Glutamate transporter 1 in the central nervous system: potential target for the treatment of alcohol dependence

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Glutamate Transporter 1 in the Central Nervous System: Potential Target for the Treatment of Alcohol Dependence

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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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Alcohol dependence is characterized by compulsive alcohol use. Chronic alcohol consumption can lead to alterations of neurotransmitter systems. The Glutamatergic system is one of the neurotransmitter systems affected as a consequence of alcohol abuse. It is suggested that increased glutamate transmission in key brain regions of the mesocorticolimbic motive circuit may promote alcohol-seeking behavior due to its reinforcing effects. We hypothesize that activation or up-regulation of the glutamate transporter 1 (GLT 1), a major transporter responsible for most of glutamate uptake would reduce extracellular glutamate and consequently attenuate alcohol consumption. Ceftriaxone (CEF), a β lactam antibiotic, has previously been identified as a drug that can elevate GLT 1 expression and reduce alcohol consumption in alcohol-preferring (P) rats (Sari et al., 2011). In the present study, we further explored the differential effects of CEF in the prefrontal cortex (PFC) and subregions of nucleus accumbens (NAc) such as the core and shell. In order to confirm the effects of upregulation/activation of GLT 1 in
the attenuation of alcohol consumption, we also tested GPI-1046, another compound known to increase GLT 1 expression.

Alcohol consumption was significantly reduced in P rats treated with CEF 50 mg/kg, CEF 100 mg/kg, GPI 10 mg/kg and GPI 20 mg/kg intra-peritoneally as compared to saline treated groups. GPI-1046 had no effect on sucrose consumption. GLT 1 expression was down regulated in P rats exposed to alcohol as compared to naïve group, in the NAc core and shell areas but not in the PFC. Upregulation of GLT 1 was observed with CEF 100 mg/kg treatment in PFC, NAc core and NAc shell and with GPI 10 mg/kg and GPI 20 mg/kg treatments in PFC and NAc Core. These results provide substantial information about the role of GLT 1 as a potential target for the treatment of Alcohol dependence.
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Contents

Abstract iii
Acknowledgments v
Contents vi
List of Figures viii

1. Introduction
1.1 Alcohol dependence 1
1.2 Current Pharmacotherapy for alcohol dependence 2
1.3 Neuropharmacology of Alcohol dependence 5
1.4 The Reward system and Alcohol dependence 14
1.5 Glutamate transporters and Alcohol dependence 16
1.6 Upregulation of GLT 1 in Alcohol dependence 23
1.7 P rats as Models of Alcoholism 29
1.8 Aims and Objectives 31

2. Material and methods
2.1 Subjects 33
2.2 Alcohol Drinking Paradigm 33
2.3 Sucrose Drinking Paradigm 36
2.4 Immunoblotting 37

3. Results
3.1 CEF groups 43
3.2 GPI-1046 groups 52

4. Discussion 60

5. References 66
List of Figures

Figure 1.1. Glutamate-Glutamine cycle.................................................................17

Figure 1.2. Cystine-glutamate exchange............................................................21

Figure 1.3. Structure of Ceftriaxone.................................................................24

Figure 1.3. Structure of Dihydrokainate .........................................................26

Figure 1.4. Structure of GPI-1046 .................................................................27

Figure 3.1. Effect of CEF treatment on alcohol consumption..........................44

Figure 3.2. Effect of CEF treatment on water consumption..............................45

Figure 3.3. Effect of CEF treatment on body weight........................................46

Figure 3.4. Effect of CEF treatment on GLT 1 expression in PFC.....................47

Figure 3.5. Effect of CEF treatment on GLT 1 expression in NAc core...............48

Figure 3.6. Effect of CEF treatment on GLT 1 expression in NAc Shell.............49

Figure 3.7. Effect of CEF treatment on alcohol intake in DHK-treated rats...........51

Figure 3.8. Effect of GPI-1046 alcohol consumption........................................53

Figure 3.9. Effect of GPI-1046 treatment on water consumption.......................55
Figure 3.10. Effect of GPI-1046 treatment on body weight……………………………………56

Figure 3.11. Effect of GPI-1046 treatment on sucrose consumption…………………………..57

Figure 3.12. Effect of GPI-1046 treatment on GLT 1 expression in PFC………………………..58

Figure 3.13. Effect of GPI-1046 treatment on GLT 1 expression in NAc core…………………59
Chapter 1

Introduction

1.1 Alcohol dependence

Chronic consumption of alcohol has been implicated in an array of diseases and disorders in human beings. The harm incurred depends on the amount of alcohol consumed and the pattern of consumption (Anderson et al., 1993). Alcohol abuse, alcohol dependence and harmful use of alcohol are collectively termed as alcohol use disorders, of which alcohol dependence, also known as alcoholism is considered as the one of high severity (Schuckit, 2009). It was estimated that about 14% of men and 5% of women in the US will experience an alcohol use disorder in their lives (Grant 1994). Alcohol dependence was found to be more prevalent among men, whites, native americans, younger and unmarried adults, and those with lower incomes in the US and only 24.1% of those diagnosed were ever treated (Hasin et al., 2007). According to the National Institute of Alcohol Abuse and Alcoholism (NIAAA), the economic loss in the US due to alcohol related problems is estimated at $185 billion each year. This includes health services and medical expenses, premature death, loss of productivity, and alcohol related crimes.

The term “Alcohol Addiction” has been supplanted by “Alcohol dependence” or “Alcoholism” over the years in alcohol related research. Alcohol dependence is a cluster of behavioral, cognitive, and physiological phenomena that may develop after repeated
alcohol use (The ICD-10 Classification of Mental and Behavioral Disorders: Diagnostic criteria for research).

The Diagnostic and Statistical Manual of Mental Disorders IV (DSM-IV) lists 7 criteria for alcohol dependence (The individuals who meet more than three of these criteria for a period of one year are diagnosed with alcohol dependence).

1. Tolerance
2. Alcohol withdrawal signs or symptoms
3. Drinking more than intended
4. Unsuccessful attempts to cut down on use
5. Excessive time related to Alcohol (obtaining, hangover)
6. Impaired social or work activities due to Alcohol
7. Use despite physical or psychological consequences

1.2 Current Pharmacotherapy for Alcohol dependence:

The current available therapy for alcohol dependence includes drugs targeting several different systems due to the non-specific nature of alcohol action in the body. Listed below are some of those that have been studied extensively.
**Disulfiram and calcium carbinide** are aversive drugs that act by inhibiting aldehyde dehydrogenase, which leads to accumulation of acetaldehyde, a metabolite of alcohol. This causes several unpleasant physiological symptoms such as tachycardia, flushing, diaphoresis, dyspnea, nausea and vomiting. The individuals refrain from alcohol intake in order to avoid these symptoms. However, clinical studies have shown that there is no significant effect of these drugs as compared to placebo (Peachey *et al.*, 1989).

**Naltrexone and other Opioid antagonists** have been shown in clinical studies to be effective in lowering alcohol craving, increasing time to first relapse and lowering overall rates of relapse in heavy alcohol drinking (Balldin *et al.*, 2003). This study suggests that the opioid antagonists may act by inhibiting alcohol-induced dopamine release, thereby reducing the reward effect to alcohol. The mechanism, however, does not fully explain their effect on alcohol consumption. Several clinical studies show that naltrexone was not beneficial in alcohol consumption. The variability in these studies may be due to different sample sizes, addiction to multiple substances of abuse in the subjects and compliance to the medication (as reviewed by Swift, 1999). Naltrexone shows some hepatotoxicity and makes the individual insensitive to safe doses of opiate analgesics for 72 hours. It has also been noted that the opiate antagonists seem to be effective only in conjunction with specific psychosocial interventions (Tambour and Quertemont, 2007).

**Acamprosate**, a synthetic GABA analog is FDA-approved drug for the prevention of Alcohol relapse. It is thought to be a functional glutamate antagonist, which regulates the
hyperglutamatergic state produced by chronic alcohol consumption. The clinical studies comparing acamprosate to placebo have shown conflicting results of efficacy as it was found in case of opioid antagonists (Whitworth et al., 1996; Boothby and Doering, 2005). Further studies are required to understand the mechanism of action underlying the activity of acamprosate.

**Tiapride** is a dopamine D2 antagonist, which is thought to help in treatment of chronic alcoholism by reducing the withdrawal symptoms (Shaw et al., 1994). Dopamine antagonists act by inhibiting the alcohol rewarding effects produced by mesolimbic dopaminergic activity and thereby preventing reinforcing effects of alcohol. Dopamine agonists such as bromocriptine were tested in alcohol relapse as it was suggested that agonists can reverse the dopaminergic hypofunction caused in alcohol withdrawal, the clinical studies, did not show efficacy compared to placebo (Naranjo et al., 1997).

**SSRIs** seem to have effects in preventing relapse in alcoholics who also suffer from depression (Cornelius et al., 1997). Buspirone, a 5 hydroxytryptamine (5HT)-1A partial agonist decreased alcohol consumption in animal studies but was found to be ineffective in clinical studies. Ondansetron, a 5HT-3 antagonist is found to be effective in alcoholics, especially those with serotonergic dysfunction. It acts by inhibiting alcohol-induced dopamine release in the mesolimbic system (Tambour and Quertemont, 2007).
Baclofen is a GABA\textsubscript{B} receptor agonist which proved to be beneficial in maintaining alcohol abstinence and preventing relapse in some preliminary clinical studies (Flannery \textit{et al.}, 2004). Baclofen is thought to act by inhibiting the GABA\textsubscript{B} receptors that are present on cell bodies of dopaminergic neurons in VTA that project to limbic areas, thereby diminishing alcohol-induced dopamine release in the limbic system (Tambour and Quertemont, 2007).

The available pharmacotherapy for alcohol dependence includes drugs acting on alcohol metabolism as well as a wide range of neurotransmitter systems including endogenous opioid system, glutamate system, dopamine system, serotonin system and GABA systems. Some drugs have been proved to be more effective than others in clinical studies. In conclusion, since alcohol has multiple mechanisms of action on the brain neurotransmitter systems, it is important to understand all the modifications it produces, identify the sequence and use this information to design therapies that could neutralize if not reverse these changes. The present study is another attempt in this direction.

**1.3 Neuropharmacology of Alcohol Dependence:**

Individuals with alcohol dependence lose reliable control of their alcohol use due to the psychological and physiological manifestations of tolerance and withdrawal effects. Long-term alcohol use induces changes in a wide range of neurotransmitter systems, neuronal proteins and membranes of neuronal cells (Chin and Goldstein, 1981).
Tolerance and withdrawal effects can be best elucidated based on alcohol action on neurotransmitters and their functional balance. In a normal functioning brain, a balance exists between excitatory and inhibitory neurotransmission. Acute alcohol consumption disturbs this equilibrium by enhancing inhibitory and attenuating excitatory neurotransmission. It has been suggested that, after long term alcohol consumption, the brain compensates for the depressant effects of alcohol, to maintain homeostasis (Valenzuela, 1997). These compensatory physiological changes play a significant role in the transition of individuals from controlled to compulsive alcohol users. The following are the effects of alcohol on various neurotransmitter systems in the CNS

**Gamma Amino Butyric Acid (GABA)** is the major inhibitory neurotransmitter in mammalian central nervous system. It acts on three classes of receptors, termed GABA_A, GABA_B and GABA_C receptors of which GABA_A and GABA_C receptors are ionotropic, while the GABA_B receptors are metabotropic. The GABA_A receptors are found in most of the neurons in brain and spinal cord and mediate the major CNS depressant effects (Johnston, 1996). Activation of GABA_A receptor by GABA leads to influx of chloride ions into the postsynaptic cell causing hyperpolarisation of the post synaptic membrane thereby decreasing neuronal excitability (as reviewed by Chebib and Johnston, 1999).

**Glycine receptors** are ligand-gated chloride channels which play a significant role in inhibitory action in the mature central nervous system, particularly in the brainstem and spinal cord. The α and β subunits are arranged on the post synaptic membrane in
pentameric form and inhibit neuronal firing by opening the Cl\textsuperscript{−} channels following agonist binding (Langosch et al., 1990).

Alcohol acts as an allosteric modulator of the GABA\textsubscript{A} receptor complex and potentiates the GABA gated current further increasing the hyperpolarisation. This potentiation was found to be due to an increase in the number of channel openings and bursts as well as the duration of channel openings (Tatebayashi et al., 1998). The alcohol-induced behavioral changes following acute consumption are enhanced by GABA agonists and antagonized by GABA antagonists (Liljequist and Engel, 1982). Ion flux studies performed in synaptoneurosomes have shown that alcohol also potentiates the inhibitory glycine receptors (Engblom and Akerman, 1991).

Ro-15-4513, a negative allosteric modulator of the GABA\textsubscript{A} receptor inhibits the potentiating effects of alcohol in studies with recombinant receptors expressed in *Xenopus* oocytes. The drug has also been shown to reverse the effects of ethanol intoxication in rats (Suzdak et al., 1986). However its anxiogenic and proconvulsant activity prevented Ro-15-4513 from being tested for clinical trials in humans.

A region of 45 amino-acid residues has been identified using chimeric receptor constructs within GABA\textsubscript{A} and glycine receptors as the putative alcohol binding site. Within this
region, two specific amino-acid residues in transmembrane domains 2 and 3 were found to be critical for allostERIC modulation of the receptors (Mihic et al., 1997).

On the other hand, prolonged exposure to alcohol leads to changes in mRNA and protein levels in the GABA\textsubscript{A} receptor subunits due to the compensatory adaptive mechanism. There is a decrease in the GABA\textsubscript{A} receptor density and region specific changes in the expression of genes for either upregulation or downregulation of $\alpha$ and $\beta$ subunits. It was also found that mRNA and protein levels of $\alpha1$ decrease and $\alpha4$ increase in certain brain regions such as cerebral cortex (Golovko et al., 2002). Chronic alcohol exposure decreases GABA\textsubscript{A} receptor $\alpha1$ subunits in the ventral tegmental area (VTA), which in turn results in an increase in dopamine release. Hence GABAergic systems are thought to be involved in mediating self-administration of alcohol in animal models by stimulating reward circuitry in the mesolimbic dopamine system (Chester and Cunningham, 2001).

The Glycine receptors also influence extracellular dopamine levels and alcohol-induced dopamine release in the NAc which is an important part of the brain reward circuit. A study conducted in male adult Wistar rats showed that reverse microdialysis of glycine into the NAc reduces alcohol intake (Molander et al., 2005). Org25935, a glycine reuptake inhibitor, was also shown to reduce alcohol intake in alcohol-preferring wistar rats by increasing extracellular glycine levels and subsequently modulating brain glycine receptors (Molander et al., 2006).
**Dopamine** release and/or synthesis increases following acute consumption of alcohol, while chronic intake results in decreased sensitivity and development of tolerance.

Alcohol consumption has been shown to increase dopamine levels in the NAc, a major component of the mesolimbic dopamine system and brain reward pathway (Weiss *et al.*, 1993). Blockade of dopamine receptors in the NAc reduced alcohol intake and knockout mice deficient in dopamine D1 and D2 receptors were found to consume less alcohol (El-Ghoundi *et al.*, 1998; Cunningham *et al.*, 2000).

Deprivation from alcohol leads to mesolimbic dopamine system hypofunction and it is presumed that this decrease in function motivates the individual to resume alcohol drinking to restore the baseline function of the system (Ahmed and Koob, 2005). Studies using rat models of chronic alcohol consumption showed underlying mechanisms of dopamine system hypofunction to be overactivity of L-type Calcium channels (Rossetti *et al.*, 1992) and a decrease in tyrosine hydroxylase (Diana *et al.*, 2003).

In a clinical study of abstinent alcoholic individuals, lower availability of dopamine receptors in ventral striatum and adjacent putamen was correlated with higher alcohol craving behavior (Heinz *et al.*, 2005). Hence, the neuroadaptative changes in the mesolimbic dopamine system play a significant role in negative reinforcing effects of alcohol in alcoholism.
**Opioid receptors**: there are three types of opioid receptors, μ, δ and κ, which are the targets for endogenous opioid peptides such as β-endorphin, enkephalins and dynorphins. Endogenous opioid peptides play a major role in the brain reward system through the modulation of the mesolimbic dopaminergic system (Spanagel *et al*., 1992).

Alcohol interferes with the opioid mechanisms both at the level of the endogenous opioid peptides by affecting their synthesis, release, or processing and at the level of the opioid receptors by altering their affinity for the opioid peptides. Acute exposure to alcohol has been shown to increase enkephalin and β-endorphin levels in the brain and higher β-endorphin levels have been correlated to alcoholism (Gianoulakis *et al*., 1996).

Opioid antagonists that are selective for μ- and δ-opioid receptors, as well as some nonselective antagonists such as Naltrexone and Naloxone have been reported to decrease alcohol intake (as reviewed by Herz, 1997). Their mechanism of action, however, is contentious. The opioid antagonists were thought to act by inhibiting alcohol-induced dopamine release in the NAc. However, in lesion studies of dopaminergic terminals in the NAc, while the lesions could not alter alcohol intake, Naltrexone was able to reduce the consumption (Koistinen *et al*., 2001). Hence the effects of opiate antagonists cannot be entirely attributed to their actions on dopaminergic transmission. As alcohol increases extracellular endorphins in the NAc, the opioid antagonists are also thought to act by inhibiting endogenous endorphin actions (Olive *et al*., 2001).
Serotonin receptors have been shown to be implicated in alcohol consumption, intoxication and withdrawal effects. 5-HT₁ receptor activity reduces baseline dopamine release in the forebrain. Hence when alcohol is consumed, there is intensified release of dopamine and thereby, increase in the rewarding properties of alcohol. Alcohol consumption has been shown to decrease the number and sensitivity of 5-HT₂ receptors over a period of time (Pandey et al., 1992). 5-HT₃ receptors are found in the NAc and are known to facilitate the dopamine release. 5-HT₃ receptor antagonists such as Ondansetron inhibit alcohol-induced dopamine release in the mesolimbic system (Tomkins and Sellers, 1995).

Acute alcohol consumption increases extracellular 5-HT levels while chronic intake causes increased reuptake by serotonin transporter (5-HTT) or decreases release from raphe nuclei. Alcohol exposure increases 5-HTT mRNA expression in regions associated with reward pathway in the brain (Heinz et al., 2003). Use of selective serotonin reuptake inhibitors was found to be beneficial in withdrawal associated anxiety and depression in alcoholics and reduce alcohol consumption to some extent (Lejoyeux, 1996).

Glutamate acts on two broad categories of receptors, ionotropic and metabotropic. The ionotropic receptors are ligand-gated ion channels and are further classified into NMDA, AMPA and Kainate receptors based on their sensitivity to respective agonists (Nakanishi, S, 1992). The metabotropic receptors are G-protein coupled receptors divided into eight mGluR subtypes, having multiple splice variants (Pin, J, 1995).
Alcohol non-competitively inhibits NMDA mediated calcium influx in the cortex, NAc, amygdala, hippocampus (HPC) and VTA (as reviewed by Gass and Olive, 2008). It also inhibits NMDA induced increase in cyclic guanosine mono phosphate (GMP) (Hoffman et al., 1989). Due to its constant inhibition of glutamate transmission, chronic alcohol administration in rats leads to a compensatory upregulation of NMDA receptors and their activity. Hence alcohol withdrawal is associated with increased excitatory amino acid transmission and manifests into symptoms such as seizures. Alcohol also inhibits AMPA and Kainate receptors, but the concentrations required to produce this effect are much higher than those that inhibit NMDA receptors (Hoffman et al., 1989).

Glutamate activity at mGluR5 a post synaptic metabotropic glutamate receptor that is abundant in areas associated with drug reward was shown to regulate motivation to self-administer alcohol in rat models (Besheer et al., 2008). The antagonists of mGluR5 were also found to be beneficial in preventing alcohol relapse in animal studies (Schroeder et al., 2005).

**Nicotinic acetyl choline receptors** (nAChRs) are present in the brain and peripheral nervous system. It was suggested that alcohol’s effects on nAChRs could be responsible for its addictive properties (Bradley et al., 1980). It was observed that alcohol has both agonistic and antagonistic actions on acetyl-choline induced responses based on the subunit combination of the nAChR. Cholinesterase inhibitors reduced alcohol
consumption in alcohol-preferring AA rats (Doetkotte et al., 2005). However, it is not clear as to which subunit composition plays a major role in this activity.

**Endocannabinoid** transmission was found to be inhibited by alcohol intake and chronic consumption was implicated in the increased levels of endocannabinoids such as Anandamide and downregulation of Cannabinoid1 (CB1) receptors (Cippitelli et al., 2008). Further, it was shown that CB1 knockout mice consumed less alcohol and CB1 agonists potentiated while antagonists diminished alcohol consumption (as reviewed by Vengeliene et al., 2008).

**Ghrelin** is a gastric peptide that regulates hunger and appetite and plays a role in maintaining body weight and energy balance. It is an endogenous ligand for growth hormone secretagogue receptor (GHS-R), which is found in several areas of brain, including those associated with reward. It has been found that alcoholic individuals showed increase in the ghrelin levels which also correlated with craving behavior (Addolorato et al., 2006). Microdialysis studies showed that ghrelin increases dopamine flow in the NAc by acting on the GHS-R in VTA and laterodorsal tegmental area. Based on these studies, GHS-R1A antagonists and GHS-R1A knockouts were tested for their effects on alcohol reward. It was reported that alcohol-induced locomotor stimulation, NAc dopamine release and conditioned place preference were inhibited in these models (Jerlhag et al., 2009).

### 1.4 The reward system and Alcohol dependence:
Reward is associated with any event (drug) for which an animal will perform an operant response (Koob, 1992). External and internal stimuli, that are capable of increasing the frequency of the operant response, are termed as reinforcers; and stimuli that decrease the frequency are termed as punishers (Spanagel and Weiss, 1999). It has been suggested that substances of abuse are self-administered due to their positive reinforcing effects, which are the direct rewarding effect of the drug or due to their negative reinforcing effect, which is to avoid unpleasant withdrawal symptoms (Kandel, 2002).

The mid-brain dopaminergic projections that arise in the VTA and project to the limbic structures, particularly the NAc, play a prominent role in regulating reward related behavior and are deemed as such to be the neurochemical substrate for reward (Wise and Rompre, 1989). The main sites in the brain that mediate alcohol reinforcement are the NAc, PFC and the VTA, which constitute the mesocorticolimbic dopamine system and the extended amygdala (AMG) (Vengeliene et al., 2008). On receiving a stimulus that is a reinforcer, the limbic system processes all the new and previously learned information associated with it (Kalivas et al., 2009). The limbic structures involved in this function include the basal lateral amygdala (BLA) which affiliates emotional processing and the HPC which makes contextual associations (Myers et al., 1994 and Yaniv et al., 2004). The role of the PFC is the determination of goal oriented action. The signals from the PFC and limbic structures activate the NAc, which articulates the received information and relays it to the motor system to produce motor actions related to the response (Sesack and Grace, 2010). The NAc does so via disinhibition of substantia nigra reticulata and
motor thalamus and further activation of motor cortex which produces motor response through its projections to spinal cord (Groenewegen et al., 1999).

There are strong dopaminergic projections from VTA to NAc and also to the PFC. Alcohol increases firing of these neurons, which lead to increase in extracellular levels of dopamine in the NAc (Di Chiara and Imperato, 1988). In addition, GABAergic inhibitory projections arising in the NAc project to the ventral palladium which further projects to VTA and the dorsal medial thalamus (DMT). The DMT sends glutamatergic efferents to PFC completing the limbic loop (Sesack and Grace, 2010). Further, the efferents between AMG and PFC and from the PFC to the NAc are glutamatergic. The PFC also sends a strong glutamatergic efferent to the dopaminergic neurons in the VTA. Activation of these neurons has been directly linked to addictive behavior (Kalivas, 2004).

Accumulation of extracellular glutamate as a result of alcohol withdrawal has been observed in the NAc, striatum and HPCs. These evidences support our hypothesis that GLT 1 may be a potential target for treating alcohol dependence as it is responsible for the reuptake of the accumulated extracellular glutamate.

### 1. 5 Glutamate transporters and Alcohol dependence
1.5.1 Glutamate-Glutamine cycle

Glutamate is an anionic amino acid present in several cell types in the body. It acts as a major excitatory neurotransmitter and a potent neurotoxin in CNS. Glutamate is synthesized in the neuronal cytoplasm with phosphate activated glutaminase (PAG) and stored in synaptic vesicles. Stimulation of glutamatergic neurons releases glutamate into the synaptic cleft where it activates ionotropic receptors for fast excitatory neurotransmission and metabotropic receptors for slower modulatory effects. The excess glutamate is then taken up by glial cells, where it is metabolized into glutamine. Subsequently, the glutamine is transported to the neuronal cell where it is converted into glutamate and taken up by vesicles. This completes the glutamate-glutamine cycle (as reviewed by Shigeri et al., 2004).

Glutamate transporters are membrane bound protein pumps found in neuronal and glial membranes. They are responsible for the uptake of glutamate at synaptic cleft and in the neuron. Accumulation of glutamate at the synaptic cleft can cause overstimulation of glutamate receptors, which in turn causes calcium homeostasis dysfunction, increased nitric oxide production, activation of proteases, increase in cytotoxic transcription factors and increase in free radicals that may subsequently lead to neuronal death (Wang and Qin, 2010). The glutamate transporters come into play here to maintain glutamate below excitotoxic level. They are of two types: sodium dependent excitatory amino acid transporters (EAATs) and vesicular glutamate transporters (VGLUTs).
1.5.2 Excitatory Amino Acid Transporters (EAATs)

The EAATs are present in both presynaptic neurons and glial cell and are responsible for terminating the activity of glutamate in the synapse and removing it from the extracellular space in order to maintain it at a non-toxic level. The glutamate transport is in conjunction with an inward sodium gradient (Kanai et al., 1995). Glutamate is co transported with two to three sodium ions and one proton along with counter transport of a potassium ion (Zerangue and Kavanaugh, 1996). There is also a substrate gated anion conductance (chloride flux) associated with EAATs (Seal and Amara, 1999). Five
subtypes of EAATs have been identified in human and rodent brains. The first three subtypes that were identified in the rat brain are termed as glutamate/aspartate transporter (GLAST), GLT 1 and excitatory amino acid carrier type 1 (EAAC1); their human counterparts are named EAAT1, EAAT2 and EAAT3 respectively. The two subtypes found subsequently were identified in both rodents and humans and named as EAAT4 and EAAT5 (Amara and Fontana, 2002).

EAAT1 and EAAT2 are primarily localized on the membranes of astroglia or Bergman glia associated with excitatory synapses. EAAT2 is also found in presynaptic neurons and oligodendrocytes. GLAST was found to be expressed predominantly in the cerebellum and moderately in the hippocampus, forebrain and spinal cord. GLT 1, on the other hand is mainly expressed in the forebrain though it is detected in all regions of the brain and spinal cord (as reviewed by Kim et al., 2011). The EAAT3 and EAAT4 are localized on postsynaptic neuronal terminals and astrocytes while the EAAT5 is present in rod and bipolar cells. EAAT3 is expressed sparsely in several regions of the brain. EAAT4 is found in the cerebellum and EAAT5 is localized in the retina (as reviewed by Kim et al., 2011).

1.5.3 Vesicular glutamate transporter (VGLUTs)

The VGLUTs are responsible for the uptake of glutamate and sequestering into the synaptic vesicles for storage after it has been synthesized in the presynaptic neuronal cytoplasm (Amara and Fontana, 2002). The uptake is driven by a proton dependent
electrochemical gradient that exists across the vesicle membrane. It depends mainly on the vesicular membrane potential gradient which is created by a vacuolar-type ATPase (Takamori, 2006).

Three isoforms of VGLUT have been identified in the mammalian CNS and were termed as VGLUT1, VGLUT2 and VGLUT3. They belong to type 1 phosphate transporter family (Herzog et al., 2006). VGLUT1 was found to be expressed in the cortex, HPC (dentate gyrus) and subiculum. VGLUT2 was found in neocortex, olfactory bulb, dentate gyrus and subiculum. It has been suggested that VGLUT1 is expressed at synapses associated with low release rate and long term potentiation, while VGLUT 2 is expressed at synapses with high release rate and long term depression (Fremeau et al., 2001). VGLUT3 was identified in somato-dendrites of neurons and glia apart from pre synaptic terminals and is distributed sparsely throughout the brain. It is thought that the expression of VGLUT outside the presynaptic terminal suggests the probability of its role in modulating glutamate signaling rather than in the exocytotic release of glutamate (Fremeau et al., 2002).

1.5.4 Cystine–glutamate antiporter

The cystine–glutamate antiporter is a source of non-vesicular glutamate release. The antiporter is a plasma membrane-bound, Na⁺-independent, anionic amino acid transporter that exchanges extracellular cystine for intracellular glutamate. It exists as two separate proteins, the light chain xCT that is unique to the cystine–glutamate antiporter and the
heavy chain 4F2 that is common to many amino acid transporters. Similar EAAT transporters, this antiporter was found to be distributed on cells throughout the body and preferentially on the glia in the brain (Baker et al., 2002). It provides cysteine for glutathione synthesis, thereby antagonizing oxidative stress and provides glutamatergic tone to mGluRs. Cystine-glutamate exchange is thought to play a prominent role in maintaining extracellular glutamate levels and excitatory neurotransmission (Murphy et al., 1989)

A study of the role of the cystine-glutamate exchange in cocaine seeking behavior by Baker and colleagues showed that reduction in the exchange increased susceptibility to relapse (Baker et al., 2003). Further, the restoration of the activity of the antiporter by intracranial perfusion of cystine or systemic administration of N-acetyl cystine was shown to decrease the cocaine seeking in the rat models. Another study in cocaine relapse by Knackstedt and colleagues showed that CEF treatment restored both GLT 1 and xCT levels, which in turn inhibited relapse (Knackstedt et al., 2009). It was suggested that glutamate transport and cystine-glutamate exchange are coregulated to maintain
glutamate homeostasis.

Figure 1.2: A representation of the cystine-glutamate exchange. Cystine (CySS) is transported inwards in a 1:1 exchange for glutamate (Glu), which travels outwards down its concentration gradient. Glutamate is co-transported with two to three sodium ions and one proton along with counter transport of a potassium ion. Intracellular cystine is converted to cysteine (Cys) (modified from McBean, 2002).

1.5.5 Role of Glutamate reuptake in Alcohol dependence

Glutamate mediated excitotoxicity has been implicate in trauma, ischemia and several other neurodegenerative disorders. Reuptake of the extracellular glutamate rapidly is necessary for the survival and normal functioning of neurons. The glial sodium dependent transporters, GLAST and GLT 1 are primarily responsible for this activity (Anderson and Swanson, 2000). GLT 1 was found to be responsible for the removal of 90% of the extracellular glutamate. Impaired glutamate uptake due to dysfunction or downregulation of EAAT2 has been implicated in a wide range of disorders such as
Amyotrophic Lateral Sclerosis (ALS), Alzheimer’s disease, epilepsy, ischemia and hepatic encephalopathy (as reviewed by Maragakis and Rothstein, 2006).

Study by Schreiber and Freund, 2000 showed a reduction in the density and function of glutamate transporter sites in synaptosomal and membrane preparations obtained from alcoholic rat models. A study of glutamate transporter expression in post-mortem human alcoholic brains showed a significant reduction in the levels of EAAT1 and EAAT2 in the BLA as compared to non-alcoholics (Kryger and Wilce, 2010). As it has previously been established that alcohol consumption has inhibitory effect on glutamate transmission, it can be inferred that glutamate transporters have an important role to play on this action of alcohol and an increase in the glutamate transporter activity would have neutralizing action on reinforcing and withdrawal effects of alcohol.

A reduction in the expression of GLAST was observed in some studies and a compensatory increase in the mRNA was observed in the post mortem human PFC samples (Flatscher-Bader and Wilce, 2008). A study of glutamate and dopamine synaptic terminals in the alcoholic rat models revealed an increase in VGLUT2 expression in NAc shell while VGLUT1 remained unchanged following deprivation of alcohol (Zhou et al., 2006). In addition, high throughput screening studies for compounds capable of upregulating GLT 1 was performed (Colton et al., 2010) since it has been previously established that GLT 1 is the key player in the removal of most of the extracellular glutamate (Rothstein et al., 1995).
Importantly, a blinded screen of 1040 FDA approved drugs and nutritionals was performed in which beta-lactam antibiotics were found to be potent modulators of GLT1 expression. Based on its favorable properties, CEF (a third generation cephalosporin) was chosen for further study in *in vitro* models of ischemic injury and motor neuron degeneration and *in vivo* animal models of ALS and showed significant positive effects (Rothstein et al., 2005). Another screen of the FDA-approved compounds using luciferase reporter assay on human astroglial cells identified harmine, a beta-carboline alkaloid, as a potent EAAT2 promoter. Further testing in cell culture and ALS animal models demonstrated that harmine effectively increased GLT1 protein and glutamate transporter activity (Li et al., 2010).

### 1.6 Upregulation of GLT 1 transporter and Alcohol dependence

#### 1.6.1 Ceftriaxone

Ceftriaxone is a third generation, semi synthetic cephalosporin having a broad spectrum of activity against Gram-positive and Gram-negative aerobic, and some anaerobic bacteria. It has a long half-life and can be administered either I.V or I.M. It exerts antibacterial action by inhibiting bacterial cell wall synthesis. It was found to be effective in complicated and uncomplicated urinary tract infections, lower respiratory tract
infections, skin, soft tissue, bone and joint infections, bacteremia/septicemia, and primarily, meningitis (Richard et al., 1984).

Ceftriaxone

Figure 1.3: Structure of CEF disodium

CEF has been extensively studied for its neuroprotective activity following the identification of its GLT 1 modulating effect. CEF was found to show a significant reduction in acute stroke mortality and improved neurological performance in animal models of stroke (Thöne-Reineke et al., 2008). CEF-mediated increase in GLT 1 expression in spinal cord was found to be beneficial in opioid-induced paradoxical pain and neuropathic pain (Ramos et al., 2010).

The effect of CEF in animal models of drug abuse has been tested and it was seen that it significantly reduced cue-induced cocaine relapse. Upregulation of GLT 1 was reported
in PFC and NAc (Sari et al., 2009; Knackstedt et al., 2009). In a study by Sari and colleagues 2011, alcohol-preferring P rats were used as models of alcoholism to test the effect of CEF. The P rats were given free choice to 15% and 30% alcohol solutions for five weeks to achieve dependence and then treated with CEF with doses ranging from 25mg/kg to 200 mg/kg for five days. A dose-dependent reduction in alcohol intake was observed but it was associated with increased in GLT 1 expression only in the higher doses, 100 mg/kg and 200mg/kg. There was no reduction in sucrose intake by the P rat models which showed specificity in the effect on alcohol consumption.

In the present study, we further explored the differential effects of the higher doses of CEF in the core and shell regions of the NAc. We have also investigated whether CEF is reversing the effects of alcohol in GLT 1 expression in NAc- shell and core, and PFC. The effect of Dihydrokainate (DHK) on CEF treatment was tested in an attempt to further elucidate its mechanism of action in alcoholism.

1.6.2 Dihydrokainate

Dihydrokainate (DHK) is a selective non-transportable inhibitor of GLT 1. GLAST and EAAC 1 were found to be relatively insensitive to inhibition by DHK. Glutamate transport in cortical, striatal, hippocampal and midbrain synaptosomes was found to be inhibited by DHK with an IC-50 value of about 100 μM (Robinson, 1998). Most of the compounds that inhibit glutamate uptake are structural analogs of glutamate receptor
agonists. They act as substrates of the transporters and elicit transporter currents in voltage clamp measurements. However, Kainic acid and DHK selectively block GLT 1 mediated uptake without acting as substrates (Arriza et al., 1994). In the present study, DHK was administered to a group of rats prior to CEF treatment to test if it could block CEF induced reduction in alcohol consumption. This would help to confirm that CEF works by increasing GLT 1 mediated glutamate uptake. The dose used was 10 mg/kg based on previous studies (Gunduz et al., 2011; Rawls et al., 2009).

1.6.3 GPI-1046

GPI-1046 is 3-(3-pyridyl)-1-propyl (2S)-1-(3,3-dimethyl-1,2-dioxopentyl)-2-pyrrolidinedinecarboxylate. It is a synthetic, non-immunosuppressive ligand of FK506 binding protein-12 (Steiner et al., 1997).
FK506 (Tacrolimus) and cyclosporin are major immunosuppressants and “immunophilins” are the high affinity protein receptors for these drugs. The FKBP-12 and cyclophilin A are prototypes of the immunophilin family. They form complexes with FK506 and cyclosporin respectively. These complexes bind to calcineurin, inhibit phosphatase activity, decrease production of cytokines such as Interleukin-2 which in turn decreases T-cell proliferation, ultimately producing immune system suppression (Schreiber et al., 1991; Emborg et al., 2001).

It has been shown that neural tissue concentrations of FKBP immunophilins are 10 to 30 fold greater as compared to immune cells, which suggested having a functional role in the nervous system. FK506 was found to potentiate nerve growth factor (NGF) induced neurite growth in vitro (Lyons et al., 1994). It later was found that FK 506 promoted neuronal survival and neurite outgrowth in cultures even in the absence of exogenous NGF at picomolar concentrations. FK 506 and its analogue L685818 also induced regeneration of a previously crushed sciatic nerve in vivo (Steiner et al., 1997). In order to exploit the potential of this neurotrophic activity, a series of non-immune suppressive
ligands of FKBP-12 which lack the ability to bind to calcineurin have been synthesized. GPI-1046 is the prototype of the non-immunosuppressive ligands.

GPI-1046 was shown to have both neuroprotective and neuroregenerative activity in vitro and in animal models of disease. It was shown to induce regeneration of crushed sciatic nerves in animals and protect serotonin neurons against parachloroamphetamine toxicity. Further, it was found that GPI-1046 can protect nigro-striatal dopamine neurons on administration with a neurotoxin in an MPTP model of Parkinson’s disease (Steiner et al., 1997).

Furthermore, a series of in vitro and in vivo studies have been conducted to study the effect of GPI-1046 on expression and function of GLT 1. It has been suggested that regulation of glutamate transporter activity plays a role in the neuroprotective activity of this compound (Ganel et al., 2006). These latter studies showed that GPI-1046 increased expression and activity of GLT 1 in spinal cord organotypic cultures and cortical primary astrocytes in vitro and in the cortex of mouse brain and cortex, thalamus and HPC of rat brain in vivo. In addition, it was also demonstrated that GPI-1046 can prevent glutamate induced neural injury in models of chronic glutamate toxicity. This effect was produced through interaction with FKBP-12 (Ganel et al., 2006). Based on these studies, we hypothesized that GPI-1046 induced upregulation of GLT 1 protein can reduce alcohol consumption in P rats exposed to a free choice alcohol drinking paradigm.

1.7 P rats as Models of Alcoholism:
The P rat lines were originally outbred from Wistar rats at the Walter Reed Army Institute for research, Washington, DC (Li et al., 1993). The original stock of Wistar rats from which they were bred were tested by a two-bottle preference test and their alcohol consumption was recorded and found to range between 0.08 to 9.24 g/kg/day. The rats with high preference were mated to develop the P rat line and a mass selection was enabled subsequently by mating the offspring with additional stock Wistar rats with high alcohol preference. The selection criteria for the P rats was alcohol consumption of greater than 5 g alcohol/kg body weight/day and a preference ratio greater than 2:1 (v/v) of alcohol to water consumed (Li et al., 1993).

Some studies of the neurotransmitter systems in P rats versus the NP rats revealed that P rats showed a deficiency of 5 HT, upregulation of 5HT1A receptors (Mc Bride et al., 1994), abnormalities in the VTA-NAc dopaminergic system (Gongwer et al., 1989) and a higher density of GABAergic terminals in the NAc (Hwang et al., 1990). However, a convergence of findings has been observed in animal and clinical studies in most of the therapies for alcoholism (Li et al., 1993).

Several criteria have been proposed in which animal models of alcoholism were expected to be tested and provide satisfactory results. Lester et al., 1973 compiled a table of 7 criteria, Cicero, 1979 proposed 6 and Keane and Leonard, 1989 proposed 4 criteria to be satisfied by the alcoholism models. However, they are very similar in their intent. It has been established that P rats satisfy all the criteria for animal models of alcoholism as proposed by Cicero, 1979.
Thus, Cicero (1979) proposed that (as reviewed in Rood et al., 2004):

1. The animal should orally self-administer Alcohol.
2. The amount of Alcohol consumed should result in pharmacologically relevant blood Alcohol levels.
3. Alcohol should be consumed for its post-ingestive pharmacological effects, and not strictly for its caloric value or taste.
4. Alcohol should be positively reinforcing, or in other words, the animals must be willing to work for Alcohol.
5. Chronic Alcohol consumption should lead to the expression of metabolic and functional tolerance.
6. Chronic consumption of Alcohol should lead to dependence, as indicated by withdrawal symptoms after access to Alcohol is terminated.

Li and colleagues summarized all the findings of P rat studies that provide evidence that they satisfy the above criteria. The summary is as follows:

1. Animals voluntarily consume 5-8 g alcohol/kg/body wt/day.
2. Animals attain blood alcohol concentrations of 50-200 mg% with free choice drinking.
3. It has been shown that P rats and alcohol non preferring rats (NP rats) react to the taste and smell of alcohol similarly; P rats self-administer alcohol intragastrically and voluntarily drink the same or greater amounts of alcohol even in the presence of highly palatable fluids and caloric sources. P rats will self-administer, through
operant responding, nanoliter quantities of alcohol at concentrations of 50-200 mg% directly into VTA.

(4) Animals bar-press to obtain alcohol orally when food and water are freely available demonstrating that alcohol is behaviorally reinforcing.

(5) Animals develop physiological tolerance towards the motor impairing effects of alcohol and tolerance to subjective aversive effects of alcohol as revealed by conditioned taste aversion. With tolerance development, P rats drink about 50% more alcohol/day

(6) Animals develop physical dependence with chronic free choice drinking.

The above unique characteristics of P rats made them the choice of subjects for the present study.

1.8 Aims and Objectives:

We intended to test the effects of CEF 50 mg/kg and CEF 100 mg/kg on alcohol consumption based on previously published data (Sari et al., 2011). We also proposed to test the effect of CEF on rats treated with DHK, an inhibitor of GLT 1 to elucidate the mechanism of action underlying the activity of CEF. We proposed to test two doses of a novel compound, GPI 1046 for effect on alcohol consumption and for specificity of action. Further, we wanted to compare the GLT 1 expression in naïve, alcoholic saline treated rats, and alcoholic drug treated rats 8 days after the first treatment, to observe the differential effects of the treatment on GLT 1 in various regions of the brain associated with reward. We hypothesized that the drugs could produce an increase of GLT 1
expression and/or activity which would result in a significant reduction of alcohol consumption in P rats.

Chapter 2

Methods and Materials
2.1 Subjects

Male Alcohol-preferring P rats were used as they are an established model of Alcoholism. They were procured from the Indiana University medical health center (Indianapolis, IN) Indiana Research center breeding colonies at 21-30 days of age. They were housed in pairs in standard plastic tubs in the DLAR (Department of Laboratory Animal Resources, University of Toledo, HSC) and acclimatized to the vivarium. The plastic tubs had Corn-cob bedding and a temperature of 21°C and 50% humidity were maintained in the room. A 12 hour light/dark cycle was followed. Food and water were given *ad libitum*. After habituation to the vivarium, the animals at 80 days of age were single housed. They were introduced to either alcohol or sucrose solutions at 90 days of age based on the paradigm.

2.2 Alcohol Drinking Paradigm

Male adult P rats at 90 days of age were given free access to food, water and Alcohol, 15% and 30% for a period of 5 weeks, to simulate chronic alcohol consumption in human beings. The alcohol was prepared by diluting 190 proof (95%) alcohol, with deionised water to make 15% and 30% concentrations. The 190 proof alcohol was purchased from PHARMCO-AAPER (Shelbyville, KY). About 100 ml of each of the diluted solutions were given to all the animals in separate bottles. The alcohol was replaced three times a week, and the bottles were weighed before and after consumption. The amount consumed was determined by subtraction of the measured bottle weights. 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 alcohol consumed per kilogram of body weight per day**. It was ensured that all the animals met the requirement of drinking ≥ 4 grams of alcohol per day consistently for at least 2 weeks before treatment. The requirement for simulation of chronic alcohol drinking behavior was the modified version of a previous model (Li, Lumeng, McBride, and Murphy, 1987). The average consumption during the last two weeks before treatment was taken as the baseline for the drinking behavior calculations.

### 2.2.1 Treatment groups

The drugs were administered to the animals intra peritoneally starting week six, for 5 days after allotting them to different treatment groups randomly. The groups included Saline (n=8), CEF 50 mg/kg (n=4), CEF 100 mg/kg (n=8), DHK 10 mg/kg + CEF 50 mg/kg (n=4), DHK 10 mg/kg + CEF 100 mg/kg (n=8), GPI 10 mg/kg (n=6) and GPI 20 mg/kg (n=7).

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 the doses of 50 mg/kg and 100 mg/kg. GPI-1046 was purchased from Key Organics, U.K. It was dissolved in saline and 2% DMSO. The dilution was made in such a way as to administer 10 mg/kg and 20 mg/kg doses, adjusted according to the animal weight. DHK was purchased as a powder in 50 mg bottles from Tocris Biosciences. It was reconstituted with phosphate buffered saline solution and diluted so as to administer
10 mg/kg dose according to the animal weight. The injections were given intraperitoneally (i.p.) for a period of 5 days. For the CEF + DHK treatment, DHK was given 1 hour prior to the administration of CEF.

We have also included a control group without alcohol exposure. The group of rats of the same age (n=6) were given only water and food during the 5 weeks. The water drinking and weights were measured 2 weeks prior treatment and during treatment. The group was treated with saline in week six to maintain the same amount of stress, thereby ruling out differences in neurochemistry due to stress.

2.2.2 Measurements

The alcohol and water consumption and weights of the animals were measured daily for eight days starting the first day of injections (during the treatment and the following three days). The measurements obtained from different groups were analyzed using two-way ANOVA and one-way ANOVA followed by Dunnett’s post-hoc test to determine statistical significant differences between control and treatment groups.

2.2.3 Brain tissue harvesting:

All the animals were euthanized by decapitation with guillotine on Day 8 following exposure to Isoflurane. The brains were dissected out, frozen immediately on dry ice and
stored at -70°C. The brains were then further dissected into left and right parts of six areas, PFC, NAc core, NAc shell, HPC and AMG based on the coordinates using the cryostat and stored for immunoblotting.

### 2.3 Sucrose Drinking Paradigm

In order to ensure that the effects of GPI-1046 on drinking behavior are specific to alcohol, a sucrose drinking paradigm was adopted. The animals of this group were given free choice to 10% sucrose, food and water. The 10% sucrose was prepared by dissolving sucrose, crystal (purchased from Mallinckrodt chemicals) in deionised water and each animal was given 100 ml of the solution per day. They were exposed to sucrose for a period of three weeks and baseline was calculated by averaging the drinking during the last two weeks. It was ensured that all animals met the requirement of drinking ≥15 milliliters of sucrose solution per kilogram of body weight per day. The animals were treated for 5 days with either saline or GPI-1046 at dose of 20 mg/kg. The measurements of drinking and weights were obtained and analyzed as described in the Alcohol drinking paradigm. Animals were sacrificed on Day 8, the brains were dissected out, frozen and stored at -70°C for future immunoblotting.

### 2.4 Immunoblotting:

#### 2.4.1 Grouping
2.4.1.1 CEF groups

Western blot assays were performed on the PFC, NAc core and shell to determine the GLT 1 protein level. The comparisons were made between the water, saline, CEF 50 mg/kg and CEF 100 mg/kg.

2.4.1.2 GPI-1046 groups

Western blot assays were performed on the PFC and NAc core to determine the GLT 1 protein level. The comparisons were made between the water, saline, GPI-1046 at doses 10 mg/kg and 20 mg/kg.

2.4.2 Protein Homogenization

Lysis buffer was prepared by mixing 1M Tris HCl, 3M NaCl, 0.5M EDTA, 10% NP-40, 10% Triton, 10% SDS and protease inhibitor tablet dissolved in Millipore water. The tissue samples were homogenized immediately using lysis buffer. The amount of lysis buffer used was adjusted based on the size of the sample and it was ground with a pestle until no solid mass remained. The homogenized suspension was incubated on ice for 30 minutes and then centrifuged at 13,200 rpm for 15 minutes in a 4°C refrigerated micro-centrifuge. The supernatant was aliquoted into labeled polypropylene tubes and the pellet was discarded. One of the aliquots was used to perform the protein quantification assay.
2.4.3 Protein Quantification Assay

This Lowry Protein Quantification assay using 96 well plates was performed to determine the amount of protein present in the homogenized samples. The protein quantification values were used to calculate the appropriate dilutions such that all the samples used for Western blot assays had the same concentration of protein per unit volume. Bovine serum albumin (1.48 mg/ml, New England Biolabs) was used as the standard. Serial dilutions of BSA were made to produce a linear standard curve. All standards and samples were assayed in triplicates and the lysis buffer was used to make up the volume. Biorad Protein assay Reagent A and Reagent S (Biorad laboratories) were combined in required concentrations and added to all the wells and mixed thoroughly. Reagent B was added as required and the plate was incubated at room temperature for 15 minutes before reading the optical density using the Multiskan FC spectrophotometer (Thermo Scientific). The optical density was read at 750 nm wavelength and Normal speed. 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.

2.4.4 Western Blot procedure

2.4.4.1 Preparation of samples
Samples were divided into four groups: water, saline, CEF 50, and CEF 100 or water, saline, GPI-1046/10 and GPI-1046/20 as required. The samples were thawed on ice and diluted with lysis buffer based on their protein quantification values to achieve a concentration of 2 ug/20 µl. A 5X Laemelli dye (IM Tris HCl,100%Glycerol, SDS, bromophenol blue, b-mercaptoethanol) was added to the sample (5 ul/20ul of the diluted sample), mixed and vortexed thoroughly. The sample mix was then incubated at 98°C for 5 minutes in a digital dry bath (Labnet International Inc.). The vials were opened and resealed to relieve pressure and vortexed again. The sample mix was centrifuged at 4°C and 13,200 rpm for 4 minutes (Centrifuge 5415R, Eppendorf Inc.).

2.4.4.2 Electrophoresis:

Tris-glycine gels (10-20%; 1.0mmX12 well) from Invitrogen were placed in the electrophoresis apparatus and immersed with 1X Laemmli buffer. The Laemmli buffer was prepared with Tris base, glycine, SDS and deionised water. The wells were flushed to avoid bubbles. The supernatant of the centrifuged sample mix was used for the procedure. A total of 20 µl of the mixed samples was added to each well and electrophoresis was performed at 200 volts for 1 hour.

2.4.4.3 Transfer of proteins:

The gels containing the separated proteins were transferred onto immobilon-P membranes in a transfer apparatus. The membranes were placed on the gels and
sandwiched between an Anode and a Cathode plate that were separated by layers of Whatman paper and sponges soaked in transfer buffer. The box was also filled with Transfer buffer (glycine, tris base, methanol and deionised water) such that the whole ensemble was submerged in it. The apparatus was run at 24 volts for 2.5 hours. At the end of the run, the membranes were numbered, placed in petri dishes and tested with Ponceau solution (1% Ponceau, 5% Acetic acid and deionised water). It was a staining method to locate the protein bands on the immobilon membrane.

### 2.4.4.4 Primary antibody

The membranes were washed immediately with deionised water until the dye disappeared and blocked with blocking buffer (dry milk and 1X TBST) and placed on agitator. After 30 minutes, the primary antibody guinea pig anti-GLT 1 (AB 1783 GP X Glutamate transporter, Millipore Inc.) was added at 1:5000 dilution. The petri dishes were sealed with parafilm and incubated overnight in refrigerator by shaking at 300 rpm.

### 2.4.4.5 Secondary antibody

The blocking buffer from the overnight was poured out and the membranes were washed with 1X TBST (Tris base, Sodium chloride and deionised water at pH adjusted to 7.4) five times for five minutes each. The membranes were blocked again with blocking buffer for 30 minutes and then incubated with horseradish peroxidase (HRP)-labeled donkey anti-guinea pig secondary antibody (Jackson Immuno research laboratories, Inc.)
at 1: 5000 dilution for 1.5 hours. The membranes were washed again with 1X TBST and prepared for developing.

2.4.4.6 Developing

The washed membranes were dried and used for chemiluminiscent detection. They were incubated with the developer solution, a mixture of SuperSignal West Pico Luminal enhancer and SuperSignal West Pico stable peroxidase solution (Developer kit, Pierce) in 1:1 ratio for about one minute. The membranes were dried again, placed in between transparent sheets and exposed to Kodak BioMax MR Film (Thermo Fischer Scientific) in a cassette for one minute. The exposed films were developed with an SRX-101A machine.

2.4.5 Normalization of Protein loading

The membranes were washed with 1XTBST and the procedure of incubating with antibodies and developing was repeated using β-tubulin primary antibody and anti-mouse secondary antibody in 1:5000 dilution.

2.4.6 Analysis of the Western blots

The developed films were used to capture images with SRX-101A Film processor and the digitized images were quantified using MCID software. The size and density of the
GLT 1 bands and the β-tubulin bands were measured for all samples. The ratios of GLT1/β-tubulin were calculated and analyzed using one-way ANOVA followed by Dunnett post-hoc test to determine statistical significant differences between control and treatment groups. Photomicrographs for GLT1 and β-tubulin bands were taken using the Q imaging DC camera and QCID software.

**Chapter 3**

**Results**
3.1 CEF Groups

3.1.2 Effect on Alcohol consumption

Alcohol consumption measurements before and after treatment were compared to determine if CEF could significantly reduce the intake in alcoholic P rats.

Alcohol consumption during treatment and the following 3 days was measured and compared with the baseline, which was the average of the consumption for two weeks before treatment. Figure 3.1 shows the average alcohol intake (g/kg body weight/day) of the animals within each group plotted against the baseline and 8 days from the day of first injection. The treatment groups were: Saline (n=8), CEF 50 mg/kg (n=4) and CEF 100 mg/kg (n=8).

A 3 x 8 (Dose by Day) two-way ANOVA performed on alcohol intake, followed by Dunnett’s t-test (two-sided), demonstrated a significant main effect of Day [F(1, 8)= 25.86, p<0.001] and a significant Day x Treatment interaction effect [F(2, 16)= 10.87, p<0.001]. One-way ANOVA analyses for each day demonstrated significant difference (F> 4.63, p<0.03) among saline and both doses of CEF from Day 2 through Day 8.

Analyses revealed significant reduction in alcohol intake in both doses of CEF groups at
Day 2 (p<0.01), and at Day 3 through 8 (p<0.001) as compared to saline groups.

Figure 3.1

**Fig. 3.1.** Daily alcohol intake of male P rats treated for 5 days with 50 mg/kg (i.p.) CEF (n=4), 100 mg/kg (i.p.) CEF (n=8), or saline (n=8). Graph represents average daily alcohol (±SEM) intake during the treatment (days 1-5) and post-treatment periods (days 6-8). Baseline was estimated as an average alcohol intake for the last 2 weeks prior to saline or CEF injections. One-way ANOVA analyses revealed significance difference (F> 4.63, p<0.03) among control and treatments groups. Dunnett’s t-test analyses revealed significant reduction in alcohol intake with both doses of CEF starting Day 2 through Day 8 as compared to saline groups. There was no significant difference between both doses of CEF. (a: p<0.01, b: p<0.001).

### 3.1.2 Effect on Water consumption

Water consumption measurements were compared before and after treatment to observe any difference in intake following CEF administration. The Water intake during treatment and the following 3 days was also measured and compared with the baseline. The figure 3.2 shows the average water intake (ml/kg body weight/day) of the animals within each group plotted against
the baseline and 8 days from the day of first injection. A 3 x 8 (Dose by Day) two-way ANOVA performed on water intake, followed by Dunnett’s t-test (two-sided), showed a significant main effect of Day \([F(1, 8)= 21.002, p<0.001]\) and a significant Day x Treatment interaction effect \([F(2, 16)= 12.45, p<0.001]\). One-way ANOVA analyses for each day revealed significant difference \((F> 8.002, p<0.01)\) in water intake between saline and both CEF doses groups starting Day 1 through Day 8. Dunnett’s (two sided) multiple comparisons test analyses revealed a significant increase in water intake starting Day 1 through Day 8 (Figure 3.2; a: \(p<0.05\), b: \(p<0.001\)) at both doses of CEF (50 and 100 mg/kg). There was no significant difference between both doses of CEF.

**Figure 3.2**

![Graph showing water intake](image)

**Fig. 3.2.** Daily water intake of male P rats treated for 5 days with 50 mg/kg (i.p.) CEF (n=4), 100 mg/kg (i.p.) CEF (n=8), or saline (n=8). Graph represents average daily water (±SEM) intake during the treatment (days 1-5) and post-treatment periods (days 6-8). One-way ANOVA analyses revealed significance difference \((F> 8.002, p<0.005)\) among control and treatments groups starting Day 1 through Day 8. Dunnett’s t-test analyses revealed significant increase in water intake starting Day 1 through Day 8 at both doses of CEF. (a: \(p<0.05\), b: \(p<0.001\)).

### 3.1.3 Effect on body weight
The effects of CEF were also evaluated on the body weight to test for any abnormal loss due to adverse effects of the drug. Figure 3.3 shows the average body weight of the animals (grams) plotted against the baseline and 8 days following day 1 of treatment. A 3 x 8 (Dose by Day) two-way ANOVA performed on body weight, which was followed by Dunnett’s (two-sided) multiple comparison test, revealed a significant main effect of Day \[F(1, 8)= 8.96, p<0.001\], but no significant Day by Treatment interaction effect has been found \[F(2, 16)= 0.62, p=0.53\] (Figure 3.3). In addition, one-way ANOVA analyses for each day did not reveal any significant differences between control and treatment groups (F< 0.27, p> 0.5) in body weight. These data demonstrate that CEF did not alter the body weight of the rats.

Fig. 3.3. Daily body weight of male P rats treated for 5 days with with 50 mg/kg (i.p.) CEF (n=4), 100 mg/kg (i.p.) CEF (n=8), or saline (n=8). Graph represents average daily body weight (±SEM) during the treatment (days 1-5) and post-treatment periods (days 6-8). CEF at both doses did not affect the body weight across the 8 days.

3.1.4 Effect on GLT 1 expression
The GLT 1 expression in Naïve, alcoholic saline treated and alcoholic CEF treated rats was compared to detect if it is the mechanism underlying the action of CEF on alcohol intake. The effects of CEF treatment on GLT 1 expression in PFC and the NAc core and the NAc shell were determined using Western blot. Figure 3.4A, an immunoblot depicts the effect on PFC. One-way ANOVA analyses revealed a significant main effect between all groups \([F(3, 21) = 3.46, p<0.05]\). Posthoc Dunnett’s multiple comparison test analyses showed significant upregulation of GLT 1 level in PFC in the CEF-100 treated group (100 mg/kg, i.p.) \((p<0.02)\) when compared to saline vehicle-treated group (Figure 3.4B). Note that there were no significant differences between naive, saline and CEF-50 treated groups. There were also no significant differences between CEF-50, CEF 100 and naïve groups.

![Figure 3.4: GLT1 in cortex](image)

**Fig. 3.4.** Effects of CEF at 50 mg/kg (CEF-50, n=4), CEF-100 at 100 mg/kg (CEF-100, n=6), saline (n=6) vehicle, naïve (water) (n=6) groups on GLT 1 expression in PFC. A) Each panel presents immunoblots for β-tubulin, which was used as a control loading protein, and GLT 1. B) Quantitative analysis revealed a significant increase in the ratio of GLT 1/β-tubulin in CEF-100 group as compared to the saline vehicle group. Error bars indicate SEM. \((* p<0.02)\)
Figure 3.5A depicts the effect on NAc core. One-way ANOVA analyses showed significant main effect between all groups \[F(3, 22)= 5.16, p<0.01\]. Dunnett’s \(t\)-test analyses showed significant increase of GLT 1 level in NAc-core in CEF-100 treated group (100 mg/kg, i.p.) \((p<0.01)\) treated groups when compared to saline vehicle group (Figure 3.5B). In addition, statistical analyses demonstrated significant down-regulation of GLT 1 level in saline group \((p<0.02)\) as compared to naive group. These results indicate that chronic alcohol consumption decreases the level of GLT 1 in the NAc-core as compared to naive group (water only exposed group).

![Figure 3.5: GLT1 in Core](image_url)

**Fig. 3.5.** Effects of CEF at 50 mg/kg (CEF-50, \(n=4\)), CEF at100 mg/kg (CEF-100, \(n=6\)), saline (\(n=6\)) vehicle, naive (water) (\(n=6\)) groups on GLT 1 expression in NAc core. A) Each panel presents immunoblots for \(\beta\)-tubulin, which was used as a control loading protein, and GLT 1. B) Quantitative analysis revealed a significant increase in the ratio of GLT 1/ \(\beta\)-tubulin in CEF-100 group as compared to the saline vehicle group. In addition, a significant down-regulation of GLT 1 expression was revealed in saline group as compared to naive group. Error bars indicate SEM. \((* \ p<0.02; ** \ p<0.01)\)

Figure 3.6A depicts the effects on NAc Shell. One-way ANOVA analyses demonstrated significant main effect between all groups \[F(3, 21)= 3.21, p<0.05\]. Dunnett’s \(t\)-test analyses
revealed significant increase of GLT 1 level in NAc-shell in CEF-100 treated group (100 mg/kg, i.p.) (p<0.05) as compared to saline vehicle group (Figure 3.6 B). Moreover, Dunnett’s t-test demonstrated significant down-regulation of GLT 1 level in saline group (p<0.05) as compared to naive group. These results demonstrate that chronic alcohol intake decreases the level of GLT 1 in the NAc-shell as compared to naive group.

Figure 3.6: GLT1 in Shell

A

Naive Saline Cef-50 Cef-100

GLT1

B-tubulin

B

3.1.5 Effect on DHK treated animals:

DHK was administered prior to CEF treatment to test if CEF induced reduction in alcohol consumption is inhibited by DHK. This was done to confirm that CEF works by increasing
GLT 1 mediated glutamate uptake. The average alcohol intake (g/kg body weight/day) of the animals within each group plotted against the baseline and 8 days from the day of first injection is shown. The treatment groups in Figure 3.7 A are saline (n=8), CEF 50 mg/kg (n=4) and DHK 10 mg/kg + CEF 50 mg/kg (n=4) and Figure 3.7 B are Saline (n=8), CEF 100 mg/kg (n=8) and DHK 10 mg/kg + CEF 100 mg/kg (n=8).

A 3 x 8 (Dose by Day) two-way ANOVA performed on alcohol intake, followed by Dunnett’s t-test (two-sided), demonstrated a significant main effect of Day \[ F(1, 8)= 43.23, \ p<0.001 \] and a significant Day x Treatment interaction effect \[ F(4,32)= 5.075, \ p<0.001 \]. One-way ANOVA analyses for each day demonstrated significant difference \( F> 4.59, \ p<0.006 \) among saline and all doses of CEF and DHK+CEF from Day 2 through Day 8.

A post hoc Dunnett’s multiple comparison test revealed no significant difference in alcohol intake, across the five days of treatment and three days post-treatment, between CEF treatments and DHK + CEF treatment groups \( p>0.05 \) with the exclusion of Day2 between CEF 100 and DHK+ CEF 100 \( p<0.05 \). These results indicate that DHK could not block CEF
induced reduction in alcohol intake.

**Figure 3.7 A**

**Figure 3.7 B**

**Fig.3.7. A)** Daily alcohol intake of male P rats treated for 5 days with Saline (n=8), CEF 50 mg/kg (n=4) and DHK 10 mg/kg + CEF 50 mg/kg (n=4). **B)** Daily alcohol intake of
male P rats treated for 5 days with Saline (n=8), CEF 100 mg/kg (n=8) and DHK 10 mg/kg + CEF 100 mg/kg (n=8).

The Graphs represent average daily alcohol (±SEM) intake during the treatment (days 1-5) and post-treatment periods (days 6-8). Baseline was estimated as an average alcohol intake for the last 2 weeks prior to injections. Independent t-test analyses revealed no significant difference in alcohol intake between CEF and DHK treated animals except Day 2 in 100 mg/kg dose (a: p<0.01).

3.2 GPI-1046 Groups

3.2.1 Effect on Alcohol consumption:

Alcohol consumption measurements before and after treatment were compared to determine if GPI 1046 could significantly reduce the intake in alcoholic P rats. The Alcohol consumption during treatment and the following 3 days was measured and compared with the baseline, which was the average of the consumption for two weeks before treatment. The Figure 3.8 shows the average alcohol intake (g/kg body weight/day) of the animals within each group plotted against the baseline and eight days from the day of first injection. The treatment groups were: Saline (n=7), GPI-1046 10 mg/kg(n=6) and GPI-1046 20 mg/kg(n=7). A 3 x 8 (Dose by Day) two way ANOVA was performed on alcohol consumption, followed by a posthoc multiple comparisons Dunnett’s test (two-sided). It revealed a significant main effect of Day [F(1, 8)= 16.27, p<0.001] and a significant Day x Treatment interaction effect [F(2, 16)= 9.25, p<0.0001]. One-way ANOVA analyses for each day demonstrated significant difference (F>5.52, p<0.02) among saline and the two doses of GPI-1046 for Day 2 through Day 8. Dunnett’s (two sided) multiple comparisons test analyses revealed significant reduction in alcohol consumption with the higher dose of GPI-1046 (20 mg/kg, i.p.) at Day 2
(p<0.01), and at Day 3 through 8 (p<0.001) as compared to saline groups. Moreover, the lower dose of GPI-1046 (10 mg/kg, i.p.) showed a significant reduction in alcohol intake, relative to saline treated group, starting at Day 3 through Day 8 of treatment (Figure 3.8; a: p<0.01, b: p<0.05, c: p<0.001). When comparing the two doses 10 mg/kg and 20 mg/kg, it is noteworthy that Dunnett’s multiple comparisons test revealed significant difference in alcohol intake at Day 3-6 (p<0.001) and Day 7 (p<0.05); however, we did not see any significant difference in the alcohol intake on Day 1-2 and Day 8.

Fig.3.8. Daily alcohol intake of male P rats treated for 5 days with 10 mg/kg (i.p.) GPI-1046 (n=6), 20 mg/kg (i.p.) GPI-1046 (n=8), or saline (n=7). Graph represents average daily alcohol (±SEM) intake during the treatment (days 1-5) and post-treatment periods (days 6-8). Baseline was estimated as an average alcohol intake for the last 2 weeks prior to saline or GPI-1046 injections. One-way ANOVA analyses revealed significance difference (F>5.52, p<0.02) among control and treatments groups. Posthoc Dunnett’s (two-sided) multiple comparison test analyses revealed significant reduction in alcohol intake with the higher dose of GPI-1046 (20 mg/kg, i.p.) starting Day 2 through Day 8 as compared to saline groups. Moreover, the lower dose of GPI-1046 (10 mg/kg, i.p.) showed a significant reduction in alcohol intake, relative to saline treated group, starting at Day 3 through Day 8 of treatment. It is noteworthy that Dunnett’s t-test
revealed significance difference in alcohol intake between both doses of GPI-1046 (10 and 20 mg/kg) from Day 3 through Day 7. (a: p<0.01, b: p<0.05, c: p<0.001).

### 3.2.2 Effect on Water consumption:

Water consumption measurements were compared before and after treatment to observe any difference in intake following GPI administration. The Water intake during treatment and the following 3 days was also measured and compared with the baseline. Figure 3.9 shows the average water intake (ml/kg body weight/day) of the animals within each group plotted against the baseline and 8 days from the day of first injection. The treatment groups were: Saline (n=7), GPI-1046 10 mg/kg (n=6) and GPI-1046 20 mg/kg (n=7). A 3 x 8 (Dose by Day) two way ANOVA was performed on water intake followed by a posthoc multiple comparisons Dunnett’s (two-sided) test. It revealed a significant main effect of Day \[F(1, 8)= 2.30, p<0.03\] and a significant Day x Treatment interaction effect \[F(2, 16)= 2.01, p<0.02\]. One-way ANOVA analyses for each day revealed significant difference \(F>4.21, p<0.05\) in water intake between saline and GPI-1046 doses groups starting Day 1 through Day 8. Dunnett’s (two sided) multiple comparisons test analyses revealed significant increase in water intake starting Day 1 through Day 8 (Figure 3.9; a: p<0.005, b: p<0.05, c: p<0.01) at higher dose of GPI-1046 (20 mg/kg), and Day 1 (p<0.005), Day 4 (p<0.05), and Days 6-8 (Figure 3.9, a: p<0.005, b: p<0.05) at lower dose of GPI-1046 (10 mg/kg). There was no significant difference in water intake between the two doses the two doses 10 mg/kg and 20 mg/kg.
**Fig. 3.9.** Daily water intake of male P rats treated for 5 days with 10 mg/kg (i.p.) GPI-1046 (n=6), 20 mg/kg (i.p.) GPI-1046 (n=8), or saline (n=7). Graph represents average daily water (±SEM) intake during the treatment (days 1-5) and post-treatment periods (days 6-8). One-way ANOVA analyses revealed significance difference (F>4.21, p<0.05) among control and treatments groups starting Day 1 through Day 8. Dunnett’s multiple comparison (two sided) test analyses revealed significant increase in water intake starting Day 1 through Day 8 at higher dose of GPI-1046 (20 mg/kg), and Day 1, Day 4, and Days 6-8 at lower dose of GPI-1046 (10 mg/kg). (a: p<0.005, b: p<0.05, c: p<0.01).

### 3.2.3 Effect on body weight:

The effects of GPI on the body weight were also evaluated to test for any abnormal loss due to adverse effects of the drug. A 3 x 8 (Dose by Day) two way ANOVA was performed on body weight (Figure 3.10), followed by a posthoc Dunnett’s multiple comparison (two sided) test. It revealed a significant main effect of Day [F(1, 8)=38.61, p<0.001], but no significant Day by Treatment interaction effect has been observed [F(2, 16)=0.93, p=0.53]. Importantly, one-way
ANOVA analyses for each day did not reveal any significant differences between control and treatment groups (F<1.25, p>0.5) in body weight. These data indicate that GPI-1046 did not alter the body weight of the rats.

**Figure 3.10**

**Fig. 3.10** Daily body weight of male P rats treated for 5 days with 10 mg/kg (i.p.) GPI-1046 (n=6), 20 mg/kg (i.p.) GPI-1046 (n=8), or saline (n=7). Graph represents average daily water (±SEM) intake during the treatment (days 1-5) and post-treatment periods (days 6-8). GPI-1046 at both doses did not affect the body weight across the 8 days.

### 3.2.4 Effect on sucrose consumption:

The effect of GPI on sucrose consumption was tested to confirm that its activity is specific to alcohol consumption and not a general reduction in intake of fluids. To determine the effects GPI-1046 at dose 20 mg/kg on sucrose consumption, the consumption during treatment and the following 3 days was measured and compared with the baseline. The baseline was the average sucrose consumption for two weeks before treatment.
The figure 3.11 shows the average sucrose intake (ml/kg body weight/day) of the animals within each group plotted against the baseline and 8 days from the day of first injection. The treatment groups were: Saline (n=5) and GPI-1046 20 mg/kg (n=5). An independent $t$-test revealed no significant difference in sucrose intake, across the five days of treatment and three days post-treatment, between saline and 20 mg/kg GPI-1046-treated group ($p > 0.05$). These results indicate that GPI-1046 did not affect sucrose consumption.

**Fig. 3.11.** Daily sucrose intake of male P rats treated for 5 days with 20 mg/kg (i.p.) GPI-1046 (n=5), or saline (n=5). Graph represents average daily sucrose intake ($±$SEM) during the treatment (Days 1-5) and post treatment periods (Days 6-8). An independent $t$-test did not show any significant difference in sucrose intake between saline and GPI-1046-treated groups ($p > 0.05$).
3.2.5 Effect on GLT 1 expression:

The effects of GPI-1046 treatment on GLT 1 expression in PFC and the NAc core were determined using Western blot to elucidate the mechanism underlying the activity of GPI in reducing alcohol intake. Figure 3.12 A, an immunoblot depicts the effect on PFC. One-way ANOVA analyses revealed significant main effect between all groups [F(3, 23)= 6.41, p<0.003]. A post-hoc multiple comparison Dunnett’s (two sided) test revealed significant increase in PFC GLT 1 expression in GPI-1046 (10 mg/kg, i.p.) (p<0.03) and GPI-1046 (20 mg/kg, i.p.) (p<0.003) treated groups as compared to saline vehicle-treated group (Figure 3.12B). There were no significant differences between Water and Saline.

Figure 3.12: GLT1 in Cortex

![A: Immunoblot for GLT1 and β-tubulin](image)

**Fig. 3.12** Effects of GPI-1046 at 10 mg/kg (GPI-10, n=6), GPI-1046 at 20 mg/kg (GPI-20, n=6), saline (n=6) vehicle, water naïve (n=6) groups on GLT 1 expression in PFC. A) Each panel presents immunoblots for β-tubulin, which was used as a control loading protein, and GLT 1. B) Quantitative analysis revealed a significant increase in the ratio of GLT 1/β-tubulin in the GPI-10 and GPI-20 groups as compared to the saline vehicle group. In addition,
a significant increase in GLT 1 expression was revealed in GPI-20 group as compared to water group. Error bars indicate SEM. (* p<0.05; ** p<0.01)

The effect on the NAc core is depicted in Figure 3.13. One-way ANOVA analyses revealed significant main effect between all groups [F(3, 23)= 11.97, p<0.001]. A post-hoc multiple comparison Dunnett’s (two sided) test revealed significant increase in PFC GLT 1 expression in GPI-1046 (10 mg/kg, i.p.) (p<0.03) and GPI-1046 (20 mg/kg, i.p.) (p<0.001) treated groups as compared to saline vehicle-treated group (Figure 3.13 B). There was also a significant decrease in the saline group as compared to the naïve group (p<0.02).

Figure 3.13: GLT1 in Core

**Fig. 3.13.** Effects of GPI-1046 at10 mg/kg (GPI-10, n=6), GPI-1046 at 20 mg/kg (GPI-20, n=6), saline (n=6) vehicle, water naïve (n=6) groups on GLT 1 expression in NAc-core. A) Each panel presents immunoblots for β-tubulin, which was used as a control loading protein, and GLT 1. B) Quantitative analysis revealed a significant increase in the ratio of GLT 1/ β-tubulin in GPI-10 and GPI-20 treated groups as compared to saline vehicle group. Significant increases in GLT 1 expression was revealed in GPI-20 as compared to GPI-10 and water naïve groups. Alternatively, statistical analyses revealed significant down-regulation of GLT 1 expression in saline group as compared to water group. Error bars indicate SEM. (* p<0.05; ** p<0.01; *** p<0.001)
Chapter 4
Discussion

Alcohol consumption in P rats was significantly reduced following the treatment with CEF 50 mg/kg, CEF 100 mg/kg, GPI 10 mg/kg and GPI 20 mg/kg as compared to saline treated groups. Down-regulation of GLT 1 expression was observed in alcoholic rats as compared to naïve group, in the NAc core and shell areas but not in the PFC. Upregulation of GLT 1 was observed on treatment with CEF 100 mg/kg treatment in PFC, NAc core and NAc shell and with GPI 10 mg/kg and GPI 20 mg/kg treatments in PFC and NAc Core.

The results in the CEF treated animals showed that the doses, CEF 50 mg/kg and CEF 100 mg/kg significantly reduced alcohol consumption when compared to the saline-treated group. There was no significant difference observed in the activity of the two doses. A compensatory increase in the water intake was observed in the treated groups while the intake remained consistent in the saline group before and during treatment. There was no effect of the drug on body weight of the animals.

Although the effect of both doses on alcohol intake was similar, on examining the changes in GLT 1 protein expression in the PFC, NAc Core and NAc Shell, a significant increase was observed only in the higher dose. This result is in agreement with the earlier study by Sari et al., 2011 which showed that GLT 1 upregulation is only achieved at doses of 100 mg/kg and above. A trend towards upregulation of the protein was observed in CEF 50 mg/kg but this effect was not statistically significant.
On the other hand in the GPI-1046 treated Animals, both doses, 10 mg/kg and 20 mg/kg significantly reduced alcohol consumption, but the higher dose was more potent in its activity. While CEF showed effect from Day2, GPI-1046 displayed significant activity from Day3. A study of CEF induced GLT 1 expression in temporal regions showed that the mRNA levels increase 48 hours after first injection and the protein levels increase after 72 hours (Chu et al., 2007). Hence, it can be inferred CEF works by other mechanisms to produce the initial effect on alcohol intake while the more sustained effect may be due to GLT 1 upregulation. GPI-1046, on the other hand, seems to act primarily by upregulation of GLT 1.

A compensatory increase in water intake was observed in the GPI-1046 group, but it was not consistently significant in the lower dose group. This could be attributed to the fact that the effect in the GPI-1046-treated animals was more gradual than, and not as drastic as, observed in the CEF-treated groups. Alternatively, GPI-1046 did not affect the body weight of animals and did not decrease sucrose intake in rats, which showed that its activity is specific to alcohol consumption.

The immunoblotting results of the PFC and NAc core displayed that both doses of GPI-1046 increased the expression of GLT 1 protein. The rationale behind harvesting the PFC and NAc for immunoblotting is that both these brain regions are considered as key plays in drug-seeking behavior (McFarland and Kalivas, 2001). It is known that the PFC is one of the
limbic structures in the reward system that is involved in processing sensory stimulus toward the motor system via the NAc (Sesack and Grace, 2010). The role of the NAc is to act as a gateway for the limbic structures to reach the motor system. It converts the limbic information into motivational action (Groenenwegen et al., 1996). The NAc has been further differentiated into subregions, core and shell. It has been suggested that the dopaminergic neurons in the core area in conjunction with the nigrostriatal system while those of shell are with the mesolimbic system (Deutch and Cameron, 1992). In addition, it has been established that the dopamine innervation in the shell is functionally associated with response to primary reward, and that of the core is associated with response reinforcement (as reviewed by Dichiara, 2004). A selective lesions study demonstrated the differential effects of core and shell in cocaine seeking behavior showed that lesions in core hindered drug seeking by disrupting response to conditioned reinforcers and lesions in the shell diminished the psychostimulant effects of cocaine in the brain (Ito et al., 2004).

In the present study, a significant decrease in the GLT 1 expression in alcohol exposed animals has been observed in both the core and the shell when compared to the naïve group. Importantly, upregulation in GLT 1 level was observed in both regions of the NAc by CEF treatment. We have also found that GPI 1046 treatment induced upregulation of GLT 1 level in the NAc core.

The PFC, however, did not show a significant downregulation of the protein in the animals exposed to alcohol as compared to the naïve group. CEF treatment at higher doses was
effective in upregulating GLT 1 level. A similar effect was observed in a study testing CEF in cocaine-seeking behavior. GLT 1 and xCT levels were lowered in the NAc but not in the PFC following self-administration of cocaine in rats (Knackstedt et al., 2010). It is suggested that these differences in GLT 1 level in cocaine-seeking model could involve neuroadaptations of the dopaminergic synapses in the PFC and the glutamatergic synapses in the NAc (Kalivas et al., 2005). Hence it could be inferred that alcohol, like other substances of abuse, causes differential neuroadaptations in the glutamatergic systems in different brain regions and the effect on the GLT 1 level is more pronounced in the NAc compared to PFC. Hence, NAc can be given primary focus in future studies in our laboratory.

The effect of CEF was found to be consistently significant even in rats treated with DHK. The dose of DHK used was 10 mg/kg based on previous studies (Gunduz et al., 2011; Chu et al., 2007). In the present study, the inability of DHK to inhibit CEF-induced GLT 1 upregulation and subsequent reduction in alcohol consumption could be explained by different conjectures. Firstly, most of the studies that demonstrated the effect of DHK as a selective GLT 1/EAAT2 inhibitor involve administration of DHK directly into the local areas of the brain or CNS. Hence it is possible that at the dose administered in the present study intra-peritoneally, DHK is unable to reach those regions of the brain in concentrations capable of producing this effect. Secondly, it is possible that the increase in GLT 1 level following CEF treatment may have been a factor is that the amount of DHK administered was not sufficient to block most of upregulated GLT 1. However, the ability of CEF 50 to reduce consumption in the presence of DHK defies this possibility. In addition, it has been shown that DHK at dose of 10 mg/kg i.p significantly blocked CEF 200 mg/kg-induced
prevention of cannabinoid tolerance (Gunduz et al., 2011). These facts support a third conjecture which was previously discussed in this thesis, which is that, though CEF upregulates GLT 1, it has other potent mechanisms by which it reduces alcohol consumption. This probability could be further substantiated by administering DHK into local regions of the brain and testing the effects of CEF on alcohol consumption.

It is noteworthy that both drugs showed significant increase in GLT 1 level, which was correlated with reduction in alcohol consumption. These data support our central hypothesis that upregulation of GLT 1 level is a potential new target for treatment of alcohol dependence. In order to further substantiate the results of this thesis, future studies may include neurochemical assays such as microdialysis to detect changes in glutamate levels following the administration of CEF and GPI-1046. It is also necessary to explore other effects of these drugs which may also play a role in countering alcohol dependence. Immunoblotting assays of other relevant proteins such as xCT, assays to detect effects on free radical scavengers such as Glutathione, and binding assays of other receptors mediating alcohol effects are warranted to be investigated in futures studies. Molecular level studies should be performed to elucidate the mechanisms of actions of CEF and GPI-1046 in the upregulation or activation of GLT 1.

CEF could be further tested in chronic alcohol consumption models such as 14 weeks. This work is underway and results will be reported elsewhere. In addition, studies in collaboration with other laboratory are focusing in testing the combination with existing FDA-approved
drug for the treatment of alcohol dependence for revealing the synergistic inhibition of alcohol-seeking behavior.

Furthermore, GPI-1046 induced increase in GLT 1 expression is in close conjunction with its effect on alcohol-seeking behavior. Hence, it could be further studied the long lasting effects of this drug. Our laboratory is planning to further investigate for potential toxicity, in particular with higher dose, in order to ensure the safety of the drug and to determine the pharmacologically relevant doses. This could be a promising new candidate for clinical studies aiming at treating alcohol dependence.

In conclusion, we report that alcohol consumption in P rats was significantly reduced following the treatment with CEF as compared to control saline treated group. Treatment with GPI-1046, another drug known to upregulate GLT1 level, has shown reduction in alcohol intake. Alternatively, P rats exposed to alcohol showed down-regulation of GLT1 expression in the NAc core and shell areas but not in the PFC as compared to naïve group. Upregulation of GLT 1 level was observed with CEF 100 mg/kg treatment in PFC, NAc core and NAc shell and with GPI-1046 10 mg/kg and GPI-1046 20 mg/kg treatments in PFC and NAc Core. These results, in conjunction with previous studies of CEF in P rats (Sari et al., 2011), provide substantial information about the role of GLT 1 as a potential target for the treatment of alcohol dependence.
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82


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