

A Thesis

Entitled

Pharmacological Studies of Compounds Targeting Glutamate Transporter 1 for the
Attenuation of Alcohol-Drinking Behavior in Alcohol Preferring Rats

By

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

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Alcoholism is a disorder involving chronic and progressive alcohol intake which leads to mental, physical and social problems. Drinking alcohol has an impact on various neurotransmitter systems within the brain. For example, alcohol self-administration increases dopamine and glutamate neurotransmission in central reward brain regions such as prefrontal cortex (PFC) and nucleus accumbens (NAC). Evidence demonstrated that glutamatergic system is implicated in the development of alcohol dependence. Extracellular glutamate levels are regulated by a number of glutamate transporters. Among these transporters, glutamate transporter 1 (GLT-1) is responsible for clearance of more than 90 % of the extracellular glutamate. Studies from our lab have shown that ceftriaxone (CEF), a beta lactam antibiotic known to upregulate GLT-1 level, reduced ethanol-drinking behavior in alcohol preferring (P) rats in both chronic and relapse-like ethanol-drinking behavior paradigms. In this study, the differential effects of CEF on

GLT-1 isoforms (GLT-1a and GLT-1b), cystine-glutamate antiporter (xCT) and glutamate-aspartate transporter (GLAST) were examined (using Western blot) in naïve (water) control, saline vehicle control (relapse-like ethanol), CEF (100 mg/kg, i.p.) treated P rat groups. Furthermore, we examined the effect of MS-153, a novel neuroprotective agent, on ethanol consumption and GLT1 expression and its signaling pathway in naïve (water) control, saline vehicle control (ethanol), MS-153 (20 and 50 mg/kg, i.p.) treated P rats. Our results showed that CEF treatment attenuated relapse-like ethanol-drinking behavior in P rats. This attenuation was associated, in part, with up-regulation in the levels of GLT-1a, GLT-1b, as well as xCT in PFC and NAC. However, we did not observe any significant change in GLAST level between all groups. Alternatively, we found that MS-153 significantly reduced ethanol intake in P rats at dose-dependent manner. Importantly, MS-153 up-regulated GLT-1 levels in NAC and amygdala (AMG) but not in PFC. Furthermore, we found that up-regulation of GLT-1 level was associated with up-regulation of translocated Nuclear Factor kappa B (NFκB) and reduction in cytosolic IκB-α levels. Our data showed for the first time that a novel compound, MS-153, targeting GLT-1 had the beneficiary effect for attenuating ethanol intake. Together, our findings demonstrated GLT-1 as a potential therapeutic target for treatment of alcohol dependence.

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Chapter 1

Introduction

1.1 Overview

Alcoholism or alcohol dependence is a disorder, which involves chronic and progressive alcohol intake leading to mental, physical and social problems (Bush et al., 1987). The alcohol use disorders are referred to as alcohol abuse and alcohol dependence (Schuckit, 2009). Alcohol abuse is an unhealthy use of alcohol that causes physical and mental effects as a result of drinking increasing amounts of alcohol. It is associated with social troubles, washout to perform major role obligations and use in dangerous conditions. Alcohol dependence, on the other hand, is a more serious condition characterized by the same signs of alcohol abuse with the presence of three or more of the followings: tolerance, withdrawal effect, spending long time to get alcohol and recover from its effects, incessant willingness or unsuccessful trials to cut off or control use and persistent use in spite of deleterious consequences (Saitz, 2005).

Craving of alcohol usually exists along with alcohol dependence. It is associated with a strong tendency to drink alcohol during and after a withdrawal period. Craving may have substantial entanglement in treating alcohol dependence. For example,

reducing craving leads to reduce alcohol consumption and vice versa (Swift, 1999).

Tolerance to alcohol-drinking behavior has been suggested as reduced response to certain amounts of alcohol upon repeated administrations. Thus, higher doses of alcohol may have the same effects in alcoholics. Accordingly, alcohol tolerance has been found to play a significant role in alcohol abuse and dependence (Miller et al., 2012). Alcohol withdrawal effects are troublesome mental and physical symptoms that may happen after stopping alcohol drinking. Thus, alcoholics have tendency to consume alcohol in order to avoid these symptoms (Finn and Crabbe, 1997).

Although moderate alcohol drinking is associated with some beneficial effects such as decreasing risk of coronary heart disease (Rimm et al., 1999), the use of alcohol is usually associated with ample deleterious impacts. The National Institute on Alcohol Abuse and Alcoholism (NIAAA) has summarized some of these impacts as shown in table 1.1 (NIAAA, 2012).

In addition to these deleterious effects, listed in table 1.1, alcohol use disorders are involved in almost all forms of violence (Schofield and Denson, 2013). Alcohol has been considered as a major agent that precipitates aggression and reduces the ability to control behavior. It is implicated in violent crimes, sexual assaults, and intimate partner violence, as well as, its association with injuries and cost for both victims and wrongdoer (Heinz et al., 2011). Furthermore, alcohol use disorders have significant ramifications, not only on the health and health care costs, but also on decreased productivity at work and puts an extra burden on the economy (Parker et al., 1987).

Table 1.1: The effects of alcohol consumption on different organs and systems

	Acute consumption (intake of excess alcohol in single occasion or short period of time)	Chronic consumption (regular intake of alcohol for long period of time)
Brain	Depression, agitation, memory loss, and seizures	Impairs many functions including motor coordination, temperature regulation, sleep, mood and many cognitive functions like learning and memory
Cardio vascular system	Arrhythmias, strokes, and hypertension	Alcoholic cardiomyopathy, arrhythmias, strokes and hypertension
Liver	Fatty liver (steatosis) and alcoholic hepatitis	Fatty liver (steatosis), alcoholic hepatitis, and cirrhosis
Pancreas		Pancreatitis
Cancer risks	Risk factor for mouth, esophagus, pharynx, larynx, liver and breast cancers	Risk factor for mouth, esophagus, pharynx, larynx, liver and breast cancers
Immune system	Suppress both the innate and adaptive immune systems, and decrease the production of cytokines	Suppress both innate and adaptive immune systems, disturb the production of cytokines, and repress the development of T-cell

Understanding the mechanisms of alcohol action, especially in the brain, would produce opportunities for treating alcohol use disorders. Many attempts have been made to find appropriate treatments for alcoholism targeting different neuronal circuitry to decrease motivation, craving and withdrawal effects of alcohol. Our studies are another endeavor regarding this aspect.

1.2 The Neurocircuitry System Mediating the Mechanism of Reward

The motivation to use alcohol and substances of abuse may be due to their reinforcing effects (Wise, 1998). Three main systems have been involved in the emergence of drug addiction and seeking behavior. These systems include: arousal, cognition and reward systems. Chronic drug addiction, including alcohol, is usually followed by cognitive impairments. On the other hand, an arousal system is required, by the organism, in order to regulate many functions such as waking and sleeping. Wakefulness may be associated with excitation of some other systems in the brain. Likewise, wakefulness has been involved in the reward properties of the brain. The reward system plays a central role in all types of behavior, including drug and alcohol addiction (Vetulani, 2001).

The behavior is modulated by reinforcement and neuroadaptation. These two factors appear to induce short term (i.e., acute) and long term (i.e., chronic) responses and craving for the drug of addiction, respectively. Reinforcement refers to the continual use of the drug either to increase the response (e.g., drug induced euphoria), which is termed as positive reinforcement or to avoid the withdrawal effects, which indicates negative reinforcement. On the other hand, drug effects may be enhanced (sensitization) or decreased (counter-adaptation or desensitization) upon repeated administration and this is called neuroadaptation (Roberts and Koob, 1997)

1.2.1 Anatomy of the Reward Neurocircuitry

1.2.1.1 The Mesocorticolimbic System

The neurochemical substrate for reward neurocircuitry involves the mid brain dopaminergic projections from the ventral tegmental area (VTA) projecting to the shell region of the nucleus accumbens (NAC) and prefrontal cortex (PFC) (Wise and Rompre, 1989). Cell bodies from VTA also project to olfactory tubercle, septum, amygdala (AMG), and hippocampus. Further, the medial VTA has projections to the medial prefrontal, cingulate and perirhinal cortices. All these connections form the mesocorticolimbic system (Wise, 2004) which is strongly implicated in reward-related behavior (Sun, 2011).

VTA projections comprise the basic reward system in the brain. In the NAC, it has been found that natural rewarding actions (e.g., eating, drinking, and sexual activities) and addictive substances (e.g., alcohol) can increase synaptic dopaminergic transmission. Long term administration of addictive substances can have consequences leading to disruption (sensitization or desensitization to some drug effects) of the reward neurocircuitry (Vetulani, 2001).

The extended amygdala, which is a neural circuitry within the basal forebrain, and its connections are also implicated in reward behavior. It is composed of the bed nucleus of the stria terminalis, central medial amygdala, and the posterior shell of the nucleus accumbens. It receives afferent fibers from limbic cortices, hippocampus, basolateral amygdala, mid brain, and hypothalamus while efferent fibers go to the posterior medial

ventral pallidum, VTA, brainstem, and to the lateral hypothalamus (Alheid and Heimer, 1988, Koob, 2003, 2009).

1.2.1.2 Regulation of Neurotransmission in Mesocorticolimbic System

The mesocorticolimbic dopamine system is regulated by both auto-regulation (by the action of dopamine auto-receptors) and afferent regulation to VTA neurons (White, 1996).

There are glutamatergic neuron projections from PFC and amygdala (AMG) to VTA (Phillipson, 1979, Gonzales and Chesselet, 1990). Moreover, NAC receives glutamatergic fibers from PFC and basolateral AMG (Hu and White, 1996). The reciprocal connections between PFC and AMG are also glutamatergic (Kalivas, 2004). These excitatory glutamatergic projections play a role in regulation of firing of mesocorticolimbic dopaminergic neurons (Gariano and Groves, 1988, Hu and White, 1996) and is strongly linked to the development of drug addiction (Kalivas, 2004).

Dopaminergic neurons are also regulated by inhibitory gamma amino butyric acid (GABA) containing neurons. In VTA, dopaminergic firing is inhibited by the action of GABA-containing neurons (Jhou et al., 2009, van Zessen et al., 2012) originating from NAC (Walaas and Fonnum, 1980) and ventral pallidum (Zahm, 1989). Moreover, GABAergic neurotransmission within central AMG plays significant role in reward related behaviors of substances of abuse (McBride et al., 1998, Koob and Le Moal, 2001).

Other neurotransmitter systems have different roles in regulation of reward neurocircuitry. For example, 5-HT_{2A} and 5-HT_{2C} subtypes of serotonin (5-HT) receptors play a significant regulatory role in dopamine neurotransmission and behavioral effects due to cocaine intake (Schmidt et al., 1992, Filip, 2005). In addition, VTA receives cholinergic fibers from pedunclopontine tegmental nucleus which act on both muscarinic and nicotinic receptors (Woolf, 1991) and induce, by action on nicotinic receptors, activation of the dopaminergic neurons (Grenhoff et al., 1986). Orexin-containing neurons projections from lateral hypothalamus in to NAC and VTA play an important role in reward motivation and function by increasing the firing of dopaminergic and glutamatergic neurons (Harris et al., 2005, Borgland et al., 2008). Moreover, Cannabinoids activate both VTA-NAC circuit (i.e., increase the dopamine concentrations in VTA and NAC) and reward related behaviors (Gardner, 2002). Furthermore, the opioid system was found to regulate dopamine levels in reward center. Beta-endorphin fibers arising from nucleus arcuatus act on mu (μ) opioid receptor in VTA to increase the dopamine release and kappa (κ) opioid receptor in NAC to decrease dopamine release. In addition, studies indicate that reward action might be mediated through a dopamine-independent mechanism involving the action on post synaptic opioid receptors in NAC (Self and Nestler, 1995, Herz, 1997). Glycine receptors in NAC act to increase the firing of dopaminergic neurons in VTA by reducing the inhibitory effect of GABA containing regulatory neurons projected from NAC to VTA (Soderpalm et al., 2009). Alternatively, Ghrelin, which is a peptide for food intake and energy regulation (van der Lely et al., 2004), binds to ghrelin receptors on both dopaminergic and GABA neurons to increase the secretion of dopamine in VTA and its turnover in NAC (Abizaid et al., 2006).

1.2.2 The Effects of Alcohol on Neurotransmission within the Reward Neurocircuitry

Drinking alcohol has an impact on various neurotransmitter systems within the mesocorticolimbic system (Fig 1-1), the structure strongly implicated in rewarding actions of the substances of abuse. Alcohol self-administration increases dopamine levels in NAC (Weiss et al., 1993) and medial PFC (Schier et al., 2013) due to activation of VTA dopaminergic neurons, however, chronic consumption of similar doses of alcohol has less effect on dopamine levels because of development of tolerance in these VTA neurons (McBride et al., 1990). Similar to dopamine, 5-HT levels are increased in NAC during acute alcohol consumption and tolerance after chronic use (De Montis et al., 2004).

Ethanol acts on the allosteric site on GABA receptors to induce hyperpolarization or enhance the effect of GABA (Koob, 2004). Acute ethanol consumption increased GABA discharge in central amygdala with an increase in the basal GABA levels after chronic alcohol treatment (Koob, 2004, Roberto et al., 2004a). On the other hand, acute ethanol administration tends to decrease GABA discharge in rat's NAC (Piepponen et al., 2002).

Regarding the glutamatergic system, ethanol has an antagonistic effect on N-methyl-D-aspartate (NMDA) receptor subtype when consumed acutely (Hoffman et al., 1989, Bhave et al., 1996). Furthermore, chronic ethanol consumption induces a compensatory upregulation of NMDA receptors (Snell et al., 1996). Moreover, acute alcohol drinking is associated with decreased glutamatergic transmission and glutamate

levels in NAC and AMG whereas increased glutamate release followed chronic alcohol drinking (Roberto et al., 2004b). With chronic ethanol drinking, extracellular glutamate reuptake decreased leading to CNS excitability after alcohol withdrawal (Melendez et al., 2005).

Alcohol drinking might affect other neurotransmitter systems. For instance: acute alcohol consumption results in increased level of beta-endorphin in blood plasma while chronic exposure results in decreases in its level (Zalewska-Kaszubska and Czarnecka, 2005). Ethanol may increase extracellular acetylcholine levels in VTA by stimulation of cholinergic afferents to VTA (Larsson et al., 2005) as well as increasing the response of high affinity neuronal nicotinic acetylcholine receptors, which are implicated in rewarding properties of alcohol (Liu et al., 2012). Alternatively, cannabinoid 1 receptors (CB1) are involved in motivation to alcohol drinking in animal models (Colombo et al., 1998). Stimulation of these receptors induces motivation to consume alcohol (Gallate et al., 1999). Furthermore, stimulation of reward neurocircuitry by alcohol requires ghrelin, which acts centrally on ghrelin receptor 1A and the blockade of these receptors may suppress alcohol intake (Jerlhag et al., 2009).

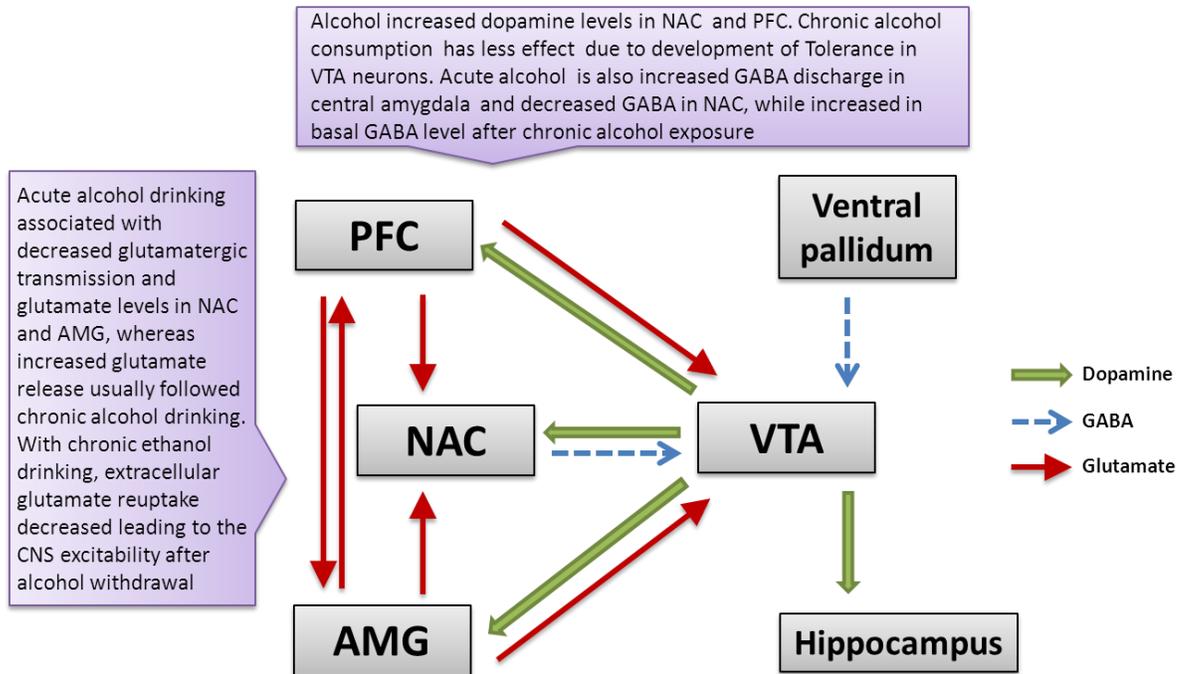


Figure 1-1 Neurocircuitry involved in development of alcohol addiction in brain

1.3 Currently Used Drugs for Treatment of Alcoholism

As a result of the complexity of alcohol mechanism of action in the body, many trials have been carried out to find treatments for alcoholism which are targeting many neurotransmitter circuits within Central Nervous System (CNS) or have been as aversive agents. Table 1.2 shows the FDA approved drugs for alcoholism:

Table 1.2: The currently FDA approved drugs for the treatment of alcoholism

Drug name	Mechanism of action	Obstacles of usage	References
Naltrexone	Not fully understood. Thought to be mediated by decreasing alcohol-induced dopamine release	Hepatotoxicity, should be used in conjugation with other behavior therapy	(Srisurapanont and Jarusuraisin, 2005);(Tambour and Quertemont, 2007)
Acamprosate	Stimulate GABA neurotransmission, glutamate antagonist	Should be used in conjugation with other behavior therapy	(Daoust et al., 1992);(Zeise et al., 1993);(Whitworth et al., 1996)
Disulfiram and calcium carbimide	Inhibit aldehyde dehydrogenase leading to acetaldehyde accumulation and arise of unpleasant symptoms like flushing, nausea and vomiting	Studies have showed that these aversive agents did not show significant effects when compared to placebo	(Peachey et al., 1989)

Many studies investigated other compounds for treatment of alcoholism including in clinical trials. The anti-convulsant, topiramate, has multiple mechanisms of action, e.g., increases GABA_A receptor activity and antagonizes AMPA and kainate glutamate receptors, leading to consequent reduction in DA release in NAC. Initial results from current clinical trials have shown topiramate to induce attenuation in alcohol drinking behavior. However, the side effects associated with topiramate treatment have limited its use. Baclofen, a GABA_B receptor agonist, decreased ethanol intake and relapse to alcohol in humans with little side effects. Several clinical studies have shown the effect of baclofen in reducing alcohol drinking, increasing rates of complete abstinence, reducing anxiety and craving, and reducing acute alcohol withdrawal symptoms. However, other studies have indicated that there are no differences between baclofen and placebo in their effects on alcohol dependence (Johnson, 2008, Garbutt et al., 2010, Addolorato et al.,

2013). Ondansetron, a 5-HT₃ receptor antagonist, is thought to decrease alcohol consumption and reduce reward based on studies conducted in humans, but seems to be effective only in some population subtypes (Addolorato et al., 2013). Selective Serotonin Reuptake Inhibitors (SSRI), that blocks serotonin transporters, reduce alcohol intake in some alcoholics, increase abstinence time, and reduce the depression state and anxiety after alcohol withdrawal in alcoholics (Sari et al., 2011a).

1.4 Glutamatergic System as a Target for Treatment of Alcoholism

The absence of a model treatment for alcoholism increases the need to search for other treatments with high efficacy and low side effects. Glutamatergic system is strongly implicated in the development of alcohol dependence. Alcohol consumption affects the level of extracellular glutamate and the level of glutamate transporters in the central reward brain regions. Alcohol withdrawal can cause increases in the level of synaptic glutamate. The number of NMDA receptors may be increased in alcohol withdrawal as well (Rao and Sari, 2012).

1.4.1 Glutamate Receptors

Glutamate is the major neurotransmitter for mediating the excitatory signals in the mammalian brain (Greuer and Rauen, 2005). Presynaptic release of glutamate activates various glutamate receptors which can be further divided into ionotropic (ligand-gated)

and metabotropic (G-protein-coupled) receptors (Wollmuth and Sobolevsky, 2004). Ionotropic glutamate receptors, including NMDA, AMPA and kainite receptor subtypes, display many pharmacological and biochemical differences (Dingledine et al., 1999).

In addition to ionotropic receptors, glutamate can regulate cell excitability and synaptic transmission by its action on metabotropic glutamate receptors (mGluRs) (Niswender and Conn, 2010). Eight mGluRs subtypes have been identified, (mGluR1-8) which are further divided into three groups: Group I includes mGluR1 and mGluR5; group II includes mGluR2 and mGluR3; and group III includes mGluR4, 6, 7 and 8 (Conn, 2003).

Both glutamate receptor subtypes are implicated in the development of alcohol dependence. Alcohol-induced sensitization may be mediated via mGluR1 and NMDA receptors (Kotlinska et al., 2006). Previous study showed the effect of NMDA receptor antagonist in inhibiting the development of alcohol tolerance (Karcz-Kubicha and Liljequist, 1995). Other studies revealed the effect of ethanol on the glutamatergic system by alteration of the function of NMDA and mGluR5 receptors (Lovinger et al., 1989, Blednov and Harris, 2008). In the hippocampus, chronic alcohol intake was shown to increase the density of NMDA receptors whereas administration of the NMDA receptor antagonist, MK801, decreased the frequency of seizures associated with alcohol withdrawal (Grant et al., 1990, Snell et al., 1996, Chen et al., 1997).

1.4.2 Glutamate Transporters

Like other neurotransmitters, extracellular glutamate levels have been regulated by a number of glutamate transporters. These transporters regulate extracellular glutamate levels after release from presynaptic neurons and prevent glutamate from reaching to the toxic levels.

Glutamate is taken up by presynaptic neuron, postsynaptic neuron or glial cells surrounding the synaptic cleft. In astrocytes, glutamate may be converted to glutamine which is further released into extracellular fluid, and up taken by the neurons and converted to glutamate. This pathway of recycling the neurotransmitter glutamate is called the Glutamine-Glutamate cycle (Danbolt, 2001).

Two major types of glutamate transporters are named as: the Excitatory Amino Acid Transporters (EAATs) and the Vesicular Glutamate Transporters (VGLUTs) (Rao and Sari, 2012). VGLUTs are located within the vesicular membrane responsible for the translocation of glutamate from the cytoplasm into the storage vesicle (Takamori, 2006). There are three subtypes are VGLUT 1, VGLUT 2 and VGLUT 3. Glutamate is transported from the synaptic vesicle to cytoplasm electrophoretically whereas one sodium ion and inorganic phosphate are co-transported in the reverse direction, i.e. from cytoplasm to vesicle (Moriyama and Yamamoto, 2004). VGLUT 1 and 2 are expressed mainly in glutamatergic neurons (Stornetta et al., 2002). On the other hand, VGLUT 3 is expressed in both glutamatergic (Takamori et al., 2002) and non-glutamatergic neurons (Gras et al., 2002). Furthermore, VGLUTs are also found in peripheral nervous system

(Moriyama and Yamamoto, 2004) and in some other peripheral non-neuronal cells (Moriyama and Hayashi, 2003).

EAATs are family of sodium-dependent transporters which transport glutamate by electrochemical gradient across the cell membrane. These transporters require sodium for glutamate binding and potassium for net transport (Roskoski, 1979, Szatkowski et al., 1991). EAATs play a major role in maintaining the concentration of extracellular glutamate below toxic levels and are responsible for clearance of glutamate after being released from presynaptic neurons. There are five subtypes of EAATs: glutamate/aspartate transporter, GLAST, (human homologue is EAAT1), GLT-1 (human homologue is EAAT2), excitatory amino acid carrier type 1, EAAC1, (human homologue is EAAT3), EAAT4, and EAAT5 (Amara and Fontana, 2002, Rao and Sari, 2012).

GLAST is expressed throughout the brain (Schmitt et al., 1997) and seems to be the major glutamate transporter in the cerebellum (Lehre and Danbolt, 1998). GLAST is also expressed in peripheral organs and serves as the major glutamate transporter in the retina and the inner ear (Lehre et al., 1997, Takumi et al., 1997). It is mainly found on the membranes of astroglial cells, where it is co-expressed with GLT-1, throughout the CNS (Berger and Hediger, 1998).

GLT-1 is highly expressed in the brain. It contributes to the maintenance of extracellular glutamate concentrations below toxic levels and it is responsible for the clearance of more than 90 % of the extracellular glutamate (Tanaka et al., 1997). GLT-1 is expressed mostly by astrocytes in the brain (Rothstein et al., 1994). However, later studies have shown the presence of mRNA for GLT-1 in some neuronal elements,

namely the CA3 neurons of mature brain hippocampus (Berger and Hediger, 1998). Further, GLT-1 exists in several splice variants which led to the presence of some variant proteins (Rauen et al., 2004). GLT-1a and GLT-1b are two isoforms of GLT-1 and are mainly expressed in astroglial but may be also expressed in neuronal terminals. GLT-1a represent the vast majority of total GLT-1 and is the predominant form in neurons. On the other hand, GLT-1b comprises much lower levels (15 times lower) than GLT-1a in adult rat brain (Holmseth et al., 2009). There are no evident differences in the transport abilities between these isoforms (Sullivan et al., 2004). Yet, these transporters are affected in different ways by different diseases and might be regulated by different mechanisms (Berger et al., 2005).

EAAC1 and EAAT4 are neuronal transporters. These transporters are principally expressed on the neurons of the hippocampus and cerebellum (Rothstein et al., 1994, Dehnes et al., 1998). EAAT4 is also found, to lesser extent, in forebrain (Furuta et al., 1997). EAAT5 is localized mainly in rod photoreceptor and bipolar cells of the retina (Arriza et al., 1997).

1.4.3 Cystine-glutamate Antiporter (xCT)

This sodium independent transporter serves to maintain the balance between cystine and glutamate. It exchanges extracellular cystine for intracellular glutamate. The cystine is then converted to cysteine which, in turn, reacts with glutamate to form gamma-glutamylcysteine (Bannai, 1986). The system x_C^- and its xCT antiporter, which

is located in glial cells (Pow, 2001), represents the major source for extra-synaptic, non-vesicular, glutamate. Furthermore, this extra-synaptic glutamate provides tone and acts on mGLU2/3 receptors which, in turn, regulate both glutamate and dopamine transmissions (Baker et al., 2002).

As mentioned above, chronic alcohol drinking is associated with increased glutamate transmission which is critical for the development of alcohol dependence. Hence, glutamate transporters, with their ability to regulate the synaptic levels of glutamate, have been regarded as a potential target for the treatment of alcohol dependence (Rao and Sari, 2012).

1.5 Glutamate Transporters and Alcoholism

1.5.1 GLT-1 and Alcoholism

Reduced expression of GLT-1 is usually associated with impaired glutamate uptake and increased extracellular glutamate as found in certain disease models (Li et al., 1997, Martin et al., 1997, Wang et al., 2003). Overexpression of glutamate transporters EAAC1 and GLT-1 resulted in increased glutamate uptake and reduced extracellular glutamate (Castaldo et al., 2007). Studies have shown down-regulation of GLT-1, in cases of ischemia (Chen et al., 2005), Amyotrophic Lateral Sclerosis (Vanoni et al., 2004), nicotine dependence (Knackstedt et al., 2009), cocaine administration (Knackstedt et al., 2010) and in alcoholic subjects (Kryger and Wilce, 2010). Our lab has shown that

chronic alcohol consumption for five weeks induced down-regulation of GLT-1 in NAC (Sari and Sreemantula, 2012).

Activation of GLT-1 results in attenuation of conditioned place preference to morphine, methamphetamine, and cocaine in mice. This suggests the inhibitory role of GLT-1 in the conditioned rewarding impacts of these drugs (Nakagawa et al., 2005). Recently, a study from our lab demonstrated that upregulation of GLT-1 levels in PFC and NAC attenuates the reinstatement of cocaine seeking behavior in rats (Sari et al., 2009).

In accordance, Sari and colleagues found that upregulation of GLT-1 levels by ceftriaxone (CEF), a beta lactam antibiotic, reduced alcohol drinking behavior in alcohol preferring rats (P rats) (Sari et al., 2011b). Moreover, upregulation of GLT-1 levels by GPI-1046, non-immunosuppressive ligands of FKBP-12, reduced ethanol intake as well (Sari and Sreemantula, 2012). Similarly, up regulation of GLT-1 level was associated with attenuation of relapse-like ethanol drinking behavior in P rats (Qrunfleh et al., 2013).

1.5.2 xCT and Alcoholism

By action of xCT, the released glutamate will act on mGLUR2 and mGLUR3 which in turn leads to decreased synaptic glutamate release (Moran et al., 2005). This indicates the possible role of xCT in modulation of glutamate transmission and alcohol dependence. Nicotine self-administration reduces xCT expression in brain regions

implicated in nicotine dependence (Knackstedt et al., 2009). Other studies have shown that cocaine produced a decrease in xCT levels in NAC, and CEF treatment normalized its expression and consequently decrease cocaine self-administration and extinction training (Knackstedt et al., 2010, Trantham-Davidson et al., 2012). xCT expression and its role in alcoholism models still needs to be further elucidated.

1.5.3 Other Glutamate Transporters and Alcoholism

Up-regulation of GLAST level has been found after repeated exposure to alcohol, this is an adaptive mechanism for a hyper-glutamatergic state which follows chronic alcohol exposure and withdrawal. This mechanism, however, fails to restore normal glutamate tone leading to escalation of alcohol drinking. On the other hand, deficiency of GLAST, due to other causes like genetic factors, may also lead to hyper-glutamatergic transmission and alcohol craving. Interestingly, deletion of GLAST results in an unexpected reduction of alcohol consumption due to impaired endocannabinoid system functions (Schreiber and Freund, 2000, Karlsson et al., 2012). In contrast to chronic, acute ethanol intake does not affect the levels of GLAST and GLT-1 level (Melendez et al., 2005). Alternatively, acute ethanol intake increased the activity of EAAT3; conversely, chronic alcohol exposure can induce decrease in EAAT3 expression (Kim et al., 2005). Similarly, acute alcohol intake decreased EAAT4 level as well (Park et al., 2008).

1.6 Target Drugs Up-regulating or Activating Glial Glutamate Transporter and xCT

1.6.1 Ceftriaxone

Using a blinded screen of 1046 FDA approved drugs and nutritional, Rothstein and colleagues found that beta-lactam antibiotics, including CEF which has been selected to be further studied, were very potent GLT-1 up-regulators (Rothstein et al., 2005). CEF is a third generation cephalosporin (Fig. 1-2) with a broad spectrum of activity against gram positive and gram negative bacteria (Richards et al., 1984). CEF has been proposed, due to its effect on GLT-1 expression, as a potential treatment for several disease models (Rao and Sari, 2012).

CEF can normalize the glutamate transport capacity and basal glutamate levels after chronic drug addiction (Trantham-Davidson et al., 2012). The exact mechanism by which CEF induce up-regulation of GLT-1 is still not fully understood, however, studies have shown that the GLT-1 promoter has four NF- κ B binding sites which are responsible for its positive and negative regulations (Sitcheran et al., 2005). In accordance, CEF exerts its effect through NF- κ B mediated GLT-1 transcriptional activation, by NF- κ B binding site at -272 position of GLT-1 promoter (Lee et al., 2008).

Sari and colleagues describe the effect of CEF on alcohol drinking, they found that CEF reduced alcohol consumption in male P rats (Sari et al., 2011b). CEF has also attenuated the relapse like behaviors in P rats due to, in part, up-regulation of GLT-1 in central brain regions associated with alcohol dependence, namely, PFC and NAC

(Qrunfleh et al., 2013). Moreover, cue-induced reinstatement of cocaine-seeking behavior has been attenuated by CEF treatment in rats (Sari et al., 2009).

CEF is also an inducer for xCT expression and increases its mRNA expression i.e., increases the activity of system x_c^- (Lewerenz et al., 2009). CEF reversed cocaine-induced deficits in system x_c^- activity leading to restoring the basal glutamate levels in cocaine addicted rats (Trantham-Davidson et al., 2012). CEF inhibited cocaine-seeking in rats by normalizing glutamate uptake and cystine-glutamate exchange (Knackstedt et al., 2010).

Taken together, these data indicate the possible role of CEF in the treatment of drug addiction.

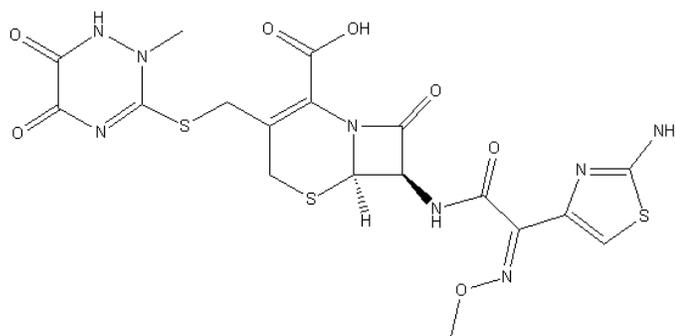


Figure 1-2 Chemical structure of CEF

1.6.2 (R)-(-)-5-methyl-1-nicotinoyl-2-pyrazoline (MS-153)

(R)-(-)-5-methyl-1-nicotinoyl-2-pyrazoline (MS-153), is a novel pyrazoline compound (Fig. 1-3) found to be a neuroprotective agent that decreases the extracellular glutamate levels in ischemic penumbra zone during permanent occlusion of the middle cerebral artery (Kawazura et al., 1997). It act as GLT-1 activator, increases the activity of GLT-1 and accelerated glutamate uptake in cell line (Shimada et al., 1999). MS-153 has the ability to enhance glutamate uptake or decrease glutamate release, which attenuate the development of behavioral sensitization to phencyclidine-induced stereotype (Abekawa et al., 2002). Studies have demonstrated the effect of MS-153 on drugs of abuse. Thus, co-administration of MS-153 decreased the development of morphine tolerance and physical dependence in mice models (Nakagawa et al., 2001). Administration of MS-153, a glutamate transporter activator, attenuated the induction of conditioned place preference to morphine, methamphetamine and cocaine without affecting acute locomotor responses (Nakagawa et al., 2001, Nakagawa et al., 2005).

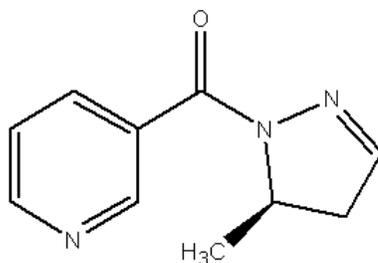


Figure 1-3 Chemical structure of MS-153

1.6.3 3-(3-pyridyl)-1-propyl (2S)-1-(3,3-dimethyl-1,2-dioxopentyl)-2-pyrrolidinecarboxylate (GPI-1046)

GPI-1046 is non-immunosuppressive ligand of FKBP-12, analog of FK506 (Fig. 1-4), which has been shown neuroprotective effect (Steiner et al., 1997a, Steiner et al., 1997b). GPI-1046 exerts its neuroprotective effect by up-regulating GLT-1 levels (Ganel et al., 2006). GLT-1 was found up-regulated in PFC and NAC after treatment with GPI-1046, this up-regulation in GLT-1 was associated in part with reduced ethanol intake in male P rats (Sari and Sreemantula, 2012).

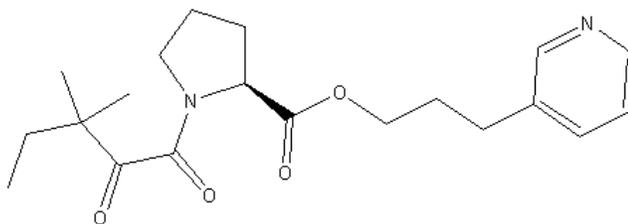


Figure 1-4 Chemical structure of GPI-1046

1.7 Alcohol-preferring Rats (P rats) Model for Alcoholism

There are efforts to design animal models for alcoholism that express similar behaviors as human. Cicero (1979) had proposed the following criteria for an animal model of alcoholism: the animal should orally self-administer ethanol; the amount of

ethanol consumed should result in pharmacologically relevant blood ethanol levels; ethanol should be consumed for its post-ingestive pharmacological effects, and not strictly for its caloric value or taste; ethanol should be positively reinforcing, i.e., the animals must be willing to work for ethanol; chronic ethanol consumption should lead to the expression of metabolic and functional tolerance; and chronic consumption of ethanol should lead to dependence, as indicated by withdrawal symptoms after access to ethanol is terminated (Rodd et al., 2004). One more criterion was added later to include relapse symptoms, animal models should express characteristics associated with relapse (McBride and Li, 1998).

P rats, which are well defined behaviorally and neurobiologically, have been regarded as a good animal models for alcoholism owing to preference for 10% (v/v) ethanol compared to water under 24 h free-choice drinking conditions (Li et al., 1993). They have been characterized and demonstrated all of the criteria for animal model of alcoholism (Bell et al., 2006). Under similar conditions, P rats drink greater than 4 g of ethanol/kg body weight/day, whereas alcohol non-preferring (NP) rats drink less than 1g/kg/day (Li et al., 1987).

Studies on the neurotransmitter systems of P rats versus the NP rats showed that P rats have abnormalities in the VTA-NAc dopaminergic system (Gongwer et al., 1989), deficiency of 5 HT and upregulation of 5HT1A receptors (McBride et al., 1994), and a higher density of GABAergic terminals in the NAc (Hwang et al., 1990).

1.8 Aims and Objectives

Recent studies from our laboratory demonstrated that CEF treatment attenuated relapse-like ethanol drinking behavior in P rats, which was associated, in part with up-regulation of GLT-1 in PFC and NAC (Qrunfleh et al., 2013). Here in this study, we investigated the effect of CEF treatment on expression of GLT-1a, GLT1b, xCT, and GLAST (using Western blot analysis), in central reward brain regions such as PFC and NAC.

We also investigated the effect of MS-153, a novel neuroprotective agent, on alcohol consumption. MS-153 has been suggested as a GLT-1 activator (Shimada et al., 1999). Previous study from our lab showed that CEF treatment attenuated ethanol-drinking behavior in P rats, which was associated with up-regulation/activation of GLT-1 level in PFC and NAC (Sari et al., 2011b). We hypothesized that MS-153 treatment may reduce ethanol consumption in P rats. We further investigated the effect of MS-153 on GLT-1 expression using Western blot analysis in NAC, AMG, and PFC. In addition, we examined NF- κ B activity, using Western bolt analysis, in NAC and PFC in order to explore the signaling pathway involved in GLT-1 expression.

Chapter 2

Materials and Methods

2.1 Animals

For both studies using CEF and MS-153, male Alcohol-preferring P rats were used as an established model of alcoholism. They were received from the Indiana University Medical Health Center (Indianapolis, IN) Indiana Research center breeding colonies at 21-30 days of age. P rats were housed in pairs in standard plastic tubs in the DLAR (Department of Laboratory Animal Resources, University of Toledo, HSC). The plastic tubs had corn-cob bedding and a temperature of 21°C and 40-60% humidity were maintained in the room on a 12 hour light/dark cycle. Food and water were given continuously. Animals at 80 days of age were single housed. They were introduced to either alcohol or sucrose solutions at 90 days of age, in addition to naïve control animals that were given food and water only, based on the paradigms. All animal procedures were approved by the Institutional Animal Care and Use committee of The University of Toledo in accordance with the guidelines of the Institutional Animal Care and Use Committee of the National Institutes of Health, and the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, Commission on Life Sciences, 1996). Animal procedures and programs are accredited by the Association

of the Assessment and Accreditation of Laboratory Animal Care International (AAALACI).

2.2 Alcohol Drinking Paradigm

2.2.1 Ceftriaxone in Relapse-like Ethanol Drinking study

A relapse like drinking behavioral was applied for this study. P rats were given continuous free choice access to food, water and two bottles that offered a choice of ethanol at different concentrations (15% and 30% v/v) for five weeks, in comparison to naïve control group that was given food and water only. The concurrent availability of different concentration of ethanol (15% and 30%) along with water necessary to achieve increase in ethanol intake by 80% on the first day of alcohol re-exposure (Rodd-Henricks et al., 2001). On week six, animals were divided into two groups and deprived from ethanol for two weeks. During the last five days of the two-week deprivation period, animals received CEF (100 mg/kg, i.p.) for five consecutive days. After five days of treatment, animals were re-exposed to free choice 15% and 30% ethanol for nine days, then euthanized on day ten. During this period of re-exposure to ethanol, body weights of animals, water and ethanol intake were recorded daily.

CEF and saline vehicle were administered intraperitoneally (i.p.) to the animals starting from the last five days of the two-week deprivation period for 5 days after dividing them to different treatment groups randomly. The groups included naïve (water) (n=6), saline vehicle (ethanol) control (n=6), and CEF treated 100 mg/kg (n=6).

CEF was purchased as powder in 250 mg bottles from the pharmacy at UTMC. It was reconstituted with saline and calculations were made according to the animal weight to administer a dose of 100 mg/kg.

Repeated measures ANOVA followed by post-hoc Dunnett's (two-sided) multiple comparison test was performed for statistical analysis of ethanol and water intake and animal body weight data.

2.2.2 MS-153 in Chronic Ethanol Drinking Study

Male adult P rats were given continuous free choice access to food, water and two bottles that offered a choice of ethanol at different concentrations (15% and 30% v/v) for five weeks, in addition to naïve control group that were given food and water only. Animals were divided into three different groups. On the first day in week six, animals received the treatment for five days then euthanized on day six.

MS-153 and saline vehicle were administered i.p. starting from the first day on Week 6 for five days, after dividing them randomly to four groups; naïve (water) control (n=7), saline vehicle (ethanol) control (n=10), MS-153 20 mg/kg (n=5), and MS-153 50 mg/kg (n=5).

MS-153 was synthesized and received from Dr. James Leighton's lab, Columbia University, Department of Chemistry, New York, NY. MS-153 was reconstituted with 1% DMSO in PBS solution as a vehicle and calculations were made according to animal weight to administer doses of 20 and 50 mg/kg body weight.

Ethanol, water consumption and body weights of animals were measured daily for five days starting 24 hours after the first injection. The measurements obtained from each group were compared using one-way ANOVA and repeated measures ANOVA followed by Dunnett's post-hoc test for comparison between control and treatment groups.

For both studies, ethanol was prepared by diluting 190 proof (95%) alcohol, with deionized water to make 15% and 30% concentrations. The 190 proof ethanol was purchased from PHARMCO-AAPER (Shelbyville, KY). The diluted ethanol solutions were given to rats in separate bottles with 100 ml ethanol each. The ethanol was replaced with freshly prepared three times a week, and the bottles were weighed every day before and after consumption. To determine the amount of ethanol consumed, we subtracted the measured bottle weights before and after consumption. Further, the animals were weighed every other day and calculations were done to report the individual drinking behavior of the animals in terms of grams of ethanol consumed per kilogram of body weight per day. All the alcohol groups' animals drank ≥ 4 grams of alcohol per day consistently for at least 2 weeks before treatment. Animals consuming less than 4 grams were discarded. The average of ethanol and water consumption during the last two weeks before treatment was taken as the baseline for the drinking behavior calculations.

2.3 Sucrose Drinking Paradigm

This paradigm was designed to assess the specific effect of MS-153 on alcohol drinking behavior. Animals were given free choice to 10% sucrose, food and water. The

10% sucrose was prepared by dissolving sucrose crystals (purchased from Mallinckrodt Chemicals) in deionized water and each animal was given 200 ml of the solution per day. They were exposed to sucrose for a period of three weeks and baseline was considered as the average of drinking during the last two weeks. It was verified that all animals met the requirement of drinking ≥ 15 milliliters of sucrose solution per kilogram of body weight per day. Animals were then treated for 5 days with either saline vehicle or MS-153 at dose of 50 mg/kg, i.p. The measurements of drinking and weights were obtained and analyzed using repeated measures ANOVA followed by Dunnett's post-hoc test to determine statistical significant differences between control and treatment group. Animals were sacrificed on day six, the brains were dissected out, frozen and stored at -70°C for further studies.

2.4 Brain Tissue Harvesting

On the last day of the drinking paradigm of each study, all the animals were euthanized by Isoflurane or CO_2 inhalation and rapidly decapitated with a guillotine; and their brains were dissected and immediately frozen on dry ice and stored at -70°C . PFC, NAC and AMG were then further micropunched stereotaxically using cryostat machine. We used stereotaxic coordinates for the rat brain Atlas established by Paxinos and Watson (Paxinos and Watson 2007) to identify all the selected brain regions. The extracted brain regions were then stored at -70°C until uses for Western blot assay.

2.5 Protein Tissue Extraction Protocol

2.5.1 Tissue lysate

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, 5 mL of dissolved protease inhibitor tablet in water, and 31.9 mL Millipore water). 250-300 μ L of lysis buffer was added to each sample in 1.5 ml eppendorf tubes and the tissue was homogenized with a pestle. 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 4° C. The supernatant was divided in to five aliquot and immediately stored in -70°C for further studies. These procedures were conducted on PFC, NAC, and AMG samples from animals sacrificed on day 10 of re-exposure (for CEF relapse study) or day 6 (for MS-153 study).

2.5.2 Nuclear and Cytoplasmic Extractions

Brain samples were weighed and homogenized in buffer A [10 mM HEPES-KOH, pH 7.9; 1.5mM MgCl₂; 10 mM KCl; 1 mM Dithiothreitol (DDT); 1 mM phenylmethylsulfonyl fluoride (PMSF); 10 uL of protease inhibitor cocktail/ml of buffer] then incubated on ice for 10 minutes before adding Nonidet P40 to final concentration of 0.1%. Samples were then incubated for 2 minutes on ice and centrifuged at 13,200 rpm for 15 minutes at 4°C. The supernatant was removed to fresh tubes and NaF, Na

vanadate, and Na pyrophosphate were added to final concentration of 50 mM, 10 mM, and 0.1 mM, respectively to obtain the cytosolic fraction.

The pellets were re-suspended in buffer B [20 mM HEPES-KOH, pH 7.9; 25% glycerol; 420 mM NaCl; 1.5 mM MgCl₂; 1 mM DDT; 1 mM PMSF; 0.2 mM EDTA; 50 mM NaF; 10 mM Na vanadate; 0.1 mM Na pyrophosphate; 10 μ L of protease inhibitor cocktail/ml of buffer] and kept on ice for 30 minutes, centrifuged at 13,200 rpm at 4°C and supernatant collected was the nuclear fraction.

2.6 Protein Quantification Assay

The Lowry Protein Quantification assay using 96 well plates was performed in order to determine the amount of protein present in the homogenized samples in both studies. All samples were assayed in quadruplicates in a 96-well plate. A standard curve was made using bovine serum albumin (BSA) (1.48mg/mL, New England Biolabs). The wells containing the proteins samples contained 1 μ L of sample diluted in 4 μ L of lysis buffer. Thereafter, 3mL reagent A (BioRad Laboratories) was mixed with 60 μ L reagent S (BioRad Laboratories) and 25 μ L of this mixture was added to each well. 200 μ L of reagent B (BioRad Laboratories) was then added to each well and the reaction was maintained at room temperature for 15 minutes. The absorbance was then measured using 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. The standard curve was obtained by plotting the BSA

optical density values against the known concentrations. The regression and line equation was obtained. The concentration of the protein in the samples was then calculated using their optical density and the line equation.

2.7 Western Blot Procedures

2.7.1 Western Blot Protocol

The samples were thawed on ice and diluted with lysis buffer based on their protein quantification values to achieve different concentrations depending on the antibody used. A 5X Laemmli dye (1M Tris HCl, 100% Glycerol, SDS, bromophenol blue, β -mercaptoethanol) was added to the samples (5 μ l/20 μ l of the diluted sample) and mixed thoroughly. The sample mix was then incubated at 98°C for 5 minutes in a digital dry bath (Labnet International Inc.). The samples were then centrifuged at 4°C and 13,200 rpm for 4 minutes (Centrifuge 5415R, Eppendorf Inc.). 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 μ L of each sample was placed into each 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. The membranes were washed with deionized water and blocked with blocking buffer (dry milk and 1X TBST) and placed on agitator. After 30-60 minutes, the primary antibody was added at different dilutions (depending on the antibody used). The petri dishes were sealed with Para film and incubated overnight at 4°C by shaking at 300 rpm. In the next day, each membrane washed with 1X TBST 5 times for 5 minutes each and then incubated again with blocking buffer for 30-60 minutes. Each membrane was then incubated in secondary antibody for 90 minutes. The membranes were washed again with 1X TBST 5 times and prepared for developing.

The washed membranes were incubated (after drying with whatman filter paper) with the developer solution, a mixture of Super Signal West Pico Luminal enhancer and Super Signal West Pico stable peroxidase solution (Developer Kit, Pierce) in 1:1 ratio for 1-2 minutes. The membranes were dried again, and exposed to Kodak BioMax MR Film (Thermo Fischer Scientific) in a cassette. The exposed films were developed with an SRX-101A machine.

2.7.2 Primary and Secondary Antibodies

Anti GLT-1 antibody, AB1783 (1:5000; Millipore), anti GLT-1a antibody (1:10000; gift from Dr. Jeffery Rothstein, Johns Hopkins University, Baltimore), anti GLT-1b antibody (1:5000; gift from Dr. Paul Rosenberg, Harvard University, Children's Hospital, Department of Neurology, Boston, MA), anti-Glyceraldehyde-3-phosphate

dehydrogenase (GAPDH) antibody clone 6C5 (1:5000; Millipore), xCT antibody (NB300-318); (1:1000; Novus Biological), SLC1A3/GLAST rabbit anti-rat polyclonal (C-terminal) antibody (1:5000; Life Span Biosciences), anti I κ B- α (C-21) (1:500; Santa Cruz Biotechnology, Inc.), anti Lamin A/C (H-110) (1:5000; Santa Cruz Biotechnology, Inc.), NF- κ B p65 antibody (1:500; Cell Signaling Technology), anti β -tubulin antibody (TUJ1) (1:5000; Convance), anti-mouse IgG, HRP-linked secondary antibody (1:5000; Cell signaling technology), donkey anti-rabbit IgG (H+L) secondary antibody (1:5000; thermo scientific), anti-Guinea pig IgG (H+L) secondary antibody (1:5000; Jackson ImmunoResearch Laboratories, Inc.).

2.7.3 Western Blot Data Analysis

A SRX-101A film processor was used to capture images from the developed films and the digitized images were quantified using MCID software. The size and density of the protein bands and the control protein bands were measured for all samples. The ratios of protein/control protein were calculated and analyzed using one-way ANOVA followed by Newman-Keuls multiple comparisons test to determine statistical significant differences between control and treatment groups.

2.8 Statistical Analysis

Repeated measure ANOVA tests were performed for statistical analysis of ethanol and water intake and animal body weight data in CEF relapse-like ethanol-drinking study. For MS-153 chronic consumption study, one-way ANOVA and repeated measure ANOVA followed by Dunnett's post-hoc test were used for comparison of ethanol and water consumption, body weights, and sucrose consumption between control and treatment groups. Western blot data were analyzed using one-way ANOVA followed by Newman-Keuls test. Statistical significance was set at $p < 0.05$ for all behavioral and immunoblot studies.

Chapter 3

Results

3.1 Ceftriaxone in Relapse-like Ethanol Drinking Study

3.1.1 Effects of CEF Treatment on Ethanol-drinking Behavior

Starting from the first day of ethanol re-exposure, ethanol consumption was measured daily as g/kg/day for the 9 days of re-exposure to alcohol after 5 days of treatment with saline or CEF 100 mg/kg. Fig 3-1 shows the average ethanol intake in saline vehicle (n=6) and CEF 100 mg/kg (n=6) treated groups. The baseline was the average of rats' ethanol consumption for the last two weeks of continuous ethanol exposure. Repeated measure ANOVA test revealed a significant main effect of Day [$F(1,6)=14.15$, $p<0.05$], and significant interaction of Treatment x Day [$F(1,6)=4.70$, $p<0.05$]. Ethanol intake was significantly reduced ($p<0.05$) in CEF 100 mg/kg treated group as compared to saline vehicle-treated group. Saline vehicle-treated group did not show significant reduction in ethanol consumption.

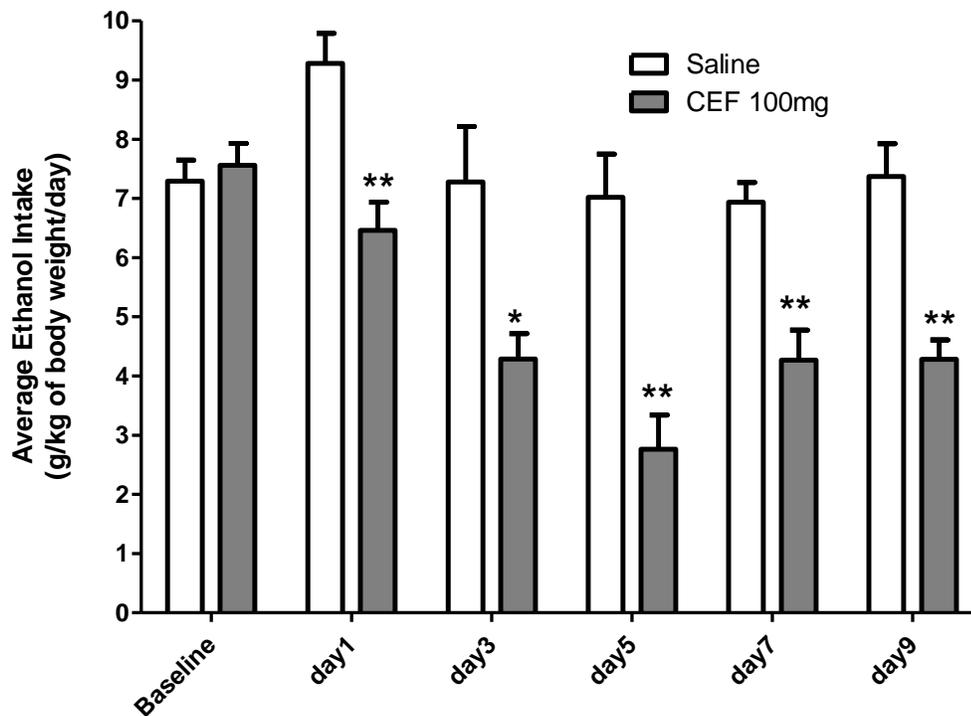


Figure 3-1 Daily ethanol intake of male P rats for nine days of ethanol re-exposure, following five days of treatment during relapse period with water (n = 6), saline vehicle (n = 6) and CEF 100 mg/kg (n = 6). The graph represents average daily ethanol intake during the nine days of ethanol re-exposure. Statistical analyses revealed a significant reduction in average daily ethanol consumption during the duration of re-exposure to ethanol for CEF-treated group compared to saline treated group. Error bars indicate SEM. (* p< 0.01; **p<0.001)

3.1.2 Effects of CEF Treatment on Water Intake

The water intake (before and after treatment for both saline and CEF treated groups) was also measured. Starting on the first day of ethanol re-exposure, water consumption was measured daily as ml/kg/day for the 9 days of re-exposure to ethanol after 5 days of treatment with either saline vehicle or CEF. Fig 3-2 shows the average water intake in saline vehicle (n=6) and CEF (n=6) treated groups. The baseline was the

average of rats' water consumption for the last two weeks of continuous ethanol exposure. Repeated measure ANOVA tests revealed significant main effect of Day [F(1,6)=5.23, p<0.05], and significant interaction of Treatment x Day [F(1,6)=9.324, p<0.05]. Water consumption did not change in saline vehicle-treated group compared to baseline. In contrast, water consumption was significantly increased in CEF-treated group compared to saline vehicle-treated group from day 5 through day 9.

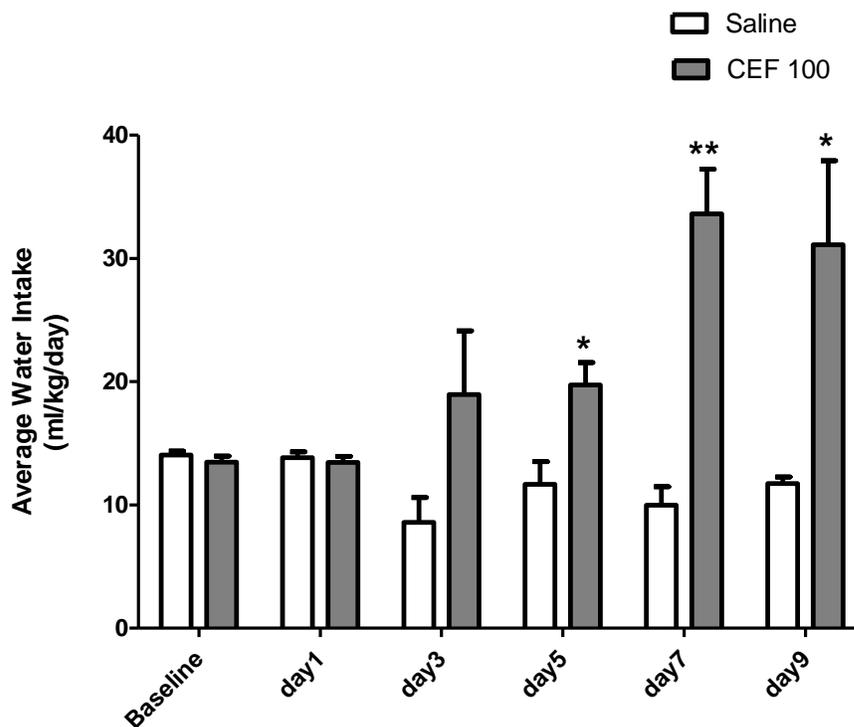


Figure 3-2 Daily water intake of male P rats for nine days of relapse paradigm, following five days of treatment with water (n = 6), saline vehicle (n = 6), or CEF 100 mg/kg (n = 6). The graph represents average daily water consumption during the nine days of ethanol re-exposure. Statistical analyses revealed a significant increase in average daily water consumption during the duration of re-exposure to ethanol for CEF-treated group compared to saline vehicle-treated groups. Error bars indicate SEM. (* p<0.05; **p<0.01)

3.1.3 Effects of CEF Treatment on Body Weight

Body weight was measured for both saline (n=6) and CEF (n=6) treated groups as (grams \pm SEM). Measures were taken for 9 days of relapse-like paradigm as well as the baseline, which represents the average of P rats body weight during the last two weeks of continuous ethanol exposure. Fig.3-3 shows average body weight plotted for baseline and 9 days followed the treatment. Repeated measure ANOVA tests revealed significant main effect of Day [$F(1,6)=22.184$, $p<0.05$], and interaction of Treatment x Day and treatment [$F(1,6)=5.148$, $p<0.05$]. There was no significant effect on CEF-treated group compared to saline vehicle-treated group,

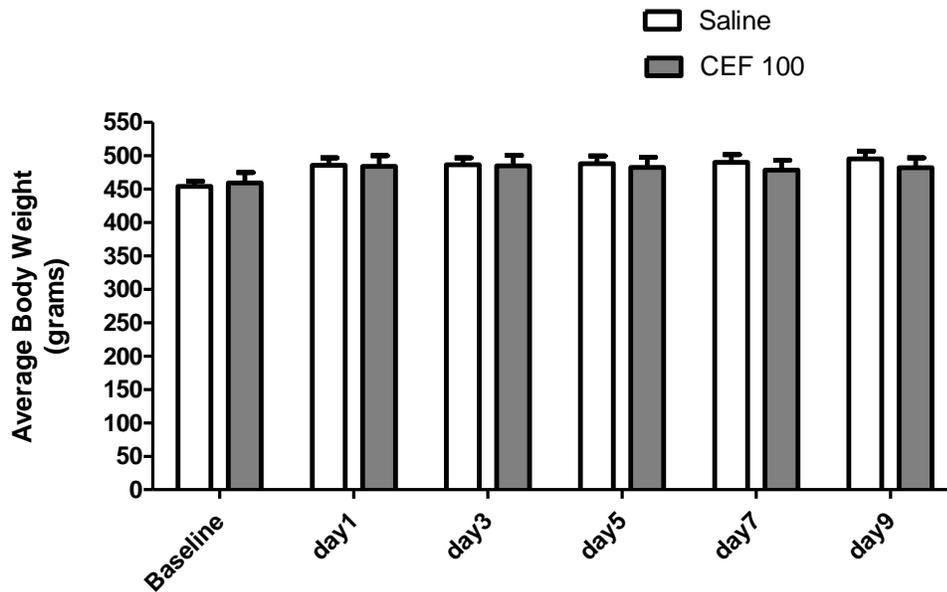


Figure 3-3 Daily body weights of P rats for nine days of relapse-like paradigm. Statistical analysis did not reveal any significant effect of CEF-treated group compared to saline vehicle-treated group. Error bars indicate SEM.

3.1.4 Effect of CEF on GLT-1a Expression

We next examined the effect of CEF treatment on GLT-1a expression in PFC and NAC using western blot. One-way ANOVA followed by Newman-Keuls multiple comparison test analyses showed up-regulation of GLT-1a levels in CEF-treated groups in both PFC (Fig. 3-4) and NAC (Fig.3-5). In PFC, expression of GLT-1a was up-regulated significantly [$F(2,17)= 6.37, p=0.009$] in CEF-treated group compared to saline vehicle and water naïve groups. There was no significant change of GLT-1a levels between saline vehicle and water naïve control groups.

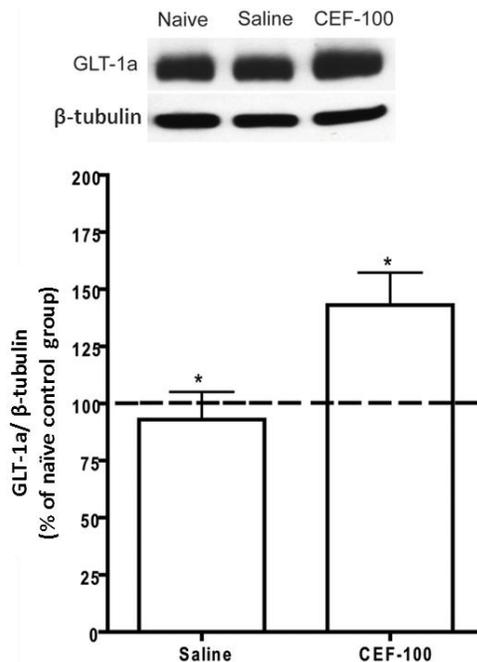


Figure 3-4 Effects of CEF 100 mg/kg (CEF-100, n = 6) on GLT-1a expression in PFC compared to saline vehicle (n = 6) and naïve (water) groups. (Upper panel): Immunoblots for GLT-1a expression, and β-tubulin, as a control loading protein. (Lower panel): Quantitative analysis of immunoblots demonstrated a significant increase in the ratio of GLT-1a/β-tubulin in CEF-100-treated group compared to naïve (100% naïve control-value) and saline vehicle control groups. There were no significant differences between saline vehicle and naïve control groups. Values are shown as means ± SEM. (* p<0.05).

In NAC, expression of GLT-1a was also up-regulated significantly [$F(2,17)= 6.89$, $p=0.0075$] in CEF-treated group compared to saline vehicle and water naïve groups. There were no significant changes of GLT-1a levels between saline and water naïve control groups.

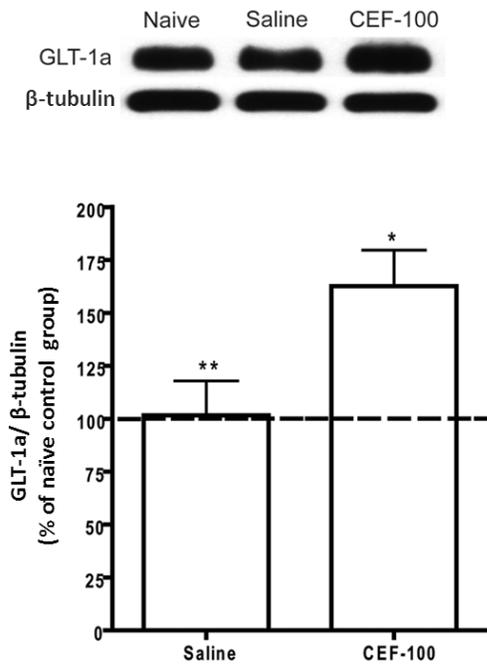


Figure 3-5 Effects of CEF 100 mg/kg (CEF-100, $n = 6$) on GLT-1a expression in NAC compared to saline vehicle ($n = 6$) and naïve (water) groups. (Upper panel): Immunoblots for GLT-1a expression, and β -tubulin as a control loading protein. (Lower panel): Quantitative analysis of immunoblots demonstrated a significant increase in the ratio of GLT-1a/ β -tubulin in CEF-100-treated group compared to naïve (100% naïve control-value) and saline vehicle control groups. There were no significant differences between saline vehicle and naïve control groups. Values are shown as means \pm SEM. (* $p<0.05$; ** $p<0.01$).

3.1.5 Effect of CEF on GLT-1b Expression

Western blot was also used to determine GLT-1b levels in PFC and NAC. One-way ANOVA followed by Newman-Keuls multiple comparison test analyses showed up-regulation of GLT-1b levels in CEF-treated group as compared to saline vehicle-treated group in both PFC (Fig. 3-6) and NAC (Fig.3-7). In PFC, expression of GLT-1b was up-regulated significantly [$F(2,17)= 6.77, p=0.008$] in CEF-treated group compared to saline vehicle and water naïve groups. There was no significant change of GLT-1b levels between saline and naïve control groups.

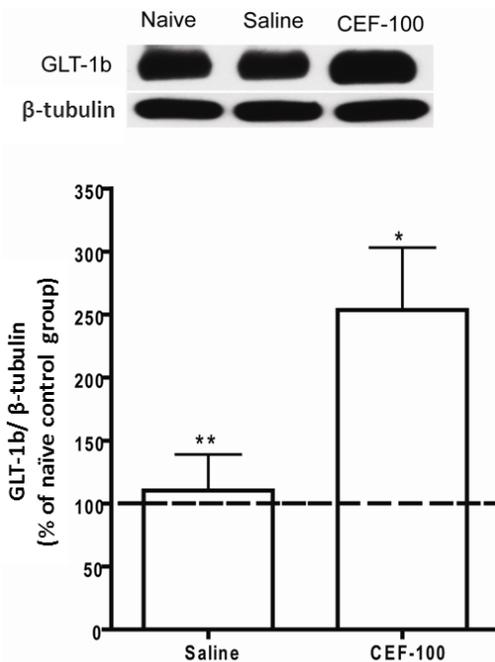


Figure 3-6 Effects of CEF 100 mg/kg (CEF-100, n = 6) on GLT-1b expression in PFC compared to saline vehicle (n = 6) and naïve (water) groups. (Upper panel): Immunoblots for GLT-1b expression, and β -tubulin as a control loading protein. (Lower panel): Quantitative analysis of immunoblots demonstrated a significant increase in the ratio of GLT-1b/ β -tubulin in CEF-100-treated group compared to naïve (100% naïve control-value) and saline vehicle control groups. There were no significant differences between saline vehicle and naïve control groups. Values are shown as means \pm SEM. (* $p<0.05$; ** $p<0.01$).

Expression of GLT-1b was also up-regulated significantly [$F(2,17)= 5.20$, $p=0.019$] in CEF-treated group compared to saline vehicle and water naïve groups in NAC. There were no significant changes in GLT-1a levels between saline vehicle and naïve control groups.

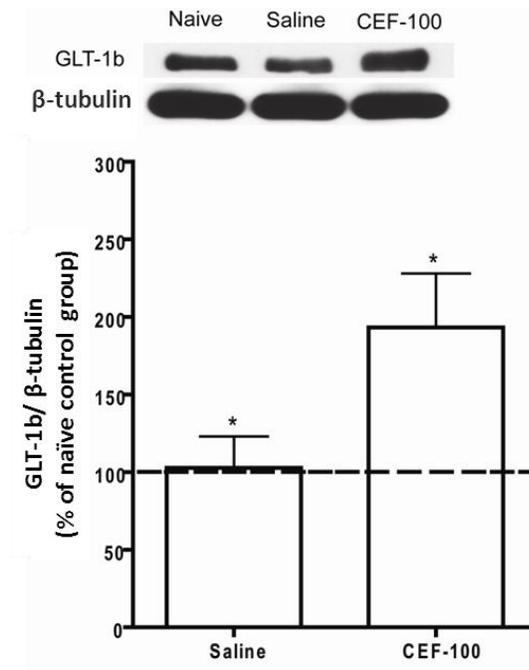


Figure 3-7 Effects of CEF 100 mg/kg (CEF-100, $n = 6$) on GLT-1b expression in NAC compared to saline vehicle ($n = 6$) and naïve (water) groups. (Upper panel): Immunoblots for GLT-1b expression, and β -tubulin as a control loading protein. (Lower panel): Quantitative analysis of immunoblots demonstrated a significant increase in the ratio of GLT-1b/ β -tubulin in CEF-100-treated group compared to naïve (100% naïve control-value) and saline vehicle control groups. There were no significant differences between saline vehicle and naïve control groups. Values are shown as means \pm SEM. (* $p<0.05$).

3.1.6 Effect of CEF on GLAST Expression

To determine the effect of CEF on GLAST expression, western blot analysis was used to compare GLAST expression levels in PFC and NAC. One-way ANOVA followed by Newman-Keuls multiple comparison test analyses showed no statistical differences between CEF (n=6), saline vehicle (n=6), and water naïve (n=6) groups in both PFC [$F(2,17)= 0.94, p=0.4$] (Fig. 3-8) and NAC [$F(2,17)= 0.11, p=0.89$] (Fig.3-9).

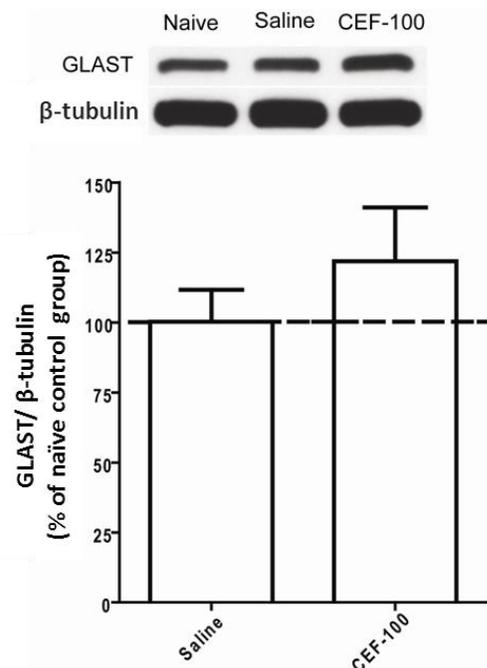


Figure 3-8 Effect of CEF (100 mg/kg, i.p.) or saline vehicle on GLAST expression in PFC. (Upper panel): Immunoblots for GLAST expression and β -tubulin as a control loading protein. (Lower panel); Quantitative analysis of immunoblots did not show a significance difference between control (100% naïve control-value), saline vehicle, and CEF treated groups. Values are shown as means \pm SEM.

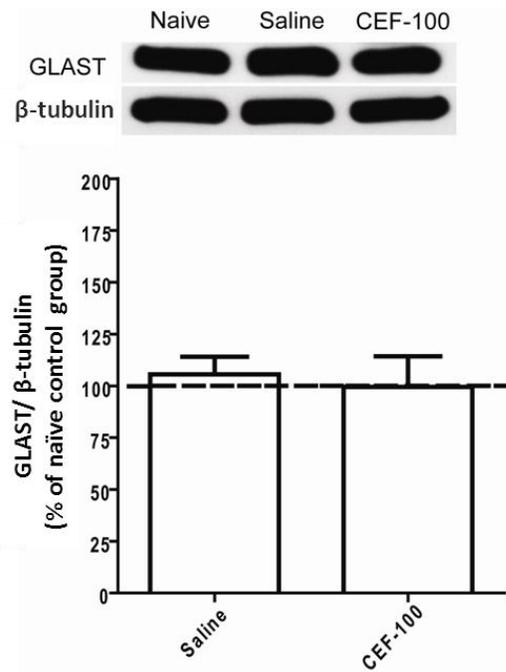


Figure 3-9 Effect of CEF (100 mg/kg, i.p.) on GLAST expression in NAC. (Upper panel): Immunoblots for GLAST expression and β -tubulin as a control loading protein. (Lower panel): Quantitative analysis of immunoblots did not show a significance difference between control (100% naïve control-value), saline vehicle, and CEF treated groups. Values are shown as means \pm SEM.

3.1.8 Effect of CEF on xCT Expression

Western blot was used to determine the effect of CEF on the expression of xCT in PFC and NAC. One-way ANOVA followed by Newman-Keuls multiple comparison test analyses showed up-regulation of xCT levels in CEF-treated group in both PFC (Fig. 3-10) and NAC (Fig.3-11). In PFC, expression of xCT was up-regulated significantly [$F(2,17)= 7.39, p=0.0058$] in CEF-treated group compared to saline vehicle and water naïve groups. There were no significant differences in xCT levels between saline vehicle and naïve control groups.

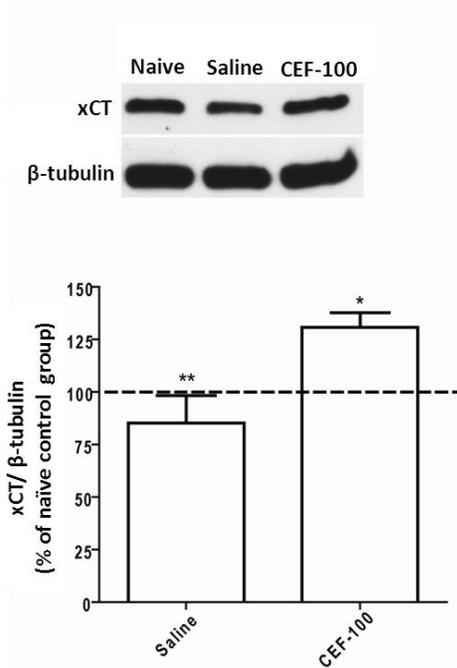


Figure 3-10 Effects of CEF 100 mg/kg (CEF-100, n = 6) on xCT expression in PFC compared to saline vehicle (n = 6) and naïve (water) groups. (Upper panel): Immunoblots for xCT expression, and β -tubulin as a control loading protein. (Lower panel): Quantitative analysis of immunoblots demonstrated a significant increase in the ratio of xCT/ β -tubulin in CEF-100-treated group compared to naïve (100% naïve control-value) and saline vehicle control groups. There were no significant differences between saline vehicle and naïve control groups. Values are shown as means \pm SEM. (* p<0.05; ** p<0.01).

In NAC, expression of xCT was also up-regulated significantly [$F(2,17)= 5.09$, $p=0.0205$] in CEF-treated group compared to saline and water naïve groups. There were no significant changes in xCT levels between saline vehicle and naïve control groups.

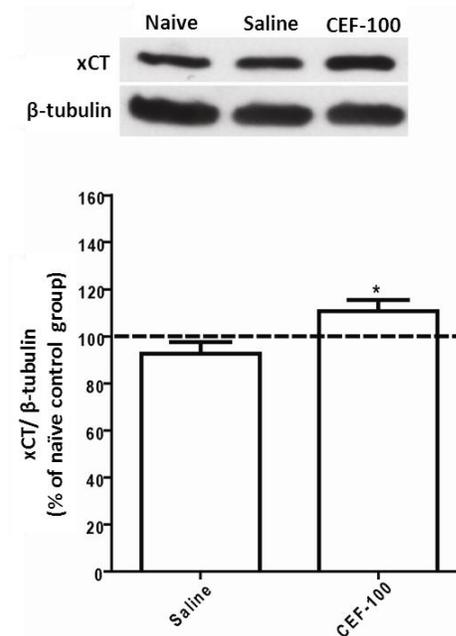


Figure 3-11 Effects of CEF 100 mg/kg (CEF-100, n = 6) on xCT expression in NAC compared to saline vehicle (n = 6) and naïve (water) groups. (Upper panel): Immunoblots for xCT expression, and β -tubulin as a control loading protein. (Lower panel): Quantitative analysis of immunoblots demonstrated a significant increase in the ratio of xCT/ β -tubulin in CEF-100-treated group compared to saline vehicle control groups. There was no significant difference between CEF-100 treated and naïve (100% naïve control-value) and saline vehicle control groups. There were no significant differences between naïve control and saline vehicle groups. Values are shown as means \pm SEM. (* p<0.05).

3.2 MS-153 in Chronic Ethanol Drinking Study

3.2.1 Effect of MS-153 on Ethanol-Drinking Behavior

The effect of MS-153 was determined by measuring ethanol consumption of P rats during the five days starting 24 hours after the first day of injections. The measurements were compared to baseline, which calculated as the average of ethanol intake during the last two weeks of continuous ethanol drinking. Fig. 3-12 shows the average ethanol intake (g/kg body weight/day) of P rats within each group plotted for

baseline and six days from the day of first injection. The treatment groups were: saline vehicle (n=10), MS-153 20 mg/kg (n=5) and MS-153 50 mg/kg (n=5). One-way ANOVA was performed followed by a posthoc Dunnett's multiple comparisons test (two-sided) revealed a significant reduction in alcohol consumption for MS-153 50 mg/kg treated group from Day 1 through Day 5 ($p < 0.05$) compared to saline vehicle-treated group. Significant reduction in alcohol consumption was observed with MS-153 20 mg/kg treated group at Day 3 and Day 4 ($p < 0.05$) as compared to saline vehicle-treated group. Repeated measure ANOVA analyses for each day demonstrated significant main effect of Day [$F(5,13) = 16.20, p < 0.05$] and significant interaction of Treatment x Day [$F(5,14) = 12.50, p < 0.05$]. There were no significant differences between MS-153 20 mg/kg and MS-153 50 mg/kg treated groups.

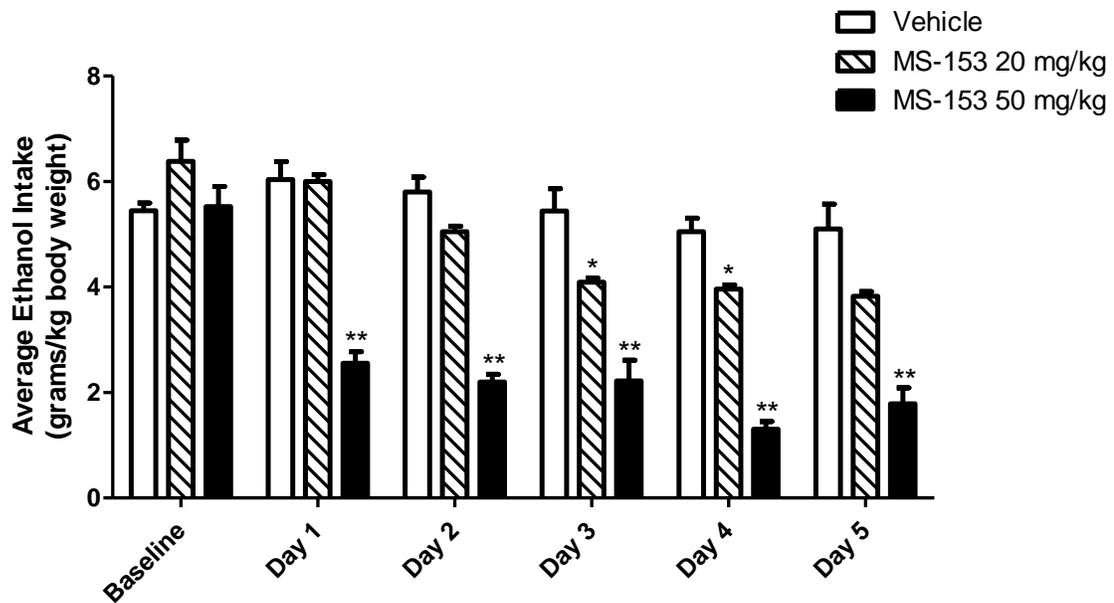


Figure 3-12 Effects of MS-153 treatment in ethanol intake in male P rats exposed to five weeks of free choice of ethanol and water. Statistical analyses exhibited a significant decrease in ethanol consumption with the higher dose of MS-153 (50 mg/kg, i.p.) from Day 1 (24 hours after the first i.p. injection) through Day 5 compared to saline vehicle-treated group. In addition, a moderate effect with the lower dose (20 mg/kg, i.p.) was observed starting on Day 3 and Day 4. Error bars

indicate SEM. (* $p < 0.05$; ** $p < 0.001$); Saline vehicle (n=10), MS-153 20 mg/kg (n=5), and MS-153 50 mg/kg (n=5).

3.2.2 Effect of MS-153 on Water Consumption

Water consumption was compared prior and after MS-153 administration. Water intake 24 hours after the first day of treatment was measured, for five days, and compared with the baseline. Fig. 3-13 shows the average water intake (ml/kg body weight/day) of the animals within each group plotted against the baseline and five days 24 hours after the first day of injection. One-way ANOVA performed on water intake, followed by Dunnett's t-test (two-sided), revealed a significant increase in water intake for MS-153 50 mg/kg treated group in Day 1, Day 3, and Day 4 ($p < 0.05$). Water intake was increased significantly in MS-153 20 mg/kg treated group in Day 4 ($p < 0.05$). Repeated measure ANOVA analyses for each day revealed significant main effect of Day [$F(5,13) = 3.46$, $p < 0.05$] and significant interaction of Treatment x Day [$F(5,14) = 5.10$, $p < 0.05$]. There were no significant differences in water consumption between groups of both doses of MS-153.

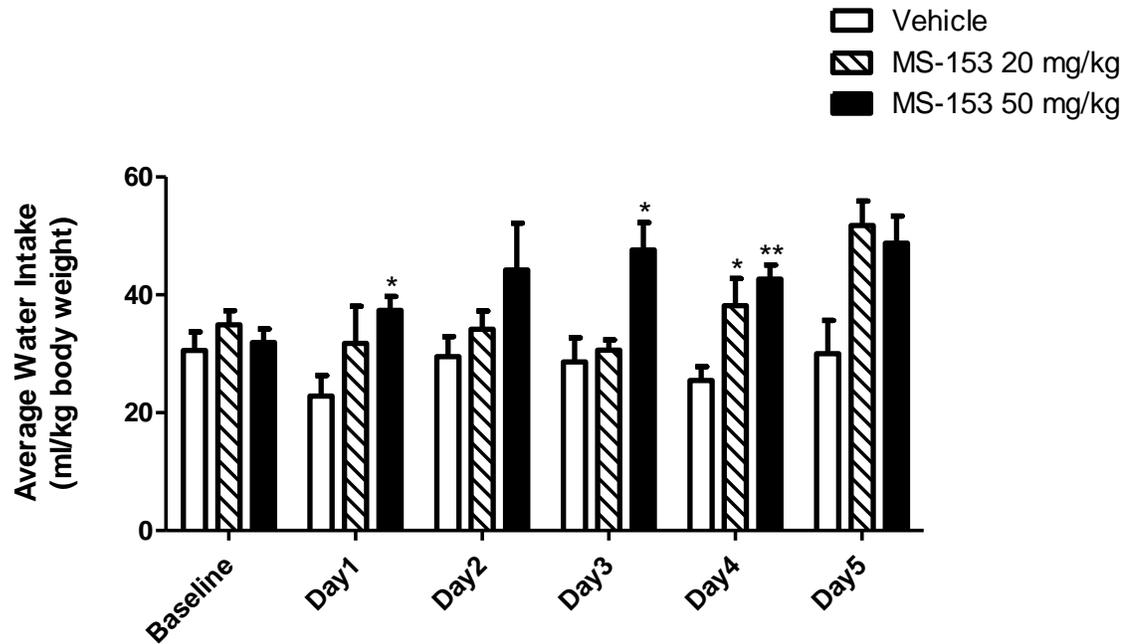


Figure 3-13 Effects of MS-153 treatment in water intake in P rats exposed to five weeks of free choice of ethanol water. Statistical analyses exhibited a significant increase in water consumption with the higher dose of MS-153 (50 mg/kg, i.p.) on Days 1, 3 and 4. In addition, an increase in water intake on Day 4 with the lower dose (20 mg/kg, i.p.) was observed. Error bars indicate SEM. (* $p < 0.05$; ** $p < 0.001$). Saline vehicle ($n=10$), MS-153 20 mg/kg ($n=5$), and MS-153 50 mg/kg ($n=5$).

3.2.3 Effect of MS-153 on Body Weight

The effect of MS-153 on the body weight was also evaluated. One-way ANOVA was performed on body weight, followed by a posthoc Dunnett's multiple comparison (two sided) test (Figure 3-14). It did not reveal any significant difference in body weight between saline vehicle and MS-153 50 mg/kg treated groups. Repeated measure ANOVA analyses for each day did not reveal significant differences in body weight between saline vehicle and MS-153 50 mg/kg treated groups.

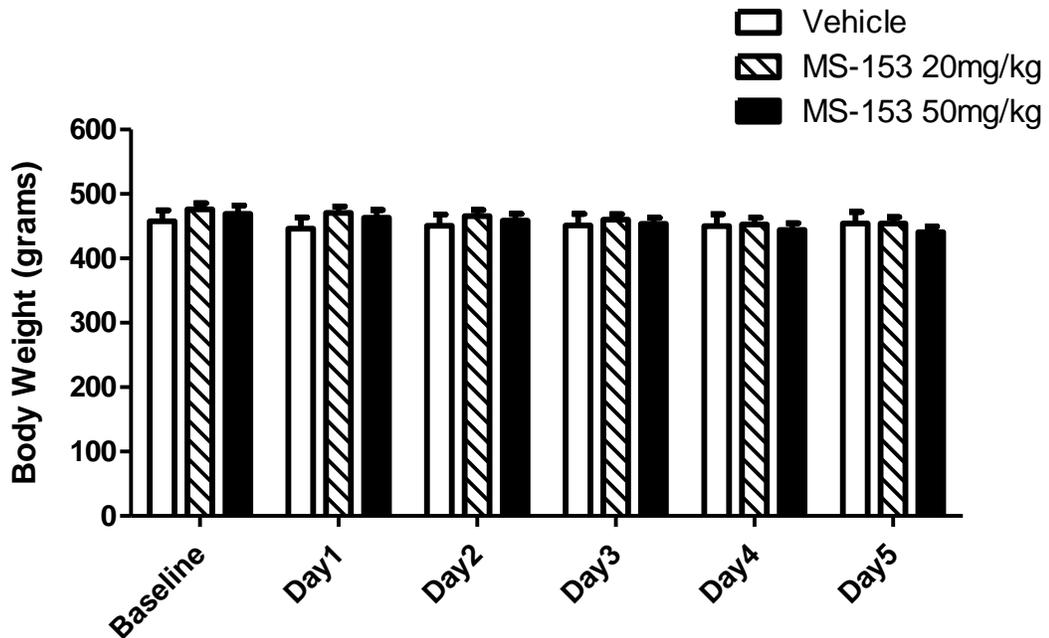


Figure 3-14 Effects of MS-153 treatment in body weight. Statistical analyses did not demonstrate a significant effect of MS-153 with the three doses on the body weight. Error bars indicate SEM. Saline vehicle (n=10), MS-153 20 mg/kg (n=5), and MS-153 50 mg/kg (n=5).

3.2.4 Effect of MS-153 on Sucrose-Drinking Behavior

The effect of MS-153 on sucrose consumption was tested to investigate the effect of MS-153 on other appetitive control fluid. Sucrose consumption was measured 24 hours after the first day of treatment and the following five days. Fig. 3-15 shows the average sucrose intake (ml/kg body weight/day) of rats within each group for baseline and five days starting from 24 hours after the first day of treatment. The treatment groups were: saline vehicle (n=5) and MS-153 50 mg/kg (n=5). Repeated measure ANOVA tests revealed no significant differences in sucrose intake between saline vehicle and MS-153 50 mg/kg treated group.

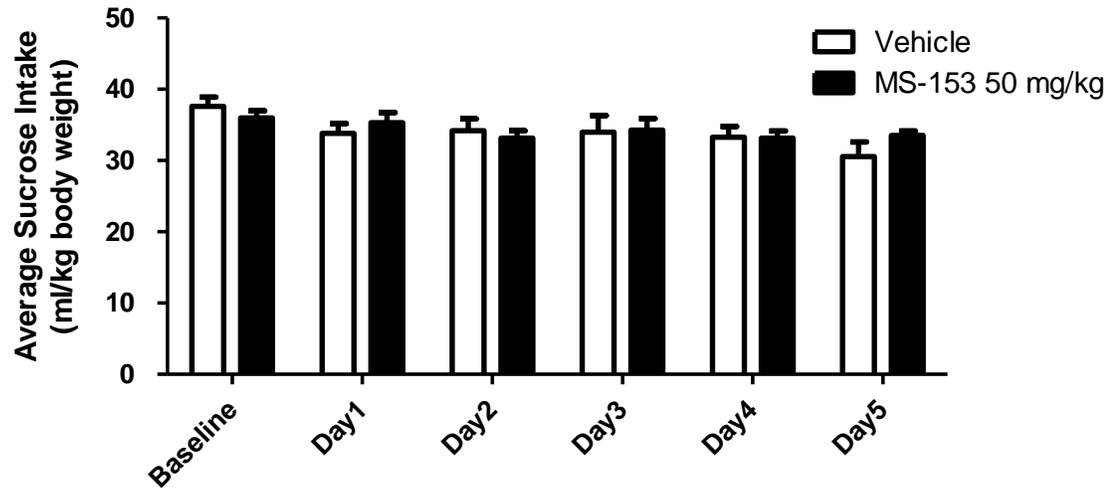


Figure 3-15 Effects of MS-153 in sucrose intake in P rats. MS-153 at higher dose (50 mg/kg, i.p.) did not induce an effect on sucrose intake. Error bars indicate SEM. Saline vehicle (n=5) and MS-153 50 mg/kg (n=5).

3.2.5 Effect of MS-153 on GLT-1 Expression

We used Western blot to determine the effect of MS-153 on GLT-1 expression in NAC, AMG, and PFC. In NAC, one-way ANOVA followed by post hoc Newman-Keuls multiple comparisons test analyses revealed significant increase [$F(3,19)= 3.86$, $p=0.0296$] in GLT-1 expression in both MS-153 doses (20 and 50 mg/kg) treated groups compared to saline vehicle-treated group. In addition, GLT-1 expression was significantly downregulated in saline vehicle-treated group compared to water naïve control group. There were no statistical differences between water naïve control and MS-153 (20 and 50 mg/kg) treated groups (Fig. 3-16).

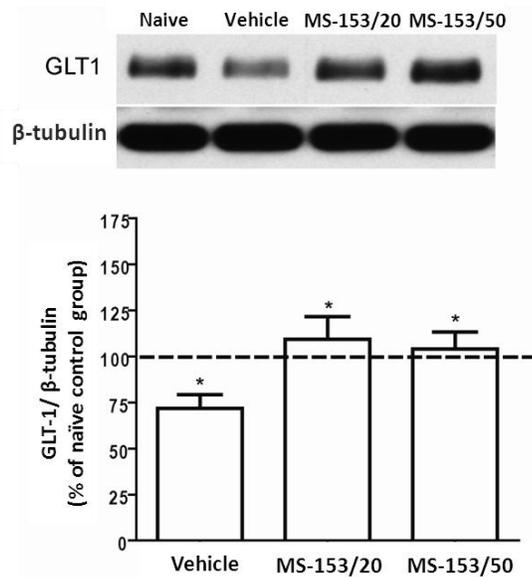


Figure 3-16 Effects of MS-153 at 20 mg/kg (MS-153, n=5), MS-153 at 50 mg/kg (MS-153, n=5), saline vehicle (ethanol group) (n=5), water naive (water group) (n=5) groups on GLUT1 expression in NAC. (Upper panel): Each panel presents immunoblots for β -tubulin, which was used as a control loading protein, and GLUT1. (Lower panel): Quantitative analysis revealed a significant increase in the ratio of GLUT1/ β -tubulin in both MS-153 doses treated groups as compared to saline vehicle-treated group. Significant downregulation of GLUT1 expression was revealed in saline vehicle-treated group compared to water naive group. Error bars indicate SEM. (* $p < 0.05$)

Furthermore, one-way ANOVA followed by post hoc Newman-Keuls multiple comparisons test analyses revealed significant increase [$F(3,19) = 6.32, p = 0.0049$] in GLUT-1 expression in both MS-153 doses (20 and 50 mg/kg) treated groups compared to saline vehicle-treated group in AMG. GLUT-1 expression was significantly downregulated in saline vehicle-treated group compared to water naïve control group. There were no statistical differences between water naïve control and MS-153 (20 and 50 mg/kg) treated groups (Fig. 3-17).

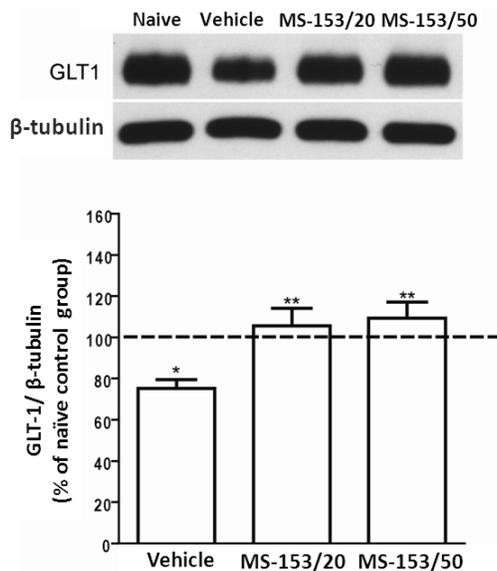


Figure 3-17 Effects of MS-153 at 20 mg/kg (MS-153, n=5), MS-153 at 50 mg/kg (MS-153, n=5), saline vehicle (ethanol group) (n=5), water naive (water group) (n=5) groups on GLUT1 expression in AMG. (Upper panel): Each panel presents immunoblots for β -tubulin, which was used as a control loading protein, and GLUT1. (Lower panel): Quantitative analysis revealed a significant increase in the ratio of GLUT1/ β -tubulin in both MS-153 doses treated groups compared to saline vehicle-treated group. Significant downregulation of GLUT1 expression was revealed in saline vehicle-treated group compared to water naive group. Error bars indicate SEM (* $p < 0.05$; ** $p < 0.01$)

In PFC (Fig. 3-18), one-way ANOVA followed by post hoc Newman-Keuls multiple comparisons test analyses revealed no significant differences [F(3,19)= 0.23, $p=0.869$] between all groups (naïve water, saline vehicle, MS-153 20 mg/kg, and MS-153 50 mg/kg).

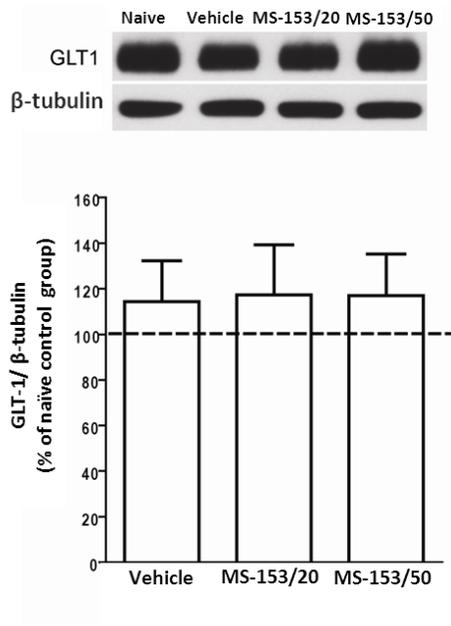


Figure 3-18 Effects of MS-153 at 20 mg/kg (MS-153, n=5), MS-153 at 50 mg/kg (MS-153, n=5), saline vehicle (ethanol group) (n=5), water naive (water group) (n=5) groups on GLT1 expression in PFC. (Upper panel): Each panel presents immunoblots for β -tubulin, which was used as a control loading protein, and GLT1. (Lower panel): Quantitative analysis did not reveal significant differences in the ratio of GLT1/ β -tubulin between all groups. Error bars indicate SEM.

3.2.6 Effect of MS-153 on NF κ B-p65 and I κ B α Levels

To demonstrate the mechanism of MS-153 action, Western blot was used to determine the effect of MS-153 on NF κ B-p65 and I κ B α levels in NAC and PFC. Fig. 3-19 shows the effect of MS-153 treatment on NF κ B-p65 and I κ B α levels in NAC. One-way ANOVA followed by post hoc Newman-Keuls multiple comparisons test analyses revealed significant main effect on NF κ B-p65 levels between all groups [F(3,19)=8.42, p=0.003] in nuclear fraction. NF κ B-p65 level was significantly increased in MS-153 50 mg/kg treated group (p<0.05) compared to water (naïve) and saline vehicle (ethanol) groups in NAC nuclear fraction. No significant changes in p65 level were detected

between water naïve, saline vehicle, and MS-153 20 mg/kg treated groups in nuclear fraction. There were also no significant changes [F(3,19)=0.29, p=0.825)] in p65 levels in all tested groups in cytoplasmic fraction (Fig. 3-19 B). One-way ANOVA revealed significant main effect on IκBα levels between all groups [F(3,19)=6.03, p=0.006] in cytoplasmic fraction. Newman-Keuls multiple comparisons test analyses revealed significant decrease in IκBα level (p<0.05) in MS-153 50 mg/kg treated group compared to water naïve group and saline vehicle-treated group in cytoplasmic fraction. No significant changes in IκBα level were detected between water naïve, saline vehicle, and MS-153 20 mg/kg groups in cytoplasmic fraction (Fig. 3-19 C).

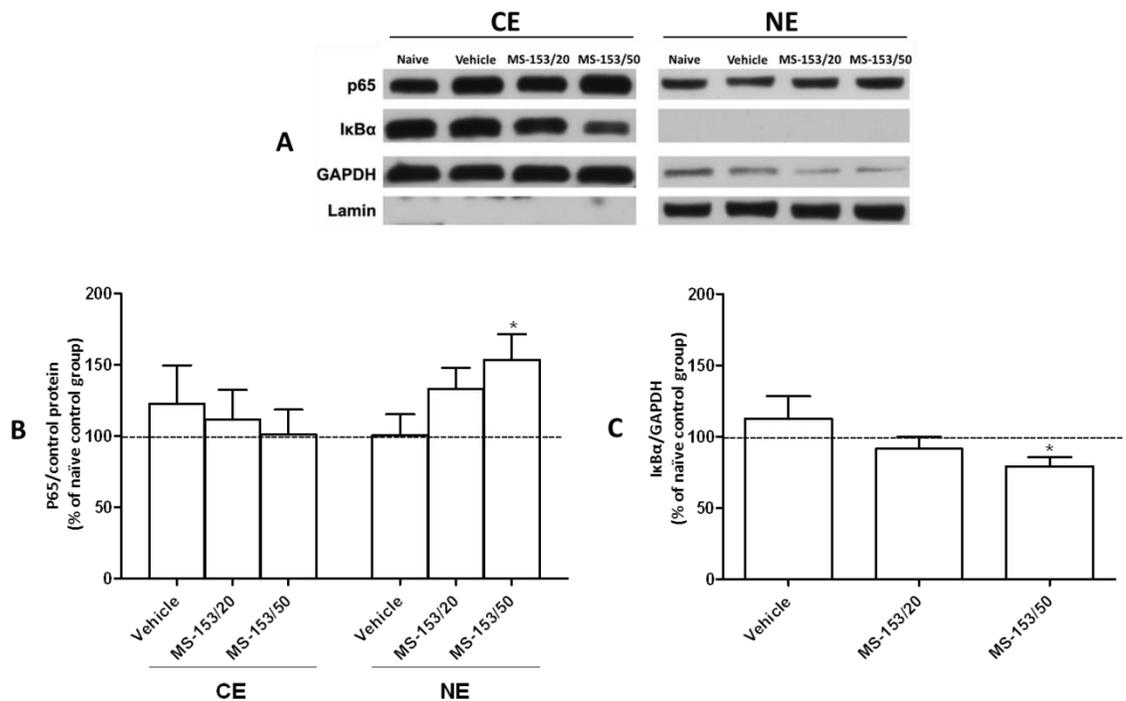


Figure 3-19 Effects of MS-153 at 20 mg/kg (MS-153/20, n=5), MS-153 at 50 mg/kg (MS-153/50, n=5), saline vehicle (ethanol) (n=5), water naïve (water group) (n=5) groups on NFκB p65 level in NAC. A) Immunoblots for GAPDH, Lamin (which were used as a control loading protein for cytoplasmic extract (CE) and nuclear extract (NE) proteins, respectively), p65, and IκBα in CE and NE. B) Quantitative analysis of p65 levels in CE and NE revealed a significant increase in the ratio of p65/Lamin level in MS-153 50 mg/kg treated group compared to water

(naïve) and saline (ethanol) vehicle treated groups in NE. No significant change of p65 levels were found between all tested groups in CE. C) Quantitative analysis of I κ B α level in CE revealed a significant decrease in I κ B α /GAPDH level in MS-153 50 mg/kg treated group compared to water naïve and saline vehicle-treated groups. Error bars indicate SEM (* p<0.05)

Furthermore, Fig. 3-20 shows the effect of MS-153 treatment on p65 and I κ B α levels in PFC. One-way ANOVA followed by post hoc Newman-Keuls multiple comparisons analyses revealed no significant change in p65 level in nuclear fraction [F(3,19)=0.10, p=0.957], and p65 level in cytoplasmic fraction [F(3,19)=0.21, p=0.887]. I κ B α level in cytoplasmic fraction was not changed [F(3,19)=0.19, p=0.901] in all tested groups (Fig 3-20 B and C).

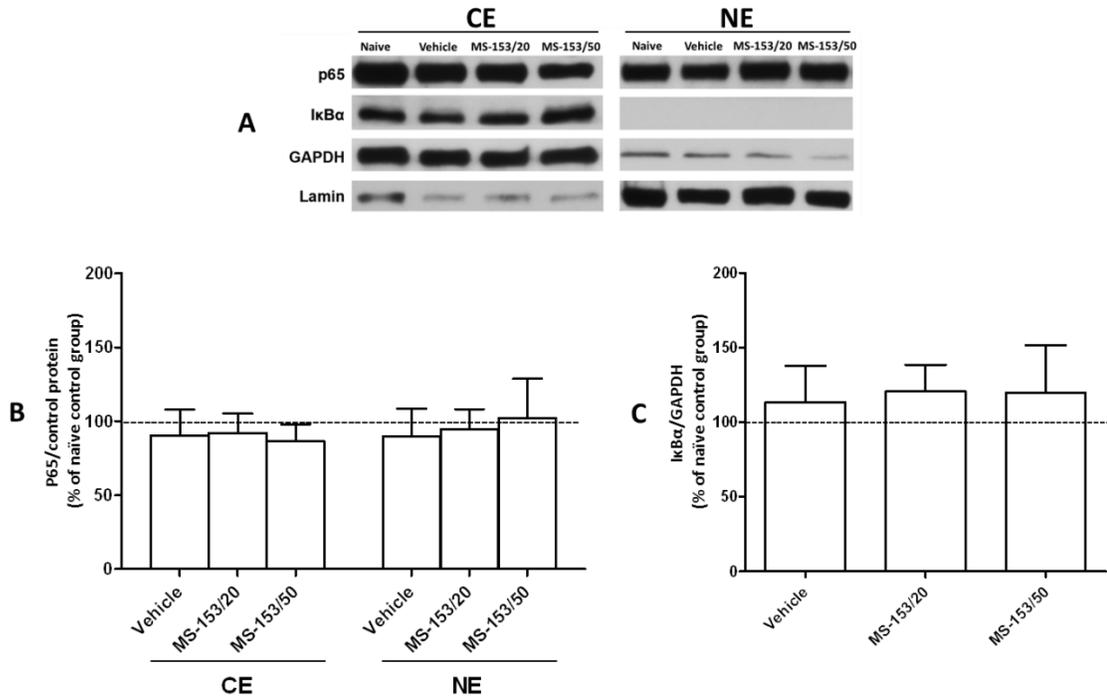


Figure 3-20 Effects of MS-153 at 20 mg/kg (MS-153/20, n=5), MS-153 at 50 mg/kg (MS-153/50, n=5), saline vehicle (ethanol) (n=5), water naive (water group) (n=5) groups on NFκB p65 level in PFC. A) Immunoblots for GAPDH, Lamin (which were used as a control loading protein for cytoplasmic extract (CE) and nuclear extract (NE) proteins, respectively), p65, and IκBα in CE and NE extracts. B) Quantitative analysis of p65 levels in CE and NE did not reveal a significant change in all tested groups in CE and NE. C) Quantitative analysis of IκBα level in CE did not reveal a significant change in IκBα level in tested groups.

Chapter 4

Discussion and Conclusions

4.1 Ceftriaxone in Relapse-like Ethanol Drinking Study

We report here that CEF treatment attenuated relapse-like ethanol-drinking behavior in male P rats. CEF treated P rats increased their water intake significantly, which is suggested to be a compensatory mechanism for decreasing their ethanol intake, however, there were no significant differences in the body weight between all groups. Recent study from our laboratory demonstrated that CEF treatment attenuated relapse-like ethanol-drinking behavior in P rats that was associated in part with upregulation of GLT-1 in PFC and NAC core (Qrunfleh et al., 2013). In this study, we tested the effect of CEF on GLT-1 isoforms (GLT-1a and GLT-1b) and other glial transporters that regulate glutamate uptake using the relapse-like ethanol-drinking paradigm. Although the two GLT-1 isoforms, i.e. GLT-1a and GLT-1b, are differentially regulated (Berger et al., 2005), our results showed that CEF treatment up-regulated both isoforms in PFC and NAC. CEF might up-regulate GLT-1a and GLT-1b through similar mechanism. It is noteworthy that GLT-1a is predominantly in astrocytes and neurons; and GLT-1b is mainly expressed in astrocytes (Berger et al., 2005). This suggests that CEF treatment induced upregulation of both neuronal and astrocytes GLT-1 isoforms.

Recent study in our laboratory showed that GLT-1 level is down-regulated in NAC core after chronic ethanol consumption for 5 weeks (Sari and Sreemantula, 2012). In the present study, we did not observe any changes in GLT-1a or GLT-1b in PFC and NAC. This may be due to the fact that neuroadaptation occurred during the two-week deprivation period that causes GLT-1a and GLT-1b in returning to their initial levels. Together, these findings suggest that both GLT-1a and GLT-1b are acting in a similar way in alcoholic P rats and their up-regulation responsible, in part, for the attenuation of relapse-like ethanol drinking behavior in P rats.

For the first time, we revealed in this study that the attenuation in relapse-like ethanol-drinking behavior was also associated in part with upregulation of xCT in both PFC and NAC. It is noteworthy that xCT plays also an important role in glutamate homeostasis. Recent studies from Kalivas's lab demonstrated that xCT was down-regulated in nicotine self-administrating rats (Knackstedt et al., 2009) and cocaine self-administrating rats (Knackstedt et al., 2010). In addition, CEF acts as a neuroprotective agent by increasing the level of xCT in cell line (Lewerenz et al., 2009). Furthermore, CEF reduces relapse to cocaine seeking behavior by normalizing xCT level in NAC (Knackstedt et al., 2010). We tested in this study the effect of CEF treatment on xCT level in PFC and NAC to determine the involvement of this glial transporter. Our results showed that CEF treatment increased xCT expression in PFC and NAC, which suggest that CEF-induced attenuation in relapse-like ethanol-drinking behavior is also mediated in part through xCT. Note that we did not observe any significant difference in xCT level between water naïve control and saline vehicle-treated rats in both PFC and NAC. These findings suggest that P rats restored their xCT level during the two-week deprivation

period. Alternatively, we did not observe any significant changes in the level of GLAST, which suggest that, the specific regulatory effect of CEF in GLT-1a, GLT-1b, and xCT expressions.

We conclude that attenuation of relapse-like ethanol-drinking behavior in male P rats is associated in part with activation of both GLT-1a and GLT-1b isoforms, and xCT in central reward brain regions such as PFC and NAC. Thus, we suggest that GLT-1a and GLT-1b isoforms, and xCT may be potential targets for attenuating relapse-like ethanol-drinking behavior and treatment of alcohol dependence. Further studies are required to investigate the effect of CEF on GLT-1a, GLT-1b, and xCT expression in chronic ethanol-drinking paradigm and the signaling pathways involved with CEF treatment effect.

4.2 MS-153 in Chronic Ethanol Drinking Study

As we expected, MS-153 treated groups showed reduction in ethanol consumption compared to saline vehicle treated group. There was a dose-dependent reduction in ethanol consumption in MS-153-treated groups. Animals treated with MS-153 20 mg/kg showed a significant decrease in ethanol consumption at Days 3 and 4. There was significant decrease in ethanol consumption in all days after treatment with MS-153 50 mg/kg compared to saline treated group. There were significant increases in water intake at the higher dose (MS-153 50 mg/kg) treated group on Days 1, 3, and 4. The water intake behavior could be explained as compensatory mechanism for decreased

ethanol intake in MS-153 treated groups. However, MS-153 did not show significant effects on body weight of all animals. Similarly, MS-153 did not show an effect on sucrose consumption, which indicates that the action of MS-153 was specific on ethanol consumption.

MS-153 was found to attenuate conditioned place preference to morphine, methamphetamine and cocaine in mice (Nakagawa et al., 2005). Our results showed, for the first time, that MS-153, novel neuroprotective agent, reduced ethanol consumption in P rats which were in ethanol drinking paradigm for 5 weeks.

MS-153 was known to enhance glutamate uptake via GLT-1 and prevented efflux of glutamate during cerebral ischemia (Shimada et al., 1999). Furthermore, MS-153 has no effect on NMDA glutamate receptors, AMPA glutamate receptors, and Ca²⁺ channels, these findings suggested that MS-153 inhibits glutamatergic system mainly by accelerating glutamate uptake (Nakagawa et al., 2005). However, the mechanism by which MS-153 induced activation of GLT-1 is still unknown. In the present study, we tested the effect of MS-153 (using two different doses, 20 and 50 mg/kg, i.p.) on GLT-1 expression in PFC, NAC, and AMG. Our findings revealed that MS-153 up-regulated GLT-1 levels in NAC and AMG but not in PFC. Furthermore, in saline vehicle treated group, GLT-1 levels were down-regulated in NAC and AMG but not in PFC. Previous study in our lab showed down-regulation of GLT-1 level in NAC but not in PFC in ethanol addicted P rats, which may be due to differences in anatomical distribution of GLT-1 in both areas (Sari and Sreemantula, 2012). AMG has numerous connections and interactions with other central reward brain regions, including PFC, NAC and VTA

suggesting its implication in drug addiction (See et al., 2003). We found GLT-1 level down-regulated in AMG, suggesting its involvement in ethanol-drinking behavior. Although there is a tight connection between AMG and PFC in performing formal tasks that require integration of information, the neuronal populations of each AMG and PFC are independent to each other; i.e. the firing of neurons in AMG was differentiated from PFC neurons in studies tested neuronal activity after gambling task and response selection (Baxter and Murray, 2002). Further, neuronal cells in AMG differ morphologically from cortical neurons (Sah et al., 2003). Moreover, AMG and PFC have distinct glutamatergic afferent and efferent projections. These observations suggest different neuroadaptation in AMG and PFC might occur after chronic ethanol consumption in P rats.

We report that MS-153 is up-regulated and normalized GLT-1 level in NAC and AMG. Up-regulation of GLT-1 in PFC and NAC was associated, in part, with attenuation of ethanol drinking behavior in P rats (Sari et al., 2011b, Sari and Sreemantula, 2012). Together, our new findings showed that MS-153, a novel neuroprotective compound, decreased ethanol consumption which is due to, in part, up-regulation of GLT-1 level in central brain reward regions, namely, NAC and AMG. Interestingly, despite the fact that MS-153 had a dose-dependent reduction in alcohol consumption, we found that both doses (20 and 50 mg/kg) of MS-153 up-regulated GLT-1 levels in NAC and AMG. These findings suggest that the higher dose of MS-153 may have other potential pharmacological effects.

To date, the mechanism of GLT-1 up-regulation by MS-153 is unknown. However, our results showed for the first time up-regulation of NF κ B in nuclear fraction of NAC but not PFC. The transcription factor NF κ B plays a critical role in various genes involved in cell growth, differentiation, and immune responses (Karin, 2006, Massa et al., 2006). NF κ B family exists in five forms including Rel (c-Rel), RelA (p65), RelB, NF- κ B1 (p50 and its precursor p105), and NF- κ B2 (p52 and its precursor p100). The transcriptional activator form is a heterodimer composed of p50 and p65, which is the most abundant activated form (Emdad et al., 2006). NF κ B is important regulator for GLT-1 expression (Sitcheran et al., 2005), which is involved in positive and negative regulation of GLT-1 expression depending on the agent administered (Su et al., 2003). Previous in vitro study has shown that CEF treatment increased GLT-1 level by activating GLT-1 promoter through activation of NF κ B signaling inducing P65 translocation by proteasomal degradation of I κ B α (Lee et al., 2008). In this present study, we demonstrated that up-regulation of GLT-1 in NAC was associated with a significant increase in NF κ B level of nuclear fraction at the higher dose of MS-153 (50 mg/kg) and a trend to increase in MS-153 (20 mg/kg) treated groups suggesting possible action of MS-153 through NF κ B signaling pathway. This might reflect another mechanism of action of MS-153 at higher doses. NF κ B up-regulation in NE was also associated with down-regulation of I κ B α in cytoplasmic fraction which indicates nuclear translocation of p65 and proteasomal degradation of I κ B α . Importantly, it has been shown that cocaine exposure induced increase in NF κ B activity in vitro (Lepsch et al., 2009). However, in vivo study showed cocaine exposure induced decrease in NF κ B activity in rat's PFC but not in hippocampus (Muriach et al., 2010). Our results did not show a change in NF κ B

level between water naïve and saline vehicle treated groups in NAC and PFC. The multi-regulatory action of NFκB in many physiological and pathological processes may underlie these results in P rats.

In conclusion, we identified MS-153, novel neuroprotective agent, as a potential drug for the treatment of alcohol dependence. MS-153 has the ability to reduce ethanol consumption in P rats, which was associated, in part, with up-regulation and activation of GLT-1 level in different central reward brain regions. Increased NFκB activity suggested the involvement of NFκB signaling pathway in GLT-1 up-regulation by MS-153.

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