpholinos prevented the ceftriaxone-induced attenuation of relapse-like alcohol drinking. We conclude that mGlu\(_5\) might contribute to the relapse-like alcohol drinking through increasing neurotransmission in nucleus accumbens and ceftriaxone can attenuate relapse-like alcohol consumption through upregulation of both GLT-1 and xCT.
Introduction:

Alcohol dependence is a complex neuropsychiatric disorder causing long-lasting neuroadaptive changes in the meso-corticolimbic system of brain and thus trigger relapse if abstinence or withdrawal is achieved. Several studies have been conducted to elucidate druggable targets for preventing relapse-like ethanol drinking. Those druggable targets included opioid receptors (antagonist, naltrexone) (Orrico et al., 2014), α1-adrenergic receptors (antagonist, prazosin) (Froehlich et al., 2015), and glutamate receptors, alcohol-derived acetaldehyde (D-penicillamine) (Marti-Prats et al., 2015). In targeting glutamatergic neurotransmission, the involvement of NMDA and AMPA receptors is considered as one of the mechanisms for inducing relapse-like ethanol drinking; and thus blockers/modulators of these receptors have been shown to prevent relapse to ethanol (Sanchis-Segura et al., 2006, Vengeliene et al., 2015). Less is known about the involvement of glutamate transporters and metabotropic receptors in relapse-like ethanol drinking.

Here, we used relapse-like ethanol drinking model with male alcohol preferring (P) rats to elucidate druggable targets among glutamate transporters and metabotropic glutamate receptors. P rats have been shown to develop alcohol deprivation effects (ADE) (Vengeliene et al., 2003). Alcohol deprivation effect (ADE) is a phenomenon when deprivation from voluntary alcohol drinking induces a pronounced, though transitory, increases in ethanol drinking upon re-exposure. Thus, ADE model with P rats is an acceptable model to study clinically relevant relapse situation in alcoholics (Vengeliene et al., 2005, Fredriksson et al., 2015). We focused our study on the nucleus accumbens.
(NAc) because of its crucial role for initiating adaptive behaviors through glutamatergic inputs from medial pre-frontal cortex (mPFC). First, we investigated the glutamate uptake in NAc as well as relative gene expression of GLT-1 and xCT. Later, we determined the relative gene expression of mGlu1/2/3/5 in NAc. Based on glutamate expression and activity results, we knocked down the expression of GLT-1 or xCT in ceftriaxone-treated rats to investigate the involvement of those transporters to prevent relapse-like alcohol drinking.

3.1. Methods and materials:

3.1.1. Subjects:

All animal housing and experimental procedures were performed in accordance with National Institutes of Health guidelines for laboratory animal use. Animal experimental protocols were approved by the Institutional Animal Care and Use Committee of The University of Toledo. Male alcohol-preferring (P) rats were obtained from Indiana University, Indianapolis, IN. P rats were singly housed with 21°C and 50% humidity in 12 hours light/dark cycle. Food and water were available unrestricted throughout the experimental procedures. All experiments were performed in light cycle.

3.1.2. Voluntary ethanol drinking procedure:

At the age of 90 days, male P rats were exposed to free choice of ethanol (15% and 30%, v/v, concurrently) and/or water for five (5) weeks. Then P rats went through ethanol abstinence for two weeks. After two-weeks of ethanol abstinence, P rats were re-exposed to voluntary ethanol drinking for one week. Body weight, ethanol drinking (as
gm/kg/day) and water drinking were measured every day during relapse-like ethanol drinking and three times a week during five week of voluntary drinking starting on third week (baseline drinking).

3.1.3. Stereotaxic surgeries for microdialysis probes and guide cannulas implantation:
Microdialysis probes were constructed as described previously (Das et al., 2015). After relapse-like ethanol drinking, microdialysis probes were implanted unilaterally (AP+1.8, ML-1.5, DV-6.5 mm) to NAc (core and/or shell) through stereotaxic surgery. On first day of ethanol abstinence, guide cannulas (Plastic One, 26G) were surgically implanted bilaterally (AP+1.8, ML±1.5, DV-5 mm) above the NAc. Cocktail (70:30) of Ketamine (75 mg/kg) and xylazine (5 mg/kg) were used for anesthesia. Flunixin (1 mg/kg, i.m.) was injected subcutaneously to relieve post-operative pain.

3.1.4. Micinjections and histology:
Vivo-morpholinos were purchased from Gene Tools, Inc. (Philomath, OR, USA). The sequences of vivo-morpholinos are as follows: anti-sense GLT-1, 5´-TGGTGCCACCCCTCCTGGATGCCAT-3´; sense GLT-1, 5´-TACCCTGATCCCACAGGATGATGCCAT-3´; anti-sense xCT, 5´-TGTTGGCACCCTCGGTTGATGCCAT-3´ and sense xCT, 5´-TGGCCACAATGGCTTCTGACCATT-3´. The base sequences for vivo-morpholinos were selected based on the previous literatures showing effective suppression of respective transporters (Reissner et al., 2012, Reissner et al., 2015). After one week of surgical recovery, vivo-morpholinos (30 pmol, 1 µl/hemisphere) were microinjected into
NAc (2 mm below the guide cannula) through internal cannulas (33 G, Plastic One) connected to Pump Name ??? with infusion rate of 0.5 µl/min. The internal cannulas were left at injection site for additional one minute for diffusion. Microinjections were carried out for three consecutive days (day 8 through day 10 of ethanol abstinence).

To investigate the upregulatory effects of GLT-1 and xCT on relapse-like ethanol drinking, ceftriaxone (a potent upregulator of GLT-1 and xCT) was i.p. injected in rats paired with anti-sense GLT-1 and anti-sense xCT vivo-morpholinos. Ceftriaxone (200 mg/kg) was injected for five consecutive days starting on third day of microinjections (last five days of ethanol abstinence).

At the end of relapse-like ethanol drinking (7 days), rats were sedated with pentobarbital (200 mg/kg, i.p.) and brains were perfused with saline followed by 4% paraformaldehyde. Brains were post-fixed with 4% paraformaldehyde, cryosectioned (45 µm) and stained with cresyl violet for verification of microinjection sites. For validation of GLT-1 and xCT suppression, sense and anti-sense vivo-morpholinos were microinjected contra-laterally into naïve P rats. Five days after third microinjections, rats were euthanized, brains frozen and NAc tissues were micro-punched.

3.1.5. Radioactive glutamate uptake assay:

Na⁺-dependent/-independent glutamate uptakes in NAc were determined using crude membrane fractionation as described previously (Shen et al., 2014). Briefly, fresh NAc samples were homogenized in 500 µl of cold 0.32M sucrose buffer containing 10 mM HEPES and 1mM EDTA (pH 7.4). The homogenized samples were centrifuged at 1000 ×
g for 10 min (4°C) and the resulting pellets were discarded. The resulting supernatants were centrifuged at 15000 × g for 20 min (4°C) to pellet down the crude membrane fractions. Pellets were re-suspended in Kreb’s-Ringer’s phosphate (KRP) buffer containing 140 NaCl, 1.2 CaCl₂, 1.2 KH₂PO₄, 5 HEPES, 1 MgCl₂ and 10 Glucose (in mM) (pH 7.4). For Na⁺-independent glutamate uptake, NaCl in KRP buffer was replaced with 140 mM Choline chloride. The glutamate uptake was initiated with the addition of ³H-glutamate (2 µCi/ml) in the presence of non-radioactive glutamate (1 µM) in final volume of 250µl. The incubation time at 37°C was 15 min and then glutamate uptake was stopped by putting samples on ice. Then samples were centrifuged at 1000 × g for 10 min (4°C) and supernatants were discarded. The pellets were further washed with ice-cold choline-containing KRP buffer to remove excess ³H-glutamate. The pellets were lysed with 1% SDS and radioactivity was counted in liquid scintillation counter. The protein concentration in each sample was determined by Lowry method (Lowry et al., 1951). The glutamate uptake was represented as disintegrations per minute (dpm/mg of protein/min).

3.1.6. RT-qPCR
Briefly, total mRNA was isolated from extracted NAc with TRIzol reagent (Invitrogen). Thermo Scientific verso cDNA kit was used for reverse transcription according to manufacturer’s protocol. The real time PCR was carried out in iCycler (Biorad laboratories) with a reaction mixture containing SYBR green as fluorescent dye. The relative gene expression was calculated using threshold cycle number according to 2⁻ΔΔCt method.
3.1.7. Statistical analyses:

The ethanol and water drinking between sense-saline and anti-sense-saline, anti-sense-saline and anti-sense-ceftriaxone groups were analyzed using two-way ANOVA followed by Bonferroni’s multiple comparison tests. Water-drinking and ethanol-drinking groups were analyzed using unpaired t-test. All data are presented as mean ± SEM. The level of significance was set to p<0.05.

3.2. Results:

3.2.1. Ceftriaxone treatment attenuated relapse-like alcohol drinking:

We determined the effect of ceftriaxone (200 mg/kg/day, i.p., last five days of alcohol abstinence) treatments on relapse-like alcohol drinking during seven days of alcohol re-exposure (Fig 3-1). Ceftriaxone treatments significantly decreased alcohol drinking from Day 3 through Day 7 of alcohol re-exposure. Two-way ANOVA, followed by Bonferroni’s multiple comparison test, revealed a significant effect of day [F (7, 42) =5.011, p=0.0003] and a significant effect of treatment [F (1, 6) =14.65, p=0.0087]. The Bonferroni’s multiple comparison tests revealed that alcohol drinking was significantly lower in EtOH-CEF group compared to EtOH-Sal on Day 3 through Day 7 of alcohol re-exposure (p<0.05 for Day 3 and 4, p<0.01 for Day 5 through Day 7) (Fig 3.2).
Fig 3-1: Experimental design for alcohol drinking paradigm and micro-injections schedules.

Fig 3-2: Effect of ceftriaxone treatments on relapse-like alcohol drinking. Ceftriaxone treatments attenuated relapse-like alcohol drinking from Day 3 through Day 7. Two-way ANOVA followed by Bonferroni’s multiple comparison tests. n=7/group. *, p<0.05; **, p<0.01. All data are expressed as mean ± SEM.
3.2.2. Relapse-like alcohol drinking did not affect glutamate uptake in NAc:

Glutamate uptake was determined through radio-active glutamate uptake assay in crude membrane fractionation of NAc. Two-tailed unpaired t-test revealed that Na\(^+\)-dependent glutamate uptake in NAc of ethanol-drinking (EtOH) group was not significantly different compared to water-drinking (control) group (t \((11) = 1.276, p=0.2284\) ) (Fig). Similarly, Na\(^+\)-independent glutamate uptake was unaffected in NAc EtOH group compared to control group (t \((10) = 0.074, p=0.9421\) ) (Fig 3-3).

**Fig 3-3:** Effects of relapse-like alcohol drinking on Na\(^+\)-dependent (A) and Na\(^+\)-independent (B) glutamate uptake in NAc. Relapse-like alcohol drinking did not affect Na\(^+\)-dependent and Na\(^+\)-independent glutamate uptake in NAc compared to water drinking control group. Two-tailed unpaired t-test. n=6/group. All data are represented as mean ±SEM.
3.2.3. Relative gene expression of GLT-1 and xCT in NAc was unaffected by relapse-like alcohol drinking:

The relative gene expression of GLT-1 and xCT was determined in NAc using RT-qPCR. Two-tailed unpaired t-test revealed that the relative gene expression of GLT-1 in NAc of EtOH group was unaffected compared to control group ($t_{(10)}=0.566$, $p=0.5833$). Similarly, two-tailed unpaired t-test revealed that relapse-like alcohol drinking for seven days did not affect the relative gene expression of xCT in NAc of EtOH group compared to control group ($t_{(10)}=2.016$, $p=0.714$).

3.2.4. Relapse-like alcohol drinking increased relative gene expression of mGlu$_5$ without affecting mGlu$_{1/2/3}$ in NAc:

The metabotropic glutamate receptors modulate the synaptic glutamate release. Thus, we determined the relative gene expression of group-I (mGlu$_{1/5}$) and group-II (mGlu$_{2/3}$) metabotropic glutamate receptors in NAc through RT-qPCR. Two-tailed unpaired t-test showed that relapse-like alcohol drinking increased the relative gene expression of mGlu$_5$ in NAc of EtOH group compared to control group ($t_{(9)}=2.347$, $p=0.0435$). However, the relative gene expression of mGlu$_1$ ($t_{(10)}=0.7183$, $p=0.4890$), mGlu$_2$ ($t_{(9)}=0.5011$, $p=0.6284$) and mGlu$_3$ ($t_{(10)}=0.2366$, $p=0.8177$) was unaffected in NAc of EtOH group compared to control group (Fig 3-4).
Fig 3-4: Effects of relapse-like alcohol drinking on relative gene expression of mGlu₁ (A), mGlu₂ (B), mGlu₃ (C) and mGlu₅ (D). Relapse-like alcohol drinking significantly increased relative gene expression of mGlu₅ with affecting relative gene expression of mGlu₁/₂/₃. Two-tailed unpaired t-test. n=5-6/group. *, p<0.05. All data are presented as mean ± SEM.

3.2.5. Effects of GLT-1 suppression on relapse-like alcohol and water drinking: 

The GLT-1 sense (control) and GLT-1 anti-sense vivo-morpholinos were microinjected into NAc of rats exposed to alcohol for three days starting on Day 8 of alcohol abstinence. Following microinjections, GLT-1 sense microinjected rats were treated with saline, and GLT-1 anti-sense microinjected rats were treated with saline or ceftriaxone (200 mg/kg/day, i.p.) for five consecutive days. Two-way ANOVA, followed by Bonferroni’s multiple comparison tests, revealed that there is no significant effect of treatment [F(2, 18) = 1.58, p=0.2329] and no significant day x treatment interaction [F(14, 126) = 1.068, p= 0.3927] in alcohol drinking (Fig 3-5).
Fig 3-5: Effect of suppression of GLT-1 during ceftriaxone treatment on relapse-like alcohol drinking. GLT-1 suppression with anti-sense vivo-morpholinos prevented the ceftriaxone-induced attenuation of alcohol drinking. Two-way ANOVA followed by Bonferroni’s multiple comparison tests. n=6-8/group. All data are expressed as mean ± SEM.

Bonferroni’s multiple comparison tests showed no significant difference in alcohol drinking between GLT-1 sense-saline (GLT-1 S-Sal) and GLT-1 anti-sense-saline (GLT-1 AS-Sal) groups from Day 1 through Day 7 (p<0.99 for Day 1, 3, 4, 5 and 6; P=0.96 for Day 2; and p=0.703 for Day 7). Bonferroni’s multiple comparison tests also revealed that the relapse-like alcohol drinking was not significantly different between GLT-1 AS-Sal
and GLT-1 AS-CEF groups from Day 1 through Day 7 (p=0.184 for Day 1; p>0.99 for Day 2, 3 and 7; p=0.07 for Day 4; p=0.186 for Day 5 and p=0.518 for Day 6). The baseline alcohol drinking is not significantly different among GLT-1 S-Sal, GLT-1 AS-Sal and GLT-1 AS-CEF groups.

We also determined the effect of GLT-1 suppression on water drinking during relapse period. Two-way ANOVA, followed by Bonferroni’s multiple comparison test, revealed that there was a significant effect of treatment [F (2, 19) =14.32, p=0.0002] and a significant day x treatment interaction [F (14, 133) = 2.721, p=0.0015] in water drinking. Bonferroni’s multiple comparison test showed that water drinking between GLT-1 S-Sal and GLT-1 AS-Sal groups was not significantly different from day 1 through day 7 (p=0.245 for Day 2 and p<0.99 for Day 1, 3, 4, 5, 6, 7). In contrast, ceftriaxone (200 mg/kg/day) treatments significantly increased water drinking in GLT-1 AS-CEF group compared to GLT-1 AS-Sal group on day 1 through 6 (p=0.0018 for Day 1; p=0.0012 for Day 2; p=0.001 for Day 3; p=0.0001 for Day 4; p=0.0003 for Day 5 and p=0.037 for Day 6). The baseline water drinking was not significantly different among GLT-1 S-Sal, GLT-1 AS-Sal and GLT-1 AS-CEF groups (Fig 3-6).
Fig 3-6: Effect of suppression of GLT-1 during ceftriaxone treatment on water drinking. Ceftriaxone treatments during the GLT-1 suppression significantly increased water drinking during day 1 through day 6 of the relapse-period. Two-way ANOVA followed by Bonferroni’s multiple comparison tests. n=6-8/group. All data are expressed as mean ± SEM.

3.2.6. Effects of suppression of xCT on relapse-like alcohol and water drinking:

The xCT sense (control) and xCT anti-sense vivo-morpholinos were microinjected into NAc of alcohol drinking rats for three days starting on day 8 of alcohol abstinence. Following microinjections, xCT sense microinjected rats were treated with saline and xCT anti-sense microinjected rats were treated with saline or ceftriaxone (200
mg/kg/day) for five consecutive days. Two-way ANOVA, followed by Bonferroni’s multiple comparison tests, revealed that there is no significant day x treatment interaction [F (14, 119) = 1.086, p= 0.378] in alcohol drinking.

Bonferroni’s multiple comparison tests showed no significant difference in alcohol drinking between xCT sense-saline (xCT S-Sal) and xCT anti-sense-saline (xCT AS-Sal) groups from day 1 through day 7 (p<0.99 for Day 1, 2, 3, 4, 6 and 7; p=0.136 for Day 5). Bonferroni’s multiple comparison tests also revealed that the relapse-like alcohol drinking was not significantly different between xCT AS-Sal and xCT AS-CEF groups from day 1 through day 7 (p=0.239 for Day 1; p=0.206 for Day 2; p<0.99 for Day 3 and 4; p=0.082 for Day 5; p=0.776 for Day 6 and p=0.062 for Day 7). The baseline alcohol drinking is not significantly different among xCT S-Sal, xCT AS-Sal and xCT AS-CEF groups (Fig 3-7).
Fig 3-7: Effect of suppression of xCT during ceftriaxone treatment on relapse-like alcohol drinking. xCT suppression with anti-sense vivo-morpholinos prevented the ceftriaxone-induced attenuation of alcohol drinking. Two-way ANOVA followed by Bonferroni’s multiple comparison tests. n=6-7/group. All data are expressed as mean ± SEM.

We also determined the effect of xCT suppression on water drinking during relapse period. Two-way ANOVA, followed by Bonferroni’s multiple comparison test, revealed that there was a significant day x treatment interaction [F (14, 126) = 3.049, p=0.0004] in water drinking. Bonferroni’s multiple comparison test showed that water drinking between xCT S-Sal and xCT AS-Sal groups was not significantly different from day 1 through day 7 (p>0.99 for Day 1 through Day 7). In contrast, ceftriaxone (200 mg/kg/day) treatments significantly increased water drinking in xCT AS-CEF group compared to xCT AS-Sal group on Day 1 through 7 (p=0.0002 for Day 1 and 4; p<0.0001 for Day 2, 3, 5, 6 and 7). The baseline water drinking was not significantly different among xCT S-Sal, xCT AS-Sal and xCT AS-CEF groups.
Fig 3-8: Effect of suppression of xCT during ceftriaxone treatment on water drinking. Ceftriaxone treatments during the GLT-1 suppression significantly increased water drinking during day 1 through day 7 of the relapse-period. Two-way ANOVA followed by Bonferroni’s multiple comparison tests. n=6-7/group. All data are expressed as mean ± SEM.

3.3. Discussion:
It is well established that alcohol exposure or withdrawal results in increased extracellular glutamate in various brain regions, including NAc. The increased extracellular glutamate within NAc might be the outcome of following phenomenon (s): decreased glutamate uptake through GLT-1 and/or increased glutamate release through xCT into extrasympathetic space and/or dysregulation in modulation of synaptic glutamate release through
mGluRs. Here, we investigated the involvement of above mentioned possibilities within NAc of P rats and determined whether or not both GLT-1 and xCT upregulation are required for CEF-induced attenuation of relapse-like alcohol drinking.

GLT-1 is the major glutamate transporter which accounts for >90% of extracellular glutamate uptake (Tanaka et al., 1997). Alternatively, xCT releases glutamate into extrasynaptic space and accounts for ~60% of that extracellular glutamate (Baker et al., 2002). Glutamate uptake by GLT-1 and glutamate release through xCT should be in equilibrium to maintain physiological extracellular glutamate concentration. Abnormality in any one of these transporters would result in imbalanced glutamate homeostasis. Here we reveal that relapse-like alcohol drinking for seven days did not alter Na+-dependent or Na+-independent glutamate uptake. These findings signify that glutamate uptake through GLT-1 or glutamate release through xCT does not play a pivotal role for hyper-neuroexcitation within NAc following relapse-like alcohol drinking. Similar results have been reported in recent years showing that repeated alcohol administration or chronic intermittent alcohol did not alter glutamate transport (Chefer et al., 2011, Griffin et al., 2015).

The unaltered glutamate uptake can be the result of unaltered gene/protein expression of GLT-1 and xCT. To test this possibility, we determined the relative gene expression of GLT-1 and xCT along with their relative expression. The RT-qPCR data revealed that the relative gene expression of GLT-1 and xCT were not significantly different following relapse-like alcohol drinking. These data explain more that glutamate uptake has less to
do with increased glutamate following relapse-like alcohol consumption. These data are consistent with our previous findings that expression of xCT and GLT-1 isoforms was unaltered following relapse-like-alcohol drinking (Alhaddad et al., 2014).

Next, we determined the relative gene expression of group-I (mGlu_{1/5}) and group-II (mGlu_{2/3}) metabotropic glutamate receptors. Relapse-like alcohol drinking for seven days did not alter the relative gene expression of mGlu_2 and mGlu_3 receptors. mGlu_{2/3} receptors are negatively coupled to adenylyl cyclase and activation of these receptors results in decreased glutamate release from pre-synaptic terminals (Nicoletti et al., 2011). Thus, unchanged gene expression of mGlu_{2/3} reveals that increased glutamate neurotransmission following alcohol might not be due to increased glutamate release through mGlu_{2/3} receptors in relapse-like alcohol drinking model. Alternatively, relapse-like alcohol drinking increased the relative gene expression of mGlu_5 receptor without affecting relative gene expression of mGlu_1. mGlu_{1/5} receptors are positively coupled to phosphoinositide hydrolysis and activation of these receptors results in increased neurotransmission (Cleva and Olive, 2012). Therefore, increased gene expression of mGlu_5 receptors signifies that relapse-like alcohol drinking might increase glutamatergic neurotransmission in NAc of P rats through mGlu_5 receptors. This finding reassures the significant role of mGlu_5 receptors in relapse-like alcohol drinking since blockade of mGlu_5 receptors was associated with decreased relapse-like alcohol drinking in rodents (Backstrom et al., 2004).

Although glutamate uptake and relative gene expression of GLT-1 and xCT remained unchanged following relapse-like alcohol drinking, we showed previously that
upregulation of both GLT-1 and xCT through CEF treatment attenuated relapse-like alcohol drinking. Here, we suppressed the expression of GLT-1 or xCT with vivo-morpholinos during CEF treatment to determine whether upregulation of both GLT-1 and xCT expression is required for CEF-induced attenuation of relapse-like alcohol drinking. GLT-1 suppression with anti-sense vivo-morpholinos prevented the CEF-induced attenuation of relapse-like alcohol drinking with increased water drinking. This finding assures that GLT-1 upregulation is mandatory in mechanism of CEF to prevent relapse-like alcohol drinking. This is also consistent with previous reports showing that deletion/blockade of GLT-1 precipitated impaired spatial memory, depression-like symptoms and anhedonia (Bechtholt-Gompf et al., 2010, John et al., 2012, John et al., 2015). Surprisingly, xCT suppression with anti-sense vivo-morpholinos also prevented the CEF-induced attenuation of relapse-like alcohol drinking with increased water drinking. Together, these data reveal that both GLT-1 and xCT are crucial to control relapse-like alcohol drinking and upregulation of both transporters is the key to prevent relapse-like alcohol drinking.

In conclusion, relapse-like alcohol drinking did not affect glutamate uptake and relative gene expression of GLT-1 and xCT in NAc of P rats. Relapse-like alcohol drinking was associated with increased relative gene expression of mGlu$_{5}$ receptors without affecting relative gene expression of mGlu$_{1/2/3}$. Upregulation of both GLT-1 and xCT can serve as a potential therapeutic intervention to attenuate relapse-like alcohol drinking.
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Chapter 4

Binge ethanol withdrawal: Effects on post-withdrawal ethanol intake, glutamate-glutamine cycle and monoamine tissue content in P rat model

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Abstract

Alcohol withdrawal syndrome (AWS) is a medical emergency situation which appears after abrupt cessation of ethanol intake. Decreased GABA-A function and increased glutamate function are known to exist in the AWS. However, the involvement of glutamate transporters in the context of AWS requires further investigation. In this study, we used a model of ethanol withdrawal involving abrupt cessation of binge ethanol administration (4 g/kg/gavage three times a day for three days) using male alcohol-preferring (P) rats. After 48 hours of withdrawal, P rats were re-exposed to voluntary ethanol intake. The amount of ethanol consumed was measured during post-withdrawal phase. In addition, the expression levels of GLT-1, GLAST and xCT were determined in both medial prefrontal cortex (mPFC) and nucleus accumbens (NAc). We also measured glutamine synthetase (GS) activity, and the tissue content of glutamate, glutamine, dopamine and serotonin in both the mPFC and NAc. We found that binge ethanol withdrawal escalated post-withdrawal ethanol intake which was associated with downregulation of GLT-1 expression level in both the mPFC and NAc. The expression levels of GLAST and xCT were unchanged in the ethanol-withdrawal (EW) group compared to control group. Tissue content of glutamate was significantly lower in both mPFC and NAc, whereas tissue content of glutamine was higher in mPFC but unchanged in NAc in the EW group compared to control group. The GS activity was unchanged in both mPFC and NAc. The tissue content of DA was significantly lower in both mPFC and NAc, whereas tissue content of serotonin was unchanged in both mPFC and NAc. These findings provide important information of the critical role of GLT-1 in context of AWS.
Introduction

Alcohol withdrawal syndrome (AWS) comprises of signs and symptoms (autonomic/neuropsychiatric) and appear up to 48 hours of abrupt cessation of binge ethanol drinking (Murdoch and Marsden, 2014). The underlying neuropathology of AWS involves decreased GABA-A inhibitory function and increased glutamatergic excitatory activity leading to rebound hyper-neuroexcitability, irritability, and seizure (Finn and Crabbe, 1997, Longo et al., 2011, Abulseoud et al., 2014). The first line treatment of AWS is benzodiazepines, which target GABAergic pathways (Mayo-Smith, 1997, Amato et al., 2011, Abulseoud et al., 2014). Other approaches to deal with AWS include blockade of the NMDA receptor (e.g. acamprosate) (Brust, 2014, Liang and Olsen, 2014) and use of conjunctive agents (cardiovascular agents such as clonidine, propranolol and magnesium sulfate; vitamins and antidotes) for symptomatic relief.

The ultimate target of the most neurochemical agents when treating the AWS is to restore balance between excitatory and inhibitory neurotransmission. The homeostasis of glutamate, the major excitatory neurotransmitter, is maintained through equilibrium between glutamate uptake and glutamate release in or out of neighboring astrocytes, respectively (Kalivas, 2009). Glutamate uptake into astrogial cells and neurons is mediated through Na⁺-dependent transmembrane proteins belonging to solute carrier 1 (SLC1) family. To date, five subtypes of excitatory amino acid transporters (EAAT) have been identified: EAAT 1-5. EAAT-1/glutamate aspartate transporter (GLAST) expressed in astroglial cells (Lehre et al., 1995), EAAT 2 (GLT-1) is expressed primarily in astroglial cells (also expressed at lower extent in neurons) (Chen et al., 2004, Fontana,
2015), EAAT 3 is expressed in neuronal cell bodies and dendrites (Rothstein et al., 1994), EAAT 4 is primarily expressed in cerebellar Purkinje cells (Gincel et al., 2007) and EAAT 5 is expressed in vertebrate retina (Arriza et al., 1997). Thus, EAAT 1/ GLAST and EAAT 2/ GLT-1 play pivotal role for tight regulation of extra-synaptic glutamate in the mPFC and NAc; and have been focused in this study. It is important to note that GLT-1 uptakes the majority of extracellular glutamate (Danbolt, 2001, Mitani and Tanaka, 2003). Furthermore, there is existence of another glial transporter protein, cystine/ glutamate exchanger (xCT), which regulates the release of glutamate from astrocytes in exchange for cystine (Warr et al., 1999, Melendez et al., 2005).

In clinics, less is known about the involvement of glutamate transporters in hyper-neuroexcitation in patients undergoing AWS, which can be a promising target of drug treatment. The effects of imported glutamate within astrocytes include: formation of glutamine by glutamine synthetase (GS) enzyme, exchanging for cystine to the extracellular space through xCT, and subsequent formation of glutathione. The formed glutamine within astrocytes is further used up by neurons, and this recycle process is termed the glutamate-glutamine cycle (Thoma et al., 2011). Thus, GLT-1, xCT and GLAST play key role for regulating extracellular glutamate concentration in the brain.

In this study, we used a model of ethanol withdrawal to simulate the context of AWS involving abrupt cessation of binge ethanol intake (4 g/kg/gavage three times a day for three days) in alcohol preferring (P) rats. We tested the effects of binge ethanol withdrawal on post-withdrawal ethanol intake as well as expression levels of GLT-1, xCT
and GLAST in medial prefrontal cortex (mPFC) and nucleus accumbens (NAc). It is noteworthy that glutamatergic projection from PFC to NAc has been shown to initiate adaptive behaviors, and stimulation of either region increases drug seeking (Moussawi and Kalivas, 2010). Thus, mPFC and NAc were areas of focus in this current study because of their crucial role in drug dependence. Beside glutamate transporter expression levels, we determined the tissue content of glutamate and glutamine as well as GS activity to test the effects of binge ethanol withdrawal on the glutamate-glutamine cycle. Since ethanol acts as a positive reinforcer, we hypothesized that binge ethanol withdrawal would affect dopamine (DA) and serotonin (5-HT) concentrations. Therefore, we also determined the tissue content of DA and 5-HT in both mPFC and NAc.

4.1. Methods and materials

4.1.1. Animals

Male P rats were acquired from Indiana University School of Medicine, Indianapolis; IN. Rats were singly housed in a room with 21°C temperature and 50% humidity in a 12h light/dark cycle. Rats had unrestricted food and water throughout the whole experimental procedure. Institutional Animal Care and Use Committee (IACUC) of The University of Toledo approved all the experimental procedures 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 of Life sciences, 1996).
4.1.2. Voluntary ethanol drinking and ethanol gavage procedures

Three-month-old male P rats were exposed to voluntary drinking of ethanol (15% and 30%, v/v, concurrently), and/ or water for two weeks (Fig. 1A). P rats then received ethanol through oral gavage needle at a dose of 4 g/kg/gavage (infusion volume: 4-5.7 ml) three times a day (12 g ethanol/kg/day, made from 40% v/v ethanol) for three days. After completion of the ethanol gavage treatment, rats went through 48 hours of ethanol withdrawal. After 48 hours of withdrawal period, ethanol-gavaged P rats were re-exposed to voluntary ethanol drinking (15% and 30%, concurrently) for one week. Control (Ctrl) rats received water through oral gavage at same time points as of ethanol-gavaged group.

4.1.3. Western Blot

After one-week of post-withdrawal voluntary ethanol drinking, P rats were euthanized with carbon dioxide inhalation and brains were removed, and flash frozen in dry ice. mPFC and NAc were dissected in a cryostat apparatus according to Paxinos and Watson (1998). GLT-1, xCT, GLAST and GAPDH expression levels were determined through western blot procedure as previously described (Alhaddad et al., 2014, Das et al., 2015). Briefly, extracted proteins were separated through SDS-PAGE and transferred onto PVDF membrane electrophoretically. Then PVDF membranes were incubated overnight at 4°C with one of the following primary antibodies: guinea pig anti-GLT-1 (Millipore), rabbit anti-xCT (Abcam), rabbit anti-GLAST (Abcam), and mouse anti-GAPDH (Millipore). Protein detection was performed using a chemiluminescent kit and developed onto HyBlot.
CL film (Denville Scientific Inc). Blots were digitized and quantified with MCID system. Data were calculated as ratios of GLT-1/, xCT/ and GLAST/GAPDH.

4.1.4. HPLC quantification of glutamate and glutamine

Glutamate and glutamine concentrations in the mPFC and NAc were simultaneously analyzed using an HPLC system with electrochemical detection as previously described (Reader et al., 1998, Das et al., 2015). Briefly, dissected brain samples were homogenized in distilled water with pestle followed by heating at 98°C for 5 min. The samples were then centrifuged at 10,000 rpm at 4°C for 5 min. After centrifugation, supernatants were collected and filtered through 0.22 μm filters, and pellets were tested for protein quantification. O-phthalaldehyde and sodium sulfite were used for pre-column derivatization of filtered supernatants with an ESA Model 540 autosampler before injecting onto a C18 column (3.0 x 50 mm, 2.5 μm particle size, Waters, Inc.). The mobile phase consisted of 0.1M Na₂HPO₄, 0.1mM EDTA, and 7.5% Methanol (pH 3.0). The CoulArray coulometric detector (model 5600A, ESA, Inc.) was used for detection of glutamate and glutamine, and the chromatograms were collected through CoulArray software. The concentrations of glutamate and glutamine were analyzed by peak area and compared with external standards. Tissue pellets were resuspended in 1N NaOH and protein concentration was determined using the Lowry method (Lowry et al., 1951). The concentrations of glutamate and glutamine were normalized relative to protein content.
4.1.5. HPLC quantification of monoamines

DA and 5-HT concentrations in mPFC and NAc were analyzed with an HPLC-EC system as previously described (Breier et al., 2006, Halpin and Yamamoto, 2012). Briefly, dissected brain regions were sonicated in 0.25N perchloric acid followed by centrifugation at 14000 x g for 20 min at 4°C. The supernatants were filtered (0.22 µm filter) and injected onto a C18 column (3.2 x 150 mm, 3µm particle size, Thermo Scientific). The mobile phase consisted of 32 mM citric acid, 54.3 mM sodium phosphate, 0.215 mM octyl sodium sulphate, and 11% methanol (pH 4.4). The CoulArray coulometric detector (model 5600A, ESA, Inc.) was used for detection of DA and 5-HT. The concentrations of DA and 5-HT were determined by peak area and compared with external standards. Pellets obtained through centrifugation were resuspended in 1N NaOH and protein content was determined using the Lowry method. The concentrations of DA and 5-HT were normalized relative to respective protein content.

4.1.6. Glutamine synthetase (GS) activity assay

GS activity was measured as previously described (Das et al., 2015). Briefly, tissue homogenates were mixed with assay mixtures followed by incubation at 37°C for 30 min. The reaction was then stopped by addition of GS stop solution. Parallel incubations of tissue homogenates with assay mixtures lacking Na-arsenate and ADP served as control.
4.1.7. Statistical analysis

All statistical analyses were performed using GraphPad Prism software. Pre- and post-withdrawal ethanol drinking was compared with paired t-test. The comparison between control and ethanol-withdrawal (EW) groups were conducted with unpaired t-test. The statistical significance was set at p < 0.05.

4.2. Results

4.2.1. Effect of binge ethanol withdrawal on post-withdrawal ethanol intake

We measured post-withdrawal ethanol intake during one-week period of relapse to ethanol drinking. Paired t-test revealed that post-withdrawal ethanol drinking was significantly
Figure 4-1. Effect of EW on post-withdrawal ethanol drinking. A) Timeline of ethanol drinking and withdrawal period. B) EW significantly increased the post-withdrawal ethanol drinking compared to pre-withdrawal drinking (*, p<0.05). All data are expressed as mean ± SEM. n = 7. EW, ethanol withdrawal.

4.2.2. Effect of binge ethanol withdrawal on GLT-1, xCT and GLAST expression levels as well as GS activity in mPFC and NAc

GLT-1, xCT and GLAST protein expression levels were determined using Western blot assay. Unpaired t-test revealed that GLT-1 protein expression level was significantly lower in mPFC of the EW group compared to control group (t (13) = 3.316, p= 0.005) (Fig. 4-2A). However, unpaired t-test showed no significant difference in expression level of GLAST (t (13) = 0.079, p=0.938) (Fig. 4-2B) and xCT (t (13) = 0.225, p= 0.825) (Fig. 4-3A) in mPFC of the EW group compared to control group.
Figure 4-2. Effects of EW on expression levels of GLT-1 and GLAST in mPFC and NAc. A) GLT-1 expression level was significantly downregulated in the EW group compared to control group in both mPFC (**, p<0.01) and NAc (*, p<0.05). B) GLAST expression level was unchanged in the EW group compared to control group in both mPFC and NAc. \( n= 7-8 \) rats/group. Ctrl, control; EW, ethanol-withdrawal.

Unpaired t-test showed no significant difference in expression level of GLAST \( (t_{(13)} = 0.507, p=0.620) \) (Fig. 2A) and xCT \( (t_{(13)} = 0.336, p=0.741) \) (Fig. 3A) in NAc of the EW group compared to control group. In addition, unpaired t-test showed no significant difference in GS activity in mPFC \( (t_{(13)} = 0.611, p=0.551) \) and NAc \( (t_{(13)} = 0.982, p=0.344) \) of the EW group compared to control group (Fig. 3B).
Figure 4-3. Effects of EW on expression level of xCT and GS activity in both mPFC and NAc.  A) The expression level of xCT was not significantly different in the EW group compared to control group in both mPFC and NAc.  B) The GS activity was not significantly different in EW group compared to control group in both mPFC and NAc. n=7-8 rats/group. Ctrl, control; EW, ethanol-withdrawal.

4.2.3. Effects of binge ethanol withdrawal on tissue content of glutamate and glutamine in mPFC and NAc

Tissue content of glutamate and glutamine was determined in mPFC and NAc using HPLC-EC system. Unpaired t-test revealed that tissue content of glutamate was significantly lower in mPFC of the EW group compared to control group (t (12) = 2.795, p=0.016) (Fig. 4-4A). However, unpaired t-test showed significantly higher tissue content of glutamine in mPFC of the EW group compared to control group (t (13) = 2.504, p=0.026) (Fig. 4-4B).

Similarly, unpaired t-test revealed that tissue content of glutamate was significantly lower in NAc of the EW group compared to control group (t (10) = 2.230, p= 0.049) (Fig. 4-4A). Surprisingly, unpaired t-test showed no significant difference in tissue content of glutamine in NAc of the EW group compared to control group (t (14) = 0.092, p= 0.927) (Fig. 4-4B).
Figure 4-4. Effects of EW on tissue content of glutamate (A) and glutamine (B) in both mPFC and NAc. A) Tissue content of glutamate was significantly lower in both mPFC and NAc of the EW group compared to control group (*, p<0.05). B) Tissue content of glutamine was significantly higher in mPFC of the EW group compared to control group (*, p<0.05). Tissue content of glutamine was unchanged in NAc of the EW group compared to control group. All data are expressed as mean ± SEM. n=6-8 rats/group. Ctrl, control; EW, ethanol-withdrawal.
4.2.4. Effects of binge ethanol withdrawal on tissue content of dopamine in mPFC and NAc

Tissue content of DA and 5-HT was measured in mPFC and NAc using HPLC-EC system. Unpaired t-test revealed that tissue content of DA was significantly lower in mPFC ($t_{(14)} = 3.185$, $p=0.006$) and NAc ($t_{(11)} = 2.462$, $p=0.031$) of the EW group compared to control group (Fig. 4-5A). However, unpaired t-test revealed no significant difference in tissue content of 5-HT in mPFC ($t_{(13)} = 0.155$, $p=0.878$) and NAc ($t_{(12)} = 1.151$, $p=0.272$) of the EW group compared to control group (Fig. 4-5B).
Figure 4-5. Effects of EW on tissue content of DA (A) and 5-HT (B) in both mPFC and NAc. A) Tissue content of dopamine (DA) was significantly lower in both PFC (**, p<0.01) and NAc (*, p<0.05) of the EW group compared to control group. B) Tissue content of serotonin (5-HT) was unaltered in both mPFC and NAc of the EW group compared to control group. All data are represented as mean ± SEM. n= 6-8 rats/group. Ctrl, control; EW, ethanol-withdrawal.

4.3. Discussion

In this study, we found that binge ethanol withdrawal significantly increased the post-withdrawal ethanol drinking compared to pre-withdrawal drinking. This withdrawal model used in this experiment has been successfully used in previous studies investigating the induction of physical dependence and withdrawal signs through abrupt cessation of binge ethanol intake (9-15 g/kg/day) for 3-4 days (Majchrowicz, 1975, Abulseoud et al., 2014). Withdrawal-induced escalation of ethanol drinking, observed in this study, is consistent with previous studies showing elevated ethanol drinking following intermittent ethanol exposure and/or withdrawal period (Carrara-Nascimento et al., 2013, Abulseoud et al., 2014).

Interestingly, we observed in this study that binge ethanol withdrawal was associated with downregulation of GLT-1 expression level in both mPFC and NAc. In contrast to this present finding, we previously reported that five weeks of voluntary ethanol drinking downregulated GLT-1 expression level in NAc, but not in mPFC (Sari and Sreemantula, 2013, Abulseoud et al., 2014).
2012, Goodwani et al., 2015). This discrepancy might be attributed to the use of binge ethanol drinking and severe withdrawal vs five weeks of voluntary ethanol drinking. Some other previous reports showed no change in expression of GLT-1 in PFC (Alele and Devaud, 2005) or NAc (Melendez et al., 2005, Ding et al., 2013) following systemic administration or voluntary drinking of ethanol. However, our present finding of down-regulated GLT-1 expression level is consistent with previous report showing downregulation of GLT-1 expression level in mPFC and NAc with similar ethanol withdrawal paradigm (Abulseoud et al., 2014). GLT-1 downregulation or knockdown was frequently reported to be associated with increased extracellular glutamate concentration (Rothstein et al., 1996, Das et al., 2015). Since AWS is well known to be associated with increased excitatory and decreases inhibitory neurotransmission in the brain, our findings provide evidence that GLT-1 could be a potential therapeutic targets for decreasing excitatory neurotransmission and alleviating AWS.

However, in this current study, we didn’t find any changes in expression levels of GLAST in mPFC and NAc. GLAST plays a predominant role for glutamate uptake in cerebellum, whereas GLT-1 plays predominant role for glutamate uptake in mPFC and NAc (Abulseoud et al., 2014). Thus, GLAST might play a different role in mPFC and NAc following voluntary ethanol drinking. Our current finding with GLAST expression is consistent with previous reports showing no change of GLAST expression following voluntary ethanol drinking (chronic or relapse-like) (Alhaddad et al., 2014) or systemic ethanol administration (Melendez et al., 2005).
Similar to GLAST expression level, the current study revealed that the expression level of xCT was unaltered in both mPFC and NAc. xCT, catalytic subunit of system X_c, pump out astrocytic glutamate into extracellular space in exchange of cystine. Thus, xCT-activity contribute about 60% to the extracellular glutamate concentration (Kalivas, 2009). There are compelling evidences that basal glutamate concentration increased in various brain regions following ethanol dependence (Melendez et al., 2005, Ding et al., 2013, Das et al., 2015). Our present finding suggests that the expression of xCT doesn’t contribute to increased basal glutamate concentration, at least not in rats undergone through severe ethanol withdrawal. Our present finding with xCT expression is consistent with previous literature showing unchanged xCT expression or activity following ethanol dependence (Ding et al., 2013, Alhaddad et al., 2014, Griffin et al., 2015). Taken together, only GLT-1 expression level was downregulated among GLT-1, GLAST and xCT expression levels following severe withdrawal in both mPFC and NAc.

GLT-1 clears the majority of extracellular glutamate into astrocytes and imported glutamate is converted into glutamine by GS. Thus, we hypothesized that GS activity would have been affected following severe ethanol withdrawal. Surprisingly, GS activity was not altered in both mPFC and NAc. Our finding differs from a previous report showing increased packing density of GS-immunoreactive astrocytes in cortex of P rats withdrawn from two- and six-months ethanol exposure (Miguel-Hidalgo, 2006). This difference can be attributed to severe withdrawal (48 hours) followed by one week relapse vs withdrawal (three days) from voluntary ethanol drinking with no relapse drinking.
However, our current finding is consistent with our previous report showing no change in GS activity following five-week of voluntary ethanol drinking (Das et al., 2015).

In this current study, we also determined the tissue content of glutamate and glutamine in both mPFC and NAc. Interestingly, tissue content of glutamate was significantly lower in both mPFC and NAc. Tissue content of glutamine was significantly higher in mPFC, but unchanged in NAc. It is well established that ethanol withdrawal is associated with increased extracellular glutamate concentration in striatum (Rossetti and Carboni, 1995, Rossetti et al., 1999), NAc (Hinton et al., 2012) and hippocampus (Dahchour and De Witte, 2003), but not in mPFC (Hinton et al., 2012). However, tissue content of glutamate doesn’t differentiate between extracellular and intracellular pool, and thus the results become difficult to interpret. The reason behind the present finding of decreased tissue content of glutamate remains unknown, but the findings confirm altered glutamate homeostasis following severe withdrawal. The increased tissue content of glutamine in PFC might be due to the decreased conversion of glutamine into glutamate since GS activity was unchanged. The unchanged tissue concentration of glutamine in NAc explains differential adaptations in mPFC and NAc following severe withdrawal. However, our current finding of decreased glutamate and increased glutamine content is consistent with a previous study conducted on human volunteers through proton magnetic resonance spectroscopy with alcohol dependence (Thoma et al., 2011).
Finally, we tested the hypothesis that relapse to ethanol following severe withdrawal would affect the tissue content of dopamine (DA) and serotonin (5-HT) in mPFC and/or NAc. Tissue content of DA was significantly lower in both mPFC and NAc. Ethanol (as a positive reinforcer) is known to increase dopamine in NAc whereas ethanol withdrawal is associated with marked decrease in DA concentration in both mPFC and NAc (Weiss and Porrino, 2002, Carlson and Drew Stevens, 2006). Our present finding with DA suggests that severe withdrawal disrupts dopamine concentration, which might be a driving force to relapse. It is also evident that depleted DA was not reversed by one-week relapse to ethanol drinking. On the other hand, tissue content of 5-HT was unchanged in both mPFC and NAc. Withdrawal from chronic ethanol intake had been shown to markedly deplete 5-HT in NAc of ethanol-dependent rats and depleted 5-HT was reversed through ethanol re-exposure (Weiss et al., 1996). Thus, unchanged tissue content of 5-HT in both mPFC and NAc of current study might be attributed to one-week relapse to ethanol drinking.

We conclude that binge ethanol withdrawal escalated post-withdrawal ethanol drinking in association with downregulation of GLT-1 in both mPFC and NAc. Besides, differential alteration in tissue contents of glutamate and glutamine in mPFC and NAc suggested the disruption of glutamate-glutamine cycle with unchanged GS activity. Binge ethanol withdrawal was also associated with marked depletion of DA in both mPFC and NAc. Taken together, the present study implicates GLT-1 as a potential target of drugs for treating AWS.
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Chapter 5

Summary

We conducted our research to investigate the druggable targets within glutamate transporters in nucleus accumbens of P rats for attenuating alcohol dependence. We used three rodent models of alcohol dependence to mimic human alcohol dependence scenarios: A) Chronic voluntary alcohol consumption, B) Relapse-like voluntary alcohol consumption following withdrawal period, and C) Relapse-like voluntary alcohol consumption following binge alcohol withdrawal. These three models have been summarized in the following figure.

A) Chronic voluntary alcohol consumption model:
B) Relapse-like voluntary alcohol consumption following withdrawal period model:

A) Outcomes of chronic alcohol consumption model:
Chronic voluntary alcohol consumption for five weeks caused two-fold increase in extracellular glutamate concentration in nucleus accumbens of P rats. Increase in extracellular glutamate concentration was associated with decrease in the expression of both GLT-1 and xCT in nucleus accumbens. Ceftriaxone treatments for five consecutive days attenuated chronic alcohol consumption with normalization of extracellular glutamate concentration. Ceftriaxone treatments also reversed the expression of GLT-1 and xCT. Reverse perfusion of dihydrokainic acid (DHK) prevented the ceftriaxone-induced decrease of extracellular glutamate concentration indicating GLT-1 mediated action of ceftriaxone. Chronic alcohol consumption did not alter the glutamine synthetase activity in nucleus accumbens of P rats.
The overall outcomes of the chronic alcohol drinking model have been depicted in the following figure-

![Diagram](image)

**Fig 5-1:** Overall outcomes of chronic voluntary alcohol consumption.

**B) Outcomes of relapse-like alcohol consumption following withdrawal model**

In relapse-like alcohol drinking model, P rats had five weeks of voluntary drinking followed by two weeks of withdrawal period. After two-week alcohol withdrawal, P rats had exposure of alcohol for one week. Relapse-like alcohol drinking did not change relative gene and protein expression of GLT-1 and xCT in nucleus accumbens. Both Na\(^+\)-dependent and Na\(^+\)-independent glutamate uptake were unaltered in nucleus accumbens. The relative gene expression of mGlu\(_5\) was significantly increased without affecting relative gene expression of mGlu\(_{1/2/3}\) in NAc. Suppression of GLT-1 or xCT expression prevented the ceftriaxone-induced attenuation of alcohol drinking indicating that
upregulation of both GLT-1 and xCT is the key to prevent relapse-like alcohol drinking. The overall outcomes of the relapse-like alcohol drinking model has been depicted in the following figure-

CEF=Ceftriaxone, NAc=Nucleus Accumbens

Fig 5-2: Overall outcomes of relapse-like alcohol drinking model.

C) Outcomes of relapse-like alcohol consumption following binge alcohol withdrawal model:

In this model, P rats had binge alcohol through oral gavage needle followed by 48 hours of alcohol withdrawal period. After binge withdrawal, P rats had one week of relapse drinking. Binge withdrawal significantly increased the relapse-like alcohol drinking. Increased alcohol drinking was associated with decreased expression of GLT-1 in both prefrontal cortex and nucleus accumbens without affecting expression of xCT and glutamate-aspartate exchanger (GLAST). The glutamine synthetase activity was
unchanged in both PFC and NAc. Relapse-like alcohol drinking decreased the tissue content of glutamate in both PFC and NAc. The tissue content of dopamine was decreased in both PFC and NAc without affecting tissue content of serotonin.

Fig 5-3: Overall outcomes of relapse-like alcohol drinking followed by binge alcohol withdrawal.

GS=Glutamine Synthetase, NAc=Nucleus Accumbens

In conclusion, it is evident that dysregulation in glutamate homeostasis within NAc of P rats promotes chronic alcohol consumption. Ceftriaxone treatment attenuated chronic alcohol consumption through normalization of extracellular glutamate concentration in GLT-1 mediated pathways. However, upregulation of both GLT-1 and xCT was critical for ceftriaxone to attenuate relapse-like alcohol consumption. Overall, upregulation of GLT-1 and xCT might be potential therapeutic targets for attenuation of alcohol dependence.
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Chapter 2


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Chapter 3


Chapter 4


Appendix A

List of Articles Published based on this Dissertation

