2012

Activation of glutamate transporter 1 attenuates relapse to alcohol-seeking behavior in rats

Abeer Qrunfleh
The University of Toledo

A Thesis

Entitled

Activation of Glutamate Transporter 1 Attenuates Relapse to Alcohol-Seeking Behavior in Rats

By Abeer Qrunfleh

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

Master of Science in Pharmacology and Toxicology

_____

Dr. Youssef Sari, Committee Chair

_____

Dr. Ming-Cheh Liu, Committee Member

_____

Dr. Zahoor Shah, Committee Member

_____

Dr. Patricia Komuniecki, Dean
College of Graduate Studies

The University of Toledo
May 2012

An Abstract of

Activation of Glutamate Transporter 1 Attenuates Relapse
to Alcohol-Seeking Behavior in Rats

By Abeer Qrunfleh

Submitted to the Graduate Faculty as a partial fulfillment of the requirement for the Master Degree in Pharmacology and Toxicology

The University of Toledo
May 2012

Relapse to alcohol use after prolonged withdrawal periods is a major problem in the treatment of alcohol addiction in humans. Recent preclinical research has focused on identifying long-term neuroadaptive changes and identifying the behavioral, environmental, and neuronal mechanisms underlying drug relapse. By doing so, potential new avenues for relapse prevention may be developed. Current research suggests that changes in glutamatergic neurotransmission may significantly contribute to alcohol relapse and alcohol addiction. Alcohol dependence has been linked specifically to the increased extracellular glutamate levels in key regions of neurocircuits mainly the mesocorticolimbic circuit. Based on the fact that glutamate transporter1 (GLT1) is responsible for the removal of the majority of extracellular glutamate, we hypothesized that the activation of GLT1 by the use of ceftriaxone, a β-lactam antibiotic, known to elevate GLT1 expression, would attenuate alcohol consumption in alcohol-preferring (P) rats and ultimately prevent relapse to alcohol-seeking behavior. Statistical analyses showed that P rats treated intraperitoneally with ceftriaxone, exhibited a significant reduction in alcohol consumption followed by a period of alcohol deprivation as compared to saline-treated P rats. Preliminary data with Western blot suggests that activation of GLT1 may play a key role in preventing relapse to alcohol-seeking behavior, and

ultimately implicate its potential role as a therapeutic-target for treatment of alcohol dependence.

Acknowledgments

Without the help and support of many people this thesis would have never been completed. Sincere thanks and gratitude goes to Dr. Youssef Sari for giving me the advice, support and the opportunity to participate in this project. I would like to extend my gratitude to the committee members, Dr. Ming-Cheh Liu and Dr. Zahoor Shah. Many thanks go to my lab colleagues Sai Nandini Sreemantula, Manal Gergis, Adnan Alazizi, and Jessica Ciesler for their help and support.

Last but not least, my greatest thanks and gratitude goes to my husband Haitham for his endless support, love and care through this journey. My father who is my idol, my inspiration and my mentor. My mother, who believed in me, and was there for me every step of the way. Your encouragement and support made me who I am today.

Contents

Abstract		iii
Acknowledgments		iv
Contents		v
List of Figures		vi
Introduction		1
1.1	Alcoholism	1
1.2	Neurotransmitter systems involved in alcohol dependence	3
1.3	Neurocircuitry of reward	8
1.4	Animal models of alcoholism	10
1.5	Animal models of alcohol relapse	12
1.6	Glutamate and glutamate transporters	16
1.7	Activation of GLT1 by ceftriaxone, a β-lactam antibiotic	19
1.8	Objectives and goals	21
Material and methods		22
2.1	Animals	22
2.2	Behavioral drinking paradigm	22
2.3	Brain tissue harvesting	24
2.4	Protein tissue extraction protocol	25
2.5	Protein quantification	25

2.6 Western blot procedures 26

Results 28

3.1 Effects of ceftriaxone treatment on alcohol drinking behavior 28

3.2 Effects of ceftriaxone treatment on water intake 30

3.3 Effects of ceftriaxone treatment on body weight 32

3.4 Effects of ceftriaxone treatment on sucrose drinking behavior 33

3.5 Effects of ceftriaxone treatment on GLT1 expression 34

Discussion 37

References 43

List of Figures

Figure 1-1. Circuitry in the development and expression of drug addiction....……..…….10

Figure 1-2. Ceftriaxone chemical structure…….…..…….....19

Figure 3-1. Effects of ceftriaxone treatment on alcohol drinking behavior…….30

Figure 3-2. Effects of ceftriaxone treatment on water intake……...31

Figure 3-3. Effects of ceftriaxone treatment on body weight……. 32

Figure 3-4. Effects of ceftriaxone treatment on sucrose drinking behavior……... 34

Figure 3-5. Effects of ceftriaxone treatment on GLT1 expression in the core…….. 35

Figure 3-6 Effects of ceftriaxone treatment on GLT1 expression in the prefrontal cortex…36

Introduction

1.1 Alcoholism

Alcoholism is a chronic relapsing disorder that is progressive and has serious detrimental health outcomes. The development of alcoholism is characterized by frequent episodes of intoxication, preoccupation with alcohol, use of alcohol despite adverse consequences, compulsion to seek and consume alcohol, loss of control in over its consumption, and emergence of a negative emotional state in the absence of the drug (American Psychiatric Association 1994). According to the National Institute on Alcohol Abuse and Alcoholism (NIAAA), more than 17 million people in the United States either abuse or are dependent on alcohol (NIAAA 2007), with a cost to U.S. society of over $180 billion annually (NIAAA 2004). Nearly 79,000 annual deaths are caused directly or indirectly by excessive alcohol consumption (Center for Disease Control and Prevention, 2008).

The Therapeutic treatments of alcoholism are not effective in alleviating this disorder, partly because alcohol is generally viewed as an unspecific pharmacological agent that has only a few known primary targets, and mainly because the neurobiology behind alcoholism is very complex. Recent work has begun to define the neurocircuits responsible for excessive ethanol drinking (Koob et al., 1998).

A neural circuit can be viewed as a group of neurons that are interconnected and relay information related to a specific function. Within the neural circuit, information is passed between neurons thru various electrochemical signaling processes. Activated

neurons release neurotransmitters that bind to specific receptors on other neurons. Depending on the neurotransmitter involved, this binding leads to the electrical excitation or inhibition of subsequent neurons in the circuit (Gilpin and Koob, 2008).

Despite the fact that drugs of abuse possess various neuropharmacological profiles, activation of the mesocorticolimbic system, particularly the ventral tegmental area, nucleus accumbens, amygdala and prefrontal cortex via dopaminergic, GABAergic and glutamatergic pathways, constitutes a common neurocircuitry by which diverse drugs of abuse mediate their acute reinforcing effects, whereas, long-term neuroadaptations in this circuitry most seemingly underlie the transition to drug dependence and cycles of relapse (Feltenstein and See, 2008).

Within the brain's central reward neurocircuits and stress circuits, alcohol interacts with several neurotransmitter systems. The interactions between alcohol and the neurotransmitter systems produce alcohol's acute reinforcing effects. However, in chronic exposure to alcohol, these interactions ultimately result in changes in neuronal function that underlie the development of tolerance, withdrawal, and dependence (Gilpin and Koob, 2008).

1.2 Neurotransmitter Systems Involved in Alcohol Dependence

Alcohol reinforcement appears to be mediated by several neurotransmitter systems including GABAergic system, opioid system, dopaminergic system, serotonergic system, and glutamatergic system (Koob et al., 1998).

γ-Aminobutyric Acid System (GABA)

γ-Aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the brain. It plays a role in regulating the neuronal excitability in the nervous system. There are two classes of GABA receptors: $GABA_A$ and $GABA_B$.

$GABA_A$ receptors are ligand-gated ion channels, whereas, $GABA_B$ receptors are G protein-coupled receptors.

Alcohol can increase GABA activity in the brain either by acting on the GABA-releasing (presynaptic) neuron, leading to an increased GABA release. Or by acting on the signal-receiving (postsynaptic) neuron, resulting in facilitating the activity of the $GABA_A$ receptor (Gilpin and Koob, 2008).

Ethanol allosterically appears to modulate the GABA receptor complex and to basically open the chloride channel and hyperpolarize cells or at least potentiates the hyperpolarization produced by GABA (Dietrich et al., 1989)

Previous studies have demonstrated that alcohol drinking is suppressed by $GABA_A$ receptor antagonists, which interferes with the actions of the $GABA_A$ receptor in the brain regions of the extended amygdala such as the nucleus accumbens, ventral pallidum, bed nucleus of the stria terminalis and amygdala (Gilpin and Koob, 2008). Of these regions, the central nucleus of the amygdala is especially sensitive to the suppression of alcohol drinking by GABAergic compounds (Hyytia and Koob, 1995). Acute alcohol exposure (short-term alcohol consumption) seems to increase the $GABA_A$ receptor function, whereas, prolonged drinking due to counteradaptive processes has the opposite effects. The effects of chronic alcohol use are linked to over-excitation due to compensatory mechanisms associated with repeated administration. Chronic alcohol use has been shown to decrease the cell surface expression of $GABA_A$ receptors and decrease their sensitivity (Mharte et al., 1993).

Opioid System

Endogenous opioids are small naturally occurring peptides that are produced in the body and have been associated in the actions of opiate drugs and alcohol for a long time. There are three classes of endogenous opioids: endorphins, enkephalins, and dynorphins (Gilpin and Koob, 2008). These endogenous opioids produce their effects by interacting with three subtypes of opioid receptors: μ, δ, and κ.

The positive alcohol reinforcement, certain properties of alcohol leading to dependence, is mediated at least in part by the release of endogenous opioids in the brain (Ulm et al., 1995). This hypothesis is indeed supported by various studies demonstrating that opioid antagonists acting on opioid receptors suppress alcohol drinking in several animal

models. Moreover, mice lacking μ-opioid receptor show reduction in alcohol intake (Roberts et al., 2000). The agent naltrexone, a pharmacological substance that binds to endorphin receptors, preventing endorphin receptor binding, and reducing the craving for abuse substances, is classified as a nonspecific opioid receptor antagonist, which is currently used as a treatment for alcoholism and narcotic addiction in humans, and is particularly effective in reducing heavy drinking to alcohol (Gilpin and Koob, 2008).

Alcohol induces an increase in the extracellular endorphin level in the nucleus accumbens (Olive et al., 2001). The opioid system influences alcohol drinking behavior by direct and indirect interactions with the mesolimbic dopamine system (Gilpin and Koob, 2008). Opioid receptor antagonists interfere with alcohol's rewarding effects by acting in various brain regions including the ventral tegmental area, nucleus accumbens, and central nucleus of the amygdala (Koob, 2003).

Dopaminergic System

Dopamine is a monoamine neurotransmitter involved in a circuit called the mesolimbic system, which projects from the ventral tegmental area to the nucleus accumbens (Gilpin and Koob, 2008). This circuit influences incentive changes in the environment, and it affects incentive motivation (Biggio et al., 2007). Studies suggest that dopamine also has a role in the incentive motivation associated with acute alcohol intoxication. Moreover, studies demonstrated that alcohol consumption can be blocked by administration of dopamine antagonist directly into the nucleus accumbens (Hodge et al., 1997). Additionally, alcohol ingestion and anticipation to the availability of alcohol

produce dopamine release in the nucleus accumbens as determined by microdialysis study (Weiss et al., 1993).

Serotonergic System

Serotonin (5-hydroxy-tryptamine or 5-HT) is a monoamine neurotransmitter that is considered a target of interest for potential therapy of alcohol dependence because of a well-established link between serotonin depletion, impulsivity, and alcohol-drinking behavior in rats, mice, and humans (Gilpin and Koob, 2008).Therapeutic compounds that target the serotonin system either by inhibiting neuronal reuptake of serotonin, therefore prolonging its actions, or by blocking specific serotonin receptor subtypes have been shown to reduce the voluntary alcohol consumption in rats (Jhonson, 2008). Serotonin release in the nucleus accumbens of rats is suppressed during alcohol withdrawal, and this reduction in serotonin release is partially reversed by self-administration of alcohol (Weiss et al., 1996). Studies have shown that SSRIs effectively maintain the attenuation of alcohol intake achieved during treatment of alcohol dependence for at least 6 months after pharmacological intervention in some alcoholics (Sari et al., 2011). For example, Escitalopram, an effective SSRI, reduces alcohol consumption in alcoholics.

Glutamatergic System

Glutamate is the chief excitatory neurotransmitter in the brain; it exerts its effects by interacting with several receptor subtypes, including the *N*-methyl-D-aspartate (NMDA) receptor (Gilpin and Koob, 2008). The glutamatergic system has long been implicated in the acute as well as chronic reinforcing actions of alcohol. In contrast to

alcohol's acute effects on GABA, alcohol inhibits glutamate activity in the brain. Furthermore, acute alcohol exposure reduces the extracellular glutamate levels in the striatum, which contains the nucleus accumbens, among other structures (Gilpin and Koob, 2008). Alternatively, chronic alcohol exposure leads to an increase in glutamate output, and impairs ability in glutamate transport, leading ultimately to excessive glutamatergic neurotransmission within the mesolimbic circuit. Importantly, alcohol affects glutamate transmission most likely by altering the functions of both NMDA receptors (Lovinger et al., 1989) and also metabotropic glutamate subtype 5 receptors (mGluR5) (Blednov and Harris, 2008).The involvement of NMDA receptors in alcoholism might be associated with neuroplasticity, a process characterized by neural reorganization that likely contributes to hyperexcitability and craving during alcohol withdrawal (Pulvirenti and Diana, 2001).

Currently, compounds targeting the glutamatergic system are being used in the treatment of alcohol dependence. The agent acamprosate blocks excessive alcohol consumption by reducing excessive glutamate activity. This process seems to depend on the involvement of genes such as Per2, which is implicated in maintaining the normal daily rhythm (the circadian clock) of an organism (Spanagel et al., 2005). The drug acamprosate has been approved for the treatment of alcoholism in humans, primarily due to its ability to suppress alcohol drinking among diverse species (Gilpin and Koob, 2008). Acamprosate tend to dampen excessive glutamate activity, and modulate glutamate transmission by acting on NMDA and/or metabotropic glutamate receptor (Littleton, 2007).

1.3 Neurocircuitry of Reward

The understanding of the neurobiological factors involved in alcohol reinforcement and dependence would lead to the development of effective pharmacotherapies for the prevention of alcohol relapse after periods of abstinence (Spanagel and Zieglgansberger, 1997). Understanding relapse lies in the same neurochemical elements that are compromised by repeated alcohol use. Studies have focused on examining motivational reward circuits through stimulation sites of specific brain regions. Most of these diverse stimulation sites later were identified to be linked through a common neural pathway; a complex bundle of axons termed the medial forebrain bundle (Phillips, 1984). Dopamine was identified later as a critical neurotransmitter in producing the rewarding effects by intracranial self-stimulation (ICSS) of the medial forebrain bundle (Liebman and Butcher, 1973; Phillips and Fibiger, 1978). It has been shown that low doses of alcohol produce a dose-dependent increase in the firing rate of dopaminergic neurons in the ventral tegmental area (Gessa et al., 1985).

The brain regions that mediate the reinforcing effects of alcohol include: the extended amygdala and the mesolimbic dopaminergic pathway, including the ventral tegmental area, the nucleus accumbens and the prefrontal cortex, these are the main sites in the brain that mediate alcohol reinforcement (Vengeliene et al, 2008). An integral part of the brain, the nucleus accumbens, which is located in the ventral striatum, has been well studied for its role in reward, as well as addiction. Currently, it is believed that the nucleus accumbens serves to integrate information contained in the mesocorticolimbic circuit and projects that information out to the motor system to produce appropriate

behavioral responses. The nucleus accumbens acts as a gateway for limbic structures to reach the motor system (Groenewegen et al., 1996: Yin and Knowlton, 2006). The limbic system is activated once a novel stimulus is encountered; it basically becomes fully engaged to process new as well as previously learned information about that novel stimulus to optimize action plans for maximizing reward outcomes (Sesack and Grace, 2010).

The limbic structures including the basal lateral amygdala and the hippocampus support a variety of functions including emotion, behavior and long term memory. The hippocampus is required for the formation of long term memories, and also implicated in maintaining contextual associations (cognitive maps). The amygdala is responsible for emotional processing. Both amygdala and hippocampus are tightly connected to the prefrontal cortex (Myers and Gluck, 1994; Yaniv et al., 2004).

The prefrontal cortex is engaged in working toward a defined goal, planning complex cognitive behaviors and ultimately producing goal oriented behavior. The nucleus accumbens then needs to convey the motor actions necessary to accomplish the intended goals; the nucleus accumbens projects to the ventral pallidum, an area that is a component of the limbic loop of the basal ganglia. The ventral pallidum receives GABAergic inhibitory efferents from the nucleus accumbens to ventral tegmental area and dorsomedial thalamus, which in turn projects to the prefrontal cortex through glutamatergic projections (Sesack and Grace, 2010). Disinhibition of the substantia nigra reticulata and motor thalamus, can lead to the activation of the motor cortex, which is involved in the execution of voluntary motor functions, and projects to the spinal cord to produce movement (Groenewegen et al., 1999; Sesack and Grace, 2010).

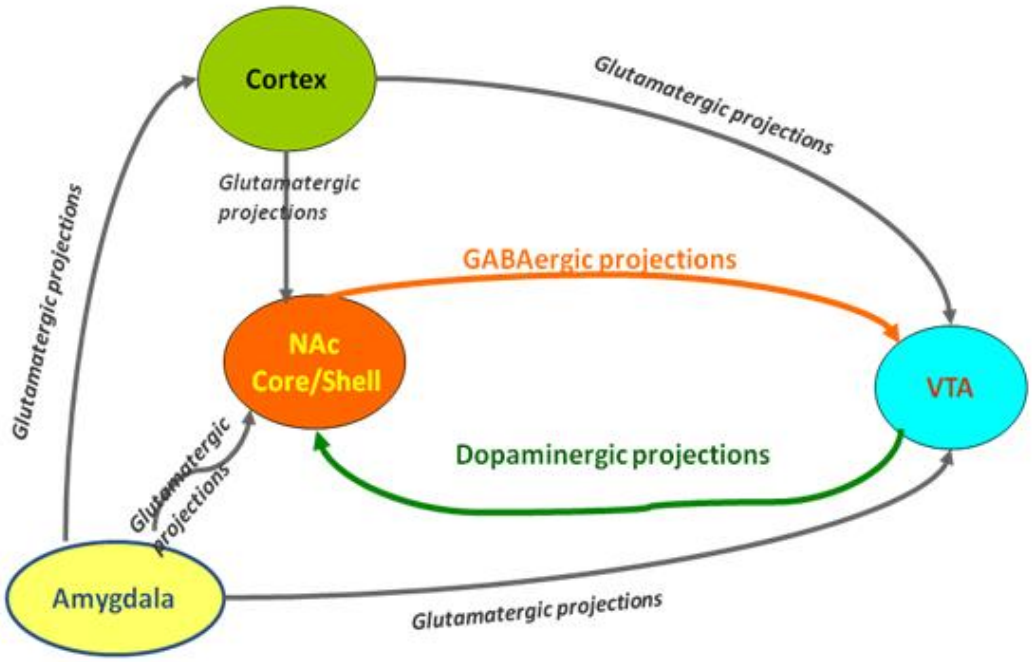


Figure 1-1 Circuitry in the development and expression of drug addiction

1.4 Animal Models of Alcoholism

The large variability in alcohol preference among animal models has set forth the selectively of breeding rats for differential alcohol preference, low or high alcohol consumption levels (Spanagel, 2000). Examples of the animal models of alcohol preference include: Alko Alcohol (AA) and Alko Nonalcohol (ANA) rats, which comprises two strains of albino rats that were selectively bred based on their selection or rejection of a 10% alcohol solution and water (Eriksson, 1968). The alcohol-preferring (P) rats, originally bred at Indiana University Institute, voluntarily consume 5 -8 grams of

alcohol per kilogram of body weight per day (g/kg/day), attaining blood alcohol concentrations of 50-200 mg/100 ml, whereas the non-alcohol-preferring rats (NP) consume less than 0.5 g/kg/day alcohol (McBride and Li, 1998). The Sardinian alcohol-preferring (sP) rats also have been selectively bred for high alcohol preference and consumption for more than 20 years (Colombo, 1997). These models have been used as a tool for characterizing the behavioral, neurochemical, and molecular correlates of differential voluntary alcohol consumption and preference (Spanagel, 2000).

Animal models attempt to parallel various aspects of human conditions, but most animal models are limited by the fact that animals do not express the wide range of behaviors that humans produce. The following criteria has been proposed for an animal model of alcoholism (Lester and Freed, 1973) : (1) the animal should orally self administer ethanol; (2) the amount of ethanol consumed should result in pharmacologically relevant blood ethanol levels; (3) ethanol should be consumed for its post-ingestive pharmacological effect, and not strictly for its caloric value or taste; (4) ethanol should be positively reinforcing, or in other words the animals must be willing to work to receive ethanol; (5) chronic ethanol consumption should lead to the expression of metabolic and functional tolerance; (6) chronic consumption of ethanol should lead to dependence, as indicated by withdrawal symptoms after access to ethanol is terminated. More recently, a 7^{th} criterion has been added that states animal model of alcoholism should also display characteristics associated with relapse (McBride, Li, 1998) because alcoholics generally go through episodes of abstinence and relapse.

The (P) rat has been well characterized both behaviorally and neurobiologically (Li et al., 1979; McBride and Li, 1998) and satisfies criteria proposed as essential for an animal model of alcoholism (Lester and Freed, 1973)

First, P rats readily self-administer greater than 5/g/kg of ethanol per day (Rodd-Henricks et al., 2000). Second, P rats can achieve blood ethanol concentrations (BECs) of 200 mg% or greater during 24-hour free-choice ethanol self administration, although typically these rats maintain BECs in the range of 50-70 mg% (Waller et al., 1982). Third, the alcohol preference of P rats appears to be stable even in the presence of dietary changes. Studies demonstrated that despite varying the carbohydrate content of the solid food (22%, 59%, or 78%), the P rats maintained high ethanol intake and preference over water (Rodd et al., 2004). Fourth, P rats will self administer ethanol under operant conditions. Using a one-lever design procedure, free-fed P rats will exceed 1000 bar presses for ethanol in a 24 hour period (Penn et al., 1978). Ethanol naïve P rats do not require fluid deprivation, food restriction, or sucrose substitution procedures to acquire ethanol self-administration under operant conditions. Furthermore, P rats will self administer ethanol intragastrically, which precludes the influence of taste (Waller et al., 1984). Fifth, P rats develop metabolic and neuronal tolerance under 24-hour free choice alcohol drinking conditions (Lumeng and Li, 1986). After chronic free-choice ethanol drinking, P rats display tolerance to the motor impairing (Gatto et al., 1987) and aversive effects of ethanol. Sixth, P rats develop dependence after chronic free-choice ethanol drinking (Waller et al., 1982).

1.5 Animal Models of Alcohol Relapse

There are three proposed animal models of alcohol relapse:

a. The Reinstatement Model.
b. The ADE (Alcohol Deprivation Effect) Model.
c. The Point Of-No-Return Model.

a. The Reinstatement Model: In the alcohol addiction field of study, Chiamulera and colleagues (1995) reported the first alcohol reinstatement study in rats. Rats were trained over several months to press a lever in order to self administer alcohol. Once stable lever pressing was obtained, the rats received water instead of alcohol in order to extinguish the lever pressing behavior. After animals extinguished their behavior, reinstatement to alcohol seeking behavior was initiated by administration of a small quantity of alcohol (Spanagel, 2000). The limitations of the reinstatement model include:

- Researchers have not conclusively proved that the rats undergoing the reinstatement procedure exhibit uncontrolled drinking behavior.
- Investigators following the alcohol extinction period present various stimuli to reinstate responding to alcohol, whereas alcoholics that undergo treatment of alcohol addiction tend to avoid exposure to alcohol during abstinence. The animal

reinstatement procedure may not accurately reflect the situation of abstinent alcoholics experiencing craving and relapse.

b. The Alcohol Deprivation Effect (ADE) Model: ADE is considered to be a long-term model of alcohol self-administration with repeated alcohol deprivation phases that can mimic abstinent alcoholics experiencing craving and relapse. Male wistar rats were exposed to three alcohol solutions (5%, 10%, 20%), for two months of continued access, and then, the rats were deprived from alcohol for several days before being offered again different alcohol solutions (Spanagel and Holter, 1999). The rats that had renewed availability of alcohol solutions following the deprivation phase demonstrated a pronounced temporary rise in alcohol intake and preference. Such pattern of relapse-like drinking is observed across several species including rats, mice, monkeys and even human social drinkers (Spanagel, 2000). The term ADE was then defined as the" temporary increase in the ratio of alcohol/total fluid intake and voluntary intake of ethanol solutions over baseline drinking conditions when ethanol is reinstated following a period of alcohol deprivation" (Spanagel, 2000).

Characteristics of ADE include:

- ADE involves changes in the animal's alcohol intake patterns; animals consume large amounts of concentrated alcohol solution at inappropriate times during their daily cycle.

- ADE can persist over long periods of abstinence indicating that there is a specific memory for alcohol. This behavior is similar to human alcoholics who can relapse even after years of abstinence.
- Animals that exhibit ADE have strong motivation for alcohol; they are motivated to perform a task to receive alcohol.
- When adulterating the taste of alcoholic solution with Quinine (a bitter tasting substance), animals that exhibited ADE would still consume large amounts of quinine containing alcohol solution. This demonstrates loss of control over drinking which a major criterion for defining addiction in animals (Spanagel, 2000).
- Additional studies demonstrated that rats undergoing ADE exhibited tolerance, physical and psychological signs of withdrawal, and stress induced drinking (Spanagel and Holter, 2000).
- The Diagnostic criteria for alcoholism listed in the 4th edition of DSMIV (Diagnostic and Statistical Manual of Mental Disorders) of the American Psychiatric Associate (1994) are covered by the alcohol deprivation model.

c. The Point Of-No-Return Model: Is an animal model on the development of loss of control in which there is an assumed irreversible point indicative of alcohol addiction. Rats were exposed to free access to water and three alcohol solutions (5%, 10%, and 20%); their drinking behavior was monitored to reflect an acquisition phase, in which the rats experimented different

alcohol doses for one to two weeks. The rats eventually developed an individual drinking pattern that was stable for months which reflects controlled behavior. However, after 6 months, rats gradually changed their alcohol drinking behavior; they demonstrated increasing alcohol consumption over the next few months (Wolffgramm et al., 1999). This transition from controlled to uncontrolled drinking, suggests that a point of No return does exist.

1.6 Glutamate and Glutamate Transporters

Glutamate is the major excitatory neurotransmitter in the brain, and the primary excitatory amino acid neurotransmitter in the central nervous system, however, it is also a potent neurotoxin that may lead to neuronal death. Glutamatergic neurons are prominently represented in the cerebral cortex and limbic regions of the brain. The concentration of glutamate in the synaptic cleft, and the duration of its action are tightly regulated in order to maintain homeostasis of glutamate. Maintaining a normal physiological level of extracellular glutamate is the key to prevent neurotoxicity that occurs under a variety of pathological conditions (Kim et al., 2010).

Under normal conditions, glutamate released from the synaptic neurons, results in activation of ionotropic glutamate receptors present on the postsynaptic neurons. This activation of glutamate receptors results in the influx of Na+ and Ca+, leading to membrane depolarization and generation of action potentials. When glutamate is released, it participates in the signaling process through different types of glutamatergic receptors, and then must be taken up from the synaptic cleft (Kanai and Hediger, 2003). The accumulation of excess extracellular glutamate will subsequently lead to

overstimulation of glutamatergic receptors, increasing the production of reactive oxygen/nitrogen species, which induce oxidative stress leading to neuronal death. Increased extracellular levels of glutamate, has been associated with the development of neurodegenerative diseases including; Huntington's diseases, Amyotrophic Lateral Sclerosis and Alzheimer's disease.

Glutamate transporters responsible for the removal of glutamate are found in neuronal and glial membranes, they are membrane bound pumps that resemble ion channels.

Classes of glutamate transporters include:

a. EAATs (Excitatory Amino acid Transporters) that are dependent on electrochemical gradient of sodium and potassium ions. In humans as well as rodents, five subtypes of glutamate transporters have been identified in rat brain: GLAST, GLT1, EAAC1 and their human homologues are EAAT1, EAAT2, and EAAT3 respectively. The two remaining human and rodent subtypes EAAT4 and EAAT5 are conserved, and share common nomenclature.

The most noteworthy transporters of all five subtypes are GLAST and GLT1. GLAST expression is prominent in the cerebellum and moderately in the hippocampus and the forebrain, whereas, GLT1 expression is mainly found in the forebrain regions and in minority in the cerebellum. EAAT3 is located on neurons and expressed at very low levels in different regions of the brain. EAAT4 and EAAT5 are conserved only in the cerebellum and retina, where they are principally localized to photoreceptors and bipolar neurons in the retina. As a

result of an action potential glutamate is released, and then rapidly removed from the extracellular space by the EAATs (Beart and O'Shea, 2007). Glutamate is taken up into Glia cells and then converted to glutamine (a molecule which does not cause excitotoxicity) and then stored in vesicles for further synaptic release. The rodent GLT1 and its human homologue EAAT2, which is primarily expressed in astrocytes, are considered selective glutamate transporters that keep extracellular glutamate levels below the excitotoxic levels by clearing glutamate from neuronal synapses in the CNS (Rothstein et al., 1996). EAAT2 is responsible for 90% of total glutamate uptake. (Mitani and Tanaka, 2003; Rothstein et al., 1995).

b. VGLUTs (Vesicular Glutamate Transporters) which are sodium independent transporters. Four types of VGLUTs are known, VGLUT 1 to 3 and the glutamate/aspartate transporter. These transporters are involved in vesicular glutamate uptake. VGLUTs are dependent on the proton gradients with the vesicles being more acidic than the cytosol.

The role of vesicular glutamate transporter 1 and 2 (VGLUT1 and VGLUT2) has been was studied in P rat model (Zhou et al., 2006). This study demonstrated that the number of VGLUT1 immunostained terminals was unchanged in the extended amygdala in either a continuously exposed alcohol group, or in a repeated deprivation alcohol group. On contrast, the number of VGLUT2 immunostained terminals increased in the shell of the nucleus accumbens of the repeated deprivation alcohol group compared to the control water group. It is noteworthy

to include that VGLUT2-bearing glutamate fibers, are associated with the motor circuit, while the VGLUT1-bearing glutamate fibers, and are associated with the cognitive circuit. The findings of this study, demonstrate that repeated alcohol deprivation may result in an increase in glutamate terminals in the nucleus accumbens shell bearing the VGLUT2, which represents the greater population of glutamate terminals. The repeated deprivation of alcohol can ultimately change the ratio of glutamate to dopamine innervations in the nucleus accumbens shell, which plays a critical role in reward related processes.

1.7 Activation of GLT1 by Ceftriaxone, a β-lactam antibiotic

Ceftriaxone is a β-lactam broad spectrum antibiotic, belonging to the third generation of cephalosporins. It works by interfering with the formation of the bacteria's cell wall so that the wall ruptures, resulting in the death of the bacteria. Ceftriaxone is mainly used by intravenous or intramuscular administration, in the treatment of many bacterial infections, such as respiratory tract infections, urinary tract infections and meningitis.

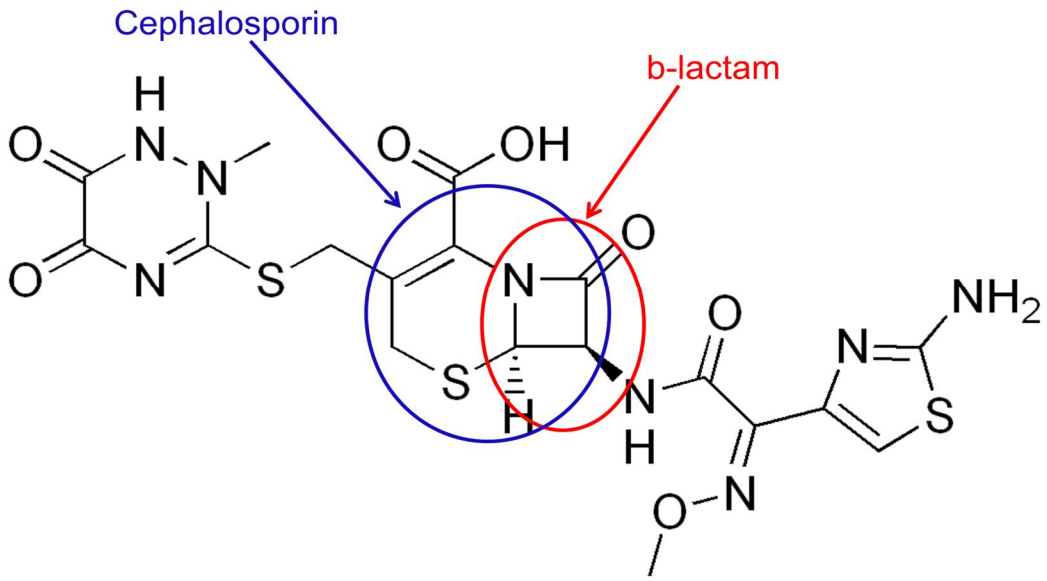


Figure 1-2 Ceftriaxone chemical structure

In an attempt to combat neurological diseases, Rothstein and his colleagues in 2005, led the mission to find therapeutic agents that increased the expression of GLT1 in a mouse model. They tested 1040 FDA approved drugs, and discovered that β-lactam antibiotics were some of the most potent activators of GLT1 expression (Rothstein et al., 2005). They further explored the biological activity of ceftriaxone in normal rats, and discovered that rats treated at a dose of 200 mg per Kg intraperitoneally (i.p) daily for five days had increased levels of GLT1 expression and functional activity in both the spinal cord and the hippocampus.

Rothstein and colleges studies documented a new property of β-lactam antibiotics, and demonstrated that indeed these β-lactam antibiotics can activate the genetic promoter for GLT1 in a dose dependent manner. Since this finding, a new era for β-lactam antibiotics uses has opened. Furthermore, In vivo and in vitro studies revealed that ceftriaxone has positive effects on GLT1 protein expression, with neuroprotective effects

based on its ability to inhibit neuronal cell death, by preventing glutamate excitotoxicity. Although the molecular mechanism of the action of GLT1 is unclear, analysis of the 2.5-kb human EAAT2 promoter revealed that NF-KB is a very important regulator of EAAT2 expression in astrocytes, the β-lactam antibiotics are transcriptional activators of EAAT2 resulting in increased EAAT2 protein levels (Kim et al., 2010). Ceftriaxone increases EAAT2 transcription in primary human fetal astrocytes through the NF-KB signaling pathway. Studies revealed that the 272 position of NF-KB binding site is critical in ceftriaxone mediated EAAT2 protein induction. Indeed, there is a promising potential utility of EAAT2 promoter for developing screening assays to identify novel regulators of glutamate transport in the brain.

1.8 Objectives and Goals

The main goal of this thesis is to investigate the role of GLT1 in relapse to alcohol seeking behavior in a high drinking alcohol-preferring (P) rat model. Based on the facts, P rats are an established model of alcoholism, and will readily consume intoxicating levels of alcohol (McBride and Li, 1998: Rodd-Henricks, et al., 2002; Rodd, et al., 2005). In addition, P rats find alcohol positively reinforcing and consume alcohol for its pharmacological effects in relevant blood alcohol concentrations (0.05-0.2) (Li e al., 1987). Further studies have revealed that chronic alcohol consumption in P rats leads to tolerance and dependence, whereas deprivation leads to relapse (McBride and Li, 1998).

In this research, we based our hypothesis on the fact that glutamate transporter GLT1 is responsible for the removal of most extracellular glutamate, we explored the hypothesis, that up-regulation or activation of GLT1 by the use of the β-lactam antibiotic ceftriaxone, known to elevate GLT1 expression, would attenuate ethanol consumption after a period of alcohol deprivation and ultimately prevent relapse. If an increase in glutamate transmission plays a critical role in alcohol relapse, then up-regulation of GLT1 should attenuate this response, and correlate with an increase of GLT1 in the nucleus accumbens and or prefrontal cortex.

Materials and methods

2.1 Animals

All the animals that were used in this study were male alcohol-preferring (P) rats. At the beginning of the experiment, the animals weighed in a range of 317 gm to 395 gm. Animals were obtained from the Indiana University Medical Center (Indianapolis, IN) at approximately 4 weeks of age and housed in groups of two in standard plastic tubs, in a temperature (21°C) and humidity (~50%) controlled vivarium maintained on a 12 hour light/dark cycle, at the main campus of the University of Toledo. The total number of animals used in the study was 44. After habituation to the vivarium, two weeks prior to

90 days old, animals were moved to the Department of Laboratory Animal Resources (DLAR) and were singly housed. All Experimental procedures began when animals were approximately 90 days old. Animal procedures were approved (animal protocol #106966) by the Institutional Animal Care and Use Committee, and in accordance with the guidelines of the National Institutes of Health.

2.2 Behavioral Drinking Paradigms

Male P rats were given free choice access to food, tap water, 15%, and 30% ethanol for five weeks at approximately three months old. 190 proof ethanol was purchased from PHARMCO-AAPER (Shelbyville, KY), diluted with distilled water to make the appropriate desired concentrations of the alcohol that was offered to rats, and then alcohol was placed in glass bottles. The daily consumption of alcohol was measured by bottle weight. Alcohol bottles were replaced three times a week (Mondays, Wednesdays, and Fridays) at noon. At that time animals were weighed by placing them in a plastic beaker on a Sartorius scale (max = 820g). Alcohol measurements were made by subtracting the weight of the bottle from the previous weight of the bottle 2 or 3 days prior. Alcohol consumption for each animal was measured as grams of alcohol consumed per kilogram of body weight per day for 5 weeks. At the end of five weeks, animals that did not meet the requirement (must have drank more than or equal to 4 grams of alcohol per kilogram body weight on baseline) for chronic alcohol drinking behavior were removed from the study. Baseline calculations were the average measurements taken across the last two weeks of the five week alcohol drinking paradigm. The requirement

for chronic alcohol drinking behavior was obtained from a previous model (Li et al., 1987). On week six, animals were separated into three different groups and deprived from alcohol for 2 weeks. During the last five days of the 2 weeks deprivation period, animals were injected intraperitoneally (i.p.) with either saline (n = 8), ceftriaxone 50 mg/kg (n = 8), or ceftriaxone 100 mg/kg (n = 8). ceftriaxone was purchased from the pharmacy of the University Of Toledo's hospital, as a powder and dissolved in a saline solution immediately before being administered to the animals. After 10 days of alcohol deprivation, ceftriaxone was administered (i.p.) once a day at noon for five consecutive days. Animal body weights and water bottles were measured daily right before injections throughout the deprivation period. After the five days of treatment, animals were re-exposed to the alcohol drinking paradigm for an additional nine days to determine the effects of ceftriaxone treatment in relapse to alcohol seeking behavior. During this time, weights of animals and their water/alcohol bottles were recorded daily as previously described. On day 10 animals were euthanized with isoflurane, rapidly decapitated with a guillotine, and their brains were dissected out and immediately frozen on dry ice and stored at (-70ºC) for immunoblotting. An additional naive control group of animals (n = 6) was treated with saline for five days to undergo a similar level of stress as the other tested animals, then after ten days were euthanized (at the same age of the other groups), decapitated, and their brains were dissected out and immediately frozen on dry ice and stored at (-70ºC) for immunoblotting. A similar experimental design was applied to sucrose consumption in order to test ceftriaxone's specificity on alcohol consumption protocol. At approximately three months old, animals (n = 14) were placed on a free-choice 10% sucrose drinking paradigm (sucrose, SIGMA; diluted with distilled water) for

three weeks before undergoing the two week deprivation period. On week four, animals were deprived of sucrose for two weeks, during the last five days of the two week sucrose deprivation period, animals were treated with either saline (n = 4), ceftriaxone 50 mg/kg (n = 5), or ceftriaxone 100 mg/kg (n= 5) once a day for five days . After the five days of treatment, animals were re-exposed to the sucrose drinking paradigm for an additional nine days to determine the long-lasting effects of ceftriaxone treatment. During this time, weights of animals and their water/sucrose bottles were recorded daily as previously described. On Day 10, animals were euthanized with isoflurane.

2.3 Brain Tissue Harvesting

In this study, we assessed GLT1 expression levels in the nucleus accumbens and prefrontal cortex, in a set of animals exposed to free choice-ethanol (15% and 30% v/v) and water for 5 weeks and then deprived for 2 weeks, afterwards treated with ceftriaxone 50, 100 mg/kg, or saline for 5 days, then re-exposed to alcohol for nine days. On Day 10, animals were euthanized by isoflurane, rapidly decapitated with a guillotine, and their brains were dissected out and immediately frozen on dry ice and stored at (-70º C). The prefrontal cortex and nucleus accumbens were punctured streotaxically using cryostat apparatus and frozen brain regions were stored for immunoblotting assays, to examine GLT1 protein levels.

2.4 Protein Tissue Extraction Protocol

Procedures were conducted on nucleus accumbens and prefrontal cortex samples from animals sacrificed on day 10 of re-exposure. Samples were homogenized using

filtered lysis buffer (2.5mL 1M Tris HCL, 2.5mL 3 M NaCl, 0.1mL 0.5M EDTA, 2.5mL 10% NP-40, 5mL 10% Triton, 0.5mL 10%SDS, 3 mL of dissolved protease inhibitor tablet in water, and 33.9 mL Millipore water). 300 µL lysis buffer was added to each sample in a 1.5 µL eppendorf tubes and the tissue was grounded with a pestle until no solid mass remained. The samples were then placed on ice for 30 minutes to allow homogenization to complete. They were then centrifuged at 13,200 RPM for 15 minutes at 4º C. The supernatant was aliquoted and immediately frozen on dry ice and the pellet was discarded.

2.5 Protein Quantification

A Lowry protein quantification assay was conducted on one aliquot of each sample. All samples were assayed in quadruplicates in a 96-well plate. Bovine serum albumin (BSA) (1.48mg/mL, New England Biolabs) was used to make a standard curve. The curve was prepared using serial BSA dilutions. The wells containing the proteins samples contained 1µL of sample diluted in 4 µL of lysis buffer. Afterwards, 3mL reagent A (Biorad Laboratories) was mixed with 60 µL reagent S (BioRad Laboratories) and 25µL of this mixture was added to each well. 200µL of reagent B (BioRad Laboratories) was then added to each well and the reaction was allowed to sit at room temperature for 15 minutes before being read on a multiskan FC spectrophotometer (Thermo Scientific) at a wavelength of 750 nm. The quadruplicate optical density values were averaged and the blank optical density was subtracted from each measurement. A standard curve was made by plotting the BSA optical density versus the diluted BSA

protein concentrations. The protein concentrations were then calculated by comparing their optical densities to the standard BSA curve.

2.6 Western Blot Procedures

Water (n = 6), saline (n = 8), ceftriaxone 50mg/kg (n = 8), and ceftriaxone 100mg/kg (n = 8) samples were used for the western blot analysis of GLT1 total protein concentration in the nucleus accumbens and prefrontal cortex. Using the protein quantification data, each sample was diluted to 8µg/20µL with the same lysis buffer. Laemmli dye was added to each sample (5µL dye per 20µL sample) and mixed well, samples were vortexed then heated for 4 minutes at 98º C in a digital dry bath (Labnet International Inc.), vortexed again, and then centrifuged for 3 minutes at 4º C at 13,200 RPM (Centrifuge 5414 R, Eppendorf).The 10-20% Tris=glycine gels (Invitrogen) were placed into an electrophoresis apparatus and submerged in 1X laemmli buffer (10X laemmli buffer = 30.2 Tris Base, 144g Glycine, 10g SDS, qsp to 1 L). A 20 µL of each sample was placed into a well of the gel and proteins were separated by electrophoresis (1 hour at 200 volts). After completion, the gels containing the proteins were removed from the electrophoresis apparatus and transferred on an immobilon-P membrane (Millipore, Fisher Scientific, Inc.) using a transfer apparatus (Idea Scientific Company, MN). Protein transfer was carried out by filling the transfer chamber with transfer buffer (3.2 L distilled water, 28.8g Glycine, 5.9g Tris Base, 800 mL methanol) which was then hooked up to electrophoresis electrodes for 2.5 hours at a 24 volts. Membranes were placed in Ponceau dye for one minute, to see the bands, then washed with water 4 times quickly, membranes were then blocked with blocking buffer for 30 minutes (10mL/blot;

3% milk made from 3 g dry milk and 100 mL 1X TBST). With the membranes still soaking in the blocking buffer, primary GLT1 antibody (AB1783 GP X Glutamate Transporter, Millipore) was added (2µL/blot, 1:5000) and was allowed to incubate overnight shaking at 300 RPM in the fridge or cold room. The next day, each membrane was washed with 1X TBST 5 times for 5 minutes each and then incubated again with blocking buffer for 30 minutes. Each membrane was then incubated in secondary anti-guinea pig (anti-guinea pig IgG HRP-linked antibody, Cell Signaling Technology, Inc.) antibody (2µL/blot, 1:5000 for 1.5 hour before being washed again with IX TBST 5 times for 5 minutes each. The membranes were dried on Whatman paper and incubated with a Super Signal West Pico developer kit (Pierce; 1mL reagent A and 1 mL reagent B per blot) for one minute to detect the chemiluminescent signal of HRP. The membranes were dried again on Whatman paper and developed on Kodak BioMax MR Film (Thermo Fisher Scientific) with an SRX-101A machine. Protein loading was then normalized using β-tubulin immunoblotting as a loading control. After exposure, membranes were washed with 1X TBST 5 times for 5 minutes each before being placed in blocking buffer for 30 minutes. The membranes were incubated in β-tubulin primary antibody (2µL/blot, 1:5000) overnight. The next day, membranes were washed with 1X TBST 5 times for 5 minutes each and then incubated again with blocking buffer for 30 minutes. Each membrane was then incubated in secondary anti-mouse (Anti-mouse IgG HRP –linked antibody (2µL/blot, 1:5000) for 1.5 hour before being washed again with IX TBST 5 times for 5 minutes each. The membranes were dried on Whatman paper and incubated with a Super Signal West Pico developer kit (Pierce; 1mL reagent A and 1 mL reagent B per blot) for one minute to detect the chemiluminescent signal of HRP. The membranes

were dried again on Whatman paper and developed on Kodak BioMax MR Film (Thermo Fisher Scientific) with an SRX-101A machine. Digitalized images of the immunoreactive proteins were quantified using an MCID system and the data are reported as percentage ratios

Results

3.1 Effects of Ceftriaxone Treatment on Alcohol Drinking Behavior

During the 9 days of re-exposure to alcohol after 5 days of treatment with either saline, ceftriaxone 50 or 100 mg/kg, alcohol consumption was measured daily as g/kg/day for 9 days, starting on the first day of alcohol re-exposure. The data presented in Figure 3-1 show the average alcohol consumption for 9 days for saline (n = 8), ceftriaxone 50 mg/kg (n = 8), ceftriaxone 100mg/kg (n = 8) with the baseline being the average of rat's alcohol consumption for the last two weeks of continuous alcohol exposure. Note that rats were initially exposed to free choice of water, ethanol 15% and 30% for 5 weeks. Alcohol intake was significantly reduced ($p < 0.001$) for the groups treated with ceftriaxone since day 1 through day 9 of re-exposure. Whereas, the saline treated group regressed back to their normal drinking behavior, as soon as they were re-exposed to alcohol (from day 1). Alcohol intake for the two groups treated with ceftriaxone was reduced at both doses, which displayed a long-lasting effect, reached the lowest alcohol intake of 2 to 3 g/kg/day on day 5 of re-exposure, progressively increased and then reached a plateau, yet overall remained around under 4g/kg/day until their

euthanization on day 10. Whereas, the saline treated group, had the highest alcohol intake on day 1 of re-exposure (9 to10gm/kg/day), and consistently consumed about 8gm/kg/day through the period of re-exposure, which is similar to their average alcohol intake through the period of 5 weeks of continuous alcohol exposure. A two-way repeated measured ANOVA followed by a Dunnett post-hoc revealed a significant interaction of day and treatment [$F(2,18) = 5.388$, $p < 0.001$], and a significant main effect of day [$F(1,9) = 23.882$, $p < 0.001$]. Simple effect analyses conducted as one-way ANOVA's for each day, demonstrated significant [$F > 15.549$, $p < 0{,}001$] differences among the doses for days 1 through 9. Dunnett's post hoc revealed that both ceftriaxone's doses, relative to saline, significantly decreased ethanol intake on day 1 through day 9. These statistical findings suggest that drinking behavior among the groups, changed from day to day, and that drinking behavior between groups was different.

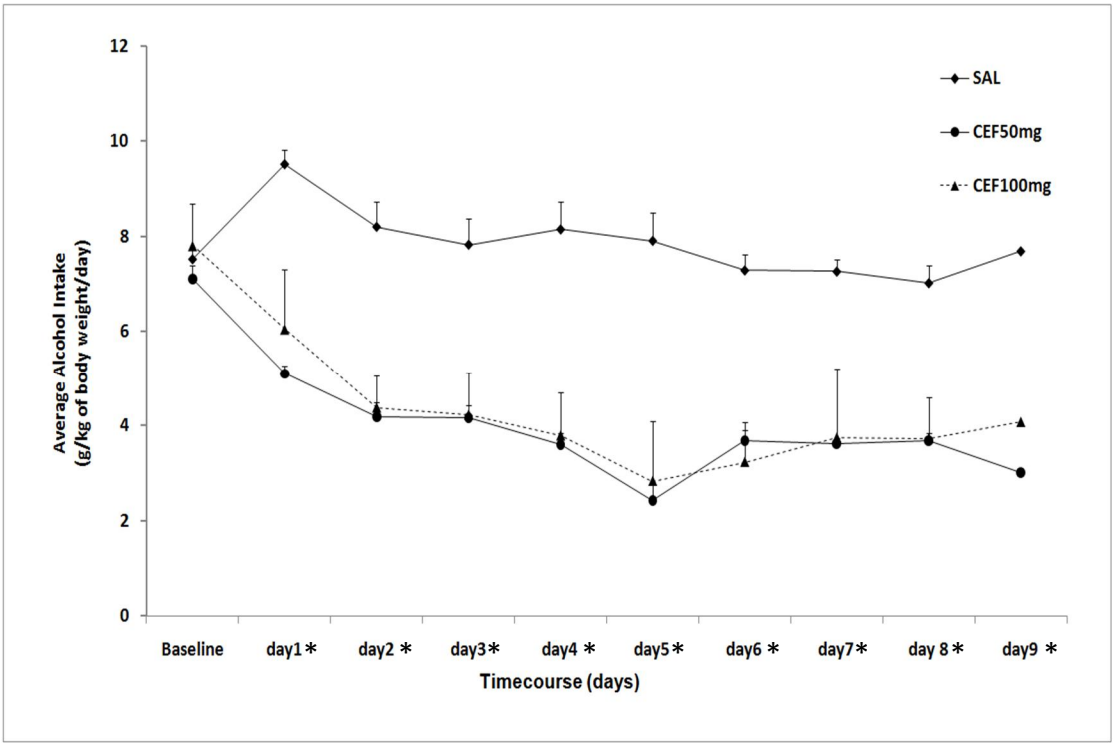


Figure 3-1:

Daily alcohol intake of male P rats for 9 days of alcohol re-exposure, following 5 days of treatment during relapse period with saline (n = 8) or ceftriaxone 50 mg/kg (n = 8)and ceftriaxone 100 mg/kg (n = 8). The graph represents average daily alcohol consumption (±SEM) during the 9 days of alcohol re-exposure. A two-way, repeated-measures ANOVA revealed a significant reduction in average daily alcohol consumption during the duration of re-exposure for both ceftriaxone-treated groups as compared to the saline treated group. *, depicts a significant ($p < 0.001$) one-way ANOVA across doses for the respective days.

3.2 Effects of Ceftriaxone Treatment on Water Intake

During the 9 days of re-exposure to alcohol, water consumption was measured daily as ml/kg/day for 9 days, for saline (n = 8), ceftriaxone 50 mg/kg (n = 8), and ceftriaxone 100mg/kg (n = 8), with baseline being the average of rat's water intake for the last two weeks of continuous alcohol exposure. The water drinking behavior was significantly reduced for the saline groups (Figure 3-2). Whereas, the ceftriaxone groups relative to saline significantly increased their water intake during the 9 days of re-exposure to alcohol. A two way repeated measured ANOVA, followed by a Dunnett post-hoc test revealed a significant main effect of interaction of day and treatment [$F(2,18) = 2.328$, $p < 0.003$], and a significant main effect of day [$F(1,9) = 6.453$, $p < 0.001$]. Simple effect analyses conducted as one-way ANOVA's for each day, demonstrated significant [$F > 3.656$, $p < 0.05$] differences among the doses for day 2 and days 4 through 9. Dunnett's post hoc revealed that both ceftriaxone's doses, relative to saline, significantly increased their water intake on day 2 and days 4 through 9.

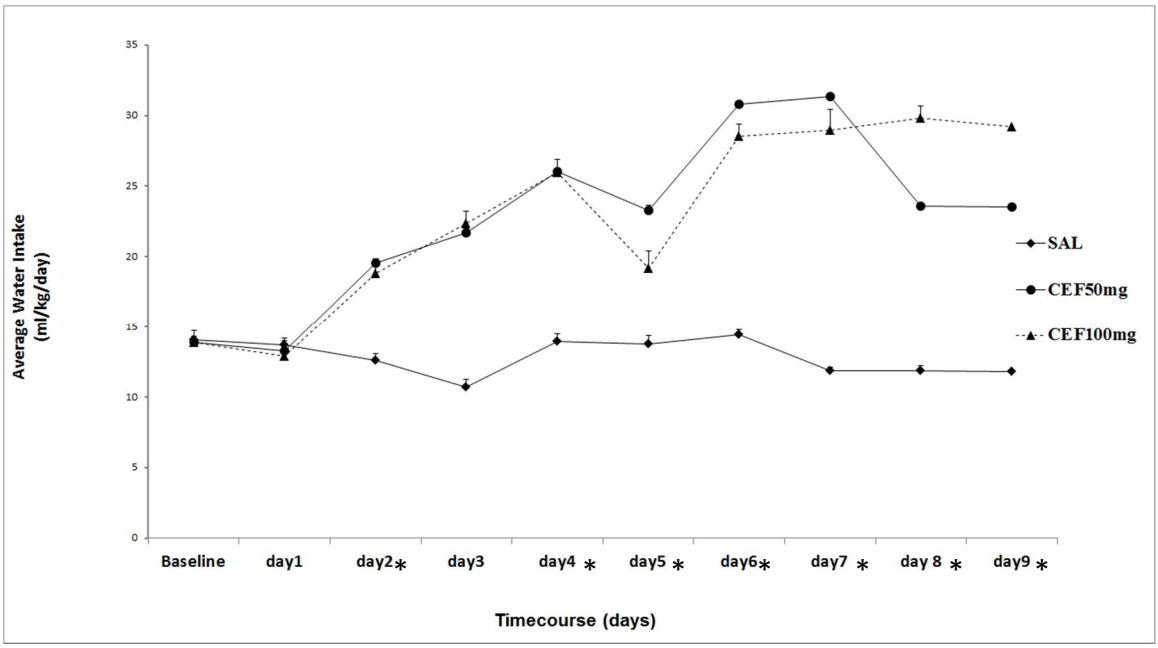


Figure 3-2:

Daily water intake of male P rats for 9 days, following 5 days of treatment with saline (n = 8) or ceftriaxone 50 mg/kg (n = 8) , and ceftriaxone 100 mg/kg (n = 8). The graph represents average daily water consumption (±SEM) during the 9 days of alcohol re-exposure. A two-way, repeated-measures ANOVA revealed a significant increase in average daily water consumption during the duration of re-exposure for both ceftriaxone-treated groups as compared to the saline treated group. *, depicts a significant ($p < 0.05$) one-way ANOVA across doses for the respective day.

3.3 Effects of Ceftriaxone Treatment on Body Weight

During the 9 days of re-exposure to alcohol, body weight was measured daily as gm (±SEM) for 9 days, for saline (n = 8), ceftriaxone 50mg/kg (n = 8), and ceftriaxone 100 mg/kg (n = 8), with baseline being the average of rat's weight for the last two weeks of continuous alcohol exposure (Figure 3-3). A two way repeated measured ANOVA, followed by a Dunnett post-hoc test revealed a significant main effect of interaction of day and treatment [$F(2,18) = 3.369$, $p < 0.001$], and a significant main effect of day [$F(1,9) = 68.251$, $p < 0.001$]. However, a simple effect analyses conducted as one-way

ANOVA's for each day, revealed no significant difference [$F < 0.725$, $p > 0.496$] differences among the doses for days 1 through 9. These data indicate that ceftriaxone did not affect body weight.

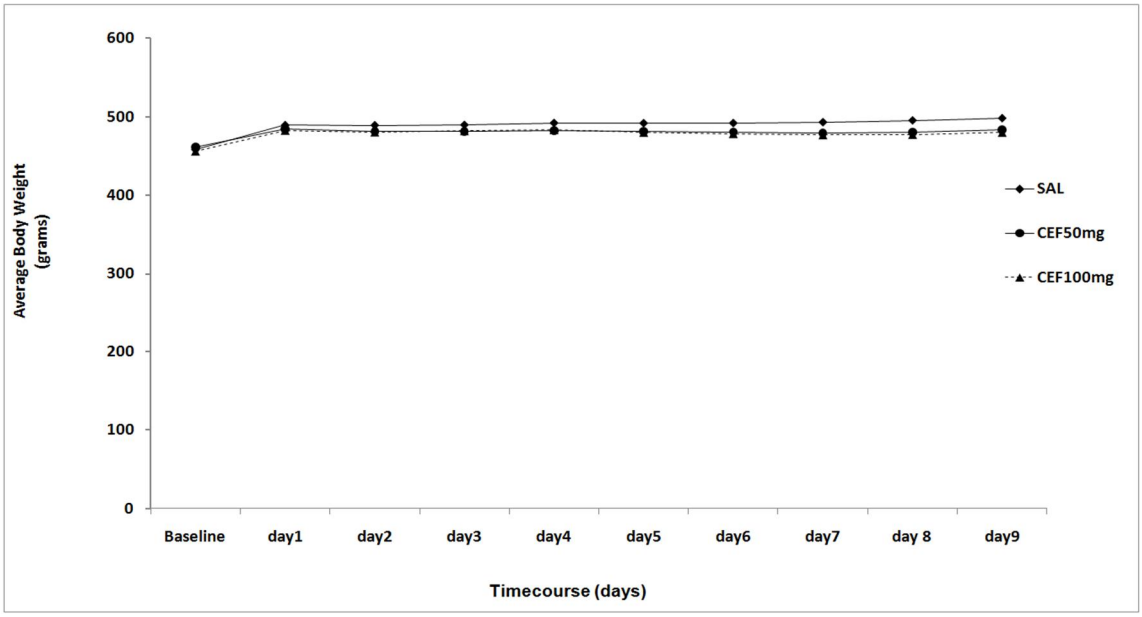


Figure3-3:

Daily body weight measurement of male P rats for 9 days, following 5 days of treatment with saline (n = 8) or ceftriaxone 50 mg/kg (n = 8) , and ceftriaxone 100 mg/kg (n = 8). The graph represents average daily weight (±SEM) during the 9 days of alcohol re-exposure. The baseline represents the average body weight of rats for the last two weeks of continuous alcohol exposure. A one-way ANOVA followed by a Dunnett post-hoc test demonstrated a non significant effect of dose and day during the duration of re-exposure for all groups, indicating that ceftriaxone did not affect body weight across the 9 days of re-exposure.

3.4 Effects of Ceftriaxone Treatment on Sucrose Drinking Behavior

As a control for motivated behavioral drinking, we further tested the effects of saline treated group (n = 4), ceftriaxone 50 mg/kg group (n = 5), and ceftriaxone 100

mg/kg group (n = 5) on sucrose (10%) consumption. Sucrose intake was examined over a period of 9 days. During the 9 days of re-exposure to sucrose (10%) after 5 days of treatment with either saline, ceftriaxone 50 mg/kg or ceftriaxone 100 mg/kg, sucrose consumption was measured daily as ml/kg/day for 9 days, starting on the first day of sucrose re-exposure. The data presented in Figure 3-4 shows the average sucrose consumption for 9 days for saline (n = 4), ceftriaxone 50 mg/kg (n = 5), ceftriaxone 100 mg/kg (n = 5) with the baseline being the average of rat's alcohol intake for the last week of continuous sucrose exposure, starting on the first day of sucrose re-exposure. A two way repeated measured ANOVA, followed by a Dunnett post-hoc test revealed a non significant main effect of interaction of day and treatment [$F(2,18) = 0.173$, $p = 1.00$], and a significant main effect of day [$F(1,9) = 4.152$, $p < 0.001$]. Simple effect analyses conducted as one-way ANOVA's for each day, demonstrated a non significant [$F < 0.514$, $p > 0.612$] differences among the doses for days 1 through 9. These data indicate that ceftriaxone did not affect sucrose intake.

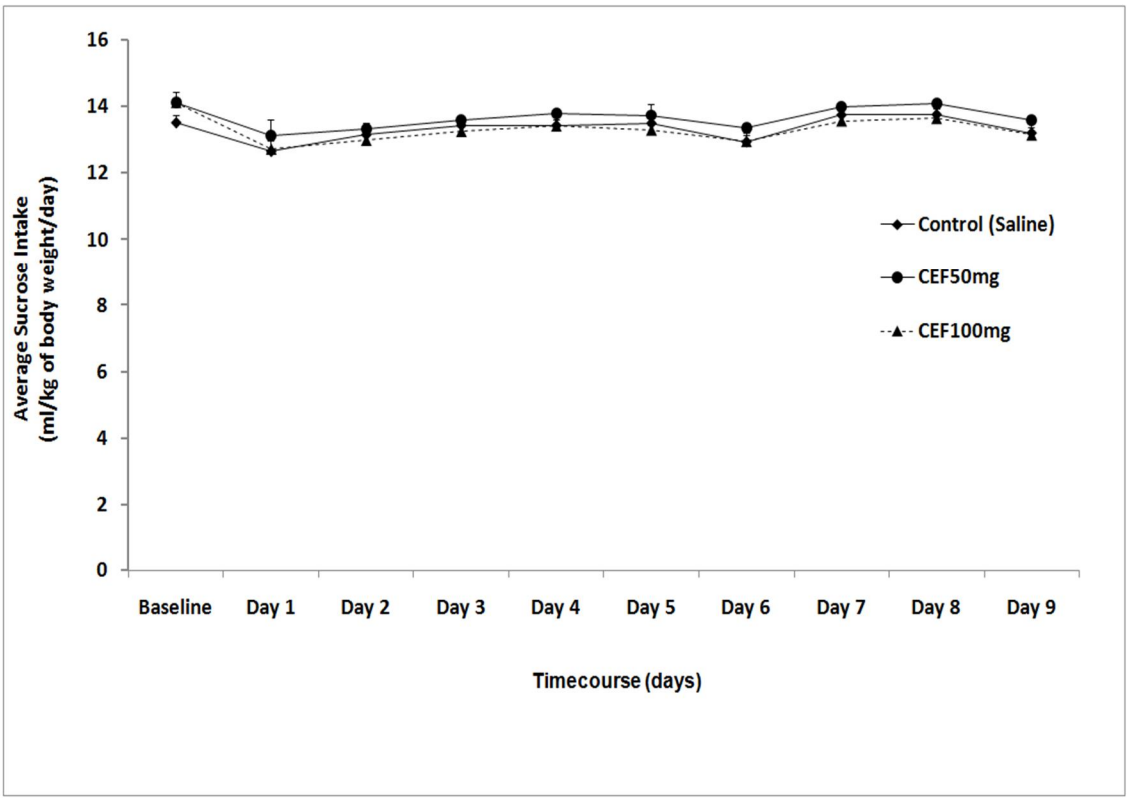


Figure 3-4:

Daily sucrose (10%)l intake of male P rats for 9 days, following 5 days of treatment with saline (n = 4) or ceftriaxone 50 mg/kg (n = 5) , and ceftriaxone 100 mg/kg (n = 5). The graph represents average daily sucrose (10%) consumption (±SEM) during the 9 days of sucrose (10%) re-exposure. While the day main effect was significant ($p < 0.001$), neither the interaction by day and treatment ($p = 1.00$) nor the day main effect ($p > 0.612$) were significant. Thus, ceftriaxone did not affect sucrose intake.

3.5 Effects of Ceftriaxone Treatment on GLT1 Expression

The expected changes in the expression of GLT1 within the nucleus accumbens core and prefrontal cortex were examined by western blot. A one way ANOVA followed by a Dunnett test showed a significant up-regulation of GLT1 in nucleus accumbens core expression in ceftriaxone 100 mg/kg treated group as compared to saline group [$F = 3.348$, $p = 0.039$] (Figure 3-5, A&B).

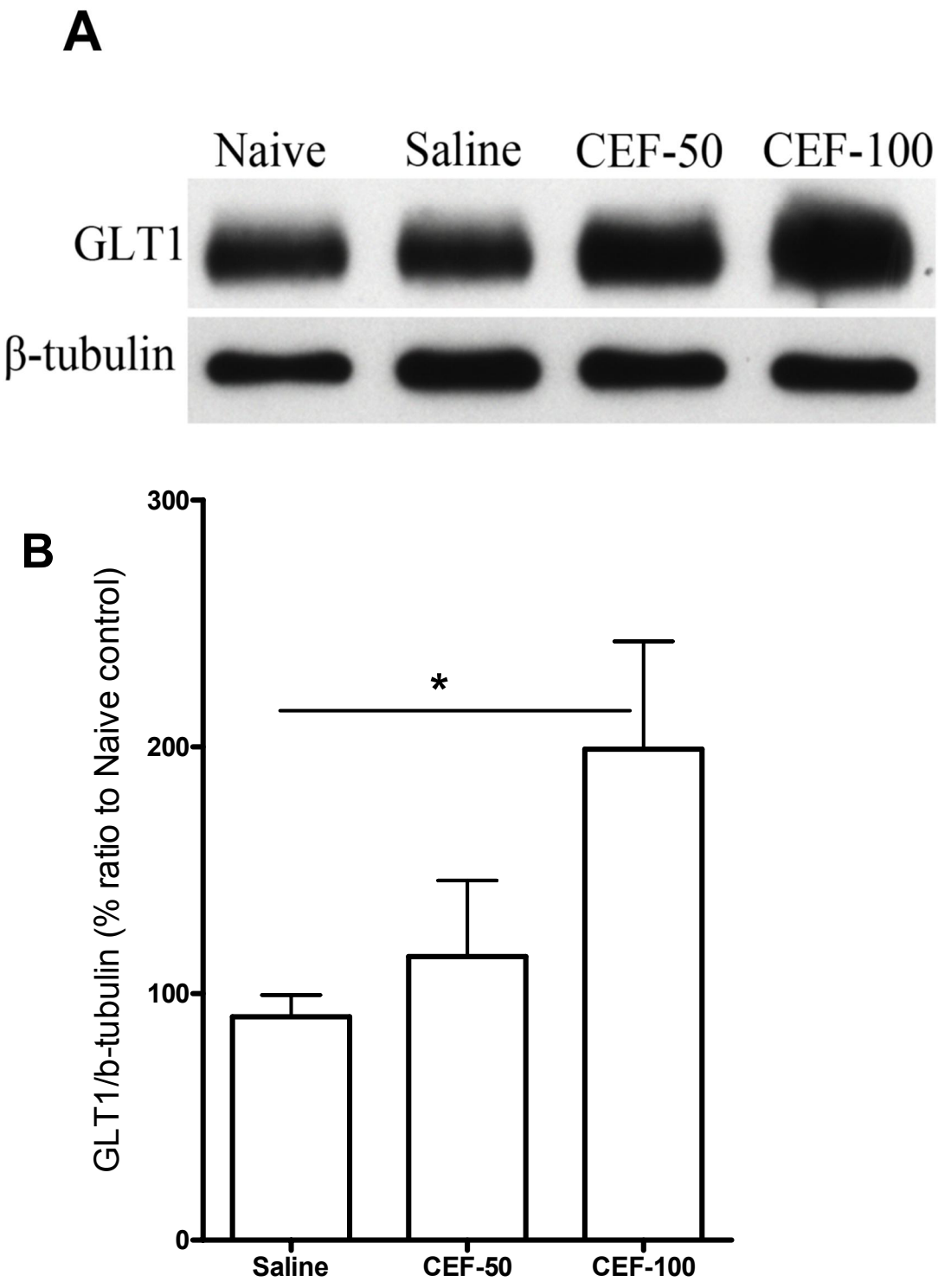
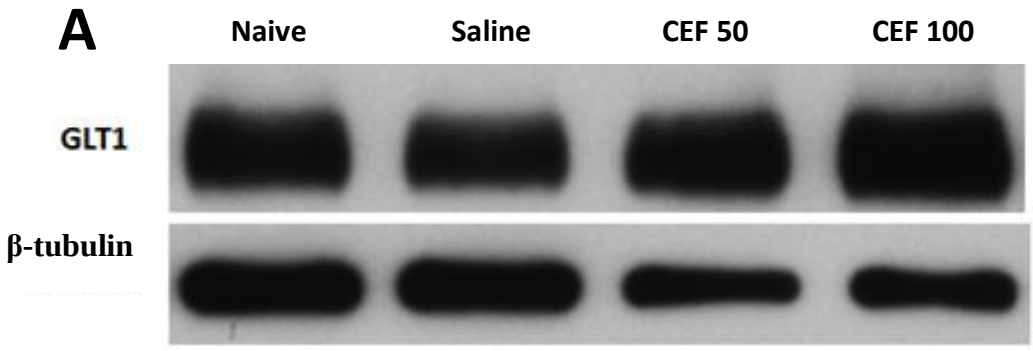


Fig. 3-5 (A&B): Effects of naive (n = 4), saline (n = 4), ceftriaxone 50mg/kg (n = 4), ceftriaxone 100mg/kg (n = 4) on GLT1 expression in the nucleus accumbens core.(A) Each panel presents immunoblots for β-tubulin, which was used as a control loading protein, and GLT1. B) Quantitative analysis revealed a significant increase in the ratio of

GLT1/ β-tubulin in CEF-100 treated group as compared to saline vehicle group. Error bars indicate SEM. (* $p<0.05$).

Preliminary data showed a trend towards up-regulation of the protein in ceftriaxone 100mg/kg dose in the prefrontal cortex, but this effect was not statistically significant (Figure 3-6, A&B). We are currently performing additional experiments to increase the sample size, to determine whether there is any significant difference in GLT1 expression between saline and ceftriaxone treated groups in prefrontal cortex, and also in other central reward brain regions such as nucleus accumbens shell and amygdala.



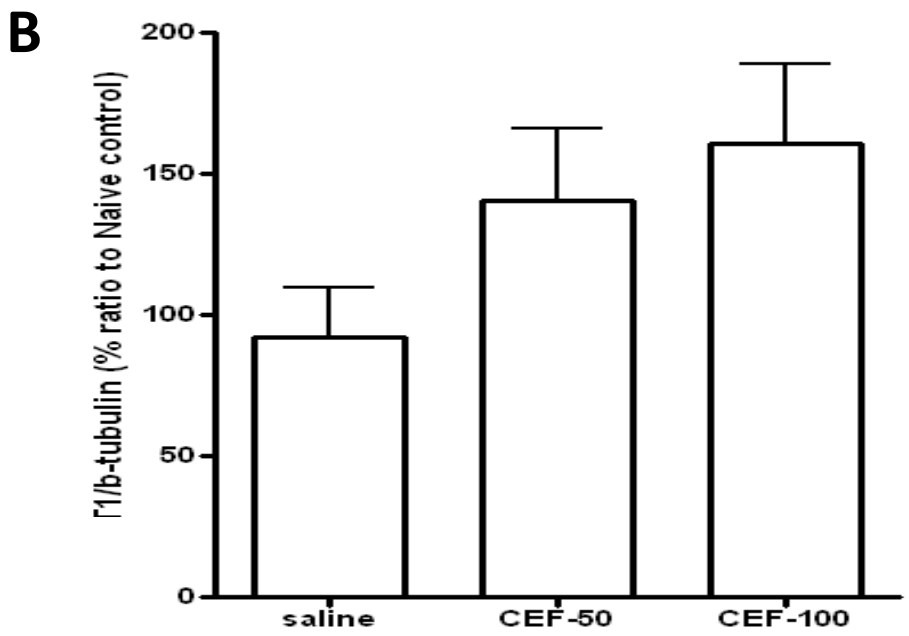


Figure 3-6 (A&B): Effects of naive (n = 4), saline (n = 4), ceftriaxone 50mg/kg (n = 4), ceftriaxone 100mg/kg (n = 4) on GLT1 expression in the prefrontal cortex. Each panel represents immunoblots for GLT1 expression and β-tubulin as a loading marker in PFC. B) Quantitative analysis revealed a trend towards up-regulation of the protein in Cef-100 treated groups but this effect was not statistically significant [F = 2.405, P = 0.10]. Error bars indicate SEM. (* $p<0.05$)

Discussion

The results of this present study demonstrate that treatment with ceftriaxone 50 mg/kg and ceftriaxone 100 mg/kg significantly attenuates relapse to alcohol seeking behavior, as compared to saline treated controls in rats. The reduction in alcohol consumption during re-exposure to alcohol (relapse) was correlated with an increase in GLT1 expression in the nucleus accumbens core, as well as a trend in up-regulation of GLT1 levels in the prefrontal cortex. Ceftriaxone treatment did not affect sucrose intake, indicating ceftriaxone's specificity in attenuating alcohol-seeking behavior. Furthermore, there were significant differences in water intake between saline and ceftriaxone treated

groups, however, there were no significant differences in the body weight between all the tested groups indicating that ceftriaxone does not affect body weight of animals. The increase in water intake in ceftriaxone treated groups could be explained by the fact that the decrease in their alcohol intake during relapse was compensated, in part, by the increase in water intake.

In this study, we investigated GLT1 protein levels in the prefrontal cortex and nucleus accumbens core. It has been established, that glutamate transmission in key brain regions of the reward circuit, plays a critical role in alcohol dependence and drug-seeking behavior. The glutamatergic system in the prefrontal cortex has been suggested to be involved in cocaine reinforcement (Sari et al., 2009). Animal studies of drugs of abuse, supports the importance of glutamate projections from prefrontal cortex to the nucleus accumbens and the ventral tegmental area (Goldstein and Volkow, 2002). It has been shown that elevations in extracellular glutamate during alcohol withdrawal from chronic alcohol exposure, are associated with increased number and function of *N*-methyl-D-aspartate (NMDA) glutamate receptors (Rossetti et al., 1999).The effect of NMDA receptor stimulation on the extracellular levels of glutamate in Sprague-Dawley rats withdrawn from chronic alcohol exposure by the administration of NMDA directly in the striatum of dependent rats, showed a significant increased glutamate output compared to sucrose controls as measured by microdialysis (Rossetti et al., 1999). The results suggest that glutamate-induced NMDA receptor-mediated elevations of extracellular glutamate during alcohol withdrawal, may contribute to the neuropathology associated with alcoholism, implicating the role of increased glutamate release in alcohol dependence (Rossetti et al., 1999). Further evidence indicates that chronic alcohol exposure leads to

an increase in glutamate output as well as an impaired ability in glutamate transport. The effect of chronic ethanol consumption on glutamate transporter binding and function was studied in the cerebral cortex of alcohol-preferring c AA rats (Schreiber and Freund, 2000). In this study, the alcohol-preferring c AA rats were chronically exposed to 10% v/v alcohol for 20 months, rats showed a down-regulation of neuronal and/or glial glutamate transporter activity, with limited re-uptake of synaptic glutamate (Schreiber and Freund, 2000).

The excessive amount of glutamate seen within the prefrontal cortex during alcohol withdrawal, causes hyperactivation of neurons that project to the nucleus accumbens, and promotes alcohol-seeking behavior. The medial prefrontal cortex, a terminal region of the mesocorticolimbic dopamine system, is composed of pyramidal glutamatergic neurons that are the output of this unit, these glutamatergic neurons are modulated by numerous neurotransmitter systems, including GABAergic interneurons that inhibit the firing of the glutamatergic neurons, preventing excessive glutamate release (Steketee, 2003). With chronic alcohol consumption, a decreased $GABA_A$ receptor density and sensitivity is observed; in contrast, an increased NMDA receptor density and sensitivity is encountered, resulting in the loss of inhibitory influence of the GABAergic interneurons over the cortical neurons. Alcohol-exposure alters glutamatergic activity in the mesocorticolimbic circuit, and the glutamatergic projections from the prefrontal cortex to the nucleus accumbens and the ventral tegmental area, are important in mediating craving for alcohol.

The specific role of GLT1 in addiction has been studied in various drug abuse models. For example, activation of GLT1 by MS-153 attenuated cocaine, morphine and methamphetamine conditioned place preference in mice (Nakagawa et al., 2005). In accordance, (Sari et al., 2009) reported that ceftriaxone attenuates cue-induced cocaine relapse in a dose-dependent manner. GLT1 is one of the major glutamate transporters, expressed predominantly in astroglial cells, and is responsible for the uptake of more than 90% of extracellular glutamate (Danbolt, 2001; Robinson, 1998). The glutamate transporters tightly regulate glutamate concentration. If an increase in glutamate transmission plays a major role in relapse to alcohol seeking behavior, then up-regulation of GLT1 should attenuate such response. We have tested this hypothesis using ceftriaxone, β-lactam antibiotic known to up-regulate GLT1, in our established male P rat model. Although, our present preliminary study in prefrontal cortex and studies from ours and others demonstrated that ceftriaxone administration for 5 days can lead to up-regulation of GLT1 levels (Sari et al., 2009, Sari et al., 2011, Rothstein et al., 2005) ceftriaxone has been shown to increase GLT1 activity independent of an increase in GLT1 expression. A study on wistar rats (Thone-Reinke et al., 2008) used a single dose of ceftriaxone treatment 200 mg/kg intraperitoneally (i.p), this treatment increased glutamate transporter activity without increasing GLT1 expression in a stroke model. It is therefore possible that ceftriaxone could reduce alcohol consumption by other mechanisms, or perhaps the GLT1 up-regulation could be secondary to an unknown primary mechanism.

Interestingly, chronic alcohol exposure is linked with a decrease in brain glutathione (GSH) and an increase in oxidized glutathione in vivo (Gotz et al., 2001).

Furthermore, alcohol withdrawal is associated with increases in oxygen-derived free radicals which by oxidation of thiol groups located on the glutamate transporters, may inhibit glutamate uptake (Volterra et al., 1994). Additionally, it has been shown that ceftriaxone increased both GSH and cysteine/glutamate exchanger (xCT) levels, leading to the reversal of the glutamate transporter deficits caused by the increased levels of free radical oxidation (Lewerenz et al., 2009). The xCT expression was increased by the use of ceftriaxone, in the nucleus accumbens of a cocaine relapse-behavior in a rat model (Knacksteddt et al., 2010). This could explain the decrease in alcohol drinking after treatment with ceftriaxone. It is possible that ceftriaxone-induced increase in xCT, and subsequent increase in the levels of GSH, corrected the deficits of the glutamate transporter, independent of GLT1 up-regulation.

Indeed this present study reports a correlation between increased GLT1 protein expression in the nucleus accumbens core, and the reduced alcohol drinking in relapse to alcohol seeking behavior, however, the next steps of our project could include:

I. Increasing the n value for the prefrontal cortex.
II. Identifying brain regions in which ceftriaxone has increased GLT1 protein expression, and other regions in which it had no effect. Additional key brain regions involved in the reward neurocircuitry such as the nucleus accumbens shell, amygdala and hippocampus need to be examined as well.
III. Additionally, brain regions of the reward circuitry, could be further separated into their sub-components to seek the possibility of any

differential effects. For example, the prefrontal cortex can be further subdivided into prelimbic cortex, infralimbic cortex and cingulated cortex.

IV. Future studies are needed to examine the mechanisms by which ceftriaxone reduces alcohol drinking. For example, dihydrokainic acid (DHK), a selective GLT1 antagonist, could be co-administered with ceftriaxone to ensure that the activation of GLT1 by ceftriaxone is the direct mechanism of action in the reduction of alcohol intake, rather than other unknown mechanisms of the drug.

V. Moreover, the role of other transporters, such as VGLUTs and GLAST in relapse to alcohol seeking behavior should be examined.

In conclusion, our findings demonstrate that activation of GLT1 by ceftriaxone attenuates relapse to alcohol-seeking behavior in male P rats. Studies revealed that the effect of ceftriaxone appears to be selective to GLT1 since other glutamate transporters are unaffected by this drug. Our results suggest that activation of GLT1 counteracts elevated extracellular glutamate in central reward brain regions that are involved in drug abuse, including alcohol. Thus, we suggest that GLT1 may be a potential target for decreasing the extracellular glutamate level, and consequently attenuating relapse to alcohol-drinking behavior.

References

Beart, P. M. and O'Shea, R. D. "Transporters for L-glutamate: An update on their molecular pharmacology and pathological involvement". British Journal of Pharmacology, 150 (2007): 5–17.

Biggio G, Concas A, Follesa P, et al. "Stress, ethanol, and neuroactive steroids". Pharmacology and Therapeutics.116 (2007): 140–171.

Blednov YA, Harris AR."Metabotropic glutamate receptor 5 (mGluR5) regulation of ethanol sedation, dependence and consumption: Relationship to acamprosate actions." International Journal of Neuropsychopharmacology. 11 (2008): 775-793.

Danbolt NC. " Glutamate uptake". ProgNeurobiol 65 (2001): 1-105.

Deitrich RA, Dunwiddie TV, Harris RA, Erwin VG. "Mechanism of action of ethanol: initial central nervous system actions". Pharmacological Reviews. 41 (1989): 489-537.

Eriksson K. "Ethyl alcohol consumption: valid measurement in albino rats." Science (New York, N. Y.). 161 (1968): 76-7.

Feltenstein, M. W. and See, R. E. "The neurocircuitry of addiction: an overview". British Journal of Pharmacology. 154 (2008): 261–274.

Gatto GJ, Murphy JM, Waller MB, McBride WJ, Lumeng L, Li TK. "Chronic ethanol tolerance through free-choice drinking in the P line of alcohol-preferring rats". Pharmacology,Biochemistry,and Behavior. 28 (1987): 111-115.

Gessa GL, Muntoni F, Collu M, Vargiu L, Mereu G. "Low doses of ethanol activate dopaminergic neurons in the ventral tegmental area."Brain Research. 348 (1985): 201-203.

Gilpin NW, Koob GF."Neurobiology of Alcohol Dependence: Focus on Motivational Mechanisms". Alcohol Research & Health. 31 (2008): 185-195.

Goldstein RZ, Volkow ND . "Drug addiction and its underlying neurobiological basis: neuroimaging evidence for the involvement of the frontal cortex." TheAmerican journal of Psychiatry.159 (2002): 1642-1652.

Gotz, M. E., Janetzky, B., Pohli, S., Gottschalk, A., Gsell, W., Tatschner, T., et al. "Chronic alcohol consumption and cerebral indices of oxidative stress: is there a link? "Alcoholism, Clinical &Expermental Research, 25 (2001): 717-725.

Groenewegen, H. J., Galis-de Graaf, Y., & Smeets, W. J. "Integration and segregation of limbic cortico-striatal loops at the thalamic level: an experimental tracing study in rats." Journal of Chemical Neuroanatomy, 16 (1999): 167-185.

Groenewegen, H. J., Wright, C. I., & Beijer, A. V. "The nucleus accumbens: gateway for limbic structures to each the motor system?". Progress in Brain Research, 107 (1996): 485-511.

Hodge CW, Samson HH, Chappelle AM. "Alcohol self-administration: Further examination of the role of dopamine receptors in the nucleus accumbens". Alcoholism: Clinical and Experimental Research.21 (1997): 1083–1091.

Hyytia P, Koob GF. "$GABA_A$ receptor antagonism in the extended amygdala decreases ethanol self-administration in rats*".* European Journal of Pharmacology*. 283 (1995)*: 151–159.

Johnson BA."Update on neuropharmacological treatments for alcoholism: Scientific basis and clinical findings." Biochemical Pharmacology.75 (2008): 34–56.

Kanai Y, Hediger MA. "The glutamate and neutral amino acid transporter family: physiological and pharmacological implications" European Journal of Pharmacology. 479 (2003): 237-247.

Kim, K., Lee, S.-G., Kegelman, T. P., Su, Z.-Z., Das, S. K., Dash, R., Dasgupta, S., Barral, P. M., Hedvat, M., Diaz, P., Reed, J. C., Stebbins, J. L., Pellecchia, M., Sarkar, D. and Fisher, P. B. "Role of Excitatory Amino Acid Transporter-2 (EAAT2) and glutamate in neurodegeneration: Opportunities for developing novel therapeutics." Journal of Cellular Physiology, 226 (2011): 2484–2493.

Knackstedt, L. A., Melendez, R. I., &Kalivas, P. W."Ceftriaxone restores glutamate homeostasis and prevents relapse to cocaine seeking". Biological Psychiatry, 67 (2010): 81-84.

Koob GF. "Alcoholism: Allostasis and beyond." Alcoholism: Clinical and Experimental Research.27 (2003): 232–243.

Koob, G. F., Roberts, A. J., Schulteis, G., Parsons, L. H., Heyser, C. J., Hyytiä, P., Merlo-Pich, E. and Weiss, F. "Neurocircuitry Targets in Ethanol Reward and Dependence". Alcoholism: Clinical and Experimental Research. 22 (1998): 3–9.

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

Lewerenz, J., Albrecht, P., Tien, M. L., Henke, N., Karumbayaram , S., Kornblum, H. I., et al. "Induction of Nrf2 and Xct are involved in the action of the neuroprotective antibiotic ceftriaxone in vitro." Journal of Neurochemistry. 111 (2009): 332-343.

Liebman, J.M., & Butcher, L. L."Effects on self-stimulation behavior of drugs influencing dopaminergic neurotransmission mechanisms." NaunynSchmiedebergsArchieves of Pharmacology, 277 (1973): 305-318.

Li TK, Lumeng L, McBride WJ, Waller MB. "Progress toward a voluntary oral consumption model of alcoholism." Drug and Alcohol Dependence. 4 (1979): 45-60.

Li TK, Lumeng L, McBride WJ, Murphy JM. "Rodent lines selected for factors affecting alcohol consumption."Alcohol and Alcoholism. 1 (1987): 91-96.

Littleton JM."Acamprosate in alcohol dependence: Implications of a unique mechanism of action". Journal of Addiction Medicine. 1 (2007): 115–125.

Lovinger DM, White G, Weight FF. "Ethanol inhibits NMDA-activated ion current in hippocampal neurons."*Science.*243 (1989): 1721–1724.

Lumeng L, Li TK."The development of metabolic tolerance in the alcohol-preferring P rats: comparison of forced and free-choice drinking of ethanol." Pharmacology, Biochemistry,and Behavior. 25 (1986): 1013-1020.

McBride, W. J., & Li T.K."Animal models of alcoholism: neurobiology of high alcohol-drinking behavior in rodents ." Critical Reviews in Neurobiology, 12 (1998): 339-369.

Mhatre MC, Pena G, Sieghart W, Ticku MK. "Antibodies specific for GABAA receptor alpha subunits reveal that chronic alcohol treatment down-regulates alpha-subunit expression in rat brain regions". Journal of neurochemistry. 61 (1993): 1620-1625.

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

Myers, C. E., & Gluck, M. A. "Context, conditioning, and hippocampal rerepresentation in animal learning." Behavioral Neuroscience, 108 (1994): 835-847.

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

Nicholas W. Gilpin, Ph.D. and George F. Koob, Ph.D."Neurobiology of Alcohol Dependence: *Focus on Motivational Mechanisms"* Alcohol Res Health. 31 (2008): 185-195.

Olive MF, Koenig HN, Nannini MA, Hodge CW."Stimulation of endorphin neurotransmission in the nucleus accumbens by ethanol, cocaine, and amphetamine". Journal of Neuroscience.21 (2001): RC184.

Penn PE, McBride WJ, Lumeng L, Gaff TM, Li TK. "Neurochemical and operant behavioral studies of a strain of alcohol-preferring rats."Pharmacology, Biochemistry,and Behavior. 8 (1978): 475-481.

Phillips, A. G. "Brain reward circuitry: a case for separate systems." Brain Research Bulletin, 12 (1984): 195-201.

Phillips, A. G., & Fibiger, H. C. "The role of dopamine in maintaining intracranial self-stimulation in the ventral tegmentum, nucleus accumbens, and medial prefrontal cortex". Canadian Journal of Psychology, 32 (1978): 58-66.

Pulvirenti L, Diana M."Drug dependence as a disorder of neural plasticity: Focus on dopamine and glutamate." Reviews in the Neurosciences. 12 (2001): 141–158.

Roberts AJ, Mcdonald JS, Heyser CJ, et al."μ-Opioid receptor knockout mice do not self-administer alcohol". Journal of Pharmacology and Experimental Therapeutics. 293 (2000): 1002–1008.

Robinson MB."The family of sodium-dependent glutamate transporters: a focus on the GLT1/EAAT2 subtype". Neurochem Int. 33 (1998): 479-91.

Rodd-Hendricks, Z. A., Bell, R. L., Kuc, K. A., Murphy, J. M., McBride, W. J., Lumeng, L., et al."Effects of ethanol exposure on subsequent acquisition and extinction of ethanol self-administration and expression of alcohol-seeking behavior in adult alcohol-preferring (P) rats: II. Adult exposure". Alcohol Clinical & Experimental Research, 26 (2002): 1642-1652.

Rodd-Henricks ZA, McKinzie DL, Shaikh SR, Murphy JM, McBride WJ, Lumeng L, Li TK.

"Alcohol deprivation effect is prolonged in the alcohol preferring (P) rat after repeated deprivations."Alcoholism, Clinical and Experimental Research. 24 (2000): 8-16.

Rodd, Z. A., Bell, R. L., McQueen, V. K., Davids, M. R., Hsu, C. C., Murphy, J. M., et al. "Prolonged increase in the sensitivity of the posterior ventral tegmental area to the reinforcing effects of ethanol following repeated exposure to cycles of ethanol access and deprivation."The Journal of Pharmacology & Experimental Therapeutics, 315 (2005): 648-657.

Rodd ZA, Bell RL, Sable HJ, Murphy JM, McBride WJ. "Recent advances in animal models of alcohol craving and relapse." Pharmacology, Biochemistry,and Behavior. 79 (2004): 439-450.

Rossetti, Z. L., Carboni, S., &Fadda, F."Glutamate-induced increase of extracellular glutamate through N-methyl-D-aspartate receptors in ethanol withdrawal." Neuroscience, 93 (1999): 1135-1140.

Rothstein JD, Dykes-Hoberg M, Pardo CA, Bristol LA, Jin L, Kuncl RW, Kanai Y, Hediger MA, Wang Y, Schielke JP, Welty DF . "Knockout of glutamate transporters reveals a major role for astroglial transport in excitotoxicity and clearance of glutamate." Neuron. 16 (1996): 675-686.

Rothstein, J. D., Patel, S., Regan, M. R., Haenggeli, C., Huang, Y. H., Bergles, D. E., et al. "Beta-lactam antibiotics offer neuroprotection by increasing glutamate transporter expression." Nature, 433 (2005): 73-77.

Rothstein, J. D., Van Kmmen, M., Levey, A. I., Martin, L. J., &Kuncl, R. W. "Selective loss of glial glutamate transporter GLT-1 in amyotrophic lateral sclerosis". Annals of Neurology, 38 (1995): 73-84.

Sari Y, Johnson VR, Weedman JM."Role of the serotonergic system in alcohol dependence: from animal models to clinics."Progress in Molecular Biology Translational Science. 98 (2011): 401-443.

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

Schreiber, R., & Freund, W.D. "Glutamate transport is downregulated in the cerebral cortex of alcohol-preferring rats." Medical Science Monitor: international medical journal of experimental & clinical research, 6 (2000), 649-652.

Seasack, S.R., & Grace, A. A. "Cortico-Basal Ganglia reward network: microcircuitry." Neuropsychopharmacology, 35 (2010): 27-47.

Spanagel R. "Recent animal models of alcoholism." Alcohol Research & Health: the journal of National Institute on Alcohol Abuse & Alcoholism. 24 (2000): 124-131.

Spanagel R, Hölter SM. "Long-term alcohol self-administration with repeated alcohol deprivation phases: an animal model of alcoholism?"Alcohol and Alcoholism. 34 (1999): 231-243.

Spanagel R, Hölter SM. "Pharmacological validation of a new animal model of alcoholism." Journal of Neural Transmission.107 (2000): 669-680.

Spanagel R, Pendyala G, Abarca C, et al. "The clock gene Per2 influences the glutamatergic system and modulates alcohol consumption." *Nature Medicine.*11 (2005): 35–42.

Spanagel R, Zieglgänsberger W."Anti-craving compounds for ethanol: new pharmacological tools to study addictive processes."Trends in Pharmacological Sciences. 18 (1997): 54-90.

Steketee, J. D."Neurotransmitter systems of the medial prefrontal cortex: potential role in sensitization to psychostimulants." Brain Research Brain Research Reviews. 41 (2003): 203-228.

Thone-Reineke, C., Neumann, C., Namsolleck, P ., Schmerbach, K., Krikv, M., Schefe, J. H., et al."The beta-lactam antibiotic, ceftriaxone, dramatically improves survival, increases glutamate uptake and induces neurotrophins in stroke". Journal of Hypertension, 26 (2008): 2426-2435.

Ulm RR, Volpicelli JR, Volpicelli LA."Opiates and alcohol self-administration in animals" The Journal of Clinical Psychiatry. 56 Suppl 7 (1995): 5-14.

Volterra, A., Trotti, D., Tromba, C., Floridi, S., &Racagani, G. "Glutamate uptake inhibition by oxygen free radicals in rat cortical astrocytes." The Journal of Neuroscience, 14 (1994): 2924-2932.

V **Vengeliene, A Bilbao, A Molander, R Spanagel.** "Neuropharmacology of alcohol addiction" British Journal of Pharmacoloy. 154 (2008): 299-315

Waller MB, McBride WJ, Gatto GJ, Lumeng L, Li TK. "Intragastric self-infusion of ethanol by ethanol-preferring and -nonpreferring lines of rats."Science. 225 (1984): 78-80.

Waller MB, McBride WJ, Lumeng L, Li TK. "Effects of intravenous ethanol and of 4-methylpyrazole on alcohol drinking in alcohol-preferring rats."Pharmacology, Biochemistry, and Behavior.17 (1982): 763-768.

Weiss F, Lorang MT, Bloom FE, Koob GF. "Oral alcohol self-administration stimulates dopamine release in the rat nucleus accumbens: Genetic and motivational determinants." Journal of Pharmacology and Experimental Therapeutics.267 (1993): 250–258.

Weiss F, Parsons LH, Schulteis G, et al."Ethanol self-administration restores withdrawal-associated deficiencies in accumbal dopamine and 5-hydroxytryptamine release in dependent rats." Journal of Neuroscience.16 (1996): 3474–3485.

Wolffgramm J, Galli G, Thimm F, Heyne A. "Animal models of addiction: models for therapeutic strategies?".Journal of Neural Transmission. 107 (2000): 649-668.

Yaniv, D., Desmedt, A., Jaffard, R., & Richter-Levin, G. "The amygdala and appraisal processes: stimulus and response complexity as an organizing factor." Brain Research Brain Research Review, 44 (2004): 179-186.

Yin, H. H., & Knowlton, B. J."The role of the basal ganglia in habit formation." Nature Reviews Neuroscience, 7 (2006): 464-476.

Zhou FC, Sahr RN, Sari Y, Behbahani K."Glutamate and dopamine synaptic terminals in extended amygdala after 14-week chronic alcohol drinking in inbred alcohol-preferring rats." Alcohol. 39 (2006): 39-49.