

ABSTRACT

Gloria E. Malpass. INTERACTION OF MEMANTINE WITH ETHANOL CONSUMPTION AND DOPAMINERGIC FUNCTION IN HIGH ETHANOL PREFERRING RATS. (Under the supervision of Dr. Brian A. McMillen) Department of Pharmacology and Toxicology, November 2009.

N-methyl-D-aspartate (NMDA) receptor antagonists have been reported to decrease ethanol consumption in rodents, but these drugs often produce adverse side effects. Memantine is a neuroprotective and low-affinity, noncompetitive NMDA receptor antagonist shown to be an effective treatment for Alzheimer's disease with a favorable clinical profile. This study investigated effects of memantine on volitional ethanol consumption in the Myers' high ethanol preferring (mHEP) rat using a two-choice 24 hour access paradigm. Memantine was found to reduce ethanol consumption in an apparent dose-dependent manner. Behavioral experiments indicated that memantine, at a dose shown to decrease ethanol consumption, did not adversely affect locomotor ability and activity, induce sedation, or add to ethanol-induced hypothermia. Previously, ethanol has been shown to alter levels of dopamine metabolism (DA) in brain regions receiving DA input in the DA reward pathway. Therefore, high performance liquid chromatography (HPLC) was used to compare levels of DA metabolism in the medial prefrontal cortex (mPFC), nucleus accumbens (NAc), and striatum (STR) of rats treated with saline, memantine (10.0 mg/kg, i.p.) and/or ethanol (1.0 or 2.5 g/kg, i.p.). No significant treatment effects were detected in levels of DA or its metabolite, 3,4-dihydroxyphenylacetic acid (DOPAC). Ethanol (2.5 g/kg) increased striatal DOPAC to levels bordering on significance, but memantine clearly produced no effect. To determine if memantine alters ethanol intake via signaling downstream of activation of the DA D1 receptor, Western blots were used to compare effects of the same treatments on levels

of DARPP-32, a protein implicated as an intracellular regulator in ethanol reward, and its phosphorylation at Thr34 and Thr75 sites in the mPFC, NAc, and STR. Bands for phospho-DARPP-32 (Thr34) in the mPFC were undetectable. All other blots indicated no significant treatment effects on levels of DARPP-32 or its phosphorylation at Thr34 and Thr75 sites. Together, these results suggest that mechanisms which do not involve glutamate and the NMDA receptor may also activate ethanol reward in the mHEP rat, and a mechanism not downstream of the DA D1 receptor is involved. The effect of memantine on consumption of ethanol does not involve modification of the DA/DARPP-32 signaling system.

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INTERACTION OF MEMANTINE WITH ETHANOL CONSUMPTION AND
DOPAMINERGIC FUNCTION IN HIGH ETHANOL PREFERRING RATS

A Dissertation

Presented To

The Faculty of the Department of Pharmacology and Toxicology
The Brody School of Medicine at East Carolina University

In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy in Pharmacology and Toxicology

by

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November, 2009

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Acknowledgements

Many people have generously provided guidance and support throughout my graduate experience. I wish to thank my thesis advisor Dr. Brian A. McMillen for providing direction and encouragement throughout this project. I am also grateful to Helen L. Williams for the assistance and support that she has kindly provided both as a laboratory technician and as a friend.

Completion of this project required numerous resources ranging from technical assistance to the use of equipment and reagents from multiple laboratories. I am grateful to everyone who contributed to this project, especially Dr. David A. Taylor, Chair of the Department of Pharmacology and Toxicology, for his guidance and support.

Many thanks to my parents, Benny E. and Joyce L. Malpass, and the countless family and friends who have graciously given their love, support, and prayers as I complete this chapter in my life. Above all, I thank God for His blessings.

To My Parents
With Love and Gratitude

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LIST OF ABBREVIATIONS

5-HT	serotonin
6-OHDA	6-hydroxydopamine
AA	Alko Alcohol rat line
AMPA	α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid
AMY	amygdala
Ca ²⁺	calcium
cAMP	cyclic adenosine monophosphate
CC	cingulate cortex
Cdk5	cyclin-dependent kinase 5
cGMP	cyclic guanosine monophosphate
CK1	casein kinase 1
CK2	casein kinase 2
CNS	central nervous system
Coc	cocaine
CREB	cAMP response element binding
Ctl	control
CTX	cortex
DA	dopamine
DARPP-32	dopamine and cyclic adenosine 3',5'-monophosphate-regulated phosphoprotein-32 kDa
DHBA	3,4-dihydroxybenzylamine
DOPAC	3,4-dihydroxyphenylacetic acid

ERK	extracellular signal-regulated kinase
EtOH	ethanol
FC	frontal cortex
GABA	γ-amino butyric acid
Glu	glutamate
HAD	High Alcohol Drinkers rat line
HCl	hydrochloric acid
HClO ₄	perchloric acid
HI	hippocampus
HIP	Hippocampal formation
HPLC	high performance liquid chromatography
HYPO	hypothalamus
iGlu	ionotropic glutamate
iGluR	ionotropic glutamate receptor
i.p.	intraperitoneally
K ⁺	potassium
KH ₂ PO ₄	potassium phosphate monobasic
KOH	potassium hydroxide
Mem	memantine
Mg ²⁺	magnesium
mGlu	metabotropic glutamate
mGluR	metabotropic glutamate receptor
mHEP	Myers' high ethanol preferring rat line
mPFC	medial prefrontal cortex
MSN	medium spiny neuron

NAC	nucleus accumbens
Na ₂ EDTA	sodium ethylene diamine tetraacetic acid
NMDA	<i>N</i> -methyl-D-aspartate
NSTR	neostriatum
OT	olfactory tubercle
P	Alcohol Preferring rat line
PFC	prefrontal cortex
PKA	cAMP-dependent protein kinase
PKC	Protein kinase C
PKG	cGMP-dependent protein kinase
PLC	phospholipase C
PP-1	protein phosphatase-1
PP-2A	protein phosphatase-2A
PP-2B	protein phosphatase-2B
PSTR	posterior striatum
Sal	saline
s.c.	subcutaneous
SDS	sodium dodecyl sulfate
Ser102	serine 102
Ser137	serine 137
Ser897	serine 897
SOS	sodium octyl sulfate
sP	Sardinian Preferring rat line
STR	striatum

Thr34	threonine 34
Thr75	threonine 75
UChB	University of Chile B rat line
VP	ventral pallidum
VTA	ventral tegmental area

CHAPTER 1

INTRODUCTION

LITERATURE REVIEW

Alcohol Use: A Historical Perspective

The production and consumption of alcohol has been prevalent throughout history. Archaeological artifacts and literary documents describe customs and regulations developed around the production and use of alcoholic beverages. Historically, alcoholic beverages have been used throughout the world for ceremonial, religious, medicinal, and social purposes. While the use of alcohol has been described as good and beneficial, there have also been reports of negative consequences associated with alcohol use. Historical writings make reference to “the evils”, mental and physical illnesses, and “all sorts of malign consequences” such as fatal accidents resulting from alcohol consumption (cf. Keller, 1979).

In the 18th century, Anthony Benezet, a Quaker teacher, and Benjamin Rush, a physician, challenged popular beliefs which held that alcohol use conveyed health benefits. Rush, who has been called “the first American authority on alcoholism and the father of the American public health movement”, referred to habitual drunkenness as a disease, and more specifically, an addiction (cf. Keller, 1979). Today, alcohol may be the most widely used drug of abuse in the world. In 2004, the World Health Organization (WHO) estimated that approximately 2 billion people worldwide consumed alcohol and 76.3 million people worldwide exhibited diagnosable alcohol use disorders while the United Nations Office on Drugs and Crime (2004) estimated that 185.0 million people, globally, used illicit drugs annually between 2001 and 2003. Furthermore, the WHO has reported a causal relationship between alcohol consumption and more than 60 types of disease and injury. Alcohol use is an underlying risk factor for diseases including mouth

and oropharynx cancers, ischemic heart disease, and cirrhosis of the liver, and for injury, including road traffic accidents, poisonings, falls, and intentional injuries, and also incurs significant social and economic costs (World Health Organization Department of Mental Health and Substance Abuse, 2004). Alcohol addiction, also called alcohol dependence and alcoholism, has been linked to the chronic consumption of dense alcoholic beverages such as brandy, whiskey, and vodka rather than “the reasonable daily consumption of light alcoholic beverages”, i.e. up to 300 g wine or 750 g beer (cf. Karkoulas et al., 2008).

Alcohol Abuse and Alcohol Dependence

In the Diagnostic and Statistical Manual, 4th Edition (DSM-IV), the American Psychiatric Association has defined alcohol abuse and alcohol dependence as maladaptive patterns of alcohol use leading to clinically significant impairment or distress. Alcohol abuse is manifested by one or more of the following in a 12-month period: recurrent alcohol use leading to a failure to fulfill major obligations at work, school, or home; recurrent alcohol use in situations in which alcohol use is physically hazardous; recurrent alcohol-related legal problems; and continued alcohol use despite its adverse effects on social or interpersonal situations. To be defined as alcohol abuse, the symptoms must have not previously met the criteria for alcohol dependence. Alcohol dependence is manifested by three or more of the following symptoms occurring at any time in a 12-month period: tolerance; withdrawal; alcohol consumption in larger amounts or over a longer period than intended; a persistent desire or failed attempts to reduce or control alcohol use; a large amount of time being spent to obtain or consume alcohol or to recover from the effects of alcohol; important activities being neglected or reduced

due to alcohol use; and continued alcohol use despite knowledge of having a persistent or recurrent physical or psychological problem likely to have been caused or worsened by alcohol (American Psychiatric Association, 1994).

Cloninger and colleagues (1988) have divided alcoholism into two subtypes based on distinct alcohol-related symptoms, personality traits, ages of onset, and patterns of inheritance. Type 1 alcoholics are characterized by anxious (passive-dependent) personality traits and rapid development of tolerance and dependence on the anti-anxiety effects of alcohol. These drinkers lose control, have difficulty ending binges once they begin, feel guilty, and experience liver complications following socially encouraged exposure to alcohol use. Type 2 alcoholics are characterized by antisocial personality traits and persistent seeking of alcohol for its euphoriant effects. These drinkers experience an early onset of inability to abstain entirely from alcohol use and are involved in fighting and arrests when drinking (Cloninger et al., 1988).

Genetics and the Development of Alcohol Use Disorders

A review by Merikangas (1990) reports that most family, twin, and adoption studies suggest that alcoholism can often be attributed to genetic factors, and that exposure, metabolism, or pharmacological effects of ethanol may be preconditions that lead to the development of tolerance and/or dependence. Mouse studies have shown that glutamate activity in the nucleus accumbens has an active role in regulating alcohol consumption, which suggests that predisposition to high alcohol intake involves genetic factors that facilitate alcohol-induced adaptations in glutamate release within the nucleus accumbens (NAc) (Kapasova and Szumlinski, 2008). Initiation and early patterns of alcohol use have been attributed to social and familial environmental factors while later

levels of alcohol use have been attributed to genetic factors (Kendler et al., 2008). Variations in the influence of genetic factors on alcoholism may be partially due to individual differences in motivations related to consumption of alcohol in social settings. Although consumption of alcohol to manage mood may overlap with a genetic risk for alcoholism, use of alcohol as a mood stabilizer is not viewed as a direct cause of alcoholism (Prescott et al., 2004). Among drinkers of alcohol, the degree of twin resemblance for consummatory behaviors is low to moderate and appears to be regulated by shared genes rather than shared environments (Prescott et al., 1994).

Costs Associated with Alcohol Abuse and Alcohol Dependence

In addition to detrimental health and injury-related consequences, alcohol use disorders severely impact the economy. In the U.S., the overall economic cost of alcohol abuse was reported as being \$185 billion for 1999 with greater than 70 percent of the cost being attributed to lost productivity and another 14.3 percent attributed to health care expenditures. Other costs involved alcohol-related automobile accidents and judicial system costs due to alcohol-related crime (National Institute on Alcohol Abuse and Alcoholism, 2001). A more recent report estimated the total economic costs of alcohol in the U.S. to be \$235 billion (Rehm et al., 2009).

Current Trends

Many reports indicate that the age at which a person first consumes alcohol is associated with the development of later alcohol dependence or alcohol abuse (Buchmann et al., 2009; Grant and Dawson, 1997; Grant et al., 2001a; Grant et al., 2001b; Pitkanen et al., 2008; Prescott and Kendler, 1999; Substance Abuse and Mental

Health Services Administration, Office of Applied Studies, 2008). In 2007, 15.9% of adults (aged 18 or older) who first consumed alcohol at age 14 or younger met the criteria for alcohol dependence or alcohol abuse while only 3.9% of adults who first consumed alcohol at age 18 or older were classified with either disorder (Substance Abuse and Mental Health Services Administration, Office of Applied Studies, 2008).

The Substance Abuse and Mental Health Services Administration defines a person's need for alcohol treatment as a person meeting the DSM-IV criteria for alcohol dependence or abuse. Based on the SAMHSA 2007 National Survey on Drug Use and Health (NSDUH), 7.8% (19.3 million) of persons aged 12 or older needed treatment for their alcohol problems in the prior year, but the majority of those needing treatment either failed to perceive the need or did not receive it for other reasons. Based on combined data from the 2004 to 2007 SAMHSA NSDUH for those who felt that they needed alcohol treatment but did not receive it, the most common reasons cited were unpreparedness to cease alcohol use (45%) and cost or insurance barriers (34.5%) (Substance Abuse and Mental Health Services Administration, Office of Applied Studies, 2008). A review by Clay and colleagues (2008) suggests that delaying treatment for an addict who is unprepared to receive help can be dangerous and detoxification alone is often ineffective. Pharmacological and cognitive-behavioral interventions with addicts have been proposed as potentially being as successful in the treatment of addiction as in the treatment of other chronic diseases (Clay et al., 2008).

Pharmacological Treatments for Alcohol Dependence

The pharmacological treatments currently used to treat alcoholism include disulfiram, an aldehyde dehydrogenase inhibitor, naltrexone, an opioid receptor

antagonist, and acamprosate, a glutamate receptor antagonist (cf. Heilig and Egli, 2006; Mark et al., 2009). These drugs are characterized by different mechanisms of action as well as limitations regarding their efficacies (cf. Heilig and Egli, 2006).

Disulfiram

For many years, disulfiram was the only medication used to aid sobriety. Disulfiram inhibits aldehyde dehydrogenase, causing acetaldehyde to accumulate when alcohol is consumed. This produces several adverse side effects which include flushing, shortness of breath, tachycardia, headache, and nausea. The rationale for treatment of alcoholism with disulfiram is based upon the belief that patients will avoid alcohol use to prevent these adverse effects. However, due to the unacceptable medical risk associated with the effects of acetaldehyde accumulation, patients who continue to consume alcohol while taking disulfiram should not be treated with this medication. While evidence for the efficacy of disulfiram is lacking, disulfiram has been found to be effective when given under supervision, which indicates that compliance is another major issue. Overall, this drug does not directly target the core phenomena of alcohol dependence and is viewed as an outmoded treatment (cf. Heilig and Egli, 2006).

Naltrexone

Naltrexone, an opioid receptor antagonist with a relative selectivity for the μ -opioid receptor at lower doses, has been shown to be a clinically effective oral treatment for alcohol dependence (cf. Heilig and Egli, 2006). It has been proposed that ethanol stimulates opiate receptors by either increasing the release of endogenous opioid peptides (Blum et al., 1982), which leads to mesolimbic dopamine release (DA), a known

contributor to the acute positive reinforcing properties of drugs of abuse (cf. Kreek et al., 2002), or by forming aberrant metabolites (Davis and Walsh, 1970; Myers, 1989). Since naltrexone blocks the endogenous opioids that mediate the acute positive reinforcement of ethanol, this drug should aid patients whose alcoholism is characterized by reward craving (Anton, 2008; cf. Heilig and Egli, 2006). This implies that naltrexone is an effective treatment for early onset, type 2 alcoholics. However, despite its well-documented efficacy and safety, naltrexone has not been widely prescribed to alcoholic patients (cf. Heilig and Egli, 2006).

Acamprosate

Acamprosate is a glutamate receptor antagonist whose precise molecular mechanisms of action are unknown. It has been proposed that acamprosate attenuates NMDA signaling by acting as a partial agonist at the spermidine site and by acting at metabotropic glutamate receptors. This drug selectively blocks dependence-induced drinking, and normalizes the progressive recruitment of elevated extracellular glutamate that occurs with repeated cycles of intoxication and withdrawal (cf. Heilig and Egli, 2006). The recommended dosage of acamprosate is up to 2 g/day divided into three doses (Saivin et al., 1998). The large doses and the dosing regimen required may limit the efficacy of acamprosate as a therapy for alcohol dependence (cf. Heilig and Egli, 2006).

Other Proposed Pharmacological Therapies

Based on the idea that naltrexone and acamprosate might produce an additive effect, combination therapy with these two drugs is being studied as a potentially more

efficacious treatment for alcoholism. Other medications proposed for the near future include the serotonin (5-HT) antagonist odansetron, the metabotropic GABA-B receptor agonist baclofen, and the anti-epileptic topiramate. Novel treatments and targets being explored as potential alcoholism therapies include cannabinoid CB₁-receptor antagonism, metabotropic glutamate receptor 5 (mGluR5) antagonism, metabotropic glutamate receptor 2/3 (mGluR2/3) agonism, corticotropin-releasing factor (CRF) receptor antagonism, neuropeptide Y (NPY) receptor antagonism, and nociceptin receptor agonism (cf. Heilig and Egli, 2006).

Animal Models and Experimental Paradigms

The development of a pharmacological therapy for alcoholism requires an appropriate animal model and an experimental paradigm that models the characteristics of addiction in man. Many animals consume low to moderate levels of alcohol for its gustatory or caloric properties, or for the modest positive reinforcing effects of its acute pharmacological actions. However, a reduction in volitional consumption of ethanol induced by a medication under these conditions does not provide adequate information as to whether the same drug will be effective in reducing drinking, craving and relapse in alcoholic patients. While many medications reduce basal alcohol consumption levels in animals lacking a history of dependence or excessive drinking due to genetic selection, these same drugs often fail to effectively decrease alcohol intake in alcoholic patients. Therefore, paradigms which model the characteristics of addiction, such as excessive, compulsive and persistent ingestion patterns, and those mimicking the conditions that precipitate craving and relapse are important when determining the potential therapeutic

efficacy of medications in the treatment of alcoholism (cf. Heilig and Egli, 2006; McMillen, 1997).

The production of animals that are genetically susceptible to high volitional consumption of ethanol and model the development of alcohol abuse and alcohol dependence involves selective breeding which increases the genetic propensity component, i.e. frequency of alleles, that influence alcohol preference and intake (Bice et al., 2008; cf. Heilig and Egli, 2006; Myers et al., 1998; Winkler et al., 1999). The most widely studied selectively-bred alcohol-preferring rat lines include the AA (Finland), P, HAD (US), sP (Italy), and UChB (Chile) which all exhibit, in varying degrees, the behavioral and physiological characteristics observed in children of alcoholics, alcohol abusers, and alcoholics, which differ from those of non-drinkers or light drinkers. In addition to high ethanol consumption and preference, selectively bred alcohol-preferring rats exhibit phenotypic characteristics, including alterations in the functions of the 5-HT and DA systems. that are evident in human alcoholics (cf. Heilig and Egli, 2006; Murphy et al., 2002).

Myers and colleagues (1998) developed a genetic strain of high ethanol preferring (mHEP) rats whose progenitors included three male alcohol-preferring P rats, obtained from T.-K. Li of the Indiana University Alcohol Research Center, and three female Sprague Dawley rats, purchased from Harlan Sprague Dawley Inc., whose selection was based on an ethanol drinking screen. The line is maintained by breeding non-sibling males and females that are selected based on an ethanol drinking screen. A model for Type 2 alcoholism, the mHEP rat is characterized by early onset of heavy alcohol consumption and a strong family history of severe alcoholism (Cloninger et al., 1988). These rats exhibit profuse volitional consumption of ethanol, a preference for

ethanol in the presence of palatable alternatives, and levels of blood ethanol that correspond essentially with the respective amounts of ethanol consumed (Myers et al., 1998).

Addiction

Addictive drugs, like natural rewards, preferentially stimulate DA transmission in the NAc shell. While the response to natural rewards undergoes one-trial habituation, addictive drugs produce a response that resists habituation, which allows addictive drugs to activate DA transmission in the shell without attenuating the response upon repeated self-administration. This process facilitates abnormal associative learning, leading to excessive motivational value being assigned to the discrete stimuli or to the contexts predictive of drug availability (cf. Di Chiara et al., 1999; cf. Di Chiara, 1999). According to this associative learning hypothesis, drug addiction results from: (1) the rewarding properties of drugs; (2) the ability of these drugs to activate DA transmission in the NAc shell; (3) the resistance of both the rewarding properties of the drugs and the activation of DA transmission in the NAc shell to properties of negative adaptive modulation, consisting of satiation and habituation, after repeated drug exposure; and (4) the adaptive changes induced by repeated drug exposure which lead to the negative emotional state of abstinence (cf. Di Chiara et al., 1999).

Ethanol and Addiction

Multiple neurotransmitter systems, including DA, 5-HT, glutamate, GABA, and opioid peptides, in the brain reward systems have been implicated in the reinforcing properties of ethanol, and it has been suggested that some of these neurotransmitters

may be acting on some of the same neural circuitry involved with the reinforcing actions of other drugs of abuse. Alcohol dependence has been associated with changes in the actions of these neurotransmitters in these reward systems as well as other neurotransmitters such as brain corticotrophin releasing factor (CRF). A pathway that connects the midbrain and forebrain and involves parts of the NAc and amygdala (AMY) has been proposed as being a central component in the neuropharmacology of alcohol reinforcement (cf. Koob, 1992; cf. Koob et al., 1994). Rassnick and colleagues (1993) demonstrated that rats with 6-hydroxydopamine (6-OHDA)-induced lesions of the mesolimbic DA system to produce major depletions of DA in the nucleus accumbens, olfactory tubercle (OT), frontal cortex (FC), and AMY, did not change ethanol self-administration as measured by total lever presses, but did show an altered pattern of responding for ethanol. This suggests that the mesolimbic DA system may contribute to ethanol reinforcement, but is not critical for maintaining the reinforcement.

Dopamine Receptors

DA is a catecholamine predominantly involved with reinforcement, reward, some aspects of craving, sustained alcohol use, and potential relapse after prolonged abstinence in an alcohol-dependent person (Anton, 2008). DA receptors are divided into two major classes based on structure and opposing modulation of adenylyl cyclase (AC) activity. The D1-like receptors include the D1 and D5 subtypes which are positively coupled to AC. The D2-like receptors include the D2, D3, and D4 subtypes which are negatively coupled to AC and inhibit the production of cyclic adenosine monophosphate (cAMP). Widely expressed in several DA-innervated areas of the brain, DA D1 and D2 receptors are highly expressed in the dorsal striatum, nucleus accumbens, and OT, with

lower levels of expression in the septum, hypothalamus (HYPO), and cortex (CTX). DA D1 and D2 receptor expression in the hippocampus (HI) is minimal or non-existent. DA D3 receptor mRNA is found in the OT, Island of Calleja complex, and in the medium spiny neurons (MSNs) of the NAc shell. DA D3 receptor mRNA is also found in the ventral tegmental area (VTA), AMY, HI, septum, and mammillary nuclei of the HYPO. DA D4 receptor mRNA is found mainly in the FC, medulla, and AMY. Low levels of DA D4 receptor mRNA are found in the STR, OT, and HI. DA D5 receptor mRNA is found only in the HI, HYPO, and parafascicular nucleus of the thalamus with low levels located in the CTX and striatal complex. DA D5 receptors are expressed in cholinergic neurons in the basal forebrain, STR, and cerebral CTX (cf. Dalley and Everitt, 2009).

Acute alcohol use stimulates neurons in the VTA, inducing DA release in the NAc. The NAc mediates reward, pleasure, and the assignment of salience to important environmental stimuli, while environmental cues associated with alcohol use may enhance this effect (cf. Anton, 2008).

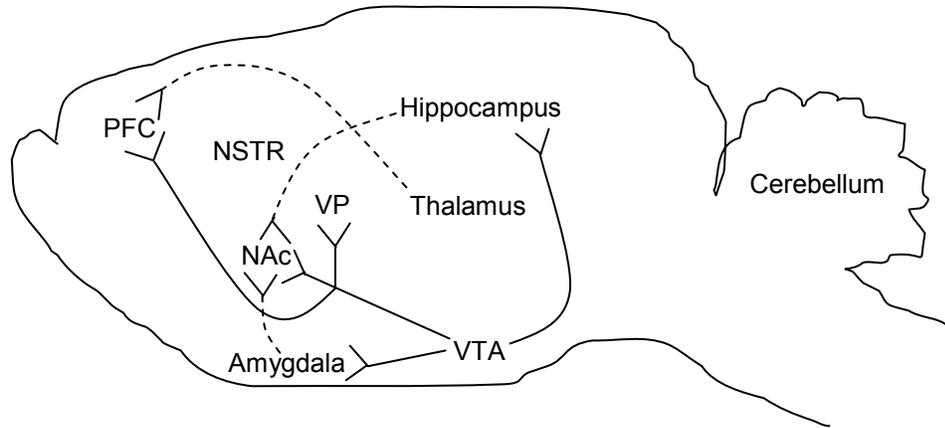
Mesolimbic and Mesocortical Pathways

The mesolimbic DA reward pathway (Figure 1.1) and the mesocortical pathway are the two major neurological pathways involved in addiction (cf. Clay et al., 2008). Many drugs act at specific receptors in specific classes of neurons. Studies of drug reward have implicated dopaminergic neurons as a major factor in brain reward. The reward circuitry is multisynaptic with multiple transmitters and neurons being involved (cf. Wise and Rompre, 1989). Most agents acting as substrates for self-administration increase DA outflow in the mesolimbic or mesocortical areas (Brodie et al., 1990).

Figure 1.1. Dopaminergic and glutamatergic projections in the limbic reward system. In this illustration of the rat brain (sagittal view), solid lines indicate dopaminergic projections and broken lines indicate glutamatergic projections. (NAc = nucleus accumbens; NSTR = neostriatum; PFC = prefrontal cortex; VP = ventral pallidum; VTA = ventral tegmental area).

[Adapted from Sigma-Aldrich; Available November 18, 2009;

http://www.sigmaaldrich.com/etc/medialib/docs/Sigma/Instructions/limbic_reward_circuit.Par.0001.File.ppt.]



The mesolimbic system involves a large dopaminergic projection from the VTA which is rich in DA-containing neurons (Brodie et al., 1990) to the NAc, tuberculum olfactorium, septum lateralis, and nuclei interstitialis stria terminalis. This circuit also consists of projections from the VTA to the amygdaloid nuclei, the olfactory nuclei, and entorhinal CTX, and a minor projection to the HI (cf. Oades and Halliday, 1987). The function of the mesolimbic DA pathway can be altered by drugs of abuse to produce uncontrolled cravings (cf. Clay et al., 2008). The mesocortical pathway involves projections to sensory, motor, limbic, and polysensory association cortices. The most significant projections from the VTA connect to the prefrontal, orbitofrontal, and cingulate cortices (cf. Oades and Halliday, 1987). The PFC, which is involved in decision-making and suppresses inappropriate reward response, can be altered by drug abuse. Genetic defects in neurotransmission within the reward pathway and stress-related developmental brain abnormalities have also been cited as possible factors that predispose a person to addiction (cf. Clay et al., 2008).

Ethanol and the Mesolimbic Dopamine System

The mesolimbic DA system is a site of action for the reinforcing effects of ethanol and ethanol self-administration (Carroll et al., 2006; Hodge et al., 1997; Lof et al., 2007; Phillips et al., 1998; Risinger et al., 2000; Thielen et al., 2004). More specifically, evidence indicates that the mesoaccumbal DA system is involved (cf. Pierce and Kumaresan, 2006; Yim and Gonzales, 2000). Activation of the mesocorticolimbic pathway in alcohol dependence and reward involves the dopaminergic projection from the midbrain VTA to several limbic structures which include the hippocampal formation

(HIP) and the AMY in addition to several cortical regions which include the FC, PFC and cingulate cortex (CC) (cf. Zhang et al., 2006).

Increased DA transmission in limbic regions of the brain, and specifically the NAc (cf. Di Chiara, 2002; cf. Di Chiara et al., 2004; cf. Pierce and Kumaresan, 2006; cf. Weiss et al., 1993; cf. Weiss et al., 2001), partially mediates ethanol reinforcement. Studies suggest that ethanol reinforcement is due to ethanol-induced elevations in the firing rates of dopaminergic neurons in the VTA producing increased DA release in the NAc (cf. Pierce and Kumaresan, 2006). It has been suggested that the ethanol-induced positive feedback loop may involve somatodentritically released DA activating D1 receptors on glutamatergic nerve terminals, thereby increasing glutamate release and subsequently leading to a rise in VTA neuronal firing which then increases DA release (Xiao et al., 2009). By regulating dopaminergic cell firing, synaptic release, or a combination of effects, ethanol may stimulate DA release which increases extracellular DA levels in the NAc (Yim and Gonzales, 2000). One report showed that a synthetic mixed DA D1/D2 receptor agonist, SDZ-205,152, decreased ethanol-reinforced responding in rats trained to orally self-administer ethanol (10% w/v) in a free-choice, two-lever operant task without altering responses for water. These results reinforce the possibility that DA neural systems may partially mediate reinforcement for volitional ethanol self-administration (Rassnick et al., 1993).

Glutamate Receptors

Glutamate, the major excitatory neurotransmitter within the central nervous system, acts at both ionotropic and metabotropic receptors (cf. Schoepp et al., 1999; cf. Watkins, 2000). The ionotropic receptors are categorized by agonist specificity into

three subtypes: *N*-methyl-D-aspartate (NMDA), α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA), and kainate (KA) receptors. Fast synaptic neurotransmission occurs when these postsynaptic ligand-gated ion channels are activated by glutamate released from presynaptic vesicles (cf. Dingledine et al., 1999). Slower transmission occurs when glutamate activates metabotropic G-protein-coupled receptors which modulate glutamate release, postsynaptic response, and the activity of other synapses. The metabotropic receptors are sub-classified into eight subtypes (mGluR1-mGluR8) with each subtype belonging to one of three groups (I-III). This classification is based upon sequence homology, transduction mechanism, and pharmacological profile (cf. Conn and Pin, 1997; cf. Pin and Duvoisin, 1995; cf. Pin et al., 1999; cf. Schoepp et al., 1999). Predominantly located postsynaptically, group I metabotropic receptors (mGluR1 and mGluR5) couple to G_q and activate phospholipase C (PLC). Activation of these receptors increases excitability. Mostly located presynaptically, group II (mGluR2 and mGluR3) and group III (mGluR4, mGluR6, mGluR7, and mGluR8) metabotropic receptors couple to G_i/G_o and inhibit AC. Activation of these receptors decreases glutamate release (cf. Pin et al., 1999; cf. Schoepp et al., 1999).

Swanson and Schoepp (2003) have identified several potential targets for novel drugs as modulators of glutamate neuronal transmission. Drugs may act at mGlu2 receptors to potentiate glutamatergic neurotransmission, while mGluR 2/3 agonists may act both presynaptically at mGluR2 and postsynaptically at mGluR 2/3 sites to block transmission. Postsynaptic mGlu1 receptors and both presynaptic and postsynaptic mGlu5 receptors may be the sites of action for antagonists that inhibit glutamatergic transmission. Postsynaptically, both potentiators and inhibitors of glutamatergic neurotransmission may target AMPA receptors, while antagonists may act at kainate

receptors and modulators act at NMDA receptors. Most iGluR antagonists have shown some efficacy in animal models of addiction, but exhibit serious adverse side effects when tested in humans (cf. Olive, 2009).

NMDA Receptor as a Potential Target for the Treatment of Alcohol Dependence

The NMDA receptor has been identified as a major target for ethanol in the brain, and the effects of ethanol on the NMDA receptor differ among areas of the brain (cf. Ron, 2004). Chronic ethanol exposure upregulates NMDA receptor function and binding in cortical cultured neurons. It has been suggested that this alteration in NMDA receptor function may be responsible for the chronic ethanol-induced behavioral consequences and withdrawal syndrome associated with chronic ethanol exposure and the subsequent increased size of evoked Ca^{2+} responses, i.e. increased intracellular Ca^{2+} concentration (Hu and Ticku, 1995).

The specific ethanol binding site or sites on the NMDA receptor protein, and the molecular mechanisms by which ethanol modulates activity of the NMDA receptor remain uncertain. One study suggests that ethanol inhibits NMDA receptors by acting at a site located in a domain exposed to, or only accessible from, the extracellular environment (Peoples and Stewart, 2000). It has been reported that ethanol does not block the ion channel, alter the ion selectivity of the channel, or interact with previously described binding sites on the NMDA/ionophore complex. Furthermore, it has been suggested that ethanol may block the NMDA-activated current via a novel type of interaction with a hydrophobic site associated with the NMDA channel (Weight et al., 1991). Other studies have shown that ethanol interactions with NMDA receptors differ among groups of neurons (Bhave et al., 1996). A study involving the effects of a novel

NMDA glycine receptor antagonist, L-701,324, a polyamine receptor antagonist, eliprodil, and a non-competitive NMDA receptor antagonist, MK 801 (dizocilpine), in rats trained to discriminate ethanol from vehicle in a two-lever discrimination paradigm demonstrated that a reduction of NMDA receptor activity, produced by the inhibition of either non-competitive NMDA recognition sites or NMDA/glycine-sensitive regulatory sites, had discriminative stimulus properties similar to those produced by ethanol. Also, L-701,324 substituted for ethanol more effectively than eliprodil which suggests that multiple NMDA receptor subunits are involved in the discriminative stimulus effects of ethanol (Kotlinska and Liljequist, 1997).

Ethanol-induced inhibition of glutamatergic neurotransmission at the ionotropic NMDA receptor (Lovinger et al., 1989; Lovinger et al., 1990; Morrisett and Swartzwelder, 1993; Peoples and Stewart, 2000) suggests that NMDA antagonists may be effective drugs for treating alcohol dependence and/or alcohol abuse. Previously, our laboratory demonstrated that LY 274614, a competitive NMDA antagonist, dizocilpine (MK 801), a non-competitive NMDA receptor antagonist, and both (+)-HA-966 and 1-aminocyclopropane-1-carboxylic acid (ACPC), antagonists of the strychnine-insensitive glycine site, reduced the volitional consumption of ethanol by male Myers' high ethanol preferring (mHEP) rats (McMillen et al., 2004). However, the dose of MK 801 that reduced ethanol consumption in mHEP rats also impaired the locomotor ability of Sprague Dawley rats (McMillen et al., 2004). Although it provides very good blockade of excitotoxicity, MK 801 has a high affinity for the Mg^{2+} binding site in the NMDA ion channel and a long "dwell time" (slow "off-rate") that causes blockade of critical normal functions and may produce side effects such as drowsiness and/or coma. Other NMDA antagonists with slightly shorter, but excessive, "dwell times" produce similar side

effects, e.g. phencyclidine (PCP) produces hallucinations and ketamine produces drowsiness (cf. Lipton, 2004a; cf. Lipton, 2004b; cf. Lipton, 2005). Furthermore, PCP and related drugs, e.g. MK 801, tiletamine, and ketamine, induced acute pathomorphological changes in CNS neurons in specific brain regions of adult rats (Olney et al., 1989).

Memantine

Excitotoxicity is defined as “excessive exposure to the neurotransmitter glutamate or overstimulation of its membrane receptors, leading to neuronal injury or death.” Excitotoxic neuronal cell damage is partially mediated via overactivation of NMDA receptors which produces excessive Ca^{2+} influx through the receptor-associated ion channel and the subsequent formation of free radicals. However, a physiological level of NMDA receptor activity is necessary to maintain normal neuronal function. Therefore, potential neuroprotective agents that block virtually all NMDA receptor activity are likely to produce intolerable side effects which has led to the failure of many potential neuroprotective drugs tested in clinical trials (cf. Chen and Lipton, 2006; cf. Lipton and Chen, 2004; cf. Lipton, 2004a; cf. Lipton, 2004b; cf. Lipton, 2005; cf. Lipton, 2006; cf. Lipton, 2007; cf. Rammes et al., 2008).

Memantine, however, is a neuroprotective drug that exhibits a clinically-tolerated mechanism of action and has been found to be an effective oral treatment for mild and moderate-to-severe Alzheimer’s disease. An adamantane derivative and a noncompetitive, low-affinity, open channel inhibitor, memantine has been shown to preferentially block excessive NMDA receptor activity without disrupting normal activity. This antagonist preferentially enters the receptor-associated ion channel when it is

excessively open, and, due to its relatively fast “off-rate”, does not substantially accumulate in the channel. Therefore, memantine does not interfere with normal physiological synaptic transmission (cf. Chen and Lipton, 2006; cf. Lipton and Chen, 2004; cf. Lipton, 2004a; cf. Lipton, 2004b; cf. Lipton, 2005; cf. Lipton, 2007; cf. Rammes et al., 2008).

In most clinical trials, memantine has been administered to patients at a dose of 20 mg which exhibits a favorable side-effect profile in patients. Excretion of memantine is through the kidney which alleviates any concerns regarding liver issues (cf. Lleo et al., 2006; cf. Roman, 2009). Also, since memantine is not metabolized by the liver, plasma levels are similar to the administered dose. Following oral ingestion, the drug is almost completely absorbed, and absorption is not altered by food. Peak plasma levels are attained in 5 to 6 hours (t_{max}) and, following a single 20 mg oral dose, peak plasma concentration (C_{max}) ranges between 22 and 46 ng/mL. Steady state levels are reached around day 11 and the terminal half-life ($t_{1/2}$) is 60 to 100 hours in humans (cf. van Marum, 2009). In the rat, the half-life ($t_{1/2}$) of memantine is 3 to 5 hours. (cf. Parsons et al., 2007).

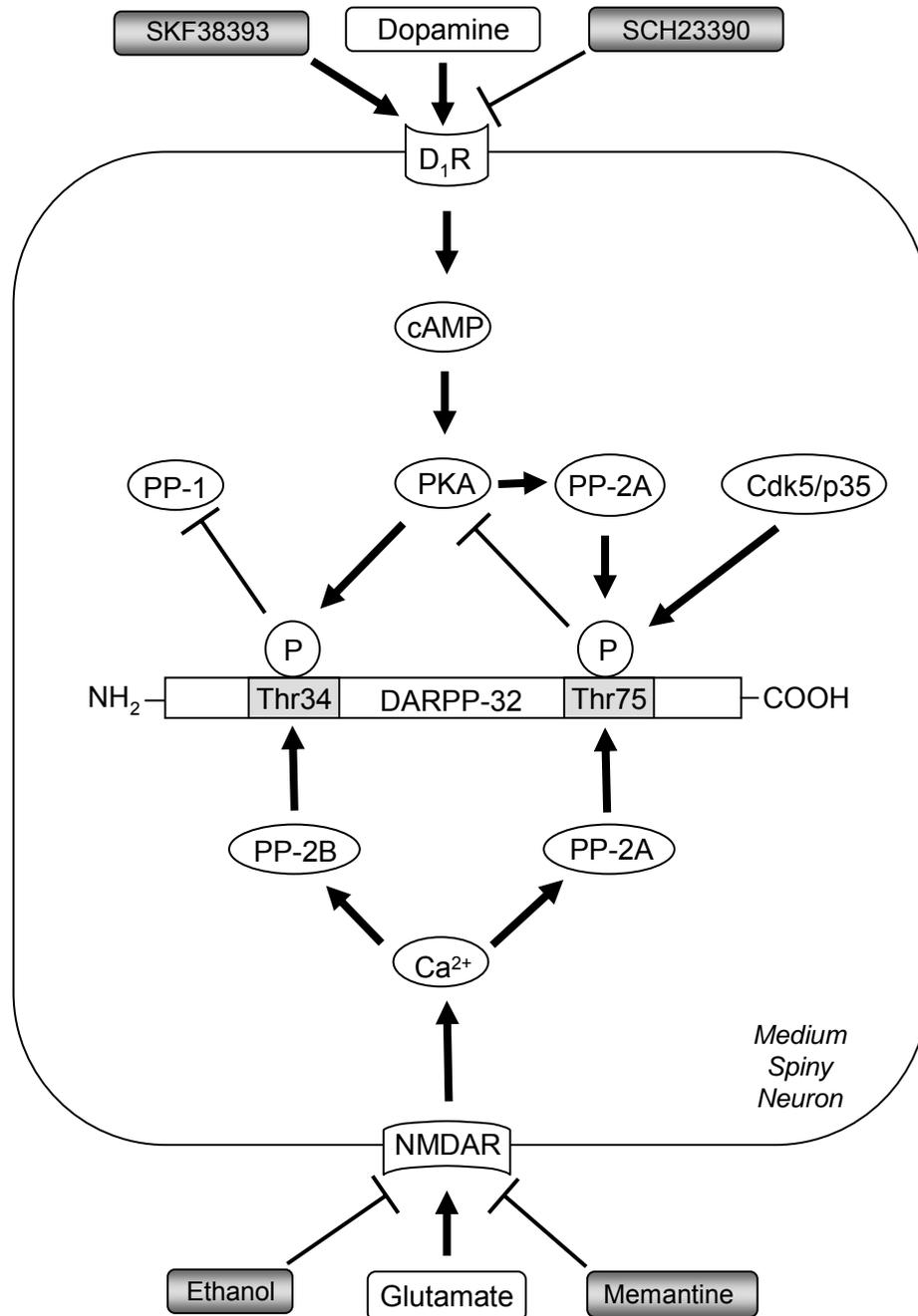
Recent reports indicate that memantine may also act at sites other than the NMDA receptor. Memantine has been shown to produce DA receptor agonism on the functional high-affinity state of the D2 receptors in the rat striatum. Since the potency is similar in magnitude to that at the NMDA receptor, this suggests that the actions at both types of receptor are involved in the clinical features of memantine (Seeman et al., 2008).

Drug addiction, which involves chronic compulsive or uncontrollable drug use followed by a withdrawal syndrome upon cessation of drug exposure (cf. Koob and Weiss, 1992), is a form of drug-induced neural plasticity in which one of the molecular mechanisms involves chronic administration of a drug of abuse which leads to an upregulation of the cyclic adenosine monophosphate (cAMP) second messenger pathway. This upregulation causes an activation of the transcription factor CREB (cAMP response element binding). Induction of the transcription factor Δ FosB produces the opposite effect, which suggests a possible association with sensitization to drug exposure (cf. Nestler, 2001). Chronic stimulation such as chronic exposure to drugs of abuse causes the transcription factor Δ FosB to accumulate and persist in the brain (Perrotti et al., 2008; Soderstrom et al., 2007). Studies also indicate that chronic, but not acute, administration of ethanol induces Δ FosB at varying levels in different regions of the brain such as the NAc, PFC, HI, and AMY (Perrotti et al., 2008).

DA induces intracellular signal transduction pathways which consist of a wide range of intracellular molecules including DA and cyclic adenosine 3',5'-monophosphate-regulated phosphoprotein-32 kDa (DARPP-32), Δ FosB, cyclin-dependent kinase 5 (Cdk5), and extracellular signal-regulated kinase (ERK) (Unterwald et al., 2003). Since the phosphorylation of DARPP-32 can be regulated by both DA and cAMP (Figure 1.2) in intact cells, DARPP-32 may be an effective marker for determining certain actions of DA that are mediated through cAMP and its associated protein kinase (protein kinase A). Immunocytochemical and biochemical studies indicate that DARPP-32 distribution in the rat brain generally follows the pattern of dopaminergic innervation. DARPP-32 is primarily present in dopaminergic neurons that possess DA-sensitive AC, i.e. DA D1

Figure 1.2. Roles of dopamine and glutamate in the phosphorylation of DARPP-32.

Arrows indicate activation and T bars indicate inhibition. (D_1R = dopamine D1 receptor; NMDAR = *N*-methyl-D-aspartate receptor; P = phosphorylation). [*Adapted from Nishi et al., 2002.*]



receptors coupled to AC, and absent from dopaminergic cells (Hemmings and Greengard, 1986; Ouimet et al., 1984; Walaas and Greengard, 1984).

Present in brain regions that receive a strong DA input, DARPP-32 is found in neuronal cell bodies and dendrites in the caudate putamen, NAc, OT, bed nucleus accumbens, bed nucleus of the stria terminalis, and parts of the amygdaloid complex. In brain regions that receive input from these nuclei, the globus pallidus, ventral pallidum (VP), entopeduncular nucleus, and the pars reticulata of the substantia nigra, DARPP-32 is localized in puncta, i.e. presumed nerve terminals, but not indigenous cell bodies and dendrites. In the rat brain, DARPP-32 is most highly concentrated in the basal ganglia (Hemmings and Greengard, 1986; Ouimet et al., 1984; Walaas and Greengard, 1984).

Protein phosphorylation is a key intracellular signaling mechanism associated with transduction and modulation of the extracellular signal-induced effects. Glutamatergic neurotransmission is modulated by a network of second messenger cascades and phosphoproteins that act by transducing the actions of DA, other neurotransmitters, and neuroactive drugs. Activation of cAMP-dependent protein kinase (PKA) or cGMP-dependent protein kinase (PKG) leads to the phosphorylation of DARPP-32 at Thr34, which then converts DARPP-32 into a potent inhibitor of protein phosphatase-1 (PP-1). Conversely, Cdk5 phosphorylates DARPP-32 at Thr75, which then converts DARPP-32 into an inhibitor of PKA. Therefore, DARPP-32 is a dual-function protein, inhibiting either PP-1 or PKA depending upon the site of phosphorylation. The phosphorylation state of the two serine residues, Ser102 which is phosphorylated by casein kinase 2 (CK2), and Ser137 which is phosphorylated by casein kinase 1 (CK1) protein, determine the phosphorylation state of DARPP-32 at Thr34. Under basal conditions, Ser102 and Ser137 are highly phosphorylated. Phosphorylation of DARPP-32 by CK1 or CK2 in

intact cells increases the state of phosphorylation of Thr34. Protein phosphatase 2B (PP-2B, or calcineurin) is the most effective phosphatase in the dephosphorylation of phospho-DARPP-32 (Thr34), while protein phosphatase 2A (PP-2A) is the most effective phosphatase in the desphosphorylation of phospho-DARPP-32 (Thr75). Due to its ability to modulate the activity of PP-1 and PKA, DARPP-32 is an important intracellular molecule in the regulation of electrophysiological, transcriptional, and behavioral responses to physiological and pharmacological stimuli, including antidepressants, neuroleptics, and drugs of abuse (cf. Svenningsson et al., 2004; cf. Svenningsson et al., 2005).

Nishi and colleagues (2000) have identified a positive feedback loop by which the Cdk5/phospho-DARPP-32 (Thr75) pathway regulates DA signaling, and conversely, DA regulates the Cdk5/phospho-DARPP-32 (Thr75) pathway. According to this model, the Cdk5 signaling and PKA signaling pathways are mutually antagonistic. Under basal conditions, Cdk5 phosphorylates DARPP-32 at Thr75 causing the inhibition of PKA which inactivates PP-2A leading to a subsequent decrease in the dephosphorylation of phospho-DARPP-32 (Thr75). The inhibition of PKA also leads to a decrease in the phosphorylation of DARPP-32 at Thr34, and therefore, causes activation of PP-1. By acting synergistically, the inhibition of PKA and the activation of PP-1 decrease the phosphorylation of various substrates. DA sequentially activates DA D1 receptors, PKA, and PP-2A, which decreases the level of phospho-DARPP-32 (Thr75).

Dephosphorylation of DARPP-32 (Thr75) by PP-2A removes the PKA-induced inhibition. The activation of PKA also increases phosphorylation of DARPP-32 at Thr34 and inhibition of PP-1. By acting synergistically, the activation of PKA and inhibition of PP-1 lead to increased phosphorylation of various substrates.

In the caudate putamen, glutamatergic cortical input and dopaminergic nigrostriatal input produce antagonistic effects on the firing rate of striatal neurons. Activation of NMDA receptors reverses the cAMP-stimulated phosphorylation of DARPP-32 in striatal slices via NMDA-induced dephosphorylation of DARPP-32. This indicates that stimulation of NMDA receptors causes activation of a neuronal protein phosphatase, most likely PP-2B, and that signal transduction in the nervous system can be mediated by protein dephosphorylation (Halpain et al., 1990).

DARPP-32 has been implicated in both acute and long-term responses to ethanol. Conditioned place preference studies of wild-type and DARPP-32 knockout (KO) mice suggest that DARPP-32 is essential for the mediation of ethanol reward (Risinger et al., 2001). DA neurons exert a major modulatory effect on the forebrain with DARPP-32 being a key factor in the regulation of DA neurotransmission. DARPP-32 is converted into a potent protein phosphatase inhibitor in response to DA. In addition to DA, other neurotransmitters also produce physiological responses and regulate the phosphorylation/desphosphorylation state of MSNs (Fienberg et al., 1998).

DARPP-32 KO mice demonstrated significantly attenuated ethanol self-administration, but a greater sensitivity to the motor stimulant effect produced by a single injection of ethanol. Another study showed that DARPP-32 regulates the ability of ethanol to inhibit NMDA receptor function which is a critical component in ethanol reinforcement (cf. Maldve et al., 2002). Ethanol exposure generally decreases NMDA synaptic currents. In brain regions containing DARPP-32, DA, via D1 receptors, activates PKA-mediated phosphorylation of the NR1 subunit of the NMDA receptor at Ser897. However, in DARPP-32 KO mice, this regulation of NMDA receptors does not occur, and activation of DA D1 receptors does not block ethanol-induced inhibition of

NMDA receptors. Moderate levels of ethanol have been found to increase phosphorylation of DARPP-32 at Thr34 in striatal slices, but the mechanism by which this occurs is unknown (cf. Nairn et al., 2004; cf. Svenningsson et al., 2005).

A plethora of scientific studies and literature support the premise that DA and glutamatergic neuronal systems are central components in the reinforcing properties of drugs of abuse, including ethanol. In animal studies, comparison of the effects of systemically and NAc-microinjected DA receptor antagonist fluphenazine with the effects of NAc-microinjected competitive NMDA receptor antagonist, 2-amino-5-phosphopentanoic acid (AP-5) in a free-choice operant task, showed that both drugs decrease ethanol self-administration. This suggests that both DA and glutamatergic neurotransmission in the NAc may regulate ethanol self-administration and its reinforcing properties (Rassnick et al., 1992).

Since the addictive properties of many drugs of abuse have been attributed to drug-induced increases in extracellular DA in the NAc, and the subsequent alteration of the plasticity of corticostriatal glutamatergic transmission, it has been proposed that major molecular alterations occur in neurons in which both DA and glutamate inputs are activated and that ERK, an enzyme important for long-term synaptic plasticity, may be a central component. ERK is activated by a multi-level protein phosphatase-controlled mechanism. This activation acts as a detector for coincident DA and glutamate signal convergence on medium-size striatal neurons and is important for maintaining the long-lasting effects of drugs of abuse (Valjent et al., 2005).

Ethanol-induced increases in DA transmission in the limbic regions of the brain involve interaction with a variety of transporters and receptors. Although increased mesolimbic DA transmission is an important component in the reinforcing effects of

ethanol, self-administration studies indicate that DA-independent processes also contribute significantly to the reinforcing effects of ethanol (cf. Pierce and Kumaresan, 2006). Ethanol is associated with changes in both excitatory and inhibitory systems of the brain, which combine to produce behavioral changes including mood elevation, anxiolytic effects, sedation, and ataxia. Ethanol positively modulates GABA-A receptors, inhibits NMDA and kainate glutamate receptors, produces both inhibitory and excitatory effects at the nicotinic acetylcholine receptors, enhances activation of Ca²⁺-activated potassium channels, inhibits N- and P/Q-type Ca²⁺ channels, and modulates inwardly rectifying K⁺ channels (cf. Davies, 2003; cf. Fleming et al., 2001; cf. Pierce and Kumaresan, 2006).

Studies have shown that ethanol produces changes in the function of second messenger proteins (cf. Davies, 2003; cf. Macdonald, 1995; cf. Pandey, 1998). The cAMP and the phosphoinositide (PI) signal-transduction pathways have been implicated as intracellular mediators for the action of ethanol and ultimate contributors to the molecular events involved in the development of ethanol tolerance and dependence. Reports have also demonstrated that various post-receptor events involving the cAMP signal transduction cascade which includes G_s protein, PKA, and CREB in the rat brain are modulated by chronic ethanol exposure. Both acute and chronic ethanol exposure have been shown to modulate the PI signal-transduction cascade in a variety of cells. Chronic ethanol exposure significantly decreased PLC activity in the cerebral CTX of mice and rats. Both acute and chronic ethanol exposure altered protein kinase C (PKC), an important component in the modulation of the function of various neurotransmitter receptors including GABA-A, NMDA, 5-HT_{2A}, and 5-HT_{2C}, and muscarinic M₁ receptors. These reports suggest that alterations in the cAMP and the PI-signaling cascades during

chronic ethanol exposure might be the critical molecular events involved in the development of ethanol dependence (cf. Pandey, 1998).

Ethanol indirectly increases GABAergic neurotransmission through elevation of endogenous GABAergic neuroactive steroids, presynaptic release of GABA, and dephosphorylation of GABA-A receptors, and subsequently causes an increase in GABA sensitivity. The effects of ethanol on intracellular signaling impact GABAergic transmission in a number of different ways across various brain regions and cell types. Adaptations in the function, expression, trafficking, and subcellular localization of GABA-A receptors all influence the effects of chronic ethanol exposure, and thereby, can be proposed to be involved in the development of ethanol tolerance, dependence, and withdrawal hyperexcitability (cf. Kumar et al., 2009).

In reward-associated behaviors, GABAergic MSNs of the NAc process and integrate information including glutamatergic input from the PFC and other limbic structures such as the HIP and dopaminergic inputs from the VTA. Drugs of abuse, including ethanol, alter the function of the MSNs, but unlike other drugs of abuse, ethanol produces widespread effects on multiple intracellular and intercellular signaling processes. Long-term alterations in the synaptic activation of these MSNs have been implicated in ethanol dependence. The antagonistic regulation of these neurons by coincident DA and NMDA inputs suggests that these neurons are critical sites of action for ethanol in the mesolimbic pathway. The co-localization of glutamatergic and dopaminergic receptors on MSNs also indicates that these two neurotransmitter systems interact in the NAc at the neuronal level during cortical information processing (cf. Zhang et al., 2006).

The expression patterns and levels of DA receptor subtypes, the association of receptor subtypes with projection patterns of MSNs, and intracellular signaling cascades triggered by the activation of postsynaptic DA receptors are critical components in the reward pathway. In the NAc, D1-like and D2-like receptors are co-expressed on a small portion of the MSNs and are localized both presynaptically and postsynaptically. By comparison, this co-expression can be found on at least 50% of MSNs in the dorsal STR (Le Moine and Bloch, 1996; cf. Zhang et al., 2006). The co-expression of D1-like and D2-like receptors suggests that receptors from the two subfamilies interact at the neuronal level. The presence of D1-like receptors on dendrites and soma of approximately 50% of the MSNs in the NAc and on presynaptic terminals suggests that DA modulation occurs at both pre-synaptic and post-synaptic sites. However, since DA terminals in the NAc do not form synaptic connections with glutamatergic afferents from the PFC, DA modulation of glutamatergic transmission is most likely non-synaptic (Shetreat et al., 1996; cf. Zhang et al., 2006).

In the NAc, both DA D1 and D2 receptors are highly expressed (cf. Zhang et al., 2006). However, only a small proportion of MSNs co-express DA D1 and D2 receptors (Aubert et al., 2000; Le Moine and Bloch, 1995). In the NAc and dorsal STR, D1 and D2 receptors are often co-expressed with different peptides in different populations of MSNs (Zhang 2006). Studies suggest that D1 receptors and substance P (SP) are co-expressed in approximately 50% of MSNs, with D2 receptors and enkephalin (ENK) being co-expressed on separate MSNs, in the ventral and dorsal STR of rodents and primates (cf. Zhang et al., 2006). In the NAc, co-expressed D1 receptors and SP are predominantly localized in the shell, while co-expressed D2 receptors and ENK are predominantly localized in the core of the NAc (Lu et al., 1998; cf. Zhang et al., 2006).

Unlike in the STR, D3 receptors are highly expressed in the NAc and are co-expressed with D1 receptors in a large number of SP-positive neurons in both the shell and core regions of the NAc (Schwartz et al., 1998). The D4 and D5 receptor expression levels are very low (cf. Zhang et al., 2006).

Generally, MSNs that selectively express D1 or D2 receptors project to different targets. Most striatal MSNs that co-express D1-like receptors and SP project to the substantia nigra while most striatal MSNs that co-express D2-like receptors and ENK project to the globus pallidus (Le Moine and Bloch, 1995). In the NAc, MSNs that co-express D1-like receptors and SP project to the VTA and VP while the MSNs that co-express D2-like receptors and ENK selectively project to the VP (Lu et al., 1997).

In MSNs, D1-like receptor activation initiates a cascade by which AC, cAMP and PKA lead to the phosphorylation of DARPP-32 at Thr34. This phospho-DARPP-32 (Thr34) blocks the phosphatase, PP-1, and subsequently the phosphorylation state and ethanol sensitivity of NMDA receptors. Therefore, D1-like receptors on MSNs may regulate the ability of NMDA receptors to modulate synaptic plasticity during ethanol exposure (cf. Zhang et al., 2006)

Reports suggest that drugs of abuse may induce NMDA receptor-dependent long term potentiation (LTP) in the mesolimbic pathway, which may be an underlying component of drug seeking behavior. However, acute ethanol has been shown to significantly and reversibly block several forms of NMDA receptor-dependent LTP in various brain regions which suggests that acute and chronic ethanol treatment may produce opposite effects on NMDA receptor-dependent LTP in the mesolimbic pathway (cf. Zhang et al., 2006).

Studies have also shown that activation of D1-like receptors enhances NMDA receptor function in the NAc, dorsal STR, and PFC, and D1-like receptor agonists dose-dependently enhance NMDA-induced responses. It has been proposed that activation of D1-like receptors may potentiate NMDA receptor function by regulating phosphorylation of the NMDA receptor subunit NR1 through the DARPP-32/PP-1 signaling pathway. The level of sensitivity of the NMDA receptor to ethanol depends upon the subunits which comprise the receptor. NR2B-containing receptors display the greatest ethanol sensitivity. However, sensitivity of NMDA receptors to acute ethanol varies by brain region. The inhibitory effects of ethanol on NMDA receptor function are regulated by the state of phosphorylation of the receptor and the extracellular concentration of Mg^{2+} . While it is uncertain which sites ethanol acts upon on the NMDA receptor, most studies indicate that ethanol acts by directly blocking the NMDA receptor channel, and reports suggest that ethanol acts directly on a receptor channel protein in the vicinity of the Mg^{2+} -binding site with the site of action being accessible only from the extracellular environment. Ethanol may also block NMDA receptor function by acting on specific amino acid residues on the transmembrane domains of the receptor (cf. Zhang et al., 2006).

PURPOSE OF THIS STUDY

As this literature review has shown, alcohol dependence is a significant health problem with many serious consequences. Due to the diverse neural systems, neurotransmitters, reward circuitry, and second messengers involved in alcohol

disorders, there remain many questions to be answered. As researchers strive to develop effective treatments for alcoholism, there is a need to understand how ethanol exerts its reinforcing effects and how different drugs alter the signaling pathways involved in ethanol reinforcement.

Based on previous reports, an NMDA receptor antagonist with neuroprotective properties may effectively reduce ethanol self-administration with minimal adverse side effects. The purpose of this study was determine if memantine, the noncompetitive NMDA receptor antagonist with neuroprotective properties, can decrease volitional ethanol consumption in a genetic high ethanol preferring rat model using an experimental paradigm that models the drinking behavior of alcoholic patients, and to further elucidate the effects of memantine on DA activity and intracellular pathways during ethanol exposure.

The specific aims of this study focused on seeking answers to the following questions:

1. Does memantine decrease volitional ethanol consumption in the mHEP rat in a two-choice 24 hour access paradigm?
2. If memantine does decrease ethanol consumption under these conditions:
 - a. Is the reduction in drinking dose-dependent?
 - b. Can a direct cause, such as an anti-caloric effect, be attributed to the reduction in drinking?
3. Does memantine, at the same dose that decreases ethanol consumption:
 - a. adversely affect locomotor ability or activity?
 - b. produce a sedating effect or anxiolytic effect?
 - c. alter ethanol-induced hypothermia?

- d. change the levels of DA metabolism in the brain reward pathway?

Specifically, does memantine alone and combined with ethanol alter the levels of DA and/or DOPAC in the mPFC, NAc, and/or STR?

Based on the role of ethanol in the mesolimbic reward pathway, we hypothesized that memantine increases metabolism of DA, which suggests that glutamate and the NMDA receptor are necessary for activation of the ethanol reward pathway. If memantine fails to increase the ethanol-induced metabolism of DA, this would suggest that glutamate acts at the NMDA receptor downstream from the drive by ethanol on the reward pathway.

- e. alter the Ca^{2+} -mediated response downstream of the DA D1 receptor?

Specifically, does memantine alone and in combination with ethanol affect the levels of DARPP-32 and phosphorylation of DARPP-32 at Thr34 and Thr75 sites in the mPFC, NAc, and/or STR? If the combination of memantine and ethanol increases phosphorylation of DARPP-32 greater than the levels of phosphorylation observed with ethanol alone, this would suggest that memantine adds to the action of ethanol. If memantine combined with ethanol does not further increase phosphorylation of DARPP-32, this would suggest that the drugs have independent actions in the CNS.

Since pretreatment with D1-like and D2-like DA receptor agonists and antagonists were previously reported to alter ethanol self-administration (Pierce and Kumaresan, 2006), this study included an evaluation of the effects of a D1 agonist and both a D1 and a D2 antagonist on ethanol drinking behavior using the mHEP rat and the

two-choice 24 hour ethanol access paradigm to validate our subjects and methods. Also, since group I metabotropic glutamate receptor (mGluR5) antagonists have been shown to effectively decrease ethanol consumption in rats (McMillen et al., 2005), the effects of a group II (mGluR 2/3) agonist LY379268 and antagonist LY341495 on volitional ethanol consumption by the male mHEP rat using the two-choice 24 hour ethanol access paradigm were investigated.

CHAPTER 2

EFFECTS OF MEMANTINE ON VOLITIONAL ETHANOL CONSUMPTION IN THE
MALE MYERS' HIGH-ETHANOL-PREFFERING RAT IN A TWO-CHOICE 24 HOUR
ACCESS PARADIGM

ABSTRACT

Potent NMDA receptor antagonists decrease volitional consumption of ethanol by rats. This study examined the effects of memantine, a low-affinity, open channel NMDA receptor antagonist, on volitional consumption of ethanol by genetic drinking rats and on potential locomotor, sedative, and hypothermic effects. Volitional consumption of ethanol in a two-choice 24 hour access paradigm was determined for male mHEP rats. Effects of memantine (0.3, 1.0, and 3.0 mg/kg, i.p., b.i.d.) or vehicle on volitional consumption of ethanol, proportion of ethanol to total fluids consumed, total fluid intake, and consumption of food were observed. To confirm that memantine dose-dependently reduced volitional consumption of ethanol, the effects of 10.0 mg/kg memantine on consumption of ethanol were determined in another group of male mHEP rats. Potential sedating and locomotor effects of memantine (10.0 mg/kg, i.p., b.i.d.) were determined using an elevated plus maze and an activity monitoring system. Rectal temperature was measured to determine if memantine (10.0 mg/kg, i.p.) produces a hypothermic effect. The results indicate that memantine dose-dependently decreased the amount of ethanol and proportion of ethanol to total fluids consumed daily by up to 48% and 24%, respectively. These effects did not appear to be anti-caloric. Memantine (10.0 mg/kg) partially reversed ethanol-induced sedation and decreases in locomotor activity. This dose did, however, produce a small, partially reversible hypothermic effect. In conclusion, memantine may decrease ethanol consumption with fewer side effects than other NMDA receptor antagonists.

INTRODUCTION

Ethanol inhibits glutamatergic neurotransmission at the ionotropic NMDA receptor (Lovinger et al., 1989; Lovinger et al., 1990; Morrisett and Swartzwelder, 1993; Peoples and Stewart, 2000) which suggests that NMDA antagonists may be effective drugs for treating alcohol dependence and/or alcohol abuse. Previously, our laboratory demonstrated that LY 274614, a competitive NMDA antagonist, dizocilpine (MK 801), a non-competitive NMDA receptor antagonist, and both (+)-HA-966 and 1-aminocyclopropane-1-carboxylic acid (ACPC), antagonists of the strychnine-insensitive glycine site, reduced the volitional consumption of ethanol by male mHEP rats (McMillen et al., 2004), a validated animal model of alcoholism (Myers et al., 1998). However, the same doses of LY 274614 and MK 801 that reduced ethanol consumption in mHEP rats also impaired the locomotor ability of Sprague Dawley rats (McMillen et al., 2004). Although it provides very good blockade of excitotoxicity, MK 801 has a high affinity for the Mg^{2+} binding site in the NMDA ion channel and a long “dwell time” (slow “off-rate”) that causes blockade of critical normal functions and may produce side effects such as drowsiness and/or coma. Other NMDA antagonists with slightly shorter, but excessive, “dwell times” produce similar side effects, e.g. phencyclidine (PCP) produces hallucinations and ketamine produces drowsiness (cf. Lipton and Chen, 2004; cf. Lipton, 2004a; cf. Lipton, 2005). Furthermore, PCP and related drugs, e.g. MK 801, tiletamine, and ketamine, induced acute pathomorphological changes in CNS neurons in specific brain regions of adult rats (Olney et al., 1989).

In contrast to these drugs, 1-amino-3,5-dimethyladamantane (memantine), a noncompetitive NMDA antagonist recently introduced for the treatment of moderate to

severe Alzheimer's disease and reported to reduce agitation as its main behavioral effect in these patients (Gauthier et al., 2005), exhibits a better therapeutic profile (Chen et al., 1992; Chen and Lipton, 1997; cf. Chen and Lipton, 2005; cf. Danysz et al., 2000; cf. Lipton and Chen, 2004; cf. Lipton, 2004a; cf. Lipton, 2005; cf. Planells-Cases et al., 2002). A low-affinity, open channel blocker, memantine only enters a channel opened by an agonist and blocks excessively activated NMDA receptors while sparing normal glutamatergic neurotransmission. Like MK 801, memantine binds at or near the Mg^{2+} site in the NMDA ion channel and exhibits a slower "off-rate" than Mg^{2+} . However, memantine has a faster "off-rate" and shorter "dwell time" than MK 801. These properties may contribute to the improved clinical tolerability of memantine in comparison to other NMDA antagonists (Chen et al., 1992; Chen and Lipton, 1997; cf. Chen and Lipton, 2005; cf. Lipton and Chen, 2004; cf. Lipton, 2004a; cf. Lipton, 2005; cf. Planells-Cases et al., 2002).

Memantine (3.75 or 7.5 mg/kg, i.p.) injected three times a day prior to intragastric ethanol administration prevented the development of ethanol dependence measured as ethanol withdrawal-induced audiogenic seizures in Wistar rats. It has been suggested that memantine, given chronically prior to ethanol administration, could take control of the NMDA receptors by blocking the receptors more stably than ethanol, preventing the influence of ethanol on the receptors. Since memantine most likely lacks abuse potential, it could prevent the development of ethanol dependence, expressed as withdrawal seizures (Kotlinska, 2001). Also, memantine has been reported to reduce *in vivo* seizures and to block alcohol withdrawal-related neurotoxicity *in vitro* (Stepanyan et al., 2008).

Furthermore, memantine suppressed relapse ethanol drinking behavior in animals following a withdrawal period. Long-term free-choice ethanol drinking rat models exhibit increased alcohol consumption and a preference for high concentrations of ethanol following a phase of ethanol deprivation. At a dose producing serum levels close to the therapeutic range in humans, memantine suppressed this ethanol deprivation effect in male Wistar rats without producing sedative, dysphoric, or stimulant side effects. This suggests that memantine produces an anti-craving effect for ethanol (Holter et al., 1996). Treatment with memantine has also been shown to completely reverse cognitive impairments associated with chronic alcohol consumption and withdrawal. In the Morris water maze test, withdrawal from ethanol produced robust deficits in the performance of male Wistar rats on the acquisition task and probe trial. Rats treated with memantine during the first four weeks following the initiation of the withdrawal period outperformed the withdrawn rats when tested ten weeks after initiation of the withdrawal period (Lukoyanov and Paula-Barbosa, 2001).

Pretreatment with memantine prior to alcohol administration in human moderate drinkers without alcohol dependence has been shown to reduce craving for alcohol before alcohol administration, but not after alcohol administration. Furthermore, memantine was reported to produce mild subjective effects including dissociation, forgetfulness, dizziness, and stimulation, but no serious adverse effects, when combined with alcohol (Bisaga and Evans, 2004).

Numerous reports indicate that ethanol produces hypothermia (Alari et al., 1987; Beleslin et al., 1997; Holloway et al., 1993; Huttunen et al., 1988; Lomax et al., 1980; Lomax et al., 1980; Pillai and Ross, 1986; Pillai and Ross, 1986; Rezvani and Levin, 2004). Dizocilpine (MK 801), a non-competitive NMDA antagonist, has been shown to

increase body temperature in male Sprague Dawley rats (Yang et al., 2000). Both MK 801 and memantine have been reported to block the development of hyperthermia associated with the development of 5-HT syndrome in rats (Nisijima et al., 2004). Memantine, administered after ethanol, has been shown to produce virtually no effect on ethanol-induced hypothermia (Beleslin et al., 1997).

In the present study, we determined whether or not memantine will decrease ethanol consumption in genetic drinking rats given 24 hour access to solutions of ethanol, and if so, whether the same doses of memantine produce locomotor, sedating, and/or hypothermic effects. Locomotor and sedating effects were evaluated in an activity monitoring system and the elevated plus maze. An increase in the number of open arm entries and in the amount of time spent on the open arms in the elevated plus maze would indicate an anxiolytic effect while an increase in the number of total arm entries would indicate an increase in activity. We also evaluated the potential effects of pretreatment with memantine on ethanol-induced hypothermia by measuring rectal temperature.

MATERIALS AND METHODS

Drugs

In all of the experiments, memantine hydrochloride (Sigma-Aldrich, Inc., St. Louis, MO) was dissolved in deionized water and doses were calculated as the free base. In the ethanol consumption experiments, deionized water was used as a vehicle. In the elevated plus maze, rectal temperature, and activity monitoring experiments, 0.9% saline was used as a vehicle. All drugs and vehicle were administered intraperitoneally (i.p.). For the volitional ethanol consumption experiment, 95% ethanol was diluted in tap water. For injections, 95% ethanol was diluted 1:5 in deionized water.

Volitional Consumption of Ethanol

Subjects and Screening

Ten male mHEP rats were selected from the F21 generation of breeding in the East Carolina University colony. The progenitor rat strains for this line were three male alcohol-preferring P rats, obtained from T.-K. Li of the Indiana University Alcohol Research Center, and three female Sprague Dawley rats, purchased from Harlan Sprague Dawley Inc., selected based on an ethanol drinking screen. The line of mHEP rats, characterized by profuse volitional consumption of ethanol and a preference for ethanol in the presence of palatable alternatives (Myers et al., 1998), is maintained by breeding non-sibling males and females that are selected based on an ethanol drinking screen. Previously, the levels of blood ethanol in these rats have been shown to correspond essentially with the respective amounts of ethanol consumed (Myers et al., 1998). The rats were initially housed with continuous access to food and water and

maintained on a 12 h on/12 h off light cycle in a temperature-controlled room. Rooms were maintained at 71° F to 75° F with humidity between 30% and 45% for all animal housing and experiments in this study.

At 60 days of age, each male mHEP rat was placed in a suspended stainless steel cage with three drinking tubes mounted on the front. One tube contained water while the other two tubes remained empty. After the one-day adaptation, one tube was filled with water, one was filled with 3% ethanol (v/v), and one remained empty. Thereafter, the concentration of ethanol was increased daily: 5, 7, 9, 11, 13, 15, 20, 25, and 30%. Rats were allowed free access to water, ethanol solution, and food at all times. Body weight and amounts of water, ethanol solution, and food consumed were recorded daily for each rat. To prevent a place preference, the positions of the bottles were rotated daily based on a randomized schedule. The empty bottle was used to prevent a side preference in drinking behavior. The concentration of ethanol producing maximal ethanol consumption with the proportion of ml ethanol consumed to total ml fluids consumed closest to 0.5 was selected as each rat's maximally preferred concentration to allow for the measurement of either an increase or a decrease in ethanol consumption during treatments. Each rat received its maximally preferred concentration of ethanol for the remainder of the experiment. Ethanol consumption was allowed to stabilize before initiating drug or vehicle injections. The average preferred ethanol concentration for these rats was 11.5%.

Experimental Procedures

During each regimen of treatments, rats underwent a 3-day baseline period preceding a 3-day period in which injections (i.p.) were administered at two hours before

and two hours following lights out and a subsequent 3-day post-treatment period. Doses of memantine (0.3, 1.0, and 3.0 mg/kg) or vehicle were administered in a counter-balanced design with part of the rats receiving 1.0 mg/kg memantine and the remaining rats receiving 3.0 mg/kg memantine initially. Upon stabilization of ethanol consumption to baseline levels following each regimen of treatments, a different dose of memantine or vehicle was injected. During the series of treatments, one rat exhibited a delayed return to baseline and did not receive the 1.0 mg/kg memantine treatment. Data for each pre-treatment, treatment, and post-treatment period for each rat were averaged, grouped, and analyzed using two-way repeated measures ANOVA and Tukey/Kramer procedure post hoc with significance taken at $P < 0.05$. Statistical analyses were performed using GB-STAT (Dynamic Microsystems, Silver Spring, MD) and graphs were generated using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA).

To verify that the reduction in ethanol consumption was dose-dependent, eight additional male mHEP rats were selected from the F24 generation of breeding in the East Carolina University colony and underwent a similar protocol. The average preferred ethanol concentration for these rats was 13.3%. Memantine (10.0 mg/kg) and vehicle were administered in a counter-balanced design. To consolidate the results from the volitional ethanol drinking experiments, data for the 0.3, 1.0, and 3.0 mg/kg treatments were compared to data for the 10.0 mg/kg treatments using the Mann-Whitney test. Data for one of the rats was omitted due to the rat deliberately spilling the ethanol solution.

Elevated Plus Maze Test

Twenty-four male mHEP rats were used in the elevated plus maze test to determine potential anxiolytic effects of memantine (10.0 mg/kg, i.p.). The elevated plus maze consisted of four arms which were 50 cm long and 10 cm wide. Two opposing arms were open while the other arms had walls which were 40 cm high. The maze was mounted 60 cm above the floor in the dimly lit corner of a room with fluorescent lighting. Illuminance at the center of the maze measured 97 lux. Each rat was handled for 5 minutes for three consecutive days. On the fourth day, memantine or vehicle was injected into each rat. Thirty minutes following the first injection, ethanol (1.0 g/kg, i.p.) or vehicle was injected into the rat. Thirty minutes following the second injection, the rat was placed in the central (neutral) region of the maze with the rat facing an open arm. Movements onto each arm and into the neutral region were recorded for 5 minutes using the computer program BEHAVIOR (Prof. L. W. Means, Dept. of Psychology, ECU). Latency to first open arm entry, frequency of open arm entries, duration of time spent in open arms, and total frequency of arm entries were recorded and analyzed using paired t-tests with significance taken at $P < 0.05$. Each rat was used in two tests separated by two weeks. Treatments were administered in a counter-balanced design. Each rat received only vehicle on one of the days of testing so that each rat served as its own control. Rats were between 56 and 61 days of age on the first day of testing.

Activity Monitor

Thirty-eight male mHEP rats were divided into five groups (three groups of eight and two groups of seven). Activity was recorded using an Auto-Track Opto-Varimex activity monitoring system (Columbus Instruments, Columbus, OH). White noise and a single red 25-watt light located at the opposite side of the testing room provided sound

and light control. The system was comprised of four chambers (each 42.2 x 42.5 x 20.5 cm). Each rat was used in two tests separated by one week. Memantine (10 mg/kg, i.p.) or vehicle was injected as the first treatment and each rat was placed in a separate activity chamber. Thirty minutes following the first injection, ethanol (1.0 or 2.5 g/kg, i.p.) or vehicle was administered and each rat was returned to its activity chamber. Thirty minutes following the second injection, rats were removed from the activity monitors. Distance traveled (cm) and resting time (sec) were recorded for each 15-minute interval spent in the activity chamber. Resting time was equivalent to total time in the chamber minus ambulatory and stereotypic time. Treatments were administered in a counter-balanced design. Each rat received only vehicle on one of the days of testing so that each rat served as its own control. Data was analyzed using paired t-tests with significance taken at $P < 0.05$. To further determine the effects of memantine on locomotor ability and sedation, activity of rats treated with vehicle and ethanol were compared to activity of rats treated with memantine and ethanol using pooled two-sample t-tests with significance taken at $P < 0.05$.

Rectal Temperature

Thirty-nine male mHEP rats were divided into five groups (four groups of eight and one group of seven). Baseline rectal temperature of each rat was recorded prior to the first injection. Memantine (10 mg/kg, i.p.) or vehicle was injected as the first treatment. Thirty minutes following the first injection, rectal temperature was recorded and ethanol (1.0 or 2.5 g/kg, i.p.) or vehicle was administered. Thirty minutes following the second injection, rectal temperature was recorded. A BD Rapid Digital Thermometer was used for all temperature checks. Each rat was used in two tests separated by one

week. Treatments were administered in a counter-balanced design. Each rat received only vehicle on one of the days of testing so that each rat served as its own control. Changes from baseline body temperature were analyzed using paired t-tests with significance taken at $P < 0.05$. To further determine the effect of memantine on body temperature, changes in body temperature of rats treated with vehicle and ethanol were compared to changes in body temperature of rats treated with memantine and ethanol using pooled two-sample t-tests with significance taken at $P < 0.05$.

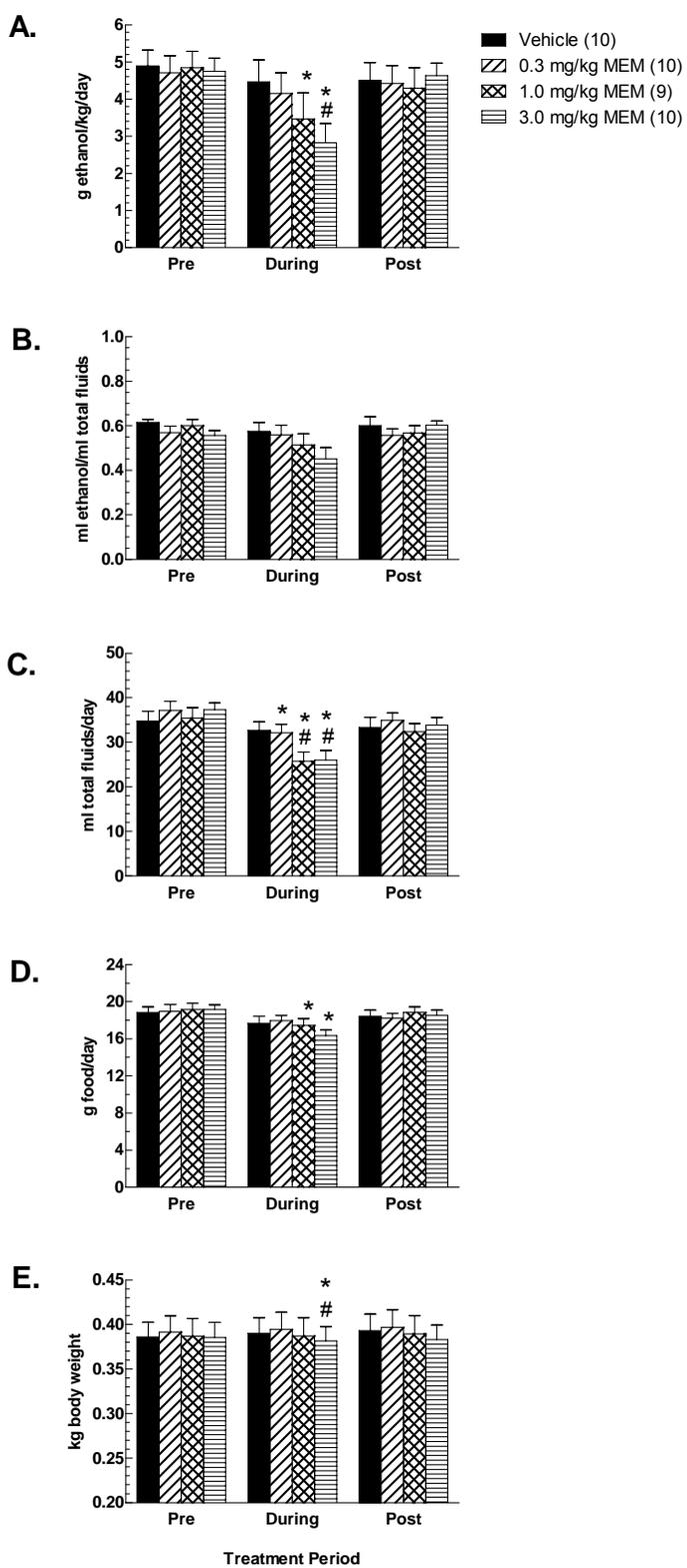
RESULTS

Effects of Memantine on Volitional Consumption of Ethanol

Figure 2.1 shows data for the effect of memantine on ethanol consumption (Figure 2.1.A), proportion of ethanol consumed to total fluid intake (Figure 2.1.B), total fluid intake (Figure 2.1.C), and food consumption (Figure 2.1.D) daily by male mHEP rats. The data indicate a dose-dependent reduction in ethanol consumption and an apparent dose-dependent decrease in proportion of ethanol to total fluid consumption during drug treatment. The two-way repeated measures ANOVA for the amount of ethanol consumed showed an effect of treatment period ($F_{2,38} = 17.99$, $P < 0.05$) and a significant interaction between dose of memantine and treatment period ($F_{6,116} = 2.90$, $P < 0.05$). Compared to pre-treatment levels, the 1.0 mg/kg and 3.0 mg/kg doses of memantine significantly reduced ethanol consumption by 28% and 41% (Tukey/Kramer, $P < 0.05$), respectively, while non-significantly decreasing proportion of ethanol consumed by 15% and 19%, respectively. Compared to vehicle, the 3.0 mg/kg dose of memantine produced a significant 37% (Tukey/Kramer, $P < 0.05$) decline in ethanol consumption and a non-significant 22% decline in proportion of ethanol consumed. Compared to pre-treatment levels, the 1.0 mg/kg and 3.0 mg/kg doses of memantine significantly decreased total fluid intake by 27% and 30%, respectively (Tukey/Kramer, $P < 0.05$). Compared to vehicle, the 1.0 mg/kg and 3.0 mg/kg doses of memantine significantly reduced total fluid consumption by 21% (Tukey/Kramer, $P < 0.05$). During each 3-day post-treatment period immediately following memantine treatments, ethanol intake, proportion of ethanol to total fluids consumed, and total fluid intake returned to within control limits.

Figure 2.1. Effect of memantine on the volitional consumption of solutions of ethanol by the male mHEP rat. All treatments administered i.p., b.i.d. Each bar represents the mean \pm S.E.M. for 9-10 rats at each dose. (MEM = memantine).

- A. Volitional consumption of ethanol. * indicates a significant difference from pre-treatment baseline, $P < 0.05$ (Tukey/Kramer). # indicates a significant difference from vehicle, $P < 0.05$ (Tukey/Kramer).
- B. Proportion of ethanol consumed to total fluids (water and ethanol solution) consumed.
- C. Consumption of total fluids (water and ethanol solution). * indicates a significant difference from pre-treatment baseline, $P < 0.05$ (Tukey/Kramer). # indicates a significant difference from vehicle, $P < 0.05$ (Tukey/Kramer).
- D. Consumption of food. * indicates a significant difference from pre-treatment baseline, $P < 0.05$ (Tukey/Kramer).
- E. Body weight. * indicates a significant difference from pre-treatment baseline, $P < 0.05$ (Tukey/Kramer). # indicates a significant difference from vehicle, $P < 0.05$ (Tukey/Kramer).



The effects of memantine on food intake (Figure 2.1.D) were smaller than the changes in ethanol consumption. The 3.0 mg/kg dose of memantine caused consumption of food to decline by 15% ($F_{2,18} = 18.35$, $P < 0.05$), which is different from pre-treatment levels, but not from vehicle. This suggested either a weak non-specific effect or a weak anti-caloric effect of this dose of drug. This effect did not persist in the post-treatment period. The 3.0 mg/kg dose of memantine also decreased body weight by 1.0% from pre-treatment levels and 2.2% from vehicle. This small, but significant reduction in weight, may have resulted from decreased fluid and food consumption.

Figure 2.2 shows the effect of 10.0 mg/kg memantine on volitional ethanol consumption. The two-way repeated measures ANOVA for the amount of ethanol consumed showed an effect of treatment period ($F_{2,13} = 13.42$, $P < 0.05$) and a significant interaction between dose of memantine and treatment period ($F_{2,41} = 9.80$, $P < 0.05$). Compared to pre-treatment levels, the 10.0 mg/kg dose of memantine decreased ethanol consumption by 48% (Tukey/Kramer, $P < 0.05$), proportion by 24% (Tukey/Kramer, $P < 0.05$), and total fluid intake by 33% (Tukey/Kramer, $P < 0.05$), but did not reduce food consumption. Compared to vehicle, the 10.0 mg/kg dose of memantine reduced ethanol consumption by 44% (Tukey/Kramer, $P < 0.05$), proportion by 23% (Tukey/Kramer, $P < 0.05$), and total fluid intake by 30% (Tukey/Kramer, $P < 0.05$). Treatment with vehicle produced a small, but significant 10% (Tukey/Kramer, $P < 0.05$) decrease in food consumption. This small reduction in food consumption may have been due to the rats being handled, and does not indicate an anti-caloric effect to the treatment. The 10.0 mg/kg dose of memantine reduced body weight by 2.5% from vehicle, but did not significantly decrease body weight from pre-treatment levels.

Figure 2.2. Effect of 10.0 mg/kg memantine on the volitional consumption of solutions of ethanol by the male mHEP rat. All treatments administered i.p., b.i.d. Each bar represents the mean \pm S.E.M. for 7 rats at each dose. (MEM = memantine).

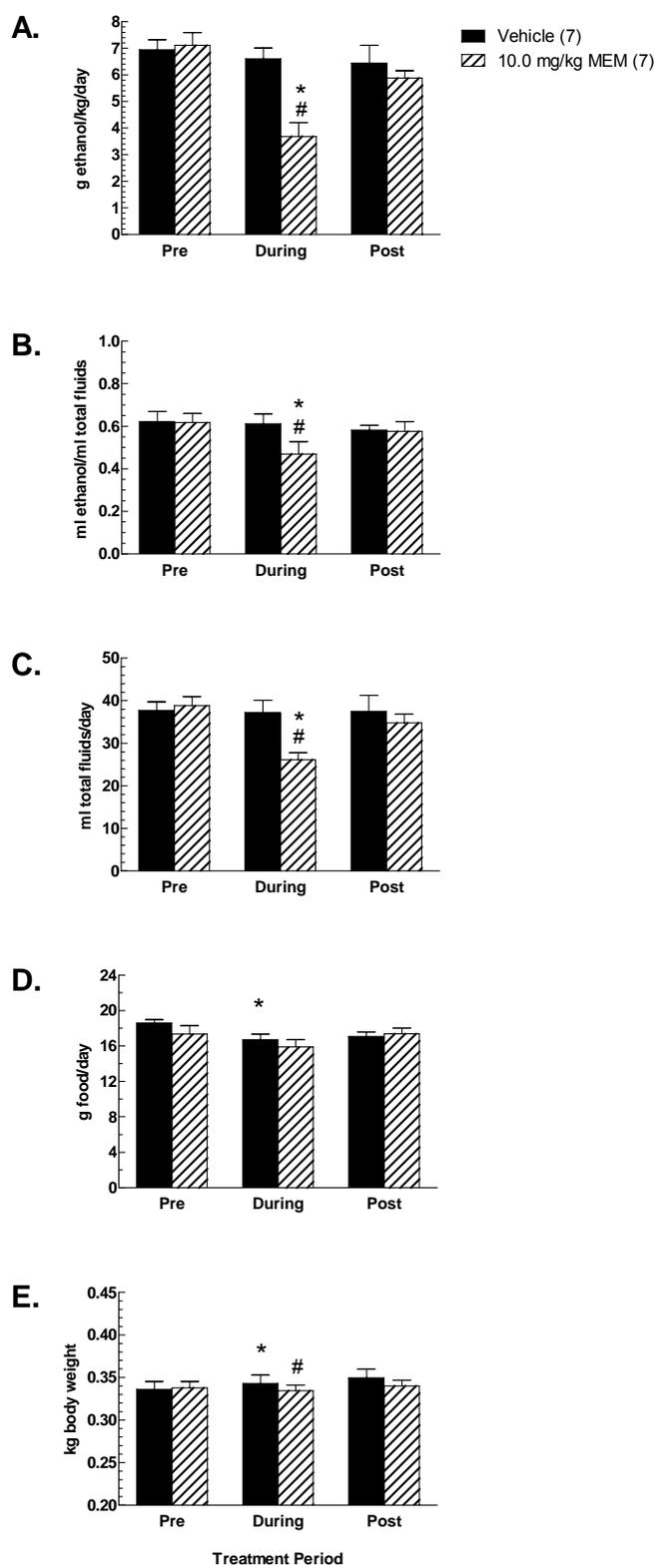
- A. Volitional consumption of ethanol. * indicates a significant difference from pre-treatment baseline, $P < 0.05$ (Tukey/Kramer). # indicates a significant difference from vehicle, $P < 0.05$ (Tukey/Kramer).

- B. Proportion of ethanol consumed to total fluids (water and ethanol solution) consumed. * indicates a significant difference from pre-treatment baseline, $P < 0.05$ (Tukey/Kramer). # indicates a significant difference from vehicle, $P < 0.05$ (Tukey/Kramer).

- C. Consumption of total fluids (water and ethanol solution). * indicates a significant difference from pre-treatment baseline, $P < 0.05$ (Tukey/Kramer). # indicates a significant difference from vehicle, $P < 0.05$ (Tukey/Kramer).

- D. Consumption of food. * indicates a significant difference from pre-treatment baseline, $P < 0.05$ (Tukey/Kramer).

- E. Body weight. * indicates a significant difference from pre-treatment baseline, $P < 0.05$ (Tukey/Kramer). # indicates a significant difference from vehicle, $P < 0.05$ (Tukey/Kramer).



Comparing the effects of the 0.3 mg/kg and 10.0 mg/kg doses of memantine indicated a significant reduction in ethanol consumption, proportion, and total fluid intake, but no significant difference in food consumption (Mann-Whitney, $P < 0.05$). Comparing the effects of both the 1.0 mg/kg and 3.0 mg/kg doses to the effects of the 10.0 mg/kg dose of memantine indicated no significant differences in ethanol consumption, proportion, total fluid intake, or food consumption. A further one-half log unit increase in memantine dose (10.0 mg/kg) produced a decrease in ethanol consumption and proportion similar to that of the 3.0 mg/kg dose. This suggested that a maximum effect was reached at the 3.0 mg/kg dose.

Effects of Memantine on Anxiety

The 10.0 mg/kg dose of memantine and/or 1.0 g/kg dose of ethanol did not alter the activity, indicated by frequency of total arm entries, of the male mHEP rats in the elevated plus maze (Table 2.1). However, the latency to enter the first open arm was significantly increased (12.9%, $t_7 = 2.80$, $P < 0.05$) in rats treated with memantine (10.0 mg/kg) followed thirty minutes later by ethanol (1.0 g/kg). While four of the rats in this group failed to enter an open arm, three rats entered an open arm within 1.5 seconds and the remaining rat entered at 42.9 seconds. Latency to enter the first open arm was similar, but the increase was insignificant, for the other two groups of rats tested. Overall, the latency to enter the open arm indicates that the doses of memantine and ethanol did not produce anxiolytic effects in this experimental protocol. There were no differences in total time spent in the open arms or in frequency of open arm entries. Furthermore, there was no significant difference in the total frequency of entries, which

Table 2.1. Effect of ethanol (1.0 g/kg, i.p.) and/or memantine (10.0 mg/kg, i.p.) on the behavior of mHEP rats in the elevated plus maze. The observation period was 300 seconds. * indicates a significant difference from "Control/Control", $P < 0.05$ (paired t-test). (Ctl = control; EtOH = ethanol; Mem = memantine).

Treatment Groups	Seconds \pm S.E.M.			Number \pm S.E.M.	
	Latency to open arm	Open arm duration	Closed arm duration	Open arm frequency	Total arm frequency
Group I ($n = 8$)					
Ctl/Ctl	83 \pm 47	20 \pm 7	246 \pm 18	2.5 \pm 0.9	18.5 \pm 4.0
Ctl/EtOH	104 \pm 47	12 \pm 3	269 \pm 5	1.5 \pm 0.4	15.0 \pm 1.8
Group II ($n = 8$)					
Ctl/Ctl	33 \pm 26	21 \pm 4	237 \pm 15	2.3 \pm 0.5	16.5 \pm 3.0
Mem/Ctl	122 \pm 52	17 \pm 6	258 \pm 11	2.0 \pm 0.7	17.0 \pm 5.9
Group III ($n = 8$)					
Ctl/Ctl	11 \pm 7	23 \pm 8	235 \pm 12	2.5 \pm 0.6	21.5 \pm 1.8
Mem/EtOH	156 \pm 55 *	22 \pm 9	249 \pm 15	2.5 \pm 1.1	19.6 \pm 5.5

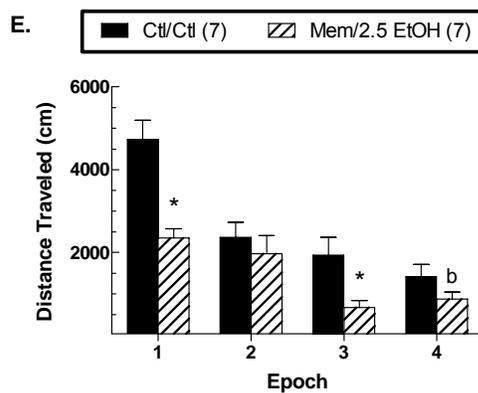
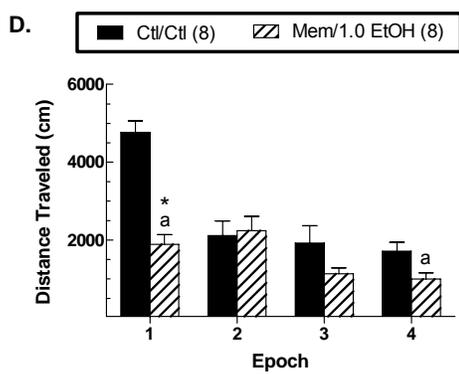
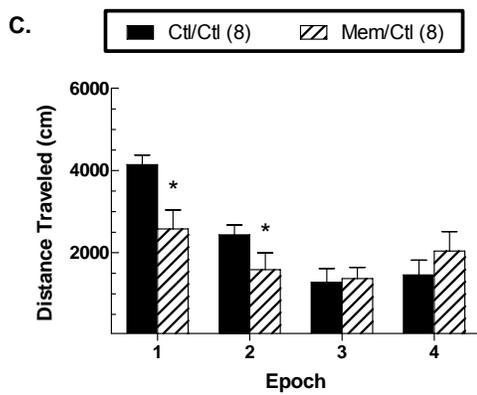
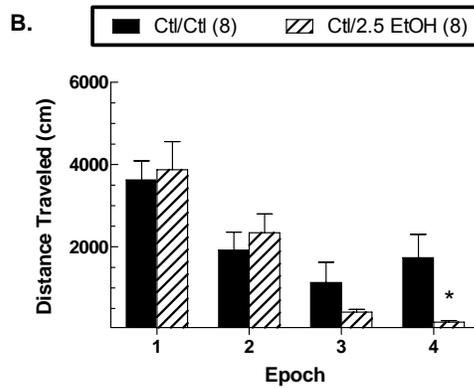
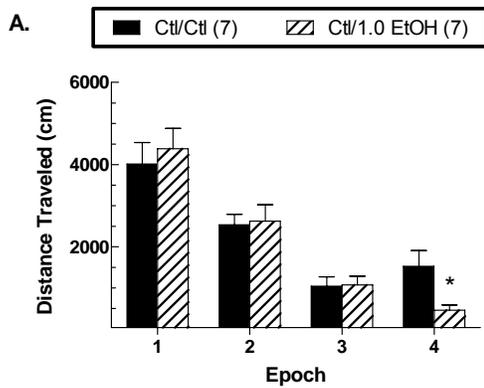
indicates that the doses tested produced neither a sedating nor an activating effect on locomotion. As the table indicates, the rats spent most of the time in the closed arms of the plus maze under the conditions of this experiment. The differences in the latency to open arm entry for the three “control/control” groups may be attributed to differences between individual rats. Within each group of rats, the latency to enter an open arm varied. Some rats entered an open arm within 1.2 seconds while others waited longer or never entered an open arm during the 5-minute testing period

Effects of Memantine on Locomotor Activity

An activity monitoring system was used as a test for non-specific sedating or locomotor altering effects of memantine. The 10.0 mg/kg dose of memantine significantly decreased the distance traveled (Figure 2.3) during the first 15-minute epoch following treatment (between 37.7% and 60.5% for three groups). During the second 15-minute epoch following treatment, only one of the three groups receiving memantine indicated a significant decrease (34.8%) in distance traveled. Rats treated with memantine (10.0 mg/kg) followed thirty minutes by ethanol (2.5 g/kg) exhibited a significant decrease (65.3%) in distance traveled during the first 15-minute epoch following ethanol treatment. Rats that received vehicle followed thirty minutes by ethanol (1.0 and 2.5 g/kg) exhibited significant decreases (70.0% and 90.4%, respectively) in distance traveled during the second 15-minute epoch following ethanol treatment while rats treated with memantine thirty minutes prior to ethanol did not exhibit significant deficits during this epoch. Comparison of memantine- and 1.0 g/kg ethanol-treated rats to vehicle- and 1.0 g/kg ethanol-treated rats exhibited a significant decrease (57.1%) in distance traveled during the first 15 minutes following memantine

Figure 2.3. Effect of ethanol (1.0 or 2.5 g/kg, i.p.) and/or memantine (10.0 mg/kg, i.p.) on the distance traveled by mHEP rats in the activity monitor. The number of rats in each group is indicated in parentheses. 1st Epoch = 1st 15-minute period following 1st injection; 2nd Epoch = 2nd 15-minute period following 1st injection; 3rd Epoch = 1st 15-minute period following 2nd injection; 4th Epoch = 2nd 15-minute period following 2nd injection. (1.0 EtOH = 1.0 g ethanol per kg; 2.5 EtOH = 2.5 g ethanol per kg; Mem = 10.0 mg memantine per kg).

- A. Control/Control vs. Control/Ethanol (1.0 g/kg). * indicates a significant difference from "Control/Control", $P < 0.05$ (paired t-test).
- B. Control/Control vs. Control/Ethanol (2.5 g/kg). * indicates a significant difference from "Control/Control", $P < 0.05$ (paired t-test).
- C. Control/Control vs. Memantine (10.0 mg/kg)/Control. * indicates a significant difference from "Control/Control", $P < 0.05$ (paired t-test).
- D. Control/Control vs. Memantine (10.0 mg/kg)/Ethanol (1.0 g/kg). . * indicates a significant difference from "Control/Control", $P < 0.05$ (paired t-test). "a" indicates a significant difference from "CTL/EtOH (1.0 g/kg)", $P < 0.05$ (pooled two-sample t-Test).
- E. Control/Control vs. Memantine (10.0 mg/kg)/Ethanol (2.5 g/kg). . * indicates a significant difference from "Control/Control", $P < 0.05$ (paired t-test). "b" indicates a significant difference from "CTL/EtOH (1.0 g/kg)", $P < 0.05$ (pooled two-sample t-Test).



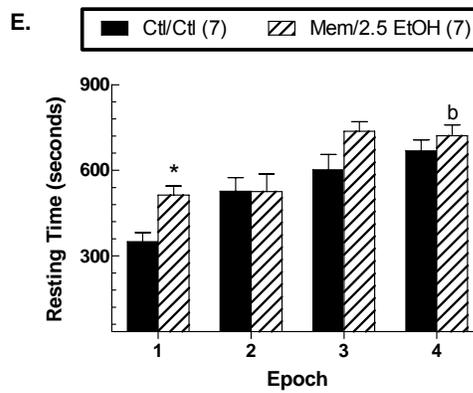
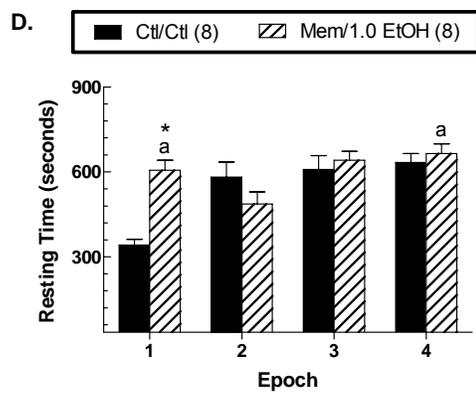
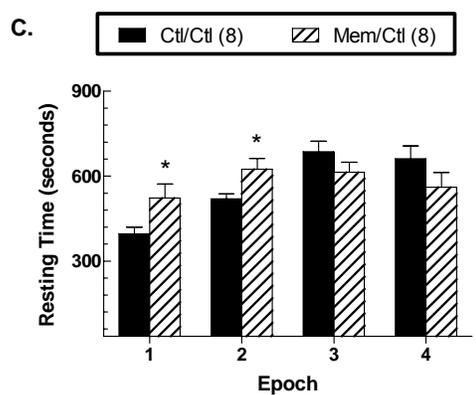
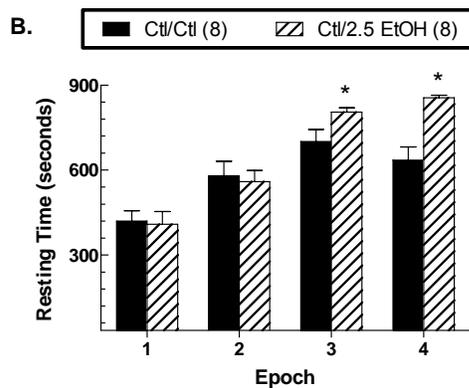
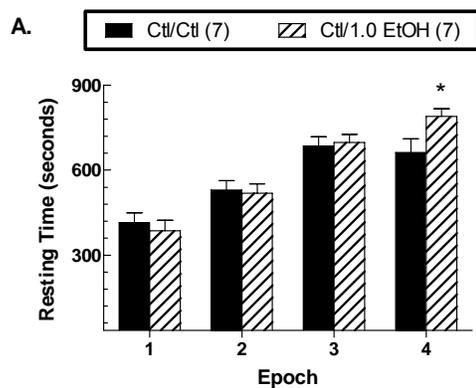
treatment, but a significant increase (116.9%) in the second 15-minute epoch following ethanol treatment. Comparison of memantine- and 2.5 g/kg ethanol-treated rats to vehicle- and 2.5 g/kg ethanol-treated rats displayed a significant increase (425.4%) in distance traveled in the second 15-minute epoch following ethanol treatment.

The 10.0 mg/kg dose of memantine significantly increased resting time (Figure 2.4) during the first 15-minute epoch following treatment (between 31.8% and 77.4% for three groups). During the second 15-minute epoch following treatment, only one of the three groups receiving memantine showed a significant change (20.2% increase) in resting time. In rats initially receiving vehicle, the 2.5 g/kg dose of ethanol increased resting time during both 15-minute epochs following treatment (14.8% and 34.7%, consecutively) while the 1.0 g/kg dose produced a similar effect only in the second 15-minute epoch following treatment (19.2% increase). Comparison of memantine- and 1.0 g/kg ethanol-treated rats with vehicle- and 1.0 g/kg ethanol-treated rats indicated a significant increase (57.1%) in resting time during the first 15-minute epoch following memantine treatment, but a significant decrease (15.9%) in the second 15-minute epoch following ethanol treatment. Comparison of memantine- and 2.5 g/kg ethanol-treated rats to vehicle- and 2.5 g/kg ethanol-treated rats indicated a significant decrease (15.7%) in resting time in the second 15-minute epoch following ethanol treatment. The effects on resting time agree with the changes observed in ambulatory and stereotypic times. Overall, these data indicate that memantine reverses, at least partially, the effects of locomotor and sedating effects of ethanol under the conditions of this experiment.

Effects of Memantine on Rectal Temperature

Figure 2.4. Effect of ethanol (1.0 or 2.5 g/kg, i.p.) and/or memantine (10.0 mg/kg, i.p.) on the resting time by mHEP rats in the activity monitor. The number of rats in each group is indicated in parentheses. 1st Epoch = 1st 15-minute period following 1st injection; 2nd Epoch = 2nd 15-minute period following 1st injection; 3rd Epoch = 1st 15-minute period following 2nd injection; 4th Epoch = 2nd 15-minute period following 2nd injection. (1.0 EtOH = 1.0 g ethanol per kg; 2.5 EtOH = 2.5 g ethanol per kg; Mem = 10.0 mg memantine per kg).

- A. Control/Control vs. Control/Ethanol (1.0 g/kg). * indicates a significant difference from "Control/Control", $P < 0.05$ (paired t-test).
- B. Control/Control vs. Control/Ethanol (2.5 g/kg). * indicates a significant difference from "Control/Control", $P < 0.05$ (paired t-test).
- C. Control/Control vs. Memantine (10.0 mg/kg)/Control. * indicates a significant difference from "Control/Control", $P < 0.05$ (paired t-test).
- D. Control/Control vs. Memantine (10.0 mg/kg)/Ethanol (1.0 g/kg). . * indicates a significant difference from "Control/Control", $P < 0.05$ (paired t-test). "a" indicates a significant difference from "CTL/EtOH (1.0 g/kg)", $P < 0.05$ (pooled two-sample t-Test).
- E. Control/Control vs. Memantine (10.0 mg/kg)/Ethanol (2.5 g/kg). . * indicates a significant difference from "Control/Control", $P < 0.05$ (paired t-test). "b" indicates a significant difference from "CTL/EtOH (1.0 g/kg)", $P < 0.05$ (pooled two-sample t-Test).

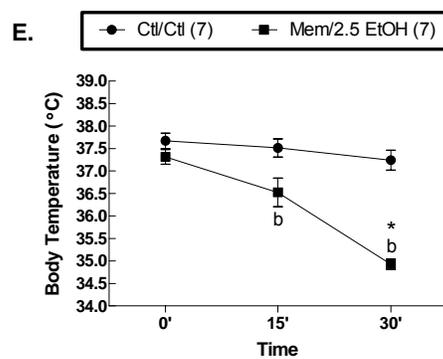
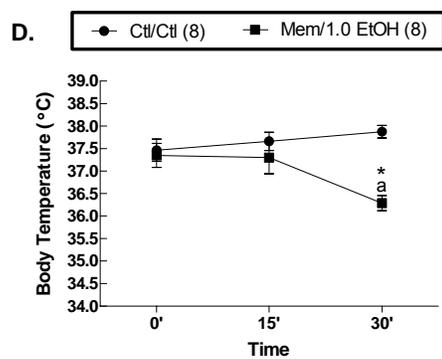
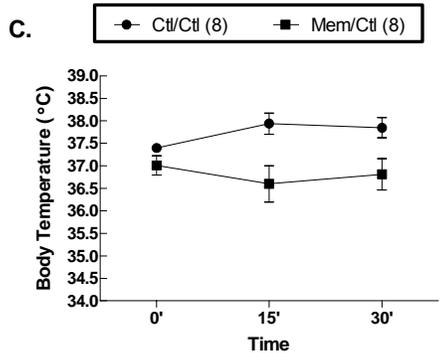
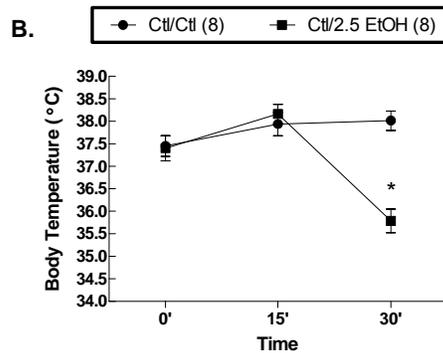
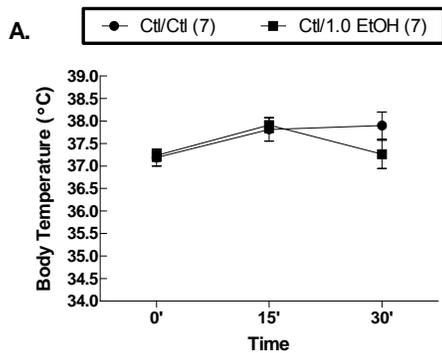


The 2.5 g/kg dose of ethanol produced significantly decreased body temperature at thirty minutes following treatment (Figure 2.5). The 10.0 mg/kg dose of memantine produced a small, but non-significant, decrease in rectal temperature at thirty minutes following treatment, but this effect was partially reversed at one hour following treatment. The 10.0 mg/kg dose of memantine administered thirty minutes before ethanol (1.0 or 2.5 g/kg) enhanced the effect of ethanol on body temperature. Furthermore, comparison of temperature changes in rats treated with vehicle and ethanol (1.0 g/kg) and rats treated with memantine (10.0 mg/kg) and ethanol (1.0 g/kg) showed a significant decrease in body temperature in the memantine-treated rats at thirty minutes following ethanol treatment ($t_{14} = 2.25$, $P < 0.05$). Comparison of temperature changes in rats treated with vehicle and ethanol (2.5 g/kg) and rats treated with memantine (10.0 mg/kg) and ethanol (2.5 g/kg) showed significant decreases in the body temperature of the memantine-treated rats at thirty minutes following vehicle or memantine treatment ($t_{14} = 6.10$, $P < 0.05$) and thirty minutes following ethanol treatment ($t_{13} = 2.45$, $P < 0.05$).

Pre-treatment with the 10.0 mg/kg dose of memantine before treatment with ethanol (1.0 or 2.5 g/kg) produced an additive or synergistic effect on the ethanol-induced hypothermia. The significant difference in body temperature of rats thirty minutes following treatment with vehicle or memantine in the comparison of rats treated with vehicle and ethanol (2.5 g/kg) and rats treated with memantine (10.0 mg/kg) and ethanol (2.5 g/kg) may be due to handling or variation among animals.

Figure 2.5. Effect of ethanol (1.0 or 2.5 g/kg, i.p.) and/or memantine (10.0 mg/kg, i.p.) on rectal temperature of the male mHEP rat. Data represents the mean \pm S.E.M. for 7-8 rats at each dose. The number of rats for each treatment group is indicated in parentheses. (1.0 EtOH = 1.0 g ethanol per kg; 2.5 EtOH = 2.5 g ethanol per kg; Mem = 10.0 mg memantine per kg).

- A. Control/Control vs. Control/Ethanol (1.0 g/kg).
- B. Control/Control vs. Control/Ethanol (2.5 g/kg). * indicates a significant difference from "Control/Control", $P < 0.05$ (paired t-test).
- C. Control/Control vs. Memantine (10.0 mg/kg)/Control.
- D. Control/Control vs. Memantine (10.0 mg/kg)/Ethanol (1.0 g/kg). . * indicates a significant difference from "Control/Control", $P < 0.05$ (paired t-test). "a" indicates a significant difference from "CTL/EtOH (1.0 g/kg)", $P < 0.05$ (pooled two-sample t-Test).
- E. Control/Control vs. Memantine (10.0 mg/kg)/Ethanol (2.5 g/kg). . * indicates a significant difference from "Control/Control", $P < 0.05$ (paired t-test). "b" indicates a significant difference from "CTL/EtOH (1.0 g/kg)", $P < 0.05$ (pooled two-sample t-Test).



DISCUSSION

These results show that memantine reduced volitional consumption of ethanol by genetic drinking rats in a dose-dependent manner as indicated by reductions in both the amount of ethanol consumed and the proportion of ethanol to total fluids (water and ethanol) consumed. Differences in baseline levels of ethanol consumption between rats used in the first and second set of experiments are likely due to the rats being from different generations. The small effect on food consumption during the 3.0 mg/kg memantine treatments may have been due to handling. Since the effect on food intake in the mHEP rats was small, the decrease in ethanol consumption was not due to an anti-caloric effect.

Memantine, at a dose of 10.0 mg/kg, lowered ethanol consumption and did not produce anxiolytic properties in the elevated plus maze test. This lack of anxiolytic properties is consistent with a previous study which demonstrated that memantine (8 and 12 mg/kg) produced no effect on ethanol withdrawal anxiety in male Wistar rats in the elevated plus maze test (Kotlinska and Bochenski, 2008). The activity monitor indicated that memantine (10.0 mg/kg) produces brief, reversible sedating effects, and this dose partially reverses the sedating effect of ethanol. A previous report indicated that a decrease in cyclic adenosine monophosphate (cAMP)-dependent protein kinase (PKA) brain activity caused an attenuation of acute ethanol-induced sedative and uncoordinated effects and reduced ethanol inhibition of NMDA-induced behavioral responses in Sprague Dawley rats (Lai et al., 2007). These results suggested that the cAMP-PKA signaling pathway may be critical in the regulation of acute ethanol actions

(Lai et al., 2007). Therefore, the partial reversal of the ethanol-induced sedating effects in our study may be due to decreased PKA brain activity.

Memantine (10 and 25 mg/kg) has been shown to decrease alcohol drinking in male C57BL/6J mice in a schedule-induced polydipsia (SIP) task. Memantine (5 and 25 mg/kg) reduced regulatory water drinking during a 1-hour limited access water task. Memantine did not significantly alter water SIP (Escher et al., 2006). In our study, male mHEP rats were allowed 24 hour access to both water and ethanol which would be normal for a human alcoholic. Our results for the intake of ethanol and total fluids indicate that memantine did not significantly alter the consumption of water at any of the doses tested.

The DA and cAMP-regulated phosphoprotein, approximate molecular weight 32 kDa (DARPP-32), has been implicated as an intracellular regulator of ethanol-seeking behavior (Risinger et al., 2001) and of DA D1 receptor function (Fienberg et al., 1998). Maldve and colleagues (2002) proposed that a positive feedback system for ethanol consumption exists that involves activation of DA D1 receptors which diminishes ethanol sensitivity of NMDA receptors via the PKA/DARPP-32 cascade in principal neurons of the nucleus accumbens (NAc). According to the mechanism suggested, ethanol exposure causes depolarization of VTA neurons, subsequently increasing DA release in the NAc, which activates D1 receptors, thereby initiating the PKA/DARPP-32 cascade. This cascade causes phosphorylation of DARPP-32, decreasing activity of protein phosphatases and increasing phosphorylation of Ser-897 on the NR-1 subunit. This phosphorylation causes decreased sensitivity of the NMDA receptor to ethanol while maintaining a near normal level of NMDA receptor function and promoting long-term

modification of glutamatergic transmission. This suggests that memantine may reduce ethanol intake by decreasing DA release thereby blocking the PKA/DARPP-32 cascade.

The 10.0 mg/kg dose of memantine also produced a mild reversible hypothermic effect when administered alone as well as enhancing the hypothermic effect of ethanol in rats. It has been previously reported that this dose of memantine prevents the development of 5-HT syndrome hyperthermia in rats (Nisijima et al., 2004). Other reports indicate that at thirty minutes following treatment with anesthetic doses of ethanol (5 g/kg), the body temperature of Wistar rats declined by 2°C and memantine (9, 18, and 35 mg/kg) did not alter the change in body temperature (Beleslin et al., 1997). Together, these data suggest that memantine enhances hypothermia when ethanol is at sedating levels, but not anesthetic levels. It has been suggested that hypothermia induced by NMDA receptor antagonists may function as a mechanism for neuroprotection against serotonergic toxicity (Farfel and Seiden, 1995a; Farfel and Seiden, 1995b). These results suggest that memantine may decrease ethanol consumption with fewer side effects than other NMDA receptor antagonists.

There are several possible mechanisms by which memantine may reduce ethanol consumption in the genetic drinking rat. Memantine may substitute for ethanol at the NMDA receptor. Male Wistar rats trained to discriminate ethanol from saline generalized the ethanol cue to compounds that differed in every property except a noncompetitive antagonism at the NMDA receptor based on activity at the ion channel (Hundt et al., 1998). Reports indicate that memantine can produce a dose-dependent and complete substitution for ethanol in rats in a drug discrimination procedure (Bienkowski et al., 1998) and suggest that the reduction in ethanol consumption produced by memantine may be due to a substitution of the stimulus properties of

ethanol at the NMDA receptor (Rammes et al., 2001). Similarly, MK 801, which dose-dependently reduces volitional ethanol consumption in mHEP rats (McMillen et al., 2004), can substitute for ethanol in a discriminative stimulus paradigm (Schechter et al., 1993). Thus, memantine can partially reduce voluntary ethanol consumption in Wistar rats in a limited access procedure (cf. Kostowski and Bienkowski, 1999). A single dose of memantine can produce a moderate and non-dose-dependent decrease in ethanol consumption in the limited access procedure while producing non-selective effects on operant behavior in rats trained to lever press for ethanol in an oral self-administration procedure (Piasecki et al., 1998). These data suggest that substitution for ethanol at the NMDA receptor may be at least partially responsible for the ability of NMDA antagonists such as memantine and MK 801 to reduce volitional ethanol consumption.

Memantine has been shown to decrease alcohol craving in the absence of alcohol (Bisaga and Evans, 2004). However, based on the results of a double-blind placebo-controlled pilot trial using human subjects seeking outpatient treatment for problems related to alcohol use, it has been suggested that memantine is not an effective treatment for reducing alcohol use or alcohol craving in actively drinking alcohol-dependent patients. Memantine was also reported to produce undesirable side effects including gastrointestinal disturbance, lightheadedness/dizziness, anxiety, and disorientation/difficulty concentrating (Evans et al., 2007). Although the patients were heavy alcohol drinkers who met the DSM-IV criteria for alcohol dependence, the amount of alcohol consumed and the age of onset for regular alcohol consumption indicate that these were heavy drinkers, but not Type 2 alcoholics. In our study, we used the mHEP rat as a model for Type 2 alcoholism characterized by early onset of heavy alcohol consumption and a strong family history of severe alcoholism (Cloninger et al., 1988).

However, one limitation of our study was the inability to determine anti-social behavior in the mHEP rat.

An alternative mechanism by which memantine decreases volitional ethanol consumption may involve interactions with the dopaminergic reward pathway. Acute administration of 20 mg/kg i.p. memantine to awake animals produces no change in DA concentrations and only a slight increase in DA metabolites in microdialysates from the PFC (Hesselink et al., 1999). This suggests that memantine may increase DA metabolism without affecting DA release. However, other reports using similar microdialysis experiments indicate that memantine increases concentrations of DA in extracellular space in the PFC and STR of animals anesthetized with halothane (Quack et al., 1995; Spanagel et al., 1994). Previously, our laboratory showed that MK 801 protects DA content from amphetamine-induced neurotoxicity (McMillen et al., 1992). Other reports suggest that excitatory amino acids (EEAs) mediate tonic inhibition of DA terminal activity in the striatum by acting through NMDA receptors and this inhibition can be reduced in rats with partially lesioned dopaminergic nigrostriatal input to the striatum as indicated by a smaller effect of NMDA antagonism on DA synthesis in the nigrostriatal-lesioned rats than in unlesioned rats (Richard and Bennett, 1995). Together, these findings suggest that NMDA antagonists such as memantine may

While possible mechanisms by which memantine decreases voluntary ethanol consumption in genetic ethanol drinking rats have been discussed, the exact mechanism is still not known. However, the findings of this study and the relative safety of this drug in Alzheimer's disease patients suggests that a clinical trial in alcohol-dependent individuals with a clear genetic history for alcoholism should be relatively safe and may demonstrate the potential usefulness of memantine as an aid in maintaining sobriety.

Memantine may also be useful as an adjunct therapy for alcohol dependence. In a previous study, a low, sub-effective dose of memantine (1.0 mg/kg) in combination with low doses of naltrexone blocked the reinforcing properties of ethanol in adult male Wistar rats. It was suggested that this combination therapy might be effective for the treatment of alcoholism in patients highly sensitive to the adverse side effects of naltrexone (Kuzmin et al., 2008). Further studies will help elucidate the mechanism by which this drug produces its effect on ethanol drinking behavior.

CHAPTER 3

EFFECTS OF DOPAMINERGIC D1 AND D2 DRUGS ON THE VOLITIONAL
CONSUMPTION OF ETHANOL BY GENETIC DRINKING MHEP RATS IN A TWO-
CHOICE 24 HOUR ACCESS PARADIGM

ABSTRACT

This study aimed to pharmacologically determine the roles of dopaminergic D1 and D2 receptors on volitional ethanol consumption by genetic drinking rats in a two-choice 24 hour access paradigm. The D1 antagonist SCH23390 (0.1 and 0.3 mg/kg) and (+)sulpiride (10.0 mg/kg), an isomer inactive at the D2 receptor, significantly decreased ethanol consumption while the D2-active antagonist (-)sulpiride (10.0 mg/kg) did not. Co-administered with SCH23390 (0.3 mg/kg), (-)sulpiride (3.0 and 10.0 mg/kg) did not alter SCH23390-induced reductions in ethanol consumption. At the doses tested, SCH23390, (+)sulpiride and (-)sulpiride altered neither proportion of ethanol to total fluids consumed nor food intake. The D1 receptor agonist SKF38393 (1.25, 2.5, 5.0 and 10.0 mg/kg) dose-dependently decreased ethanol consumption. Co-administered SCH23390 and SKF3839 produced effects similar to those of each drug alone. These data suggest that D1 receptors may exhibit an optimal level of activation and any deviation from that level of activity reduces volitional ethanol consumption.

Previous experiments indicated that the NMDA receptor antagonist memantine decreased volitional ethanol consumption in the mHEP rat, a validated animal model of alcoholism. In this study, co-administered memantine (1.0 mg/kg) and SCH23390 (0.3 mg/kg) reduced ethanol consumption, but did not produce an additive or synergistic effect.

INTRODUCTION

The mesolimbic DA system is a site of action for the reinforcing effects of ethanol and ethanol self-administration (Carroll et al., 2006; Gonzales et al., 2004; Hodge et al., 1997; Lof et al., 2007; Phillips et al., 1998; Risinger et al., 2000; Thielen et al., 2004). More specifically, evidence indicates that the mesoaccumbal DA system is involved (cf. Pierce and Kumaresan, 2006; Yim and Gonzales, 2000). Increased DA transmission in limbic regions of the brain, and specifically the NAc, partially mediates ethanol reinforcement. Studies suggest that ethanol reinforcement is due to ethanol-induced elevations in the firing rates of dopaminergic neurons in the VTA producing increased DA release in the NAc (cf. Pierce and Kumaresan, 2006). It has been suggested that the ethanol-induced positive feedback loop may involve somatodendritically released DA activating DA D1 receptors on glutamatergic terminals, thereby increasing glutamate release and subsequently leading to a rise in VTA neuronal firing which then increases DA release (Xiao et al., 2009). By regulating dopaminergic cell firing, synaptic release, or a combination of effects, ethanol may stimulate DA release which increases extracellular DA levels in the NAc (Yim and Gonzales, 2000). Together, these reports suggest that DA receptor agonists and antagonists can be used to influence ethanol reinforcement.

Self-administration studies in which animals were trained to emit an operant response to obtain a drug reinforcer have demonstrated that pretreatment with DA D1-like and D2-like receptor agonists and antagonists alter ethanol self-administration, thereby suggesting that accumbal DA D1 and D2 receptors play a critical role in the reinforcing effects of ethanol (cf. Pierce and Kumaresan, 2006). Furthermore, D1-like

and D2-like receptors in the NAc may interact to regulate ethanol self-administration in a manner similar to their interactive regulation of other behaviors (Hodge et al., 1997). In a limited 4 hour ethanol access paradigm using high-alcohol-drinking (HAD) rats and subcutaneous (s.c.) drug injections, quinpirole, a DA D2 receptor agonist, dose-dependently decreased ethanol consumption throughout the access period. Spiperone, a D2 and 5-HT_{2A} receptor antagonist, produced no effect initially, but increased ethanol intake at lower doses and decreased intake at higher doses by the end of the 4-hour access period. SCH23390, a DA D1 receptor antagonist, dose-dependently decreased ethanol consumption during the first hour of access. SKF38393, a DA D1 receptor agonist, SKF38393, also reduced ethanol intake, but less effectively than SCH23390 (Dyr et al., 1993). In another study, local microinfusion of the DA D2 receptor antagonist sulpiride increased ethanol intake by alcohol-preferring (P) rats (McBride et al., 1993).

It has been suggested that inconsistent findings among studies may be due partially to the animal model of ethanol consumption used and the type of experiment performed. The route by which ethanol is administered may also be involved in differential effects of ethanol in the CNS and DA release (cf. Tupala and Tiihonen, 2004). In the present study, we pharmacologically examined the roles of dopaminergic D1 and D2 receptors on volitional consumption of ethanol by genetic drinking rats in a two-choice 24 hour access paradigm. Our experiments determined the effects of (+)sulpiride, an inactive isomer at the DA D2 receptor, (-)sulpiride, an active isomer at the DA D2 receptor, SCH23390, and SKF38393 on volitional intake of ethanol by the male mHEP rat. A validated animal model of alcoholism, the mHEP rat is characterized by the volitional consumption of profuse volumes of ethanol, a high level of blood ethanol corresponding to the high volume of ethanol consumed, and a preference for ethanol in

the presence of palatable alternatives (Myers et al., 1998). The doses of drugs used were based on doses previously used in other laboratories.

Previously, the noncompetitive NMDA receptor antagonist memantine had been shown to decrease volitional ethanol consumption in the mHEP rat. In this study, we aimed to determine if the combination of memantine and a DA receptor selective drug shown to decrease ethanol consumption produces an additive or synergistic effect on the decrease in drinking. Since we found that the DA D1 antagonist SCH23390 greatly decreased volitional ethanol intake in the mHEP rat, we tested the combination of this drug with a dose of memantine that reduced ethanol consumption.

MATERIALS AND METHODS

Drugs

SCH23390 maleate (Schering Corporation, Bloomfield, NJ), (+)sulpiride (Ravizza, Milano, Italy), and (-)sulpiride (Sigma-Aldrich, Inc., St. Louis, MO) were dissolved in deionized water and acetic acid. SKF38393 hydrochloride (Sigma-Aldrich, Inc., St. Louis, MO) and memantine hydrochloride (Sigma-Aldrich, Inc., St. Louis, MO) were dissolved in deionized water and doses were calculated as the free base. In all experiments, deionized water or 0.9% saline was used as the vehicle. Memantine was injected i.p. All other drugs and vehicle were administered via s.c. injections. Ethanol provided for consumption was derived by diluting 95% ethanol in tap water.

Subjects and Screening

Male mHEP rats were selected from the F23, F24, and F25 generations of breeding in the East Carolina University colony. This line of rats was originally derived from the cross-breeding of three male alcohol-preferring (P) rats (T.-K. Li of the Indiana University Alcohol Research Center) and three female Sprague Dawley rats (Harlan Sprague Dawley Inc.) with the selection of rats based on an ethanol drinking screen. The line of mHEP rats is maintained by breeding non-sibling males and females whose selection is based on an ethanol drinking screen. The rats were initially housed with continuous access to food and water and maintained on a 12 h on/12 h off light cycle in a temperature-controlled room. All procedures were in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals and approved by the Brody

School of Medicine at East Carolina University Institutional Animal Care and Use Committee.

At 60 days of age, each male mHEP rat was placed in a suspended stainless steel cage with free access to three glass drinking tubes mounted on the front. One tube was filled with water and the other two tubes remained empty while the rats underwent a one-day adaptation period. On the second day, one tube was filled with water, one was filled with 3% ethanol (v/v), and one remained empty. Thereafter, the concentration of ethanol was increased daily to include 5, 7, 9, 11, 13, 15, 20, 25, and 30%. Rats were allowed free access to food at all times. Body weight and amounts of water, ethanol solution, and food consumed were recorded daily for each rat. To prevent a place preference, the positions of the bottles were rotated daily according to a randomized schedule. Three bottles, of which one remained empty, were used to prevent a side preference. For each rat, the maximally preferred concentration of ethanol was determined as the concentration of ethanol that produced maximal ethanol consumption with the proportion of ethanol consumed to total fluids consumed being closest to 0.5. The maximally preferred concentration of ethanol for each rat was provided for the remainder of the experiment. The average preferred ethanol concentration for each group of rats used in this study was 14%. Ethanol consumption was allowed to stabilize before the onset of drug or vehicle injections. Rats were continuously maintained on the same 12 h on/12 h off light cycle in a temperature-controlled room.

Experimental Procedures

For each regimen of treatments, rats underwent a 3-day baseline period followed by a 3-day treatment period and a subsequent 3-day post-treatment period. During each

3-day treatment period, drug(s) or vehicle was/were injected two hours before and two hours following lights out. In all experiments, drugs and vehicle were administered in a counter-balanced design. Upon stabilization of ethanol consumption to baseline levels, a different dose of drug, a different drug or drug combination, or vehicle was administered. For each drug or vehicle treatment, data for each pre-treatment, treatment, and post-treatment period for each rat were averaged, grouped, and analyzed. Results from each experiment included volume of ethanol consumption, proportion of ethanol to total liquids consumed, volume of total liquid consumption, volume of food consumption, and body weight. Statistical analyses were performed using GB-STAT (Dynamic Microsystems, Silver Spring, MD) and graphs were generated using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA).

Initially, (+)sulpiride (10.0 mg/kg), (-)sulpiride (3.0 and 10.0 mg/kg), SCH23390 (0.1 and 0.3 mg/kg), combinations of (-)sulpiride (3.0 and 10.0 mg/kg) and SCH23390 (0.3 mg/kg), or vehicle were administered to the first group of rats. Each rat underwent up to four different treatment regimens with seven to ten rats receiving each treatment. Another eight rats received injections of SKF38393 (1.25, 2.5, 5.0 and 10.0 mg/kg) and vehicle to determine the effect of a DA D1 receptor agonist on volitional ethanol consumption. To examine the combined effect of a DA D1 receptor agonist and a DA D1 receptor antagonist, SKF38393 (5.0 mg/kg) and SCH23390 (0.3 mg/kg) were co-administered and vehicle was administered to a group of six rats. Data from these experiments were analyzed using two-way repeated measures ANOVA and Tukey/Kramer procedure post hoc with significance taken at $P < 0.05$. Results for SKF38393 (5.0 mg/kg) and SCH23390 (0.3 mg/kg) administered alone and co-administered were converted to percentages with pre-treatment values being

established as 100%. Percent change from pre-treatment baseline was determined for individual and combination drug treatments and compared using the Kruskal-Wallis one-way ANOVA with significance taken at $P < 0.05$.

To examine the combined effects of memantine and SCH23390 on ethanol drinking behavior, six male mHEP rats selected from the F24 generation were treated with vehicle or co-administered memantine (1.0 mg/kg) and SCH23390 (0.3 mg/kg) in a counter-balanced design. Data from these experiments were analyzed using two-way repeated measures ANOVA and Tukey/Kramer procedure post hoc with significance taken at $P < 0.05$.

RESULTS

Figure 3.1 shows the effects of (+)sulpiride, (-)sulpiride, and SCH23390 on volitional consumption of ethanol, total fluids, and food by male mHEP rats. (+)Sulpiride (10.0 mg/kg) decreased ethanol consumption by 21% ($F_{2,9} = 6.08$, $P < 0.05$) and total fluid consumption by 11% ($F_{2,9} = 4.20$, $P < 0.05$). SCH23390 (0.1 and 0.3 mg/kg) reduced ethanol consumption by 32% ($F_{2,7} = 26.36$, $P < 0.05$) and 53% ($F_{2,7} = 25.52$, $P < 0.05$), respectively, and total fluid consumption by 23% ($F_{2,7} = 13.70$, $P < 0.05$) and 38% ($F_{2,7} = 42.29$, $P < 0.05$), respectively. (-)Sulpiride (3.0 and 10.0 mg/kg) alone did not significantly alter ethanol consumption or total fluid consumption. However, co-administration of (-)sulpiride (3.0 or 10.0 mg/kg) and SCH23390 (0.3 mg/kg) decreased ethanol consumption by 23% ($F_{2,6} = 23.59$, $P < 0.05$) and 43% ($F_{2,7} = 9.86$, $P < 0.05$), respectively, and total fluid consumption by 32% ($F_{2,6} = 23.02$, $P < 0.05$) and 35% ($F_{2,6} = 38.54$, $P < 0.05$), respectively. Treatment with vehicle reduced ethanol consumption by 22% ($F_{2,6} = 6.99$, $P < 0.05$) and total fluid consumption by 16% ($F_{2,6} = 28.30$, $P < 0.05$). The effects observed during vehicle treatment may be due to handling. None of the treatments significantly altered the proportion of ethanol to total fluids consumed or the consumption of food. During treatment with vehicle, (+)sulpiride (10.0 mg/kg), (-)sulpiride (3.0 mg/kg), SCH23390 (0.1 and 0.3 mg/kg), and co-administered (-)sulpiride (3.0 mg/kg) and SCH23390 (0.3 mg/kg), rats gained small, but significant amounts of weight. None of the treatments produced significant body weight loss.

Figure 3.2 shows the effects of SKF38393 (1.25, 2.5, 5.0, and 10.0 mg/kg) on ethanol, total fluid, and food consumption. SKF38393 significantly decreased ethanol intake relative to pre-treatment levels ($F_{2,33} = 71.45$, $P < 0.05$) at each dose tested with

Figure 3.1. Effects of (+)sulpiride, (-)sulpiride, and SCH23390 on the volitional consumption of solutions of ethanol by the male mHEP rat. Each bar represents the mean \pm S.E.M. Number of rats for each treatment is indicated in parentheses. [(+)SULP = (+)sulpiride; (-)SULP = (-)sulpiride; SCH = SCH23390].

- A. Volitional consumption of ethanol. * indicates a significant difference from pre-treatment baseline, $P < 0.05$ (Tukey/Kramer).
- B. Proportion of ethanol to total fluids (water and ethanol) consumed.
- C. Consumption of total fluids (water and ethanol). * indicates a significant difference from pre-treatment baseline, $P < 0.05$ (Tukey/Kramer).
- D. Consumption of food.
- E. Body weight. * indicates a significant difference from pre-treatment baseline, $P < 0.05$ (Tukey/Kramer).

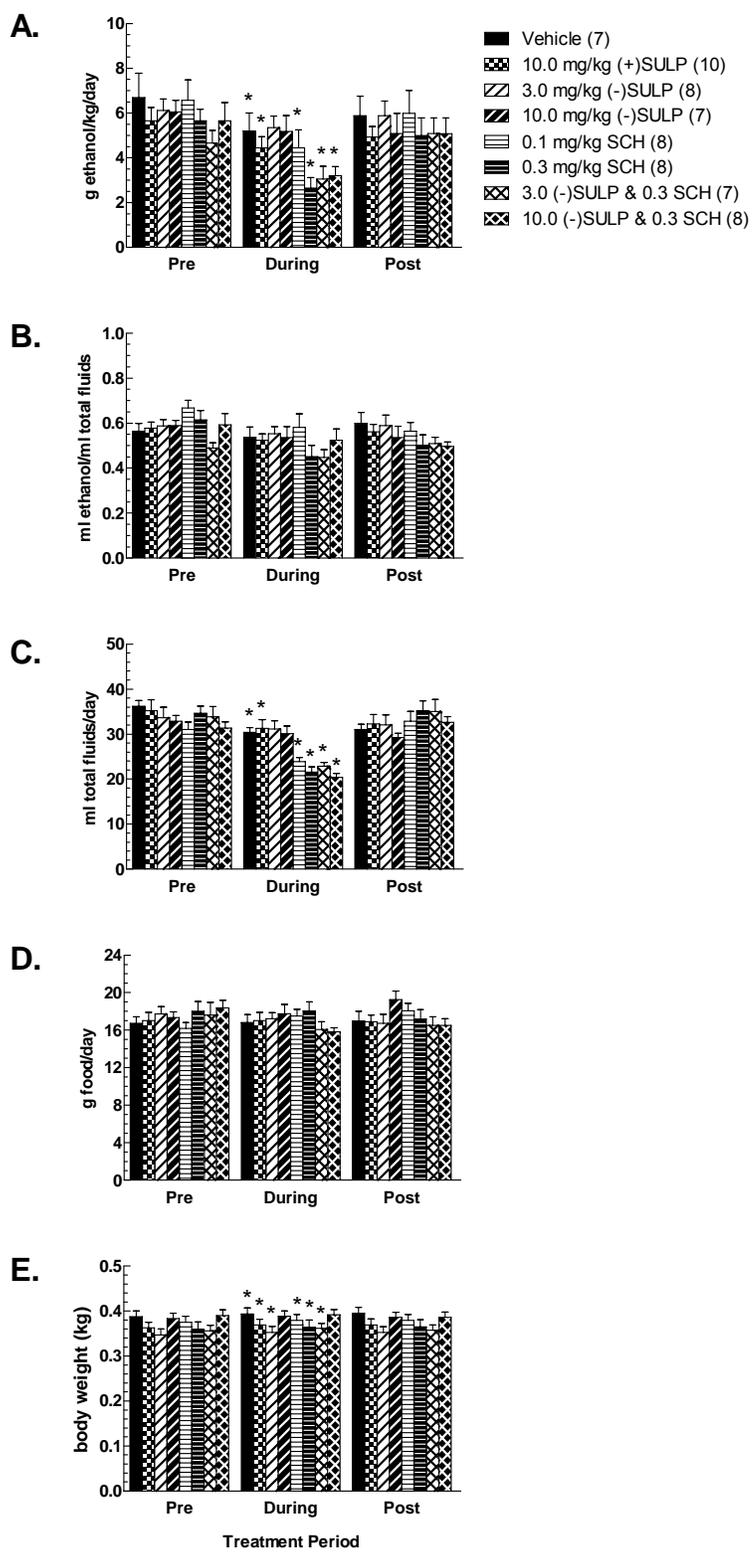
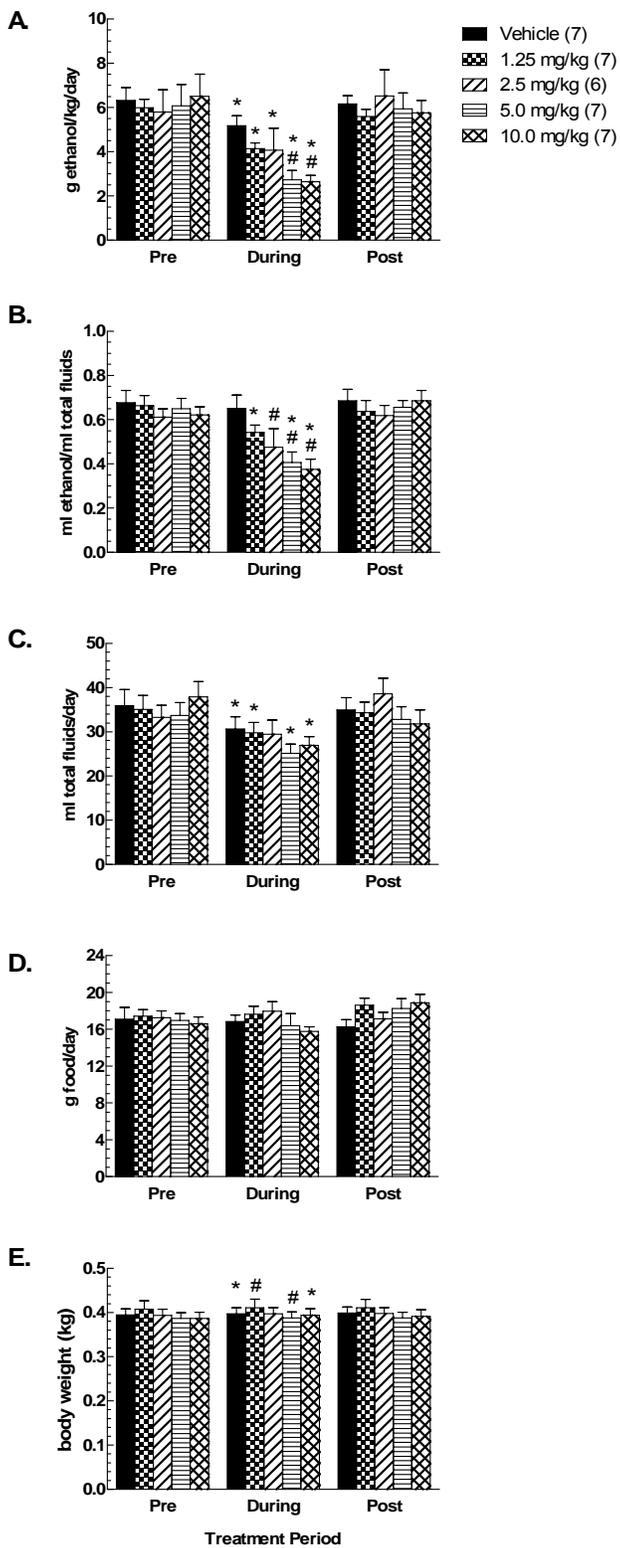


Figure 3.2. Effect of SKF38393 on the volitional consumption of solutions of ethanol by the male mHEP rat. Each bar represents the mean \pm S.E.M. Number of rats for each treatment is indicated in parentheses.

- A. Volitional consumption of ethanol. * indicates a significant difference from pre-treatment baseline, $P < 0.05$ (Tukey/Kramer). # indicates a significant difference from vehicle treatment, $P < 0.05$.
- B. Proportion of ethanol to total fluids (water and ethanol) consumed. * indicates a significant difference from pre-treatment baseline, $P < 0.05$ (Tukey/Kramer). # indicates a significant difference from vehicle treatment, $P < 0.05$.
- C. Consumption of total fluids (water and ethanol). * indicates a significant difference from pre-treatment baseline, $P < 0.05$ (Tukey/Kramer).
- D. Consumption of food.
- E. Body weight. * indicates a significant difference from pre-treatment baseline, $P < 0.05$ (Tukey/Kramer). # indicates a significant difference from vehicle treatment, $P < 0.05$.

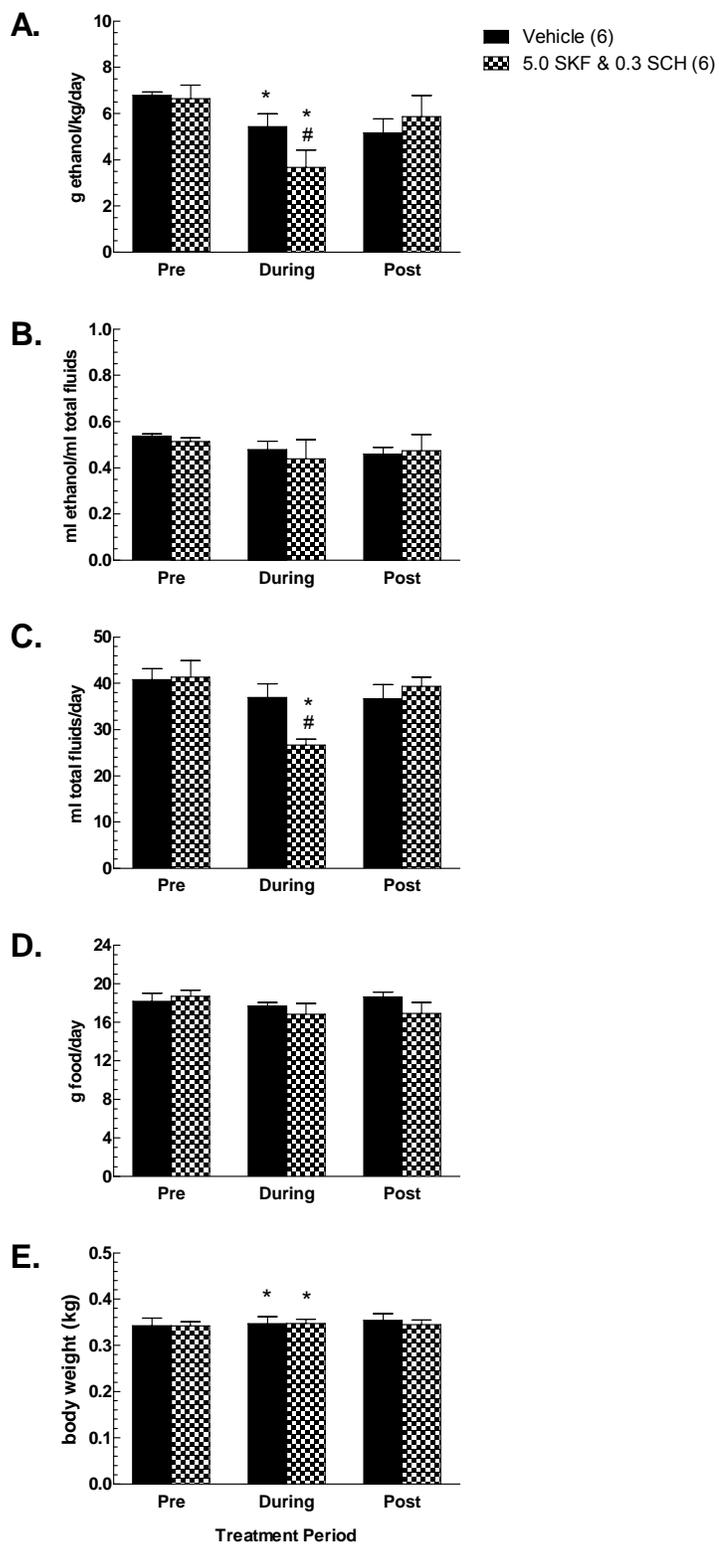


the 10.0 mg/kg dose producing a 58% decrease. At both 5.0 and 10.0 mg/kg doses, SKF38393 significantly reduced ethanol consumption relative to vehicle treatment by 47%, indicating a treatment effect ($F_{8,101} = 3.47$, $P < 0.05$). At the 1.25, 2.5, 5.0, and 10.0 mg/kg doses, SKF38393 decreased the proportion of ethanol to total fluids consumed compared to pre-treatment levels with the highest dose producing a 39% reduction ($F_{2,33} = 60.26$, $P < 0.05$). The 5.0 and 10.0 mg/kg doses also produced a treatment effect on proportion, indicated by reductions of 38% and 42%, respectively, from vehicle levels ($F_{8,101} = 5.16$, $P < 0.05$). The 1.25, 5.0, and 10.0 mg/kg doses of SKF38393 also decreased total fluid intake compared to pre-treatment levels with the highest dose producing a 29% reduction ($F_{2,33} = 40.37$, $P < 0.05$). Treatment with vehicle decreased ethanol consumption by 18% and total fluid consumption by 15% from the pre-treatment level, possibly caused by handling. None of the treatments significantly altered food consumption. During treatments with vehicle and the 10.0 mg/kg dose of SKF38393, rats gained small, but significant, amounts of weight ($F_{2,33} = 16.11$, $P < 0.05$). Differences in body weight between vehicle treatment periods and SKF38393 (1.25 and 5.0 mg/kg) treatment periods are most likely due to similar differences existing during the 3-day pre-treatment periods.

Figure 3.3 shows the effects of co-administered SKF38393 (5.0 mg/kg) and SCH23390 (0.3 mg/kg) on ethanol, total fluid, and food consumption. Co-administration of these drugs reduced ethanol consumption and total fluid consumption from pre-treatment levels by 45% ($F_{2,11} = 30.56$, $P < 0.05$) and 35% ($F_{2,11} = 23.63$, $P < 0.05$), respectively. This combination also decreased ethanol consumption and total liquid consumption from vehicle treatment levels by 32% ($F_{2,35} = 10.06$, $P < 0.05$) and 28% ($F_{2,35} = 12.50$, $P < 0.05$), respectively. Vehicle treatment produced a 20% reduction from

Figure 3.3. Effect of co-administered SKF38393 and SCH23390 on the volitional consumption of solutions of ethanol by the male mHEP rat. Each bar represents the mean \pm S.E.M. Number of rats for each treatment is indicated in parentheses. (SKF = SKF38393; SCH = SCH23390).

- A. Volitional consumption of ethanol. * indicates a significant difference from pre-treatment baseline, $P < 0.05$ (Tukey/Kramer). # indicates a significant difference from vehicle treatment, $P < 0.05$.
- B. Proportion of ethanol to total fluids (water and ethanol) consumed.
- C. Consumption of total fluids (water and ethanol). * indicates a significant difference from pre-treatment baseline, $P < 0.05$ (Tukey/Kramer). # indicates a significant difference from vehicle treatment, $P < 0.05$.
- D. Consumption of food.
- E. Body weight. * indicates a significant difference from pre-treatment baseline, $P < 0.05$ (Tukey/Kramer).



the pre-treatment level for ethanol intake. No significant effects on proportion or food consumption were observed. Rats treated with vehicle and with co-administered SKF38393 (5.0 mg/kg) and SCH23390 (0.3 mg/kg) gained small, but significant amounts of weight ($F_{2,11} = 30.39$, $P < 0.05$).

Figure 3.4 compares the effects of SKF38393 (5.0 mg/kg) and SCH23390 (0.3 mg/kg) administered alone and together on ethanol, total fluid, and food consumption. Results indicate no significant treatment effect (Kruskal-Wallis, $P < 0.05$) with differences of 7% for ethanol consumption, 22% for proportion, 15% for total liquid consumption, 11% for food consumption, and 1% for body weight.

Figure 3.5 shows the effects of co-administered memantine (1.0 mg/kg) and SCH23390 (0.3 mg/kg) on ethanol, total fluid, and food consumption. The repeated measures two-way ANOVA indicated an effect of treatment period on ethanol consumption ($F_{2,21} = 29.38$, $P < 0.05$), total fluid consumption ($F_{2,21} = 55.63$, $P < 0.05$), and body weight ($F_{2,21} = 96.03$, $P < 0.05$). The ANOVA also indicated a significant interaction between drug treatment and treatment period for ethanol intake ($F_{2,65} = 12.53$, $P < 0.05$), total fluid intake ($F_{2,65} = 45.90$, $P < 0.05$), and body weight ($F_{2,65} = 11.61$, $P < 0.05$). Co-administration of these drugs reduced ethanol consumption and total fluid consumption from pre-treatment levels by 41% and 37%, respectively. This combination also decreased ethanol consumption and total liquid consumption from vehicle treatment levels by 36% and 30%, respectively. Vehicle treatment produced a small, but significant reduction of food intake by 6% from pre-treatment levels. No significant effects on proportion of ethanol to total fluids consumed were observed. During treatments with both vehicle and co-administration of memantine and SCH23390, rats gained small, but significant amounts (2%) of weight. The small, but significant

Figure 3.4. Percent change from pre-treatment baseline for SKF38393 and SCH23390 administered alone and co-administered. Each bar represents the mean \pm S.E.M. Number of rats for each treatment is indicated in parentheses. No significant difference was observed between treatments ($P < 0.05$, Kruskal-Wallis). [SKF = SKF38393; SCH = SCH23390; 5.0 SKF = SKF38393 (5.0 mg/kg); 0.3 SCH = SCH23390 (0.3 mg/kg)].

- A. Volitional consumption of ethanol.
- B. Proportion of ethanol to total fluids (water and ethanol) consumed.
- C. Consumption of total fluids (water and ethanol).
- D. Consumption of food.
- E. Body weight.

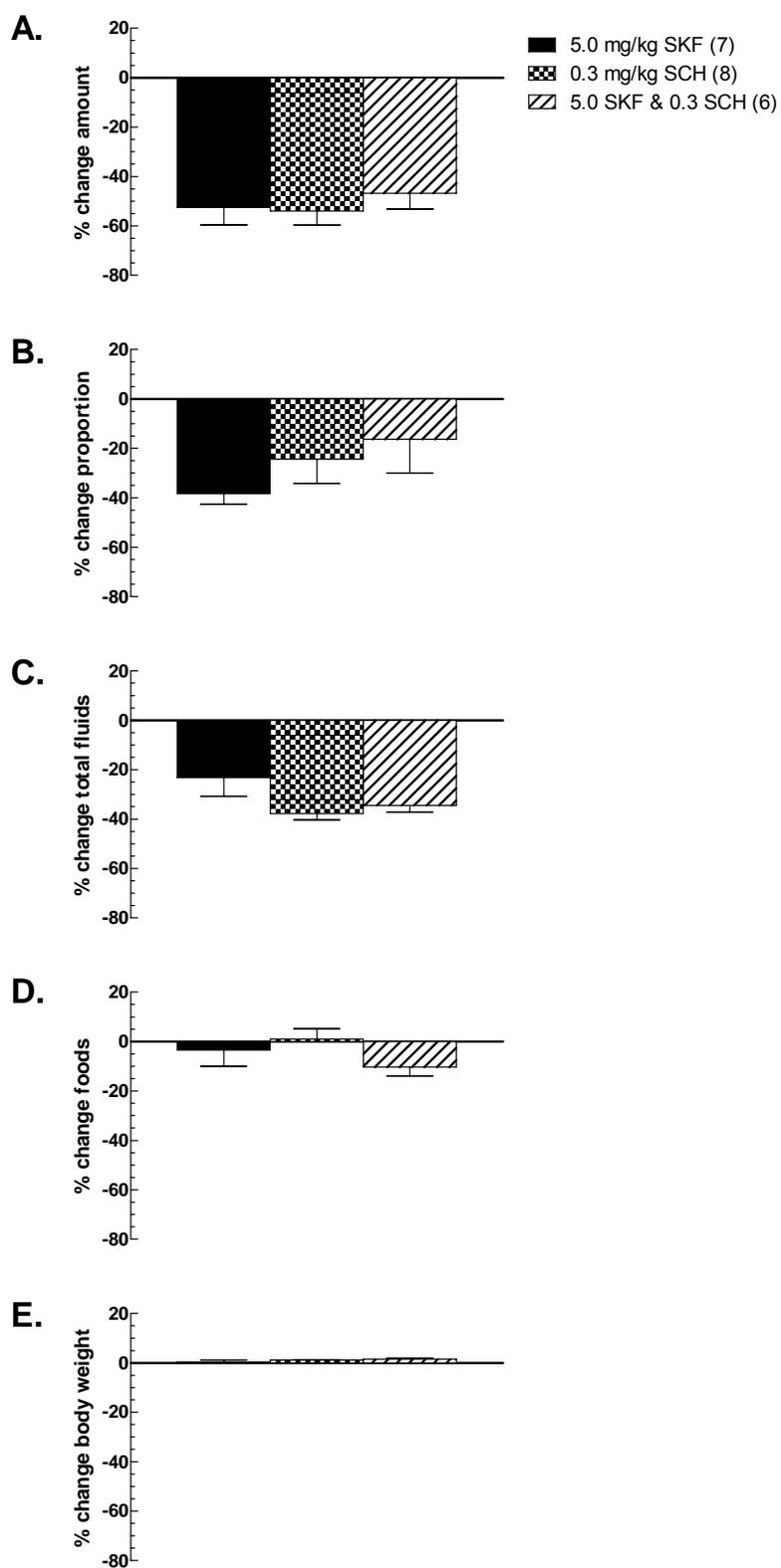
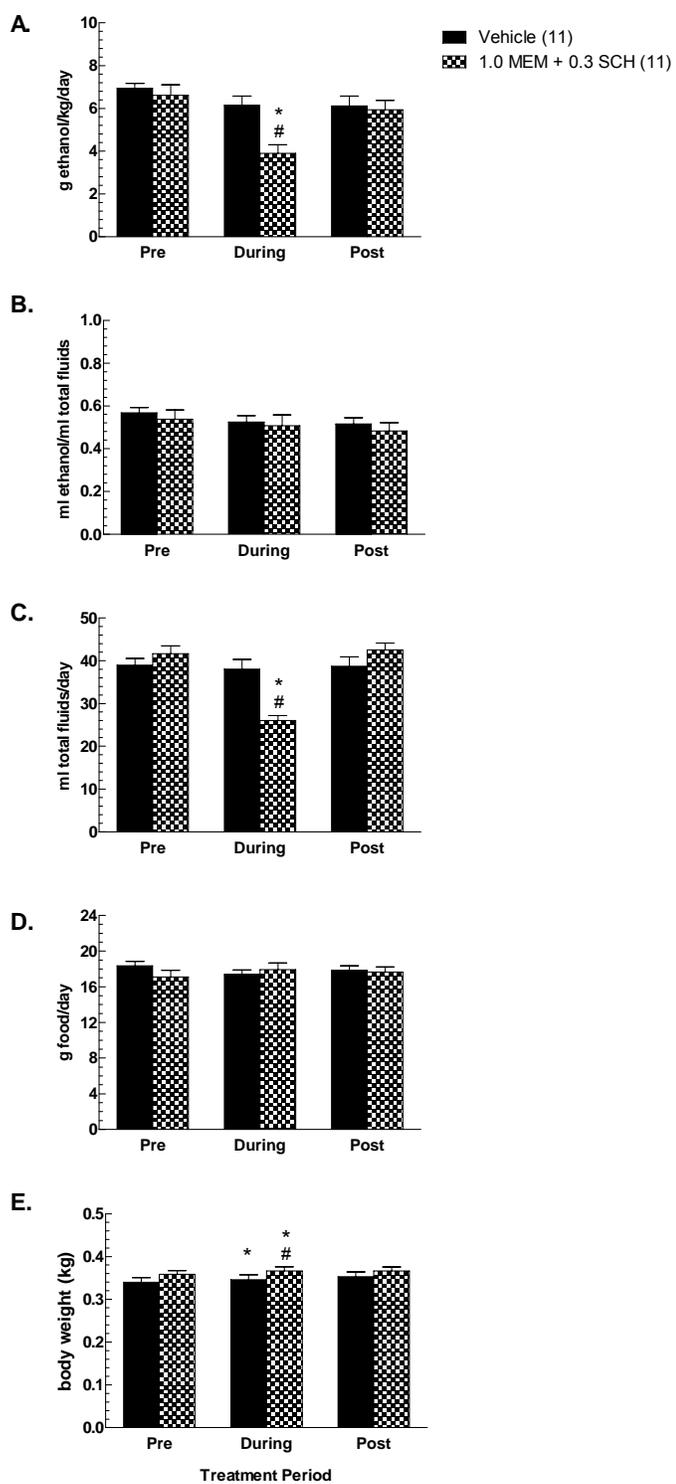


Fig. 3.5. Effect of co-administered memantine and SCH23390 on the volitional consumption of solutions of ethanol by the male mHEP rat. Each bar represents the mean \pm S.E.M. Number of rats for each treatment is indicated in parentheses. [1.0 MEM = Memantine (1.0 mg/kg); 0.3 SCH = SCH23390 (0.3 mg/kg)].

- A. Volitional consumption of ethanol. * indicates a significant difference from pre-treatment baseline, $P < 0.05$ (Tukey/Kramer). # indicates a significant difference from vehicle treatment, $P < 0.05$.
- B. Proportion of ethanol to total fluids (water and ethanol) consumed.
- C. Consumption of total fluids (water and ethanol). * indicates a significant difference from pre-treatment baseline, $P < 0.05$ (Tukey/Kramer). # indicates a significant difference from vehicle treatment, $P < 0.05$.
- D. Consumption of food.
- E. Body weight. * indicates a significant difference from pre-treatment baseline, $P < 0.05$ (Tukey/Kramer). # indicates a significant difference from vehicle treatment, $P < 0.05$.



difference (3%) in body weight for the two treatment groups was due to the same difference (3%) being present during the pre-treatment period.

DISCUSSION

The reductions in ethanol consumption and total fluid consumption do not appear to be anti-caloric since no significant reductions in food consumption were observed for any of the treatment groups. Furthermore, no significant reductions in body weight were observed. While the DA D1 antagonist SCH23390 (0.1 and 0.3 mg/kg) decreases volitional ethanol intake, co-administration of the DA D2 antagonist (-)sulpiride (3.0 and 10.0 mg/kg) with SCH23390 (0.3 mg/kg) neither enhances nor inhibits this effect. The DA D1 agonist SKF38393 dose-dependently reduces volitional ethanol consumption with the 5.0 mg/kg dose producing effects on ethanol consumption similar to those resulting from treatment with SCH23390 (0.3 mg/kg). Co-administration of SK38393 (5.0 mg/kg) and SCH23390 (0.3 mg/kg) produce effects that do not differ from those resulting from administration of each drug alone. These data indicate that the two drugs neither block nor enhance the effect of each other when co-administered at the doses tested. The combined results of this study suggest that the DA D1 receptors exhibit an optimal level of activation and any deviation from that level of activity produces a decrease in volitional ethanol consumption.

In previous experiments, memantine decreased volitional ethanol consumption in an apparent dose-dependent manner with the 1.0 mg/kg dose producing a 28% reduction. In this study, the DA D1 antagonist SCH23390 (0.3 mg/kg) decreased volitional ethanol consumption by 53%. Co-administration of these two drugs at these doses produced a 41% reduction in ethanol consumption which suggests that these drugs are not producing an additive or synergistic effect. Since SCH23390, alone, decreased total fluid intake, but did not change proportion of ethanol to total fluids

consumed, while memantine, alone, decreased ethanol intake and proportion of ethanol to total fluids consumed, this suggests that the effects on ethanol and total fluid intake observed when the two drugs are combined are caused by SCH23390. As previously indicated, SCH23390 did block the effects of agonist SKF38393 on ethanol consumption. Therefore, the combination of memantine and SKF38393, which remain to be tested, would be expected to produce an additive or synergistic effect on ethanol consumption.

Previous studies indicate that acute ethanol exposure acts within the mesolimbic DA system, ongoing ethanol intake alters the effects of ethanol on the mesolimbic DA system, and past ethanol consumption produces prolonged neuroadaptations within the mesolimbic DA system (Carroll et al., 2006). Microdialysis studies with freely moving, conscious male Sprague Dawley rats indicate that an acute administration i.p. of a moderate dose (1.0 g/kg) of ethanol increases the concentration of extracellular DA in the nucleus accumbens, with no dramatic blockade of DA uptake. This suggests that ethanol increases extracellular DA by increasing DA release from the terminal (Yim and Gonzales, 2000). In a previous study, DA uptake experiments indicated that chronic ethanol consumption increases the function of the DA transporter (DAT) in the High-Alcohol-Drinking replicate line 1 (HAD-1) rats. However, it is uncertain if the change is due to a change in the number of available transporters or a change in the velocity of uptake (Carroll et al., 2006). Together, these reports suggest that rats with unlimited access to ethanol, as in our experimental paradigm, may undergo altered DA transport in the mesolimbic system. The mechanisms of action for the D1- and D2-receptor agonists and antagonists do not involve DA uptake.

The reward associated with drugs of abuse activates the central reward circuitry directly, rather than via peripheral nerves. Although drugs are transported by circulation to all brain regions, many drugs act only at specific receptors restricted to specific classes of neurons. Many studies have implicated dopaminergic neurons as one of the central components in brain reward. Although the specific site in the brain at which ethanol produces its rewarding effect is uncertain, evidence indicates that ethanol activates the mesolimbic DA system and has psychomotor stimulant actions. However, reward circuitry is multisynaptic with DA being one of several possible reward transmitters and DA neurons being one of several pathways for rewards (cf. Wise and Rompre, 1989). Multiple reports have indicated that drugs known to act at DA receptors may also function at other types of receptors to reduce ethanol consumption. Studies suggest that systemically administered SKF38393 most likely reduces ethanol consumption by acting through DA or other receptor systems, i.e. 5-HT, in brain regions other than the NAc (Hodge et al., 1997). Acute and chronic administration of two DA receptor partial agonists, SDZ 208-911 and terguride, have been shown to significantly decrease ethanol consumption without affecting water intake in male albino Wistar rats using a free-choice limited access paradigm (Bono et al., 1996). Both SDZ 208-911 and terguride have a high affinity for both D2 and central alpha receptors and a somewhat lower affinity for 5-HT_{1A} receptors (Svensson et al., 1991).

In our experiments, we observed a significant reduction in ethanol consumption induced by the partial agonist SKF38393. In a previous study, SKF38393, along with several other DA D1 receptor agonists, has been shown to decrease ethanol self-administration at doses lower than those that significantly increase locomotor activity in

rats and has supported the involvement of DA D1 receptors in reward processes (Cohen et al., 1999).

Another study reported that microinjection of the D1 antagonist SCH23390 in the nucleus accumbens of female alcohol-preferring (P) rats did not significantly alter ethanol intake (Levy et al., 1991). Other reports indicated that SCH23390, at doses of 0.03 and 0.1 mg/kg, significantly decreased water intake, but did not alter ethanol intake in male Sprague Dawley rats in a 1 hour food- and ethanol-limited access paradigm with 24 hour free access to water. The reduction in water consumption was attributed to a non-specific decrease in consummatory behavior (Silvestre et al., 1996). In our experiments, we found that SCH23390, at the doses we tested (0.1 and 0.3 mg/kg), significantly reduced ethanol consumption in the mHEP rat using a two-choice free access paradigm. The differences between the results from our study and the previous reports may be due to the different animal strains and experimental paradigms.

Additional experiments are needed to test the effects of DA D2 receptor agonists and the combination of memantine and the DA D1 receptor agonist SKF38393 on ethanol consumption in mHEP rats using the two-choice 24 hour access paradigm. Further studies are also needed to determine potential locomotor and sedating effects of DA agents at doses shown to decrease volitional ethanol consumption in mHEP rats.

CHAPTER 4

EFFECTS OF GROUP II METABOTROPIC GLUTAMATE AGONIST AND ANTAGONIST DRUGS ON VOLITIONAL ETHANOL CONSUMPTION BY GENETIC DRINKING RATS

ABSTRACT

Antagonists of both NMDA and group I metabotropic glutamate receptors reduce the consumption of ethanol by rodents. This study aimed to determine the effects of group II metabotropic glutamate receptor (mGluR 2/3) agonist and antagonist drugs on volitional consumption of ethanol by genetic drinking rats. Volitional ethanol consumption in a two-choice paradigm was determined for male Myers' high ethanol-preferring (mHEP) rats. After establishment of the preferred ethanol concentration for each rat in a 10-day 'step-up' test of 3-30% v/v ethanol versus water, the concentration producing a proportion closest to 50% was used as each rat's fixed concentration. Rats underwent 3-day baseline, 3-day drug injection b.i.d., and 3-day post-treatment periods and, after recovery of drinking, the sequence was repeated with a different dose of drug. Order of administration of drugs and vehicle was made in a counter-balanced design. Rats received s.c. injections of vehicle and mGluR 2/3 agonist LY379268 (0.3, 1.0, and 3.0 mg/kg) or mGluR 2/3 antagonist LY341495 (0.3, 1.0, and 3.0 mg/kg). At the 3.0 mg/kg dose, LY379268 decreased ethanol consumption by 32%, food intake by 33%, and total liquid (ethanol and water) consumption by 22% from baseline. At the 3.0 mg/kg dose of LY341495, ethanol consumption and total liquid consumption declined, respectively, by 44% and 32% while food intake increased non-significantly by 5%. Furthermore, LY341495 reduced the proportion of ethanol to total liquids consumed. The 3.0 mg/kg dose of LY379268 did not significantly affect the ability of Sprague Dawley rats to walk on a rotating rod. These data indicate that the mGluR 2/3 agonist produced an aphagic effect while the antagonist caused an anti-ethanol consumption effect. These results suggest that a metabotropic glutamate group II antagonist may be

a viable agent for decreasing volitional ethanol consumption. Additional experiments may be necessary to differentiate effects on ethanol intake versus caloric intake by both agonists and antagonists of the mGluR 2/3 receptors.

INTRODUCTION

Based upon a review by Palucha and Pilc (2007), it is apparent that localization of the metabotropic glutamate receptors (mGluRs) in the brain determines their roles. Group II mGluRs (mGluR 2/3) are highly expressed in brain regions related to emotional states, including the forebrain and limbic areas, such as the AMY, HI, and PFC (Ohishi et al., 1993a; Ohishi et al., 1993b; Tamaru et al., 2001; Wright et al., 2001). Since these receptors are negatively linked to the AC signal transduction pathway and are mostly located at the extrasynaptic sites of terminal axons on glutamatergic neurons, they act as autoreceptors, reducing release of the excitatory neurotransmitter glutamate, especially when excess glutamate exists in the synapse (cf. Schoepp, 2001). Presynaptic group II mGluRs also regulate the release of other neurotransmitters and/or neuromodulators such as GABA, DA, 5-HT, purines, and others (Cartmell et al., 2001). By modulating K^+ , Ca^{2+} , and other ion channels, postsynaptic group II mGluRs help regulate neuronal excitability (cf. Pin and Duvoisin, 1995). Activation of mGlu3 receptors widely expressed in glial cells, including astrocytes, enhances glutamate uptake (Yao et al., 2005).

In a study of post-mortem human brains, high levels of mGluR2 were observed in the dorsolateral PFC, anterior CC, orbitofrontal CTX, parietal CTX, and occipital CTX with lower levels of expression in the caudate nucleus, NAc, and cerebellum, and the lowest levels in the thalamus. Relatively high levels of mGluR2 were expressed in the HI. Generally, mGluR3 was more evenly distributed within cortical regions, with the highest levels expressed in the HI. In contrast to mGluR2, mGluR3 was highly expressed in the caudate nucleus and NAc, with moderate levels being detected in the

thalamus and cerebellum. Expression of both mGluR2 and mGluR3 was relatively high in the CTX (Ghose et al., 2009).

Numerous areas in which mGluR2 and mGluR3 are found correspond to major components in the mesocorticolimbic pathway, also known as the dopaminergic reward pathway. The mesocorticolimbic system has been implicated as a common neural substrate for the reinforcing properties of drugs of abuse (cf. Maldonado, 2003), including alcohol dependence and reward. In the mesocorticolimbic pathway, dopaminergic projections from the midbrain VTA innervate numerous limbic structures including the NAc, HIP, and AMY and cortical regions including the FC, PFC, and CC (cf. Pierce and Kalivas, 1997; cf. Zhang et al., 2006).

Reports indicate that the mGluR 2/3 antagonist LY341495 (1-30 mg/kg, i.p.) does not alter ethanol self-administration in C57BL/6J mice using a lever-pressing paradigm (Hodge et al., 2006). Other studies reveal that the mGluR 2/3 agonist LY404039 decreases alcohol-seeking during reinstatement following alcohol deprivation, but does not reduce alcohol self-administration in alcohol-preferring (P) rats using a lever-pressing paradigm (Rodd et al., 2006). Reports also indicate that mGluR 2/3 agonists are anxiolytic-like (Linden et al., 2005b; O'Neill et al., 2003; Rodd et al., 2006; Rorick-Kehn et al., 2006; Tizzano et al., 2002) while mGluR 2/3 antagonists inhibit anxiolytic effects (Linden et al., 2005b). Furthermore, the mGluR 2/3 antagonist LY341495 increased anxiety in the elevated plus maze in a dose-dependent manner in a mouse model, but did not alter total ambulations (Linden et al., 2005a). Other studies indicate that group II mGluR antagonists increase locomotor activity (David and Abbraini, 2001; O'Neill et al., 2003). Similar to the mGluR 2/3 agonists, several mGluR5 antagonists produce anxiolytic effects (Carroll, 2008; cf. Palucha and Pilc, 2007; cf. Spooen et al.,

2000). Also, the mGluR5 antagonist 2-methyl-6-(phenylethynyl)pyridine (MPEP) will reduce consumption of ethanol by a genetic high drinking rat line using a two-choice 24 hour access paradigm (McMillen et al., 2005).

Both mGluR 2/3 agonists and ethanol increase DA metabolism as indicated by an increased production of the dopaminergic metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA). Systemic administration of the selective group II metabotropic receptor agonist LY379268 increased extracellular levels of DA, DOPAC and HVA, and the major 5-HT metabolite, 5-hydroxyindoleacetic acid (5-HIAA), in the rat mPFC. The levels of the metabolites were raised in a dose-dependent manner. LY379268 (10 mg/kg, s.c.) also increased tissue levels of the metabolites DOPAC, HVA and 5-HIAA in the nucleus accumbens and striatum (Cartmell et al., 2000). Acute administration of ethanol increased the levels of DOPAC and HVA in the cerebral CTX and STR, while chronic ethanol consumption did not alter monoamine levels and metabolism in P rats (Murphy et al., 1983). Acute treatment with high dose ethanol (2.5 g/kg, i.p.) increased the levels of DA in the posterior striatum (PSTR) after 60 minutes while raising the levels of DOPAC and/or HVA in the NAc and HYPO after 15, 30, and 60 minutes, and in the PSTR, lateral septal nucleus (LSN), and FC after 60 minutes. The levels of DOPAC and/or HVA were not altered by the high dose of ethanol in either the thalamus or olfactory bulbs (Khatib et al., 1988).

This study aimed to determine if either mGluR 2/3 agonists or antagonists will effectively decrease volitional consumption of ethanol by genetic drinking rats using a two-choice 24 hour access paradigm. The agonist LY379268 and antagonist LY341495 were used due to their high selectivity for the mGluR 2/3 receptors.

MATERIALS AND METHODS

Drugs

The mGluR 2/3 agonist LY379268 (Eli Lilly and Company) was dissolved in deionized water. The mGluR 2/3 antagonist LY341495 (Eli Lilly and Company) was dissolved in deionized water and 0.10 ml 1.0 N sodium hydroxide. Drugs and vehicle (deionized water) were administered via s.c. injections. Ethanol solutions were prepared by diluting 95% ethanol in tap water.

Volitional Ethanol Consumption Experiment

Subjects and Screening

The mHEP rat is a validated model of alcoholism characterized by profuse volitional consumption of ethanol, a preference for ethanol in the presence of palatable alternatives, and levels of blood ethanol corresponding essentially with the respective amounts of ethanol consumed (Myers et al., 1998). Therefore, the mHEP rat was used as the animal model to test the effects of the group II metabotropic receptor agonist and antagonist on volitional ethanol consumption.

At 60 days of age, mHEP rats were placed in a suspended stainless steel cage to be screened for preferred ethanol concentrations. Each day, volumes of fluids and food consumed and body weights were recorded. Initially, rats received 3% ethanol (v/v) and tap water in graduated bottles. The concentration of ethanol was increased daily: 5, 7, 9, 11, 13, 15, 20, 25, and 30%. The concentration of ethanol which produced maximal ethanol consumption with the proportion for consumption of ethanol to total fluids closest to 0.5 was selected as each rat's maximally preferred concentration. Two

groups of male rats were selected from the F22 generation for testing the effects of LY379268 and LY341495 on volitional ethanol consumption. Each rat received its maximally preferred concentration of ethanol throughout the experiment. The average preferred ethanol concentration was 12% for rats treated with LY379268, 13% for rats treated with LY341495, and 14% for rats treated with co-administered LY379268 and LY341495.

Experimental Procedures

During each regimen of treatments, rats underwent a 3-day baseline period followed by a 3-day period during which injections were administered two hours before and two hours following lights out and a subsequent 3-day post-treatment period. For one group of rats, doses of LY379268 (0.3, 1.0 and 3.0 mg/kg) and vehicle were administered in a counter-balanced design with some rats receiving 0.3 mg/kg LY379268 and the remaining rats receiving 1.0 mg/kg LY379268 initially. Upon stabilization of ethanol consumption to baseline levels, a different dose of LY379268 or vehicle was injected. Similarly, the second group of rats received doses of LY341495 (0.3, 1.0 and 3.0 mg/kg) or vehicle in a counter-balanced design. Seven male mHEP rats received co-administered 3.0 mg/kg LY379268 and 3.0 mg/kg LY341495. For each group of rats, data for each pre-treatment, treatment, and post-treatment period for each rat were averaged, grouped, and analyzed using two-way repeated measures ANOVA and Tukey/Kramer procedure post hoc. Statistical analyses were performed using GB-STAT (Dynamic Microsystems, Silver Spring, MD).

Rotating Rod Experiment

Two groups of male Sprague Dawley (Harlan, Frederick, MD) rats were used to test the effects of 3.0 mg/kg LY379268 and 3.0 mg/kg LY341495 on the ability of the animals to walk on the rotorod. Rats were trained to walk continuously on a 12 rpm rotating rod (4 Station Rat Rota Rod, Med Associates, Inc., Georgia, VT) for 180 s. The ability of the rats to walk on the rotorod was tested 30 minutes and 60 minutes after drug or vehicle was injected. A 60 s criterion was used to indicate minimal or no effect of drug.

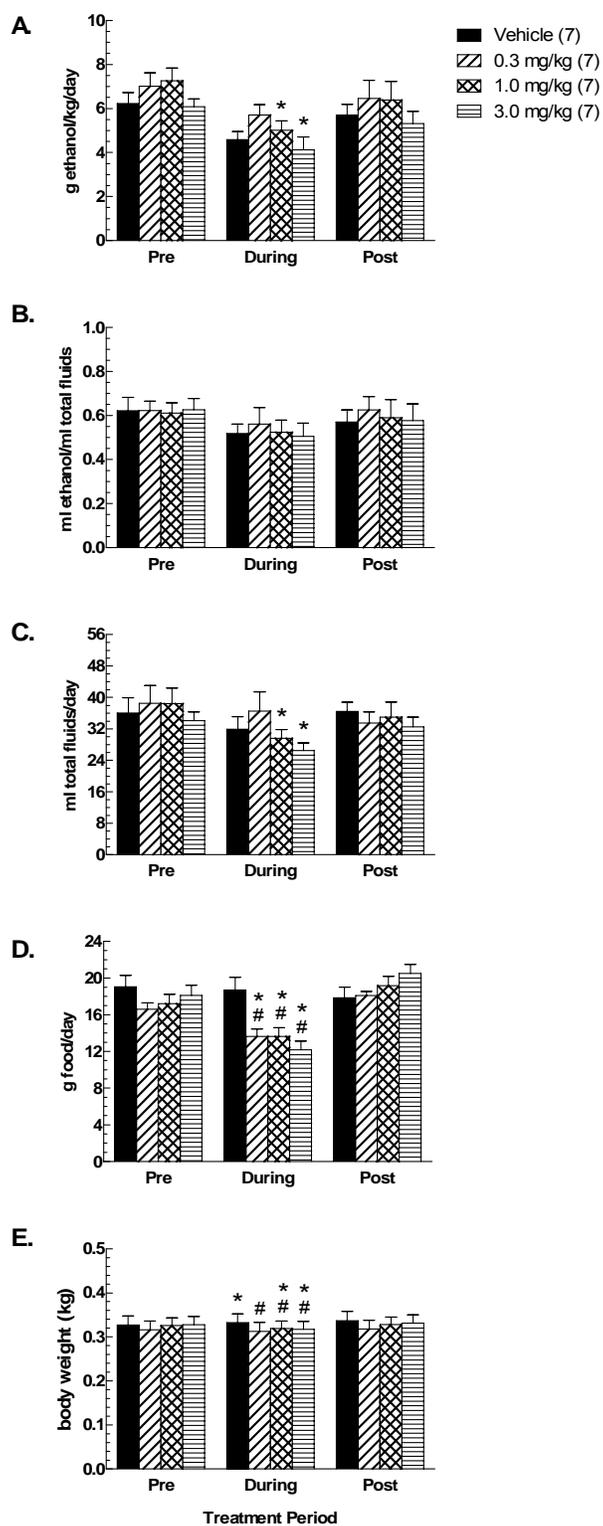
RESULTS

Effects of LY379268 and LY341495 on Volitional Ethanol Consumption

Figure 4.1 shows the effects of LY379268 (0.3, 1.0, and 3.0 mg/kg) on ethanol, total fluid, and food consumption. The repeated measures two-way ANOVA indicated an effect of treatment period on ethanol consumption ($F_{2,27} = 15.95$, $P < 0.05$), total fluid consumption ($F_{2,27} = 12.75$, $P < 0.05$), food intake ($F_{2,27} = 104.63$, $P < 0.05$), and body weight ($F_{2,27} = 62.40$, $P < 0.05$). The ANOVA also indicated a significant interaction between dose of LY379268 and treatment period for food intake ($F_{6,83} = 19.20$, $P < 0.05$) and body weight ($F_{6,83} = 11.28$, $P < 0.05$). The 1.0 and 3.0 mg/kg doses of LY379268 significantly decreased ethanol intake relative to pre-treatment levels by 31% and 32%, respectively, but no effect relative to vehicle treatment was observed. Similarly, the 1.0 and 3.0 mg/doses of LY379268 significantly decreased total fluids consumed relative to pre-treatment levels by 23% and 22%, respectively, but no effect relative to vehicle treatment was observed. At the doses tested, LY379268 produced no effect on the proportion of ethanol to total fluids consumed. LY379268 appears to dose-dependently decrease food consumption (18 to 33%) relative to pre-treatment levels at all doses with a 33% decrease at the highest dose. LY379268 also reduced food intake relative to vehicle treatment at all doses with a 35% decrease at the highest dose. During treatment with vehicle, rats gained weight. During treatment with the 1.0 and 3.0 mg/kg doses of LY379268, rats underwent weight loss of 2% and 3%, respectively, relative to pre-treatment levels. Relative to vehicle treatments, all doses of LY379268 produced a weight loss of 4 to 6%. Reductions in body weight coincide with decreased food

Figure 4.1. Effect of LY379268 on the volitional consumption of solutions of ethanol by the male mHEP rat. Each bar represents the mean \pm S.E.M. Number of rats for each treatment is indicated in parentheses.

- A. Volitional consumption of ethanol. * indicates a significant difference from pre-treatment baseline, $P < 0.05$ (Tukey/Kramer).
- B. Proportion of ethanol to total fluids (water and ethanol) consumed.
- C. Consumption of total fluids (water and ethanol). * indicates a significant difference from pre-treatment baseline, $P < 0.05$ (Tukey/Kramer).
- D. Consumption of food. * indicates a significant difference from pre-treatment baseline, $P < 0.05$ (Tukey/Kramer). # indicates a significant difference from vehicle treatment, $P < 0.05$.
- E. Body weight. * indicates a significant difference from pre-treatment baseline, $P < 0.05$ (Tukey/Kramer). # indicates a significant difference from vehicle treatment, $P < 0.05$.



consumption which suggests that the weight loss may be an anti-caloric effect.

Figure 4.2 shows the effects of LY341495 (0.3, 1.0, and 3.0 mg/kg) on ethanol, total fluid, and food consumption. The repeated measures two-way ANOVA indicated an effect of treatment period on ethanol consumption ($F_{2,30} = 30.70$, $P < 0.05$), proportion of ethanol to total fluids consumed ($F_{2,30} = 14.89$, $P < 0.05$), total fluid consumption ($F_{2,30} = 25.46$, $P < 0.05$), and body weight ($F_{2,30} = 67.20$, $P < 0.05$). The ANOVA also indicated a significant interaction between dose of LY341495 and treatment period for ethanol consumption ($F_{6,92} = 3.29$, $P < 0.05$), total fluid consumption ($F_{6,92} = 3.36$, $P < 0.05$), and body weight ($F_{6,92} = 4.72$, $P < 0.05$). Vehicle and both the 0.3 mg/kg and 3.0 mg/kg doses of LY379268 significantly decreased ethanol intake relative to pre-treatment levels. The vehicle-treated rats decreased ethanol consumption by 12% while the 0.3 mg/kg and 3.0 mg/kg LY341495-treated rats reduced ethanol intake by 36% and 44%, respectively. Relative to vehicle treatment, 3.0 mg/kg LY341495 produced a 37% decrease in volitional ethanol consumption. The 0.3 mg/kg dose of LY341495 produced a 27% reduction in proportion of ethanol to total liquids consumed relative to pre-treatment levels, but no treatment differences were observed. LY341495 appears to have dose-dependently decreased total fluid consumption (15 to 32%) relative to pre-treatment levels at all doses with a 32% decrease at the highest dose. The 1.0 mg/kg and 2.0 mg/kg doses of LY341495 also reduced total fluid intake relative to vehicle treatment by 25% and 27%, respectively. The mGlu 2/3 antagonist did not significantly alter food consumption. Relative to pre-treatment levels, rats underwent significant weight gain during treatment with vehicle and 0.3 mg/kg LY341495. Differences between body weight during vehicle treatment and LY341495 treatment can most likely be attributed to differences in pre-treatment weights for these groups.

Figure 4.2. Effect of LY341495 on the volitional consumption of solutions of ethanol by the male mHEP rat. Each bar represents the mean \pm S.E.M. Number of rats for each treatment is indicated in parentheses.

- A. Volitional consumption of ethanol. * indicates a significant difference from pre-treatment baseline, $P < 0.05$ (Tukey/Kramer). # indicates a significant difference from vehicle treatment, $P < 0.05$.
- B. Proportion of ethanol to total fluids (water and ethanol) consumed. * indicates a significant difference from pre-treatment baseline, $P < 0.05$ (Tukey/Kramer).
- C. Consumption of total fluids (water and ethanol). * indicates a significant difference from pre-treatment baseline, $P < 0.05$ (Tukey/Kramer). # indicates a significant difference from vehicle treatment, $P < 0.05$.
- D. Consumption of food.
- E. Body weight. * indicates a significant difference from pre-treatment baseline, $P < 0.05$ (Tukey/Kramer). # indicates a significant difference from vehicle treatment, $P < 0.05$.

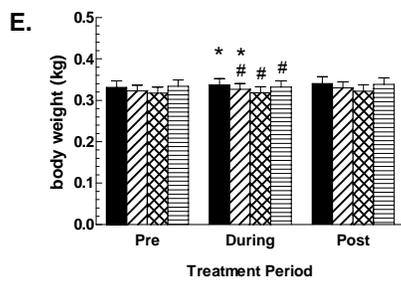
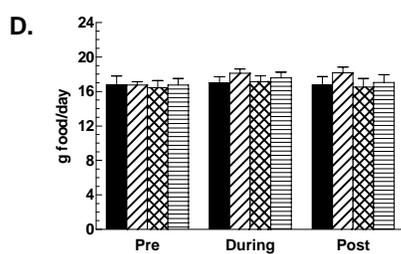
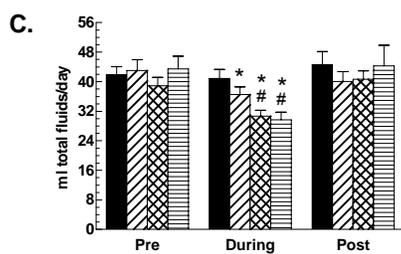
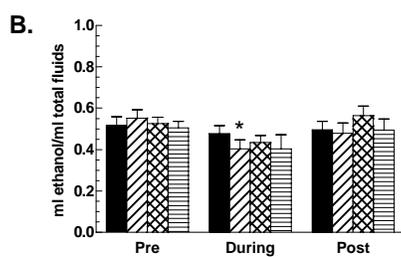
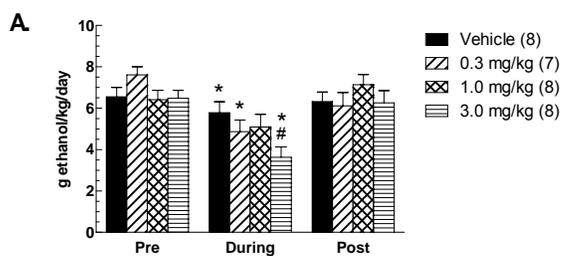


Figure 4.3 shows the effects of co-administered LY341495 (3.0 mg/kg) and LY379268 (3.0 mg/kg) on ethanol, total fluid, and food consumption. The repeated measures one-way ANOVA indicated an effect of treatment period on ethanol consumption ($F_{2,6} = 54.70$, $P < 0.05$), proportion of ethanol to total fluids consumed ($F_{2,6} = 18.53$, $P < 0.05$), total fluid consumption ($F_{2,6} = 9.74$, $P < 0.05$), food intake ($F_{2,6} = 43.73$, $P < 0.05$), and body weight ($F_{2,6} = 35.49$, $P < 0.05$). Relative to pre-treatment levels, treatment with the co-administered drugs significantly decreased ethanol intake by 60%, proportion of ethanol to total fluids consumed by 42%, total fluid consumption by 44%, food intake by 27%, and body weight by 3%.

Effects of LY379268 and LY341495 on Locomotor Activity

The rotorod experiments indicated that neither LY379268 (3.0 mg/kg) nor LY341495 (3.0 mg/kg) produced a significant effect on locomotor activity of Sprague Dawley rats at 30 or 60 minutes following treatment (Table 4.1).

Figure 4.3. Effect of co-administered LY341495 and LY379268 on the volitional consumption of solutions of ethanol by the male mHEP rat. Number of rats is indicated in parentheses.

- A. Volitional consumption of ethanol. * indicates a significant difference from pre-treatment baseline, $P < 0.05$ (Tukey/Kramer).
- B. Proportion of ethanol to total fluids (water and ethanol) consumed. * indicates a significant difference from pre-treatment baseline, $P < 0.05$ (Tukey/Kramer).
- C. Consumption of total fluids (water and ethanol). * indicates a significant difference from pre-treatment baseline, $P < 0.05$ (Tukey/Kramer).
- D. Consumption of food. * indicates a significant difference from pre-treatment baseline, $P < 0.05$ (Tukey/Kramer).
- E. Body weight. * indicates a significant difference from pre-treatment baseline, $P < 0.05$ (Tukey/Kramer).

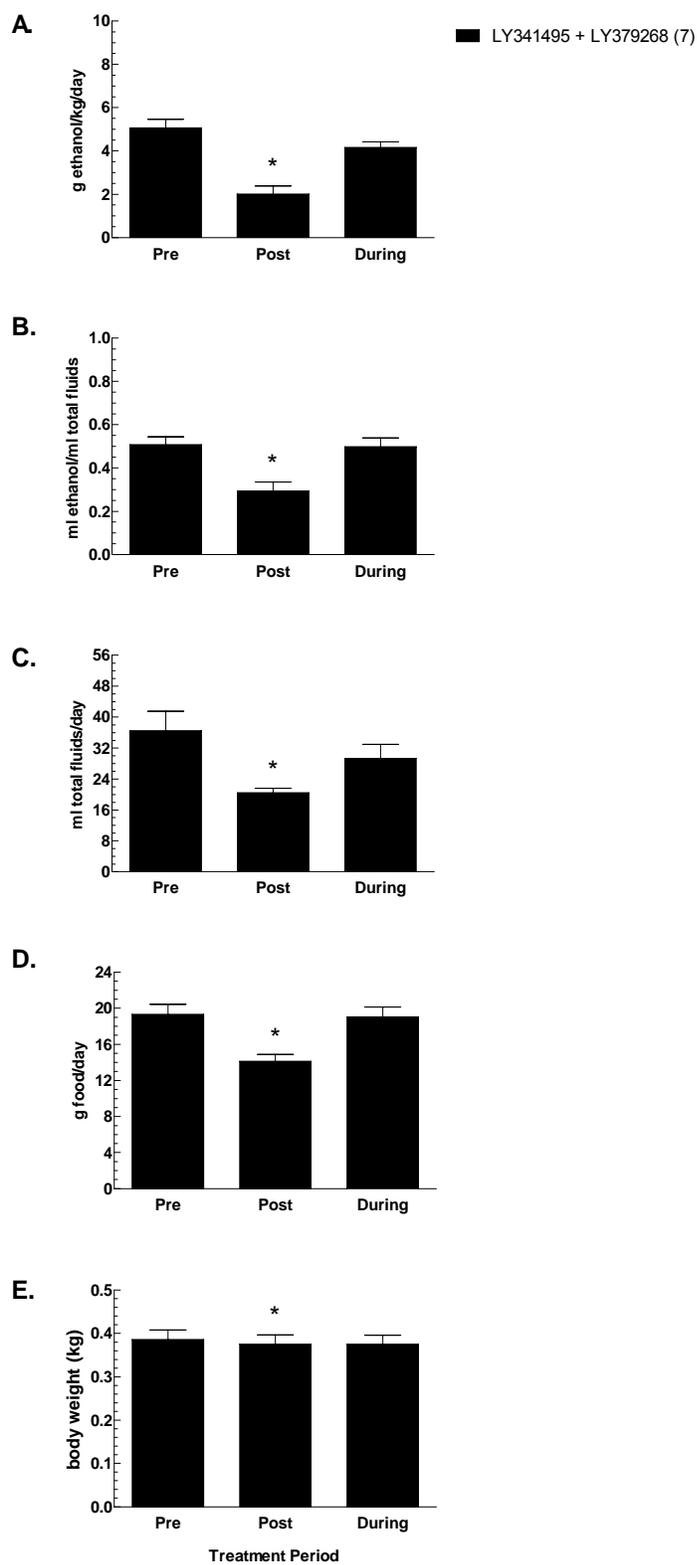


Table 4.1. Effects of LY379268, LY341495, or vehicle on the ability of Sprague Dawley rats to walk on a rotorod. Ability to walk for 60 seconds indicates minimal or no effect of each treatment. No effect was observed at the doses tested. Number of animals tested is shown in parentheses.

Treatment	Seconds \pm S.E.M.	
	30 Minutes Post-Treatment	60 Minutes Post-Treatment
LY379268		
3.0 mg/kg (8)	119 \pm 15.5	160 \pm 13.1
Vehicle (8)	156 \pm 16.3	172 \pm 8.3
LY341495		
3.0 mg/kg (9)	180 \pm 0.0	176 \pm 4.4
Vehicle (9)	180 \pm 0.0	180 \pm 0.0

DISCUSSION

The mGluR 2/3 agonist LY379268 decreased both volitional ethanol consumption and food consumption which suggests that the drug may be having an aphagic or anorexic effect. These findings are in harmony with the observations of Peters and Kalivas (2006) that demonstrated a decrease in operant responding for food following injections of LY379268. Conversely, the reduction in volitional ethanol consumption by the mGluR 2/3 antagonist LY341495 does not appear to be an aphagic or anti-caloric effect. Furthermore, LY341495 does not inhibit or reverse the reduction in food consumption caused by LY379268. Therefore, if LY379268 is producing an aphagic effect, LY341495 does not reverse the effect at the dose tested. To determine if LY341495 does reverse the effect, several additional doses would need to be tested. This would require numerous additional male mHEP rats. The weight loss in LY379268-treated rats during treatment may be attributed to the decline in caloric consumption. During the post-treatment period of all experiments, the rats appear to recover from deficits in drinking and feeding incurred during treatment. This suggests that the consummatory effects of LY379268 and LY341495, at the doses used, are neither long-term nor irreversible. Although the results from this study suggest that the reduction in ethanol intake associated with each of these drugs may be dose-dependent, further studies with more animals are needed to determine if the decrease in ethanol consumption is actually dose-dependent.

In summary, LY341495 exhibits a favorable profile as a candidate for the treatment of alcohol dependence while LY379268 may produce serious consummatory side effects. In the rat brain, the mGlu2 receptors are extensively expressed

presynaptically in most forebrain structures including the CTX (Gu et al., 2008) which functions to regulate neurotransmitter release (Pin and Duvoisin, 1995) while mGlu3 receptors are expressed in glial and cells in the the GABAergic reticular nucleus of the thalamus (Gu et al, 2008). This suggests that drugs with greater receptor specificity may allow for determination of which receptor site is more important for consummatory effects. These drugs do not differentiate between mGlu2 and mGlu3 receptors so as more drugs are developed, it may be beneficial to examine the effects of drugs with greater receptor subtype specificity on ethanol consumption.

The dichotomy between our results from the volitional ethanol consumption experiments and those previously published may be due to differences between the paradigms and/or the animal models used. In our study, we used a two-choice 24 hour protocol to better simulate the access to alcohol experienced by alcohol-dependent patients. In contrast to other studies which did not account for potential caloric effects, we reported total fluid and total food intake.

The results from the rotorod experiment suggest that these drugs do not affect locomotor ability at the doses tested. However, the behavior of Sprague Dawley rats rather than mHEP rats was measured on the rotorod. Also, this experiment measured locomotor ability rather than locomotor activity. Further studies are needed to determine the effects of the mGluR 2/3 agonist and antagonist on locomotor ability and activity in mHEP rats.

Acknowledgement

LY379268 and LY341495 were generous gifts provided by David L. McKinzie of Lilly Research Laboratories.

CHAPTER 5

EFFECTS OF MEMANTINE ON DOPAMINE METABOLISM AND PHOSPHORYLATION OF DARPP-32 IN THE DOPAMINERGIC REWARD PATHWAY OF MALE MYERS' HIGH ETHANOL PREFERRING RATS

ABSTRACT

Previously, we showed that memantine, a partial NMDA receptor antagonist, decreases volitional ethanol consumption in the male mHEP rat in an apparent dose-dependent manner without adversely affecting locomotor ability or activity. Multiple neurotransmitters and pathways are involved in ethanol reinforcement. Previous studies indicate that ethanol alters levels of monoamines and their metabolites, including DA and its metabolite, DOPAC, in various regions of the brain in rats. Reports also implicate DARPP-32 as an intracellular regulator involved in ethanol reward. This part of our study focused on the two following aims: (1) To determine if memantine increases the ethanol-induced metabolism of DA; and (2) to determine if the mechanism by which memantine reduces ethanol drinking involves signaling downstream of the activation of the DA D1 receptors. Male mHEP rats were used for all experiments.

HPLC analysis showed that memantine (10.0 mg/kg, i.p.) and ethanol (1.0 or 2.5 g/kg, i.p.), both alone and together, failed to significantly alter levels of DA and DOPAC in the mPFC, NAc, and STR of male mHEP rats. However, ethanol (2.5 g/kg) did produce changes in levels of striatal DOPAC close to being significant. Overall, these results show that memantine does not alter levels of DA in the mPFC, NAc, and STR.

Western blots were used to measure the effects of memantine (10.0 mg/kg, i.p.) and ethanol (1.0 or 2.5 g/kg, i.p.), both alone and together, on the abundance of DARPP-32, phospho-DARPP-32 (Thr34), and phospho-DARPP-32 (Thr75) in the mPFC, NAc, and STR. Bands for phospho-DARPP-32 (Thr34) in the mPFC for all treatment groups were undetectable in all blots. Results from all other blots indicated that memantine and ethanol, alone and combined, failed to significantly alter levels of

DARPP-32 and phospho-DARPP-32 (Thr75) in the mPFC, NAc, and STR and failed to change levels of phospho-DARPP-32 (Thr34) in the NAc and STR.

Together, the data from these experiments suggest that mechanisms which involve neither glutamate nor the NMDA receptor can also activate the ethanol reward pathway in the mHEP rat, and a mechanism other than one downstream from the DA D1 receptor activation is involved.

INTRODUCTION

Ethanol inhibits glutamatergic neurotransmission at the ionotropic NMDA receptor (Lovinger et al., 1989; Lovinger et al., 1990; Morrisett and Swartzwelder, 1993; Peoples and Stewart, 2000) which suggests that blockade of the NMDA receptor may interrupt the ethanol reward pathway. Previously, we demonstrated that the low-affinity, noncompetitive NMDA receptor antagonist memantine decreases volitional ethanol consumption in a dose-dependent manner in the male mHEP rat in a two-choice 24 hour access paradigm. Memantine is a neuroprotective drug effective in the treatment of Alzheimer's disease and previously shown in clinical trials to not produce intolerable side effects. The neuroprotective properties of memantine are attributed to the drug only binding excessively open channels, thereby not inhibiting normal physiological levels of NMDA receptor activity, and to its relatively fast "off-rate" (cf. Chen and Lipton, 2006; cf. Lipton, 2004a; cf. Lipton, 2005; cf. Lipton, 2006; cf. Lipton, 2007; cf. Rammes et al., 2008).

Ethanol produces many different effects in multiple intracellular and intercellular signaling cascades in the CNS (cf. Zhang et al., 2006). Acute and chronic ethanol has been shown to alter levels of DA metabolism in various brain regions in the rat (Lucas and McMillen, 2002; Vasconcelos et al., 2004). If memantine substitutes for ethanol, the NMDA antagonist may increase the effects of ethanol on the metabolism of DA.

DARPP-32 has been implicated as an intracellular regulator of ethanol-seeking behavior (Risinger et al., 2001) and of DA D1 receptor function (Fienberg et al., 1998). One proposed positive feedback system for ethanol consumption involves activation of DA D1 receptors diminishing ethanol sensitivity of NMDA receptors via the PKA/DARPP-

32 cascade in principal neurons of the nucleus accumbens (NAc). According to this mechanism, ethanol exposure causes depolarization of VTA neurons which increases DA release in the NAc, thereby activating DA D1 receptors and initiating the PKA/DARPP-32 cascade. This causes phosphorylation of DARPP-32 which decreases activity of protein phosphatases and increases phosphorylation of Ser-897 on the NR-1 subunit of the NMDA receptor. This phosphorylation causes decreased sensitivity of the NMDA receptor to ethanol while maintaining a near normal level of NMDA receptor function and promoting long-term modification of glutamatergic transmission (cf. Maldve et al., 2002). This suggests that memantine may reduce ethanol intake by decreasing DA release, thereby inhibiting the PKA/DARPP-32 cascade. Furthermore, the FC, parietal CTX, HI, and neostriatum (NSTR) of rats contain neurons co-expressing DARPP-32 with NMDA receptor subunits which suggests that DA and glutamate receptors may interact in dopaminoceptive neurons with distinct receptor compositions and may be involved in modulation of neuronal properties and excitotoxicity in the mammalian forebrain (Wang et al., 2004).

A validated animal model for alcoholism, the mHEP rat is characterized by profuse volitional consumption of ethanol, a preference for ethanol in the presence of palatable alternatives, and levels of blood ethanol corresponding essentially with the respective amounts of ethanol consumed (Myers et al., 1998). The purpose of this study was to determine if memantine and ethanol, individually or combined, produce additive effects on DA metabolism and DA-stimulated phosphorylation of DARPP-32 at the Thr34 and Thr75 sites. More specifically, the two aims were: (1) To determine if memantine increases the ethanol-induced metabolism of DA, detected as an elevation in levels of the DA metabolite, DOPAC, which would suggest that glutamate and the NMDA receptor

are necessary for activation of the ethanol reward pathway; and (2) to determine if the mechanism by which memantine reduces ethanol drinking involves signaling downstream of the activation of the DA D1 receptors. The brain regions studied included the mPFC, NAc, and STR since they are known to receive DA projections from the VTA in the reward pathway and have been shown to contain DARPP-32 (Hemmings and Greengard, 1986; cf. Nairn et al., 2004; Ouimet et al., 1984; cf. Svenningsson et al., 2004; cf. Svenningsson et al., 2005; Walaas and Greengard, 1984; Wang et al., 2004).

MATERIALS AND METHODS

Drugs and Reagents

Memantine hydrochloride (Sigma-Aldrich, Inc., St. Louis, MO) was dissolved in deionized water and doses were calculated as the free base. In all experiments, deionized water was used as the vehicle and 95% ethanol was diluted in deionized water. Memantine, ethanol, and vehicle were administered i.p.

Subjects

Male mHEP rats (55 to 80 days old) were selected from the breeding colony in the East Carolina University colony. This line of rats was originally derived from the cross-breeding of three male alcohol-preferring (P) rats (T.-K. Li of the Indiana University Alcohol Research Center) and three female Sprague Dawley rats (Harlan Sprague Dawley Inc.). The line of mHEP rats is maintained by breeding non-sibling males and females whose selection is based on an ethanol drinking screen. The rats were single-housed with continuous access to food and water and maintained on a 12 h on/12 h off light cycle in a temperature-controlled room. All procedures were in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals and approved by the Brody School of Medicine at East Carolina University Institutional Animal Care and Use Committee.

Treatment Groups

Male mHEP rats were weighed and allowed a minimum of 45 minutes to acclimate to the laboratory before initiation of injections. Rats were divided into six

treatment groups designated as “1st treatment/2nd treatment”: (1) Control/control; (2) control/1.0 g ethanol per kg; (3) control/2.5 g ethanol per kg; (4) memantine/control; (5) memantine/1.0 g ethanol per kg; and (6) memantine/2.5 g ethanol per kg. For the first injection, rats received control (0.9% saline) or memantine (10.0 mg/kg). Thirty minutes following the first injection, rats received control (0.9% saline) or ethanol (1.0 or 2.5 g/kg). Thirty minutes following the second treatment, rats were euthanized.

Tissue Samples

Rats were lightly anesthetized with isoflurane (Webster Veterinary Supply, Charlotte, NC) and decapitated by guillotine. Brains were extracted, placed in ice-cold 0.9% saline, blotted, and dissected using a modified (McMillen et al., 1988) procedure of Glowinski and Iversen (1966). Areas removed for analysis included mPFC, NAc, and STR. Tissues were immediately frozen over dry ice and stored at -70°C until analyzed by HPLC or Western blotting.

HPLC Analysis

HPLC was used to determine the levels of DA and its metabolite, DOPAC. 3,4-dihydroxybenzylamine (DHBA) was used as an internal standard. Each sample was weighed, placed in a glass homogenizing tube containing 0.8 ml of 0.4 N perchloric acid (HClO₄) and 50 ng DHBA, and homogenized using a Teflon pestle and a 115V homogenizing power unit (Eberbach Con-Torque Homogenizer, Ann Arbor, MI). Homogenates were transferred to fresh, labeled microcentrifuge tubes. Standards containing 0.4 N HClO₄, DA (50 ng or 250 ng), DOPAC (50 or 250 ng), and DHBA (50 ng) were prepared to be run as verification of retention times for analyte compounds. An

alumina extraction (Appendix C) was performed on all homogenates and standards. The extracted samples and standards were autoinjected into an HPLC system consisting of a Coulochem 5100A carbon electrode detector (ESA, Bedford, MA) and a Brownlee Velosep Column (RP-18, 3 μ m, Shelton, CT). The oxidizing and reducing electrodes were set at +0.50 volts and -0.35 volts, respectively. The mobile phase for the HPLC consisted of a 0.1 M potassium dihydrogen phosphate (KH_2PO_4) buffer containing 0.1 mM disodium ethylenediamine tetraacetate (Na_2EDTA), 1.12 mM sodium octyl sulfate (SOS), and 9% methanol, with the final pH adjusted to 3.9. The mobile phase was degassed by vacuum filtration and run through the HPLC at a flow rate of 0.6 ml/min. Chromatograms were recorded on paper and peak heights were measured for analysis.

Western Blots

Samples and Blotting

Western blotting was used to determine the levels of DARPP-32, phospho-DARPP-32 (Thr34), and phospho-DARPP-32 (Thr75). Each sample was weighed and homogenized in a lysis buffer (PBS [pH 7.4], 1% SDS, 2 mM EDTA, 5 mM ethylene glycol tetraacetic acid (EGTA), 25 mM sodium fluoride, 2 mM sodium orthovanadate) at a volume of 1.0 ml per 100 μ g tissue. Homogenates were microcentrifuged at 14,000 rpm for 10 to 20 minutes at 4°C. Supernate for each sample was transferred to a fresh labeled microcentrifuge tube and protein concentration was determined using a Bicinchoninic Acid Solution (BCA) protein assay test (Thermo Scientific Pierce, Rockford, IL) (Appendix E).

Antibody Procedure

Samples were boiled for 10 minutes in Laemmli stop buffer (95% Laemmli Sample Buffer; Bio-Rad, Hercules, CA; 5% v/v 2-mercaptoethanol). Duplicate samples were separated by electrophoresis on a 10% polyacrylamide gel (10% Tris-HCl; Bio-Rad) and transferred to a nitrocellulose membrane (0.2 μm ; Bio-Rad). Concentrations of sample loaded for each set of experiments are shown in Table 5.1. Membranes were blocked in 5% non-fat milk (Bio-Rad blotting grade non-fat milk diluted in phosphate buffered saline) for 1 h at room temperature. Following five quick rinses in PBST-T (phosphate buffered saline with 0.1% Tween-20), membranes were incubated with primary antibodies overnight at 4°C. Membranes were probed with two primary antibodies: (1) mouse anti-glyceraldehyde-3-phosphate dehydrogenase (anti-GAPDH) (1:1,000,000; Millipore, Temecula, CA) as an internal control and (2) rabbit anti-DARPP-32 (Cell Signaling Technology, Inc., Danvers, MA), rabbit anti-phospho-DARPP-32 (Thr34) (Thermo Scientific Pierce), or rabbit anti-phospho-DARPP-32 (Thr75) (Cell Signaling). Dilutions for antibodies against DARPP-32, phospho-DARPP-32 (Thr34), and phospho-DARPP-32 (Thr75) are shown in Table 5.2. After incubation with primary antibodies, membranes were washed five times with PBST-T for 5 minutes per wash and incubated with two secondary antibodies: (1) goat anti-rabbit IRDye® 800CW (concentrations shown in Table 5.2; Li-Cor Biosciences, Inc., Lincoln, NE); and (2) goat anti-mouse IRDye 680® (1:30,000; Li-Cor) for 1h at 4°C. All antibodies were diluted in PBST-T at concentrations determined by previous optimization studies. After incubation with secondary antibodies, membranes were washed five times with PBST-T for 5 minutes per wash. Antibody binding was detected using an Odyssey infrared imaging system (Li-Cor).

Table 5.1. Concentrations of protein loaded on gels for Western blots.

Phosphoprotein	Concentration of protein (μg) by brain region		
	Prefrontal cortex	Nucleus accumbens	Striatum
DARPP-32	50	30	10
phospho-DARPP-32 (Thr34)	60	40	25
phospho-DARPP-32 (Thr75)	50	30	20

Table 5.2. Concentrations of primary and secondary antibodies used for immunoblotting.

	Concentrations of antibodies by brain region		
	Prefrontal cortex	Nucleus accumbens	Striatum
Anti-DARPP-32	1:750	1:1000	1:1000
Goat anti-rabbit IR Dye® 800CW	1:20,000	1:25,000	1:25,000
Anti-phospho-DARPP-32 (Thr34)	1:250	1:250	1:500
Goat anti-rabbit IR Dye® 800CW	1:10,000	1:10,000	1:10,000
Anti-phospho-DARPP-32 (Thr75)	1:250	1:500	1:1000
Goat anti-rabbit IR Dye® 800CW	1:10,000	1:10,000	1:10,000

Densitometry

Immunoreactivity was quantitated using Odyssey Infrared Imaging System Application Software Version 3.0 (Li-Cor). Levels of total and phosphorylated DARPP-32 were normalized to GAPDH. Data are presented as the ratio of integrated intensity between the DARPP-32, phospho-DARPP-32 (Thr34), or phospho-DARPP-32 (Thr75) band and the GAPDH band, expressed as a percentage of controls (control/control).

Statistical Analysis

Statistical analyses for all experiments were performed using GB-STAT (Dynamic Microsystems, Silver Spring, MD) and graphs were generated using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA). Data were analyzed using a one-way ANOVA and Tukey/Kramer procedure post hoc with significance taken at $P < 0.05$.

RESULTS

Effects of Ethanol and/or Memantine on Levels of Dopamine and DOPAC

No significant differences were observed between treatment groups in levels of DA or DOPAC in the mPFC, NAc, or STR (Figures 5.1-5.3). However, the 2.5 g/kg dose of ethanol did produce changes in the levels of striatal DOPAC that were close to being significant. A comparison of striatal samples from rats treated with vehicle only and rats that received vehicle and ethanol (2.5 g/kg) displayed a 46% elevation of DOPAC levels in the rats treated with ethanol (2.5 g/kg). Similarly, a comparison of striatal tissue from rats that received memantine and vehicle with tissue from rats treated with memantine and ethanol (2.5 g/kg) showed a 30% elevation of DOPAC levels in rats treated with memantine and ethanol (2.5 g/kg). DA levels in the NAc of rats treated with memantine and vehicle were 30% lower than those of NAc samples from rats treated with vehicle only. NAc samples from rats treated with memantine and ethanol (2.5 g/kg) exhibited DA levels that were 24% below those of NAc samples taken from rats treated with vehicle only.

Effects of Ethanol and/or Memantine on Levels of DARPP-32 and Phosphorylation of DARPP-32 at Thr34 and Thr75

Bands for phospho-DARPP-32 (Thr34) binding in the mPFC samples for all treatment groups were undetectable in all blots. Results from all other blots (Figures 5.4-5.11) indicated that memantine (10.0 mg/kg) and ethanol (1.0 or 2.5 g/kg), both alone and combined, failed to significantly alter levels of DARPP-32 and phospho-

Figure 5.1. Effects of ethanol (1.0 or 2.5 g/kg, i.p.) and/or memantine (10.0 mg/kg, i.p.) on the levels of dopamine (DA) and the DA metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) in the medial prefrontal cortex (mPFC) of male mHEP rats. The number of rats for each treatment group is shown in parentheses. No significant differences between treatment groups were observed for DA or DOPAC for the brain region studied. (CTL = control; EtOH = ethanol; MEM = memantine).

A. Levels of DA.

B. Levels of DOPAC.

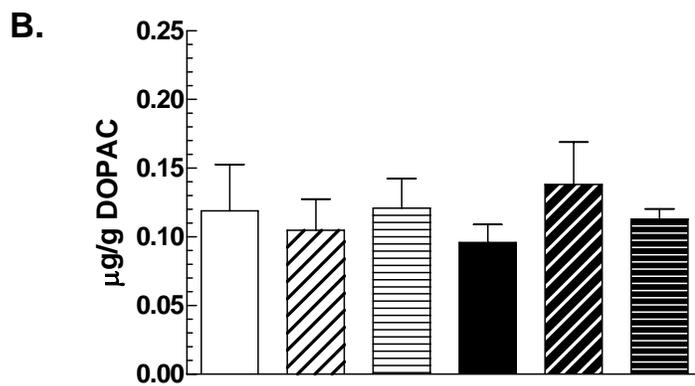
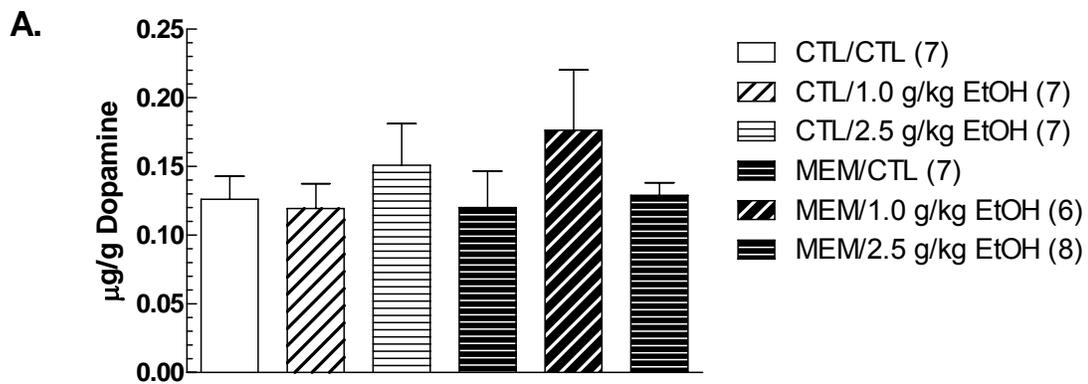


Figure 5.2. Effects of ethanol (1.0 or 2.5 g/kg, i.p.) and/or memantine (10.0 mg/kg, i.p.) on the levels of dopamine (DA) and the DA metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) in the nucleus accumbens (NAc) of male mHEP rats. The number of rats for each treatment group is shown in parentheses. No significant differences between treatment groups were observed for DA or DOPAC for the brain region studied. (CTL = control; EtOH = ethanol; MEM = memantine).

A. Levels of DA.

B. Levels of DOPAC

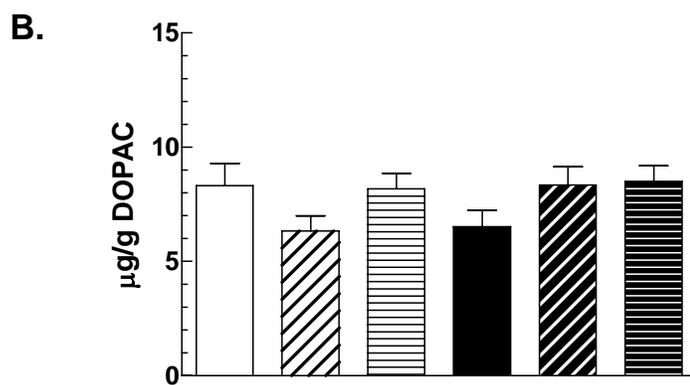
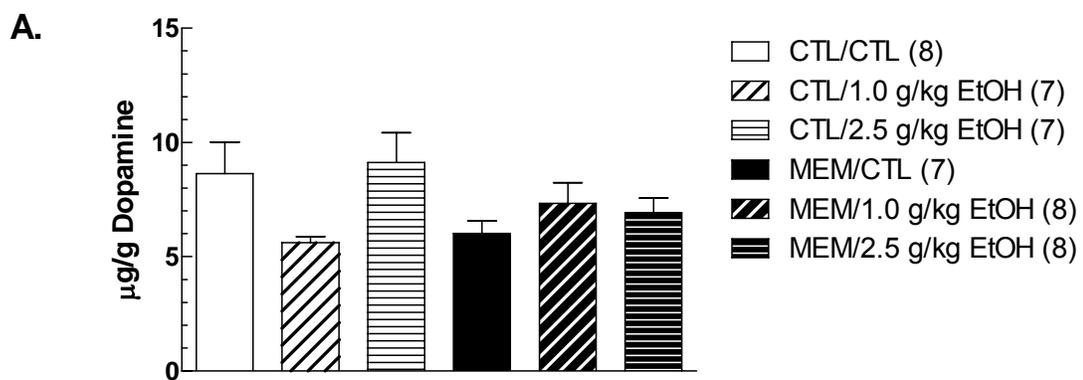


Figure 5.3. Effects of ethanol (1.0 or 2.5 g/kg, i.p.) and/or memantine (10.0 mg/kg, i.p.) on the levels of dopamine (DA) and the DA metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) in the striatum (STR) of male mHEP rats. The number of rats for each treatment group is shown in parentheses. No significant differences between treatment groups were observed for DA or DOPAC for the brain region studied. (CTL = control; EtOH = ethanol; MEM = memantine).

A. Levels of DA.

B. Levels of DOPAC.

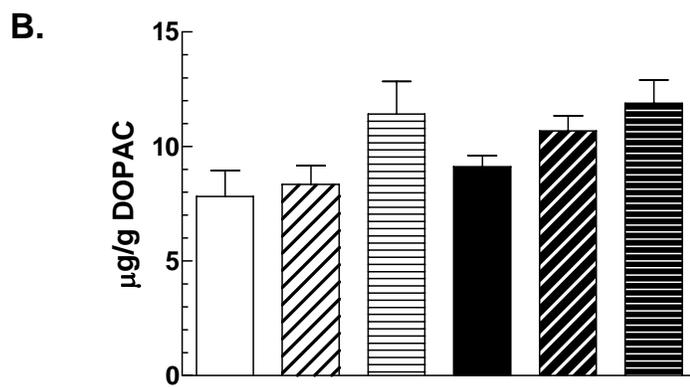
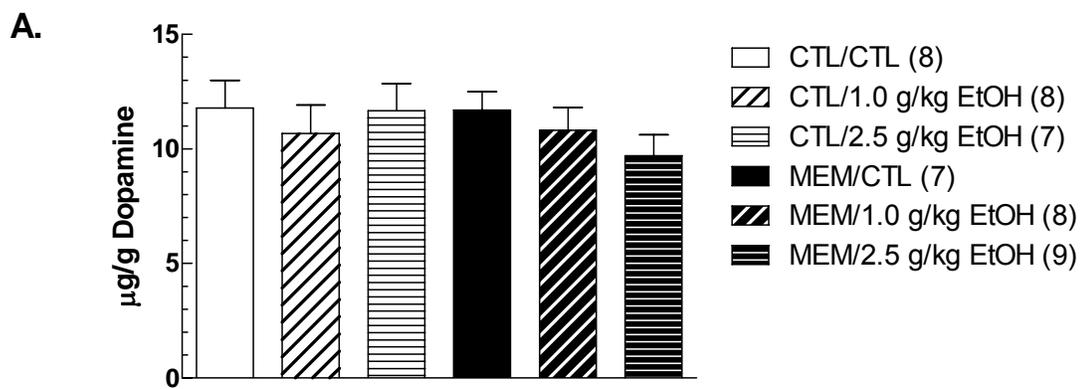


Figure 5.4. Effects of ethanol (1.0 or 2.5 g/kg, i.p.) and/or memantine (10.0 mg/kg, i.p.) on the abundance of DARPP-32 in the medial prefrontal cortex (mPFC). The number of samples for each treatment group is indicated in parentheses. Each sample contained tissue obtained from a single male mHEP rat. Data are presented as the ratio of integrated intensity between the DARPP-32 band and the GAPDH band, expressed as a percentage of controls (control/control). No significant differences were observed between treatment groups for the protein and brain region studied. (CTL = control; EtOH = ethanol; MEM = memantine). A blot representative of the bands detected is shown below the graph.

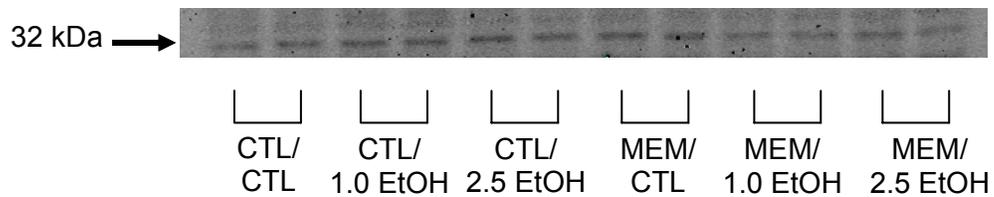
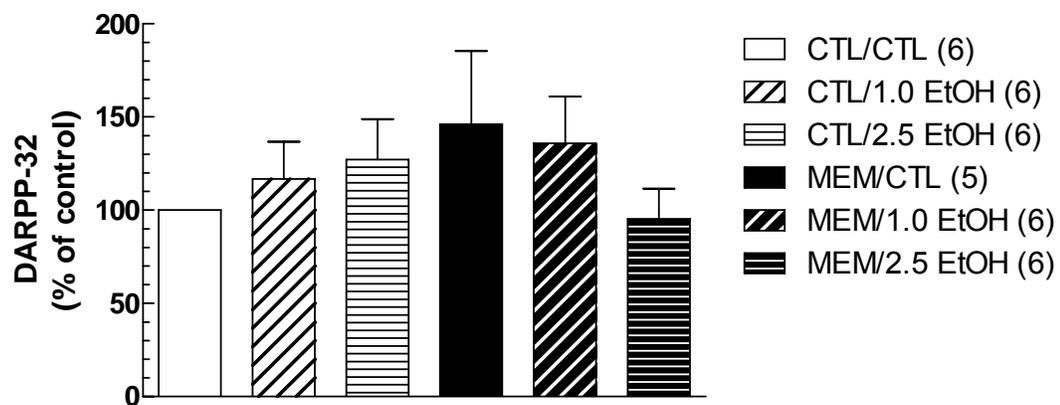


Figure 5.5. Effects of ethanol (1.0 or 2.5 g/kg, i.p.) and/or memantine (10.0 mg/kg, i.p.) on the abundance of phospho-DARPP-32 (Thr75) in the medial prefrontal cortex (mPFC). The number of samples for each treatment group is indicated in parentheses. Each sample contained tissue obtained from a single male mHEP rat. Data are presented as the ratio of integrated intensity between the phospho-DARPP-32 (Thr75) band and the GAPDH band, expressed as a percentage of controls (control/control). No significant differences were observed between treatment groups for the protein and brain region studied. (CTL = control; EtOH = ethanol; MEM = memantine). A blot representative of the bands detected is shown below the graph.

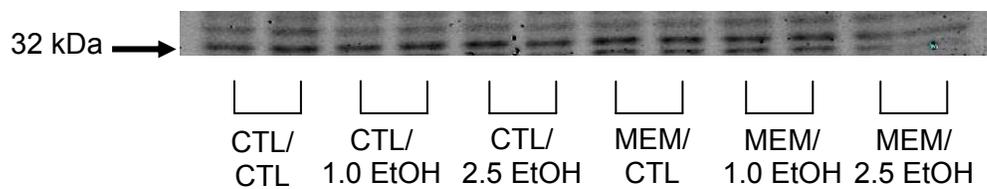
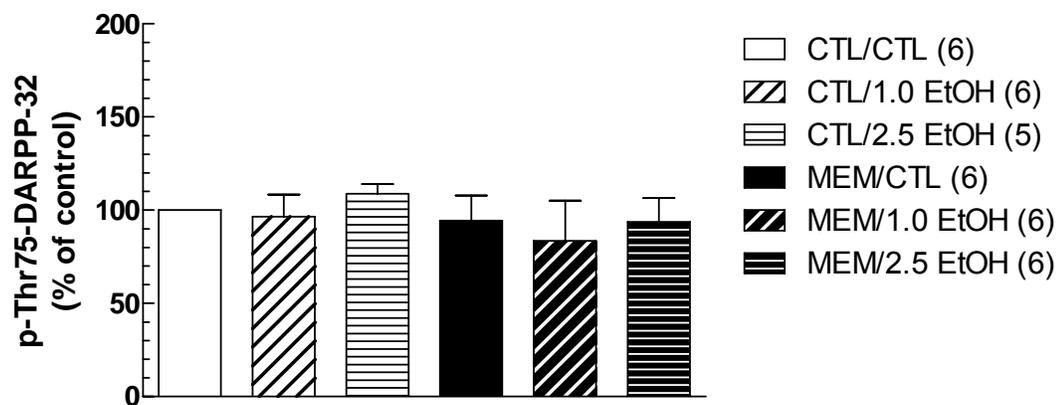


Figure 5.6. Effects of ethanol (1.0 or 2.5 g/kg, i.p.) and/or memantine (10.0 mg/kg, i.p.) on the abundance of DARPP-32 in the nucleus accumbens (NAc). The number of samples for each treatment group is indicated in parentheses. Each sample contained tissue obtained from 2 to 4 male mHEP rats. Data are presented as the ratio of integrated intensity between the DARPP-32 band and the GAPDH band, expressed as a percentage of controls (control/control). No significant differences were observed between treatment groups for the protein and brain region studied. (CTL = control; EtOH = ethanol; MEM = memantine). A blot representative of the bands detected is shown below the graph.

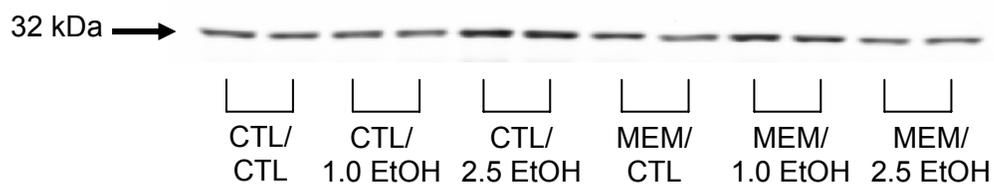
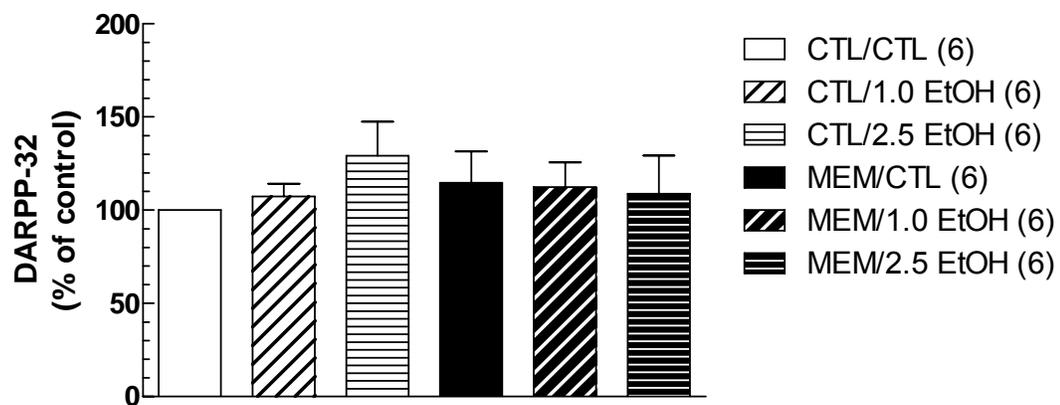


Figure 5.7. Effects of ethanol (1.0 or 2.5 g/kg, i.p.) and/or memantine (10.0 mg/kg, i.p.) on the levels of phospho-DARPP-32 (Thr34) in the nucleus accumbens (NAc). The number of samples for each treatment group is indicated in parentheses. Each sample contained tissue obtained from 2 to 4 male mHEP rats. Data are presented as the ratio of integrated intensity between the DARPP-32 or phospho-DARPP-32 (Thr34) band and the GAPDH band, expressed as a percentage of controls (control/control). No significant differences were observed between treatment groups. (CTL = control; EtOH = ethanol; MEM = memantine). A blot representative of the bands detected is shown below the graph.

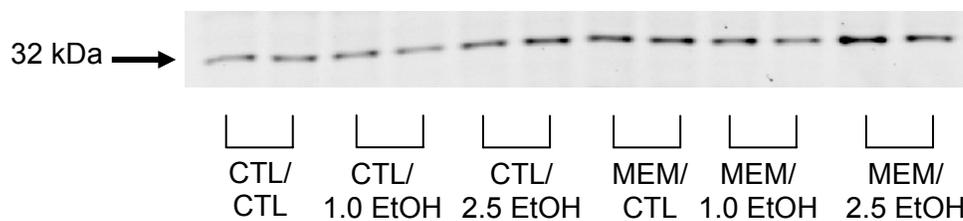
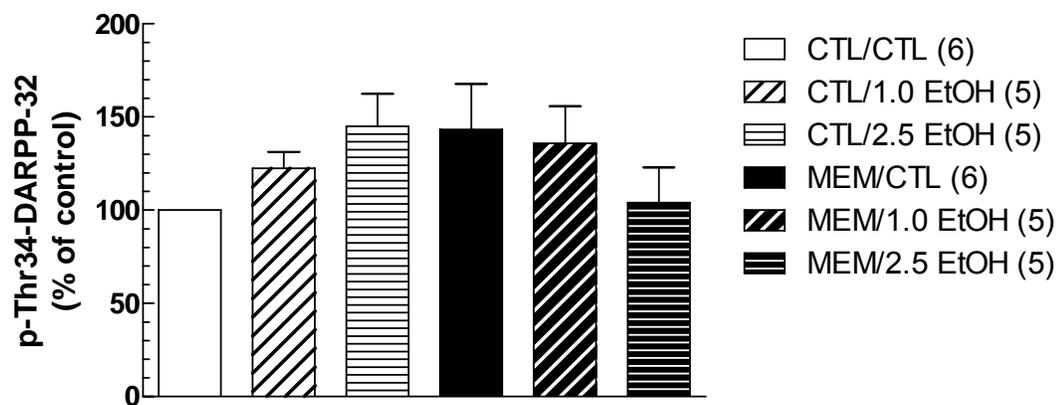


Figure 5.8. Effects of ethanol (1.0 or 2.5 g/kg, i.p.) and/or memantine (10.0 mg/kg, i.p.) on the levels of phospho-DARPP-32 (Thr75) in the nucleus accumbens (NAc). The number of samples for each treatment group is indicated in parentheses. Each sample contained tissue obtained from 2 to 4 male mHEP rats. Data are presented as the ratio of integrated intensity between the DARPP-32 or phospho-DARPP-32 (Thr34) band and the GAPDH band, expressed as a percentage of controls (control/control). No significant differences were observed between treatment groups. (CTL = control; EtOH = ethanol; MEM = memantine). A blot representative of the bands detected is shown below the graph.

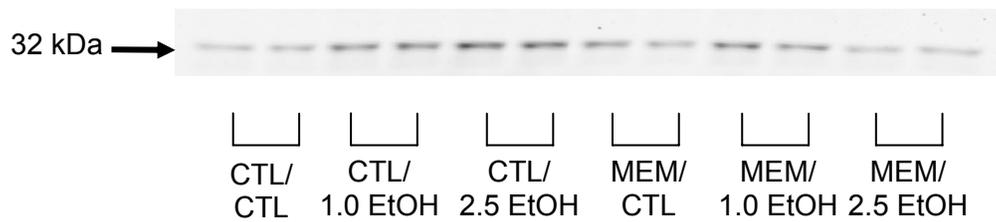
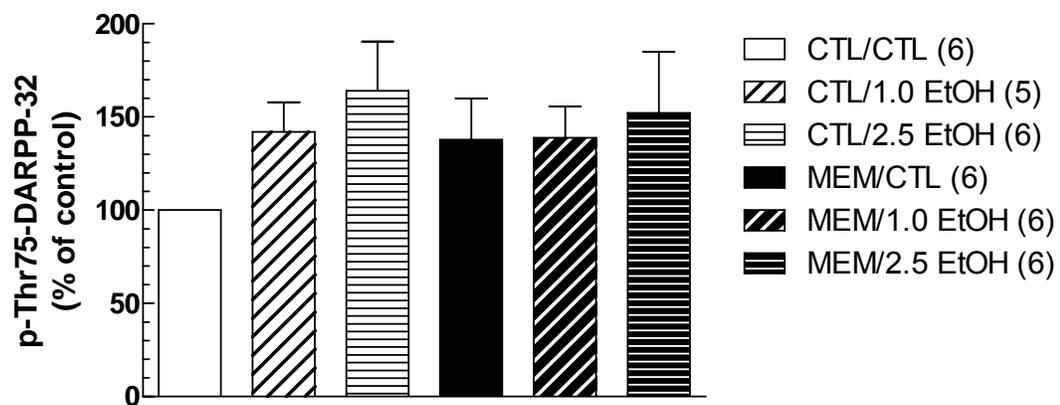


Figure 5.9. Effects of ethanol (1.0 or 2.5 g/kg, i.p.) and/or memantine (10.0 mg/kg, i.p.) on the levels of DARPP-32 in the striatum (STR). The number of samples for each treatment group is indicated in parentheses. Each sample contained tissue obtained from a single male mHEP rat. Data are presented as the ratio of integrated intensity between the DARPP-32 band and the GAPDH band, expressed as a percentage of controls (control/control). No significant differences were observed between treatment groups for the protein and brain region studied. (CTL = control; EtOH = ethanol; MEM = memantine). A blot representative of the bands detected is shown below the graph.

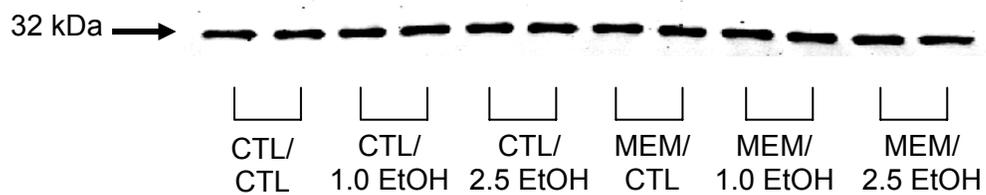
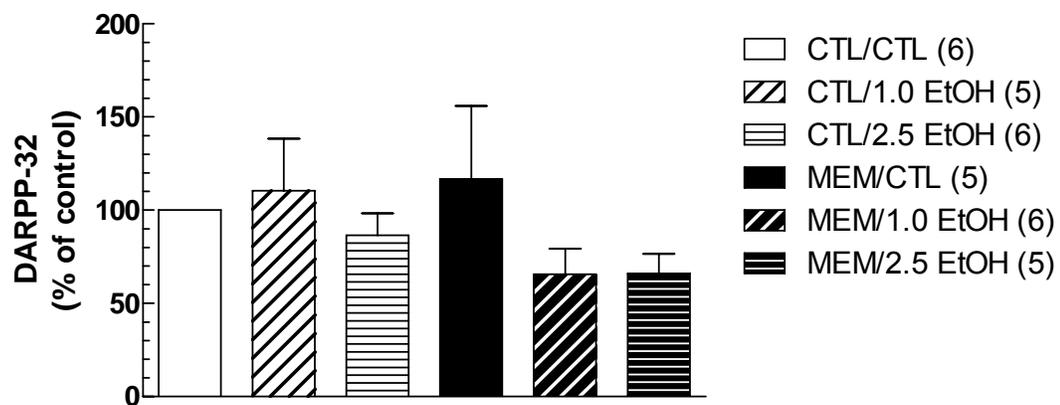


Figure 5.10. Effects of ethanol (1.0 or 2.5 g/kg, i.p.) and/or memantine (10.0 mg/kg, i.p.) on the levels of phospho-DARPP-32 (Thr34) in the striatum (STR). The number of samples for each treatment group is indicated in parentheses. Each sample contained tissue obtained from a single male mHEP rat. Data are presented as the ratio of integrated intensity between the DARPP-32 or phospho-DARPP-32 (Thr34) band and the GAPDH band, expressed as a percentage of controls (control/control). No significant differences were observed between treatment groups. (CTL = control; EtOH = ethanol; MEM = memantine). A blot representative of the bands detected is shown below the graph.

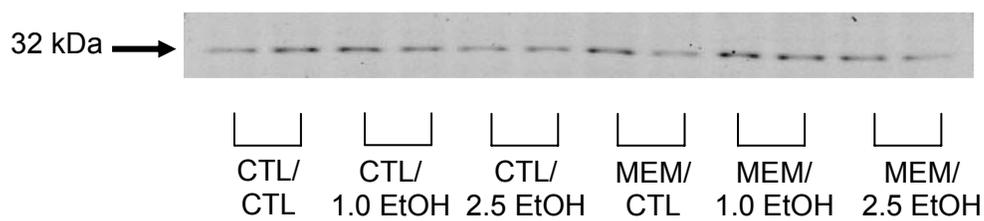
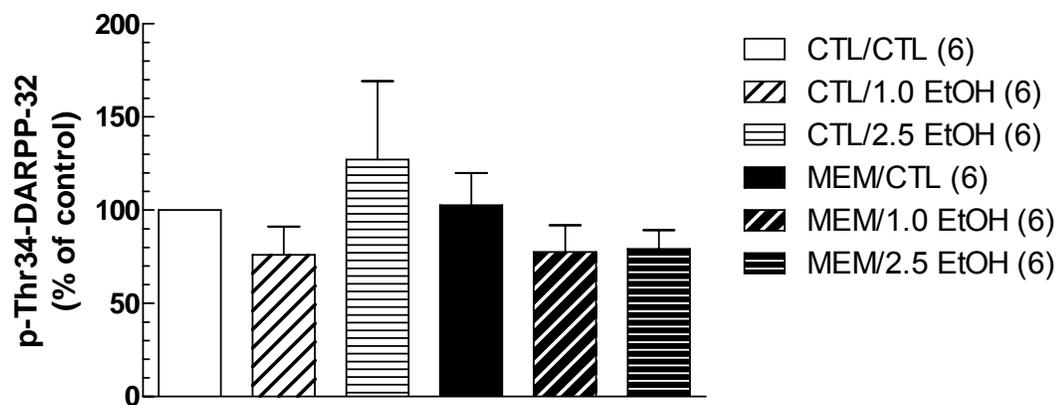
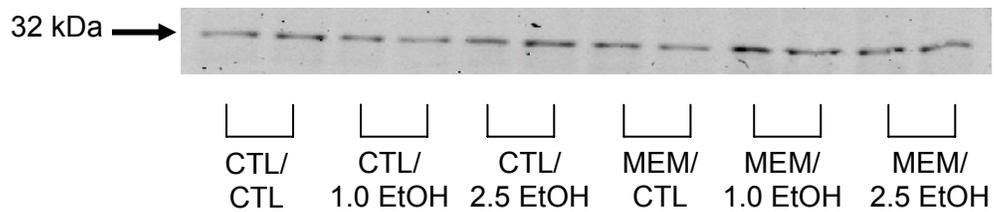
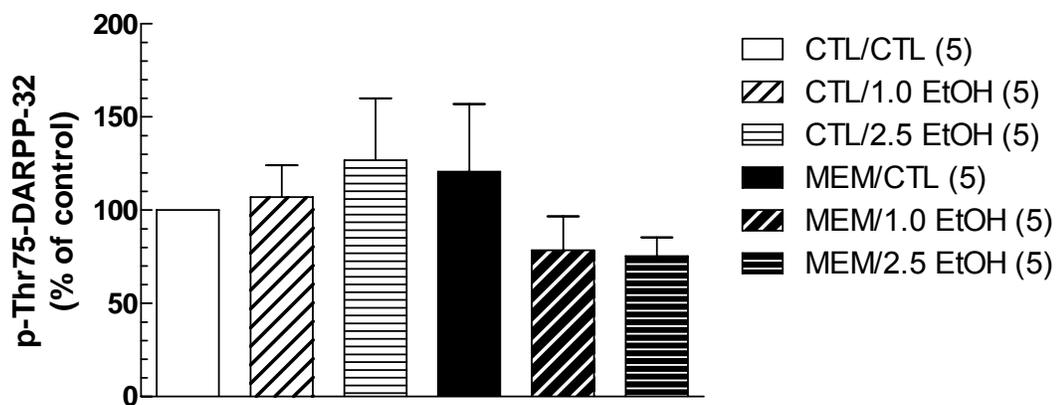


Figure 5.11. Effects of ethanol (1.0 or 2.5 g/kg, i.p.) and/or memantine (10.0 mg/kg, i.p.) on the levels of phospho-DARPP-32 (Thr75) in the striatum (STR). The number of samples for each treatment group is indicated in parentheses. Each sample contained tissue obtained from a single male mHEP rat. Data are presented as the ratio of integrated intensity between the DARPP-32 or phospho-DARPP-32 (Thr75) band and the GAPDH band, expressed as a percentage of controls (control/control). No significant differences were observed between treatment groups. (CTL = control; EtOH = ethanol; MEM = memantine). A blot representative of the bands detected is shown below the graph.



DARPP-32 (Thr75) in the mPFC, NAc, and STR and failed to change levels of phospho-DARPP-32 (Thr34) in the NAc and STR.

Western blot results were re-analyzed by calculating the proportion of phospho-DARPP-32 at Thr34 to DARPP-32 and the proportion of phospho-DARPP-32 at Thr75 to DARPP-32. Prior to these calculations, concentrations of phospho-DARPP-32 at Thr34, phospho-DARPP-32 at Thr75, and DARPP-32 were normalized to corresponding levels of GAPDH as previously described. Data were expressed as a percentage of controls (control/control). All data were analyzed using a one-way ANOVA and Tukey/Kramer procedure post hoc with significance taken at $P < 0.05$. The results of this reanalysis (Appendix H) were similar to the data from the original analysis with no significant effects being observed among the control and treatment groups for any of the brain regions or proteins studied.

DISCUSSION

The failure of ethanol to significantly alter the metabolism of DA in the mPFC, NAc, as STR was unexpected. Based on previous reports, we expected some changes in DOPAC levels following treatment with ethanol (2.5 g/kg) and the increases in concentration obtained were within the range of other reports, but did not reach levels of significance (Dar and Wooles, 1984; Honkanen et al., 1994; Khatib et al., 1988). This lack of altered metabolism may be due to ethanol-induced hypothermia (McMillen and Shore, 1978; McMillen, 1981). Previously, we showed that this dose of ethanol produced a significant hypothermic effect which was enhanced by pre-treatment with memantine (10.0 mg/kg) in the mHEP rat. This suggests holding the room temperature at 32°C might have helped the rats maintain their normal basal body temperature. Based on earlier reports, other drugs have been shown to produce different effects on DOPAC levels in rats when the room is maintained at 32°C versus being maintained at normal room temperature (McMillen and Shore, 1978; McMillen, 1981). The failure of memantine to alter DA metabolism in any of the brain areas studied suggests that glutamate and the NMDA receptor may not be necessary components in the ethanol reward pathway.

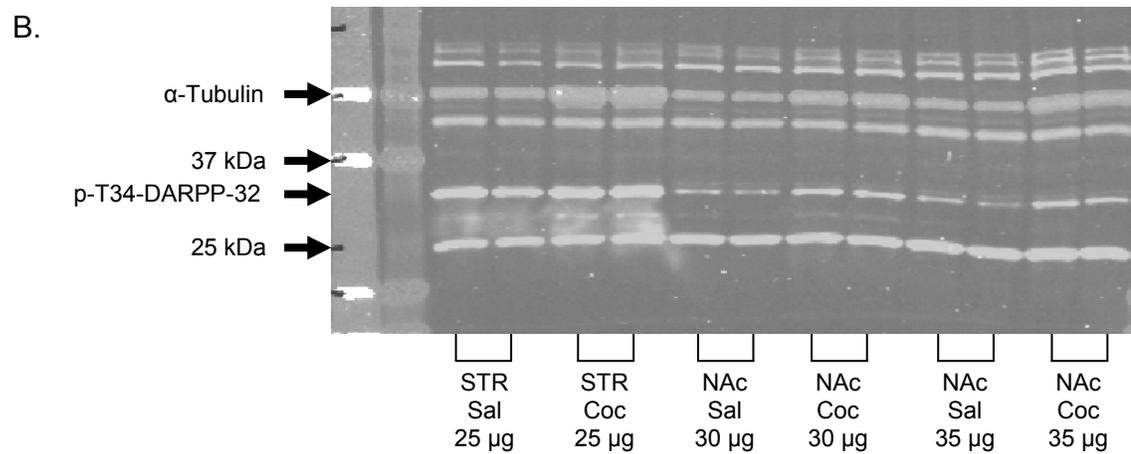
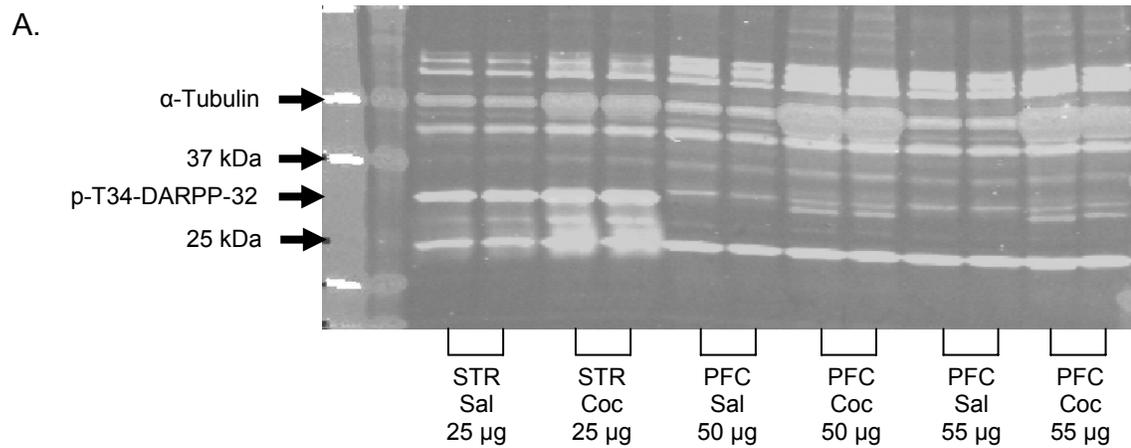
In this study, bands indicating phosphorylation of DARPP-32 at the Thr34 site in homogenates obtained from the mPFC were undetectable. This is likely due to low abundance of DARPP-32 in this region. Numerous studies have indicated that the STR contains high levels of DARPP-32 and, compared with the STR, the FC and neocortex of the rat CNS contains low to moderate levels of DARPP-32 (Hemmings and Greengard, 1986; cf. Nairn et al., 2004; Ouimet et al., 1984; cf. Svenningsson et al., 2004; cf.

Svenningsson et al., 2005; Walaas and Greengard, 1984). The lower level of expression in the frontal FC has been attributed to diffuse DA innervation which predominantly projects to the basal layers of this region, with concomitant localization of DARPP-32 to a subpopulation of neurons. In comparison to the PFC, the whole cerebral CTX contains a slightly higher level of DARPP-32 (Walaas and Greengard, 1984). A single acute dose of cocaine has been shown to increase levels of DARPP-32 in the PFC of rats (D'Addario et al., 2007). While optimizing conditions for the Western blots, we compared STR, mPFC, and NAc samples obtained from saline-treated and cocaine-treated (15.0 mg/kg, i.p) male mHEP rats to confirm the presence of phospho-DARPP-32 (Thr34) in each of these regions. As Figure 5.12 indicates, cocaine-induced increases in phospho-DARPP-32 (Thr34) were detected in homogenates from each of the regions. This suggests that another method may be needed to measure the levels of phosphorylation of DARPP-32 at the Thr34 site in the mPFC. An alternative may be to use immunoprecipitation (IP) to separate out the phospho-DARPP-32 (Thr34) followed by a Western blot. After concentrating the homogenate, an anti-phospho-DARPP-32 (Thr34) antibody covalently attached to beads may be used to separate out the phosphoprotein which may then be analyzed via Western blotting. Another alternative may be to measure levels of a protein downstream of DARPP-32 that is activated or inhibited by phosphorylation of DARPP-32 at Thr34. Protein phosphatase 1 (PP-1) would be a likely candidate.

The failure of ethanol and/or memantine to alter levels of DARPP-32, and the phosphorylation of DARPP-32 at Thr34 and Thr75 was also unexpected. Ethanol increases phosphorylation of DARPP-32 (Wang et al., 2004), and we would expect to observe these molecular changes in the mPFC, NAc, and STR. A previous study in

Figure 5.12. Demonstration of cocaine-induced increase in levels of phospho-DARPP-32 (Thr34) in the striatum (STR), medial prefrontal cortex (mPFC), and nucleus accumbens (NAc) of male mHEP rats. Each sample comprised pooled tissue from 5 rats. Protein concentration (μg) loaded on gels is shown in the figure. (Sal = 0.9% saline; Coc = 15.0 mg cocaine per kg body weight).

- A. STR and mPFC.
- B. STR and NAc.



which transgenic mice overexpressing an ethanol-sensitive isoform of type VII human AC (AC7) in the brain and wild-type mice were exposed to ethanol (2.0 g/kg, i.p.) showed increased abundance of phospho-DARPP-32 (Thr34) in the NAc and AMY of both strains, but with a greater effect in the transgenic mice. No increase in the abundance of phospho-DARPP-32 (Thr34) was observed in either type of mouse. Ethanol increased phospho-DARPP-32 (Thr75) in the NAc and STR of both types of mice, and in the AMY of only the wild-type mice (Donohue et al., 2005). Together, the data from these experiments suggest that mechanisms which do not involve both glutamate and the NMDA receptor can also activate the ethanol reward pathway in the mHEP rat, and a mechanism other than one downstream from the DA D1 receptor activation is involved.

CHAPTER 6

GENERAL DISCUSSION

Alcohol abuse and alcohol dependence are serious health issues with many adverse consequences. These disorders have been associated with many physical illnesses, psychological and social problems, serious accidents which are often fatal, and numerous criminal activities. Alcohol research involves elucidating the behavioral effects of ethanol and the mechanisms by which ethanol produces its effects, and ultimately, developing effective treatments for alcohol-use disorders.

Alcohol research requires the use of animal models which exhibit the appropriate characteristics of the alcohol disorder being studied and experimental paradigms which best simulate the environmental stimuli that will influence alcohol use disorders (McMillen, 1997). This experimental plan takes into account genetic and environmental factors which are both associated with alcohol use disorders. In this study, the alcohol use disorder being studied was Cloninger's Type 2 alcoholism which involves the early onset of heavy alcohol consumption and a strong family history of severe alcoholism (Cloninger et al., 1988). The mHEP rat was selected as the test system due to its validation as an animal model for Type 2 alcoholism. These selectively bred rats exhibit the early onset of heavy alcohol consumption, profuse volitional consumption of ethanol, a preference for ethanol in the presence of palatable alternatives, and levels of blood ethanol that correspond essentially with the respective amounts of ethanol consumed (Myers et al., 1998). Since the mHEP rat line is maintained by breeding non-sibling males and females that are selected based on an ethanol drinking screening procedure, these rats also reflect a strong family history of alcoholism.

Numerous studies have implicated various neurotransmitters, neuromodulators, receptor types and subtypes, and brain regions in the development and manifestation of alcohol dependence. The mesolimbic DA system, which involves DA projections from

the VTA to various limbic regions, is a major neurological pathway involved in the reinforcing effects of ethanol and ethanol self-administration (Carroll et al., 2006; Gonzales et al., 2004; Hodge et al., 1997; Lof et al., 2007; Phillips et al., 1998; Risinger et al., 2000; Thielen et al., 2004). More specifically, the projections from the VTA to the NAc have been implicated as a major pathway involved in ethanol reward (cf. Pierce and Kumaresan, 2006; Yim and Gonzales, 2000). Other studies have suggested that the glutamatergic NMDA receptor is a major target of ethanol (cf. Ron, 2004). Together, the involvement of the mesolimbic DA system and the NMDA receptor in the actions of ethanol served as the basis for this study.

The potential adverse side effects of neuroprotective NMDA receptor antagonists shown to decrease ethanol consumption in genetic ethanol drinking rats (McMillen et al., 2004) suggest that these drugs may not be clinically tolerable treatments for humans. However, the low-affinity, noncompetitive NMDA receptor antagonist memantine exhibits both neuroprotective properties and a tolerable clinical profile (cf. Chen and Lipton, 2006; cf. Lipton and Chen, 2004; cf. Lipton, 2004a; cf. Lipton, 2004b; cf. Lipton, 2005; cf. Lipton, 2007; cf. Rammes et al., 2008). Together, these findings suggest that memantine might effectively decrease ethanol consumption without producing intolerable clinical side effects. An alternative approach to modify glutamatergic activity is to use drugs which target the metabotropic glutamate receptors (Swanson and Schoepp, 2003).

This project focused on memantine as a potential treatment for alcohol dependence with minimal adverse side effects and the potential effects of this drug on the ethanol reward pathway. First, it was demonstrated that memantine exhibited a dose-dependent decrease in consumption of ethanol in the genetic animal model. It was

further demonstrated that a mGlu 2/3 glutamate receptor antagonist, which would decrease glutamate release, also decreased consumption of ethanol. Secondly, this study investigated the possibility that blockade of the NMDA receptor alters DA metabolism and/or alters the Ca²⁺-mediated response downstream of the DA D1 receptor. Previous studies indicate that activation of DA receptors converts DARPP-32 into a potent protein phosphatase inhibitor in response to DA receptor activation in the forebrain (Fienberg et al., 1998) and that moderate concentrations of ethanol increase phosphorylation of DARPP-32 at Thr34 in striatal slices (cf. Nairn et al., 2004; cf. Svenningsson et al., 2005). Therefore, ethanol-induced alterations in the phosphorylation levels of DARPP-32 in the limbic regions may serve as markers to indicate that memantine and ethanol are influencing the same intracellular cascade downstream of the DA D1 receptor. The three limbic regions studied included the NAc, mPFC, and STR.

Summary of Findings

Findings from Behavioral Experiments

Behavioral experiments provided information on the effects of memantine on ethanol consumption, locomotor coordination and activity, and rectal temperature in the mHEP rat. Additional studies showed the effects of various DA receptor drugs, a mGlu 2/3 receptor agonist, and a mGlu 2/3 receptor antagonist on ethanol consumption in the same animal model using the same ethanol drinking paradigm. The results and conclusions from these behavioral studies can be summarized as follows:

1. Memantine, at doses of 1.0, 3.0, and 10.0 mg/kg, i.p., significantly decreased volitional ethanol consumption in an apparent dose-dependent manner in the

male mHEP rat in the two-choice 24 hour paradigm. The effect on ethanol intake does not appear to be an anti-caloric effect.

2. Results from the activity monitor demonstrate that the 10.0 mg/kg dose of memantine does not significantly alter locomotor activity or ability. The data indicate that this dose of memantine is non-sedating, and induces neither hypoactivity nor hyperactivity.
3. Results from the elevated plus maze demonstrate that the 10.0 mg/kg dose of memantine does not produce an anxiolytic effect. Furthermore, the total number of arm entries indicates that this dose of memantine is non-sedating.
4. The 10.0 mg/kg dose of memantine produced a mild reversible hypothermic effect when administered alone. When co-administered with ethanol, this dose of memantine enhanced the ethanol-induced hypothermic effect in rats.
5. The DA D1 receptor antagonist SCH23390 (0.1 and 0.3 mg/kg) and (+)sulpiride (10.0 mg/kg), an isomer inactive at the D2 receptor, significantly decreased ethanol consumption while the DA D2-active antagonist (-)sulpiride (10.0 mg/kg) did not. Co-administered with SCH23390 (0.3 mg/kg), (-)sulpiride (3.0 and 10.0 mg/kg) did not alter SCH23390-induced reductions in ethanol consumption. The effects produced by SCH23390, (+)sulpiride, and (-)sulpiride do not appear to be anti-caloric. The DA D1 receptor agonist SKF38393 (1.25, 2.5, 5.0 and 10.0 mg/kg) dose-dependently decreased ethanol consumption. While SCH23390 appears to produce an aphagic effect, SKF38393 decreases ethanol consumption without an aphagic effect. Co-administered SCH23390 and SKF38393 produced effects

similar to those of each drug alone which suggests that the predominant effect observed is due to SCH23390-induced aphagia.

6. Co-administered memantine (1.0 mg/kg) and SCH23390 (0.3 mg/kg) reduced ethanol consumption but did not produce an additive or synergistic effect.

The predominant effect appears to be an aphagic effect of SCH23390 which means nonspecific effects of the memantine or the combination of SCH23390 and memantine is observed.

7. The mGluR 2/3 agonist, LY379268, decreased both ethanol and food intake which suggests that the drug may be producing an anti-caloric or aphagic effect that impacts on ethanol consumption. These results are in harmony with a previous report indicating that rats decreased operant responding for food following injections of LY379268 (Peters and Kalivas, 2006).

Conversely, the mGluR 2/3 antagonist, LY341495, reduced ethanol consumption, but the effect did not appear to be anti-caloric or aphagic.

Furthermore, LY341495 (3.0 mg/kg) did not reverse or inhibit the effect of LY379268 (3.0 mg/kg) on food consumption. To determine if LY341495 does reverse the effect, additional doses would need to be tested.

For convenience, summaries of the data from the ethanol drinking, activity monitor, and rectal temperature experiments are presented in tables in Appendix B.

Findings from Biochemical Experiments

Biochemical experiments were performed to determine the effects of memantine on DA metabolism and on levels of DARPP-32 and its phosphorylation at Thr34 and

Thr75 sites in limbic regions receiving DA input from the VTA. The results and conclusions from these biochemical studies can be summarized as follows:

1. HPLC analysis showed that:
 - a. Memantine, at a dose of 10.0 mg/kg, did not alter the levels of DA or its metabolite, DOPAC, in the PFC, NAc, or STR.
 - b. Ethanol, at doses of 1.0 and 2.5 g/kg, did not alter the levels of DA or its metabolite, DOPAC, in the PFC, NAc, or STR. However, the 2.5 g/kg dose of ethanol produced increases in striatal DOPAC which bordered on being significant and were in the range of expected levels of change based on previous reports (Dar and Wooles, 1984; Honkanen et al., 1994; Khatib et al., 1988).
 - c. The combination of ethanol, at a dose of 1.0 or 2.5 g/kg, and memantine, at a dose of 10.0 mg/kg, did not alter the levels of DA or its metabolite, DOPAC, in the PFC, NAc, or STR. Memantine neither blocked nor added to the increase in striatal DOPAC produced by the 2.5 g/kg dose of ethanol which bordered on being significant.

These results indicate that at the doses tested, neither memantine nor ethanol, alone, significantly alter DA metabolism in the brain regions studied. However, the results suggest that the addition of more rats to the experiment might cause the increase in striatal DOPAC levels to become significant. More importantly, pre-treatment with memantine did not influence the levels of DA or DA metabolism in the mHEP rats treated with ethanol. This suggests that glutamate acts at the NMDA receptor downstream from the drive by ethanol on the reward pathway.

2. Western blot analysis showed that memantine did not alter the levels of DARPP-32 or phosphorylation of DARPP-32 at Thr34 or Thr75 in the NAc and STR. Memantine also did not alter the levels of DARPP-32 or phosphorylation of DARPP-32 at Thr75 in the mPFC. The levels of phosphorylation of DARPP-32 at Thr34 in the mPFC were undetectable using the Western blotting technique. These results indicate that ethanol and memantine, at the doses tested, may be acting in different intracellular cascades downstream of the DA D1 receptor.

For convenience, summaries of the data from the HPLC and Western blot analyses are presented in tables in Appendix G.

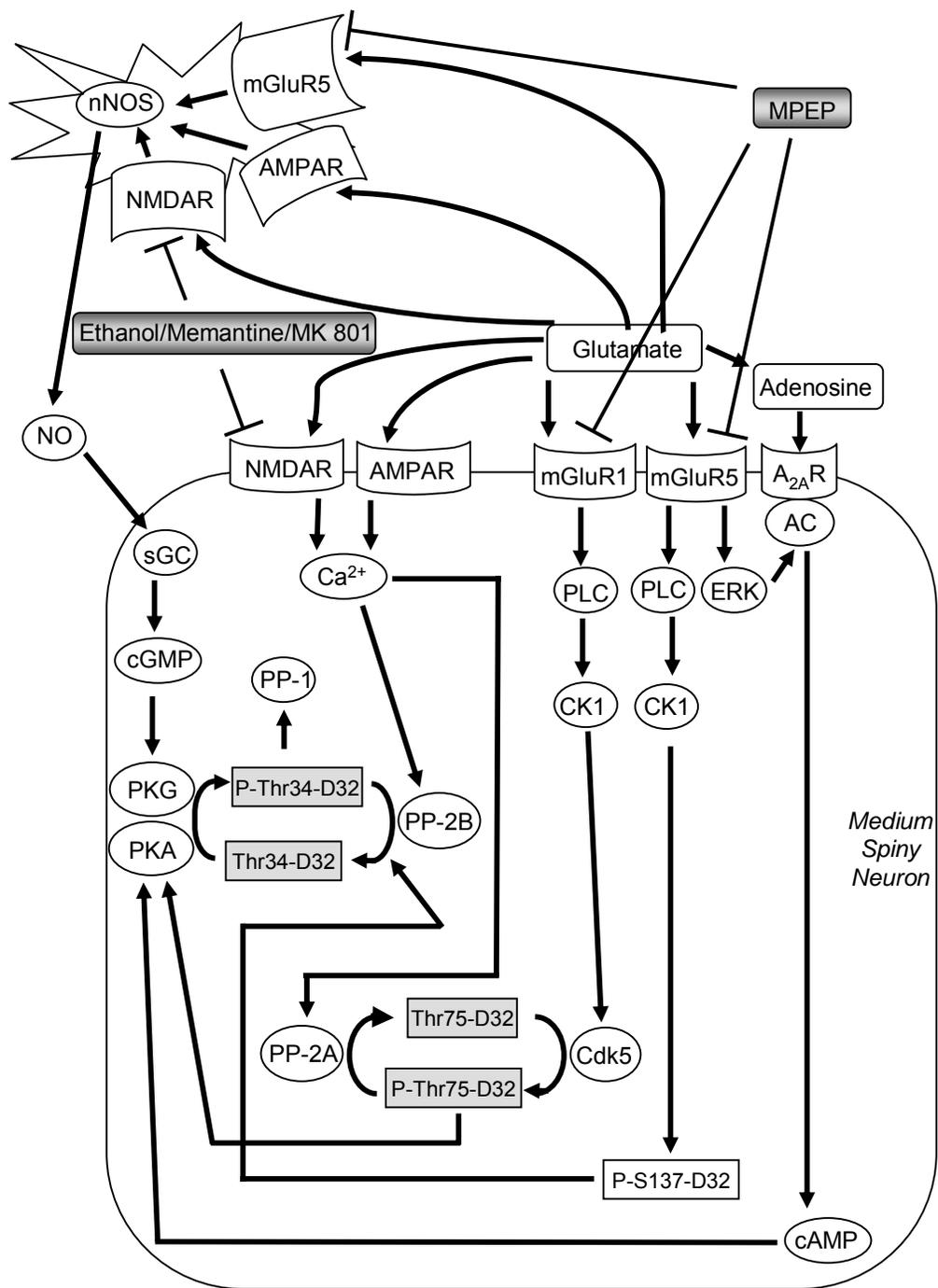
Overall Summary of Findings

Overall, the results from this study suggest that memantine and the mGluR 2/3 antagonist, LY341495, will reduce ethanol consumption in a genetic high ethanol drinking rat model with minimal adverse side effects. Data from the biochemical experiments suggest that mechanisms which do not involve glutamate and the NMDA receptor may activate the ethanol reward pathway in the mHEP rat. Also, the effect of memantine on the ethanol intake does not involve modification of the DA/DARPP-32 signaling system.

Conclusions and Proposed Mechanisms

Previous studies have shown that glutamate modulates dopaminergic signaling via the activation of at least five different signaling cascades (Figure 6.1) with different time dependencies in the NSTR (Nishi et al., 2005): (1) Activation of the NMDA, AMPA,

Figure 6.1. Signaling cascades activated by NMDA, AMPA, mGlu1, and mGlu5 receptors and the phosphorylation of DARPP-32 at Thr34 and Thr75. Arrows indicate activation and T bars indicate inhibition. [NMDAR = N-methyl-D0-aspartate receptor; AMPAR = α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptor; $A_{2A}R$ = adenosine A_{2A} receptor; D32 = DARPP-32; S137 = Ser137; NO = nitric oxide; nNOS = neuronal nitric oxide synthase; sGC = soluble guanylyl cyclase; MPEP = 2-methyl-6-(phenylethynyl)pyridine]. [Adapted from Nishi et al., 2005.]



and mGlu5 receptors mediate a neuronal nitric oxide synthase (nNOS)/nitric oxide (NO)/cGMP/PKG signaling cascade that leads to increased phosphorylation of DARPP-32 at the Thr34 site (Nishi et al., 2005); (2) Activation of NMDA and AMPA receptors leads to Ca^{2+} -dependent activation of PP-2B, which decreases phosphorylation of DARPP-32 at the Thr34 site (Nishi et al., 2002); (3) Activation of NMDA and AMPA receptors leads to Ca^{2+} -dependent activation of PP-2A, which decreases phosphorylation of DARPP-32 at the Thr75 site (Nishi et al., 2002); (4) Activation of the Group I mGlu5 receptors stimulates the A_{2A} receptor-mediated formation of cAMP in an ERK-dependent manner which leads to an increase in the phosphorylation of DARPP-32 at the Thr34 site (Nishi et al., 2003); (5) Activation of Group I mGlu receptors increases the phosphorylation of DARPP-32 at the Thr75 site and the Ser137 site. In the NSTR, the Ser137 site is known to be phosphorylated by CK1 (Liu et al., 2001) Activation of Group I mGlu receptors stimulates PLC and CK1 which activates Cdk5 via an unknown mechanism, and subsequently, increases the phosphorylation of DARPP-32 at the Thr75 site (Liu et al., 2002). The time courses for these signal transduction pathways are: (1) Rapid (15-30 s) activation of nNOS/NO/cGMP/PKG signaling; (2) intermediate (1-5 min) activation of the Ca^{2+} /PP-2B and Ca^{2+} /PP-2A signaling; and (3) slow (5-10 min) activation of mGluR5/PLC and ERK and Group I mGluR/PLC signaling. Furthermore, the nNOS/NO/cGMP/PKG signaling and mGluR5/PLC and ERK signaling cascades which lead to increased phosphorylation at Thr34 and Ca^{2+} /PP-2A signaling which leads to decreased phosphorylation at Thr75 contribute positively to DA/D1 receptor/phospho-Thr34 DARPP-32 signaling. Conversely, the Ca^{2+} /PP-2B signaling which leads to decreased phosphorylation at Thr34 and Group I mGluR/PLC signaling which leads to

increased phosphorylation at Thr75 are antagonistic to DA/D1 receptor/phospho-Thr34 DARPP-32 signaling (Nishi et al., 2005).

Both ethanol and memantine inhibit NMDA receptors, which suggests that these drugs, alone or combined, should increase the phosphorylation of DARPP-32 at the Thr34 and Thr75 sites via PP-2B- and PP-2A-mediated pathways, respectively. Memantine attenuates Ca^{2+} influx, allowing levels of Ca^{2+} associated with a low degree of NMDA receptor stimulation, which supports the premise that this drug allows for physiological levels of NMDA receptor-mediated responses, but inhibits excessive NMDA-evoked activity (Chen et al., 1992). Ethanol is also an inhibitor of NMDA (Lovinger et al., 1989; Simson et al., 1991; Wirkner et al., 2000). This suggests that memantine or ethanol, alone, should decrease intracellular Ca^{2+} levels, and a combination of the two drugs should produce the same, if not an additive or synergistic, effect.

DARPP-32 is enriched in dopaminergic neurons which contain D1 receptors, which are DA receptors positively coupled to AC. Phosphorylation of DARPP-32 can be regulated by both DA and cAMP. These findings suggest that DARPP-32 is involved in mediating certain trans-synaptic effects of DA on dopaminergic neurons (Walaas et al., 1983). DA, via its activation of DA D1-like receptors, stimulates AC which leads to increased formation of cAMP and activity of PKA. This leads to phosphorylation of DARPP-32 at the Thr34 site, which converts it into a potent inhibitor of the phosphatase, PP-1 (cf. Snyder et al., 1998). DA D2-like receptors produce opposing effects on the phosphorylation of DARPP-32 (Nishi et al., 1997). Glutamate, via activation of NMDA receptors, increases Ca^{2+} influx and activity of the Ca^{2+} /calmodulin dependent protein phosphatase calcineurin (PP-2B). This leads to the dephosphorylation of DARPP-32,

and subsequent inactivation of the phosphoprotein (King et al., 1984; Nishi et al., 1997). DA increases phosphorylation of the NR1 subunit of NMDA receptors via a PKA-dependent pathway, and it has been proposed that the effect of PKA is partially mediated by regulation of the DARPP-32/PP-1 signaling cascade (Snyder et al., 1998).

A previous study of DA alteration of sensitivity of NMDA receptors to ethanol indicates that D1-like receptors, postsynaptically, activate the cAMP/PKA/DARPP-32 signaling cascade, which subsequently leads to phosphorylation of the NMDA receptor NR1 subunits and a strong decrease in the sensitivity of NMDA receptor-mediated synaptic transmission to ethanol (cf. Maldve et al., 2002; Zhang et al., 2005). Furthermore, the findings of Zhang and colleagues (2005) suggest that D1-like receptor regulation of ethanol sensitivity at the NMDA receptor involves both presynaptic and postsynaptic components of NMDA receptor-mediated synaptic transmission. SKF38393, a DA D1 receptor agonist, reversed ethanol inhibition of NMDA while SCH23390, a DA D1-selective receptor antagonist, completely inhibited both presynaptic and postsynaptic actions of ethanol. These results led to the suggestion that DA D1-like receptors modulate presynaptic and postsynaptic effects of ethanol on NMDA receptor-mediated synaptic transmission in the NAc, and these interactions may contribute to ethanol-induced neuroadaptation of the ethanol reward pathway (Zhang et al., 2005). This suggests that an increase in DA levels in the NAc, resulting from an ethanol-induced increase in the firing of dopaminergic neurons in projections from the VTA, might alter the sensitivity of the NMDA receptors to ethanol, thereby decreasing ethanol blockade of the NMDA receptor, and subsequently increasing intracellular Ca^{2+} levels and decreasing phosphorylation of DARPP-32 at Thr34 and Thr75. This suggests an alternative mechanism which might counteract a mechanism that increases

phosphorylation or blocks the dephosphorylation of DARPP-32. It is likely that ethanol produces actions on multiple signaling cascades at several different sites.

A review by Greengard and colleagues (1999) illustrates the central role of the DARPP-32/PP-1 pathway in signal transduction in MSNs and demonstrates that this pathway influences the phosphorylation state of a wide range of proteins. In MSNs that co-express DA D1-like and D2-like receptors and that co-express adenosine A_{2A} receptors and D2 receptors, activation of D2 receptors decreases cAMP levels. Activation of D2 receptors produces an increase in intracellular Ca^{2+} levels, through an unknown mechanism, and an increase in PP-2B activity. Neurotensin increases phosphorylation of DARPP-32 by increasing the release of DA. Conversely, cholecystinin (CCK) decreases phosphorylation of DARPP-32 by increasing glutamatergic neurotransmission. Phospho-DARPP-32 inhibits PP-1, thereby acting synergistically with different protein kinases, predominantly PKA and PKC, to increase the phosphorylation levels of various downstream effector proteins. The increased levels of phosphorylated proteins is associated with increased activity of NMDA and AMPA receptors, voltage-gated ion channels including L-, N-, and P- type Ca^{2+} channels, Na^+/K^+ -ATPase, and CREB. Phosphorylation of CREB alters activation of the transcription factor FRA (Fos-related antigen) (cf. Greengard et al., 1999).

Levels of DOPAC, a metabolite of DA, were analyzed in this study as an indicator of DA release. Elevated DOPAC levels would indicate higher levels of DA metabolism which would suggest higher levels of DA release. The data in this study indicated an ethanol-induced increase in striatal DOPAC which bordered on being significant. This suggests that ethanol increases DA release in the STR, but not in the NAc and mPFC.

Since memantine failed to alter levels of DOPAC, it appears that memantine did not increase the release of DA.

Since acute alcohol use stimulates neurons in the VTA, inducing DA release in the NAc (cf. Anton, 2008; cf. Di Chiara, 2002; cf. Di Chiara et al., 2004; cf. Kreek et al., 2002; cf. Pierce and Kumaresan, 2006; cf. Weiss et al., 1993; cf. Weiss et al., 2001), this suggests that phosphorylation of DARPP-32 at Thr75 will increase via Cdk5 and the positive feedback loop by which DA regulates the Cdk5/phospho-Thr75 DARPP-32 pathway, and conversely, the Cdk5/phospho-Thr75 DARPP-32 pathway regulates DA signaling (Nishi et al., 2000). According to this mechanism, DA activates DA D1 receptors, which leads to the progressive activation of PKA and PP-2A, and a reduction in the levels of phosphorylation of DARPP-32 at Thr75. PP-2A dephosphorylates DARPP-32 at Thr75, thereby removing the inhibition of PKA. The activation of PKA leads to increased levels of phosphorylation of DARPP-32 at Thr34 and inhibition of PP-1. Together, activation of PKA and inhibition of PP-1 produce a synergistic increase in the phosphorylation of various substrates. However, no significant alterations in levels of phosphorylation of DARPP-32 at Thr34 or Thr75 were observed in the STR or NAc in this study and no changes were observed in the phosphorylation at Thr75 in the mPFC. Since results for the phosphorylation of Thr34 in the mPFC were undetectable, no conclusions can be drawn about that particular mechanism.

Blockade of glutamate activation at the NMDA receptor by memantine and ethanol should reduce Ca^{2+} influx. Less intracellular Ca^{2+} would mean less activation of PP-2A and PP-2B. Decreased activation of PP-2A would reduce the dephosphorylation of DARPP-32 at Thr34. Decreased activation of PP-2B would reduce the dephosphorylation of DARPP-32 at Thr75. Based on this premise, samples from

memantine- and/or ethanol-treated rats should show increased levels of phosphorylation at Thr34 and Thr75. Since the levels of phosphorylation of DARPP-32 at Thr34 and Thr75 did not change following NMDA inhibition, this suggests that perhaps glutamate increases its activity at other receptors. For example, increased activation of AMPA receptors would increase the dephosphorylation of DARPP-32 at Thr34 and Thr75 via the same Ca^{2+} /PP-2B and Ca^{2+} /PP-2A pathways, respectively, involved in NMDA receptor activation. Increased activation of the mGlu5 receptor by glutamate should increase the dephosphorylation of DARPP-32 at Thr34 via the PLC/CK1/Ser137 cascade. Alternatively, if DA acts at the D2 receptor, phosphorylation of DARPP-32 would be inhibited, which might offset the reduction in dephosphorylation potentially caused by the memantine and ethanol blockade of the NMDA receptor.

In a review by Cepeda and Levine (2006), five mechanisms for D1-NMDA receptor interactions, called synaptic complexes or triads, which have been observed in several brain regions including the STR, CTX, and NAc, are demonstrated. Three of the mechanisms are physical while the other two are mediated by second messengers. The physical interactions include the following: (1) Activation of NMDA receptors traps diffusible DA D1 receptors. The functional outcome of this interaction is unknown. (2) DA D1 receptors and the NMDA receptor NR1 subunit form an oligomer at the synapse, blocking the receptor internalization that is normally produced by the activation of D1 receptors. These oligomers are transported from the endoplasmic reticulum to the synapse where they could potentiate NMDA receptor function. (3) The NMDA receptor NR1 and NR2A subunits contact with the C terminus of the DA D1 receptor. During blockade of PKA and PKC cascades, activation of DA D1 receptors attenuates NMDA currents and NMDA receptor-mediated excitotoxicity. The NMDA-D1 interactions

mediated by second messengers include the following: (1) Activation of DA D1 receptors by DA leads to the potentiation of NMDA receptor function via the cAMP/PKA/DARPP-32 signaling cascade. (2) Activation of DA D1 receptors upregulates the abundance of NMDA receptor subunits at the synapse via activation of the cytoplasmic-protein tyrosine kinase Fyn.

One or more of these D1-NMDA receptor interactions may also be influencing the levels of phosphorylation of DARPP-32 observed following treatments with memantine and/or ethanol. For example, since memantine allows a basal level of NMDA activation, potentiation of DARPP-32 function might increase the level of excitation. This would mean a greater influx of Ca^{2+} which would increase the levels of phosphorylation of DARPP-32 at Thr34 and Thr75, and offset a potential decrease in dephosphorylation caused by memantine and/or ethanol.

Since the mGluR 2/3 antagonist, LY341495, decreased ethanol consumption without adversely affecting food consumption, this suggests that a mGluR 2/3 antagonist may be an effective treatment for alcohol dependence. However, potential adverse effects such as anxiety, sedation, hypoactivity, hyperactivity, hypothermia, and hyperthermia remain to be evaluated in the mHEP rat. Since the mGluR 2/3 agonist, LY379268, appears to have produced an aphagic or anti-caloric effect, mGlu2/3 agonism may not be a viable treatment for alcohol dependence.

The mGluR 2/3 agonist LY379268 has been shown to exhibit high potency and selectivity for the mGlu2/3 receptors (Monn et al., 1999). Studies have indicated that LY379268, at doses of 1.0 and 3.0 mg/kg (i.p.), reverses phencyclidine (PCP)-induced hyperactivity in animal models predictive of antipsychotic activity. The reversal of PCP-induced hyperactivity was observed in wild-type (WT) and mGluR3 KO mice, but not in

mGluR2 KO mice which suggests that mGluR2 mediates the actions of LY379268 in the mouse model predictive of antipsychotic activity (Woolley et al., 2008). This would also suggest that LY379268 may decrease locomotor activity in the mHEP rat. The mGluR 2/3 antagonist LY341495 exhibits nanomolar potency and selectivity for the mGlu2/3 receptors with its greatest potency at mGluR3 and equal or less potency at mGluR2 (Kingston et al., 1998). LY341495, at a dose of 2.5 mg/kg (s.c.), has been shown to increase locomotor activity in mice (O'Neill et al., 2003). This would suggest that LY341495 may increase locomotor activity in the mHEP rat.

The Group II mGluR 2/3 receptors, which are predominantly located presynaptically, couple to G_i/G_o and inhibit AC. Activation of these receptors decreases glutamate release (cf. Pin et al., 1999; cf. Schoepp et al., 1999). This suggests that the mGluR 2/3 antagonist should increase glutamate release. Based on the pathways involving glutamate regulation of DARPP-32 phosphorylation, glutamate activates NMDA and AMPA receptors leading to decreased phosphorylation of DARPP-32 at the Thr34 and Thr75 sites. Glutamate also activates mGlu1 and mGlu5 receptors leading to an increase in phosphorylation of DARPP-32 at Thr34 and Thr75. However, ethanol blocks NMDA receptors, which would decrease the dephosphorylation of DARPP-32 at Thr34 and Thr75. This suggests that LY341495 and ethanol combined produce increased levels of phosphorylation at DARPP-32 at Thr34 and Thr75. Since this study did not involve biochemical experiments to determine potential mechanisms by which LY341495 decreases ethanol consumption in the mHEP rat, it is uncertain what effects this drug has on the metabolism of DA and the phosphorylation of DARPP-32. However, it is possible that this drug will produce different effects from memantine since the two drugs act at different receptors.

Ultimately, the goal of alcohol research is to develop pharmacological treatments that can be combined with some type of cognitive or behavioral therapy to aid in helping alcohol-dependent patients maintain sobriety. Several studies indicate that the effectiveness of therapy depends upon the drug and the type of cognitive or behavioral therapy used (Jones et al., 1982; Longabaugh et al., 2005; Longabaugh et al., 2009). A desirable pharmacological treatment will reduce alcohol consumption with minimal adverse side effects and minimal compliance issues.

Future Directions

While providing answers, research also raises new questions. Several items that remain to be determined include:

1. The effect of co-administered memantine and SKF38393 on volitional consumption of ethanol in the male mHEP rat.
2. Determination of blood alcohol concentration (BAC) levels in male mHEP rats exposed to both chronic and acute ethanol.
3. The effects of memantine (10.0 mg/kg, i.p.) and acute ethanol (1.0 and 2.5 g/kg, i.p.), alone and together, on the levels of phosphorylation of DARPP-32 at the Thr34 site in the mPFC of the male mHEP rat. The bands indicating immunoreactivity were undetectable in the Western blots performed in this study. Alternative techniques include:
 - a. Immunoprecipitation (IP) followed by Western blotting.
 - b. Concentration of the homogenate and use of an anti-phospho-DARPP-32 (Thr34) antibody covalently attached to beads to separate out the phosphoprotein followed by Western blotting.

- c. Measure levels of a protein downstream of DARPP-32 that is activated or inhibited by phosphorylation of DARPP-32 at Thr34.
Protein phosphatase 1 (PP-1) would be a likely candidate.
4. The effects of memantine on the levels of DA and DA metabolism in the limbic regions of the male mHEP rat exposed to chronic ethanol.
5. The effects of memantine on the levels of DARPP-32 and phosphorylation of DARPP-32 at the Thr34 and Thr75 sites in the limbic brain regions of the male mHEP rat exposed to chronic ethanol.
6. The effects of memantine on the rectal temperature of the male mHEP rat exposed to chronic ethanol.
7. The effects of ethanol, both acute and chronic, and memantine, alone and combined with ethanol, on levels of cAMP in the limbic regions of the mHEP rat. This can be measured via a cAMP radioimmunoassay.
8. The effects of DA D2 receptor agonists on ethanol consumption in the mHEP rat in the two-choice 24 hour access paradigm.
9. The potential locomotor and sedating effects of DA agonists and antagonists at doses shown to decrease volitional ethanol consumption in the mHEP rat.
10. The potential of the mGluR 2/3 antagonist, LY341495, at doses not tested in this study, to reverse the reduction in food consumption produced by the mGluR 2/3 agonist, LY379268.
11. The potential locomotor and sedating effects of the mGluR 2/3 agonist and antagonist, LY379268 and LY341495, respectively, at doses shown to decrease volitional ethanol consumption in the mHEP rat.

12. The effects of LY341495, both alone and combined with ethanol, on DA metabolism.
13. The effects of LY341495, both alone and combined with ethanol, on the levels of DARPP-32 and phosphorylation of DARPP-32 at the Thr34 and Thr75 sites.

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APPENDIX A

ANIMAL USE APPROVAL LETTER

**Animal Care and Use Committee**

East Carolina University
212 Ed Warren Life Sciences Building
Greenville, NC 27834
252-744-2436 office • 252-744-2355 fax

February 5, 2009

Brian McMillen, Ph.D.
Department of Pharmacology
Brody 6S-10
ECU Brody School of Medicine

Dear Dr. McMillen:

Your Animal Use Protocol entitled, "CNS Transmitters and Metabolites in Alcohol Addiction," (AUP #W129e) was reviewed by this institution's Animal Care and Use Committee on 2/5/09. The following action was taken by the Committee:

"Approved as submitted"

A copy is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies.

Sincerely yours,

A handwritten signature in black ink, appearing to read 'Robert G. Carroll, Ph.D.'.

Robert G. Carroll, Ph.D.
Chairman, Animal Care and Use Committee

RGC/jd

enclosure

APPENDIX B

DATA TABLES FROM BEHAVIORAL EXPERIMENTS

EFFECTS OF MEMANTINE ON ETHANOL CONSUMPTION

Treatment	Ethanol consumed (g/kg/day)		
	Pre-treatment	During treatment	Post treatment
Vehicle (10)	4.90 ± 0.43	4.46 ± 0.59	4.50 ± 0.49
0.3 mg/kg (10)	4.71 ± 0.46	4.16 ± 0.56	4.42 ± 0.48
1.0 mg/kg (9)	4.85 ± 0.44	3.47 ± 0.70 *	4.30 ± 0.54
3.0 mg/kg (10)	4.75 ± 0.36	2.82 ± 0.52 *†	4.64 ± 0.33

Treatment	Proportion of ethanol consumed (ethanol (ml)/[ethanol (ml) + water (ml)])		
	Pre-treatment	During treatment	Post treatment
Vehicle (10)	0.62 ± 0.01	0.58 ± 0.04	0.60 ± 0.04
0.3 mg/kg (10)	0.57 ± 0.03	0.56 ± 0.04	0.56 ± 0.03
1.0 mg/kg (9)	0.60 ± 0.03	0.51 ± 0.05	0.57 ± 0.03
3.0 mg/kg (10)	0.56 ± 0.02	0.45 ± 0.05	0.60 ± 0.02

Treatment	Total fluids consumed (ml/day)		
	Pre-treatment	During treatment	Post treatment
Vehicle (10)	34.8 ± 2.2	32.7 ± 1.9	33.3 ± 2.3
0.3 mg/kg (10)	37.2 ± 2.1	32.1 ± 1.9 *	34.9 ± 1.7
1.0 mg/kg (9)	35.5 ± 2.3	25.8 ± 2.0 *†	32.4 ± 1.8
3.0 mg/kg (10)	37.3 ± 1.5	26.0 ± 2.2 *†	33.9 ± 1.7

Treatment	Food consumed (g/day)		
	Pre-treatment	During treatment	Post treatment
Vehicle (10)	16.8 ± 0.6	17.7 ± 0.8	18.4 ± 0.7
0.3 mg/kg (10)	19.0 ± 0.7	17.9 ± 0.6	18.2 ± 0.5
1.0 mg/kg (9)	19.2 ± 0.7	17.4 ± 0.7 *	18.8 ± 0.6
3.0 mg/kg (10)	19.2 ± 0.5	16.3 ± 0.6 *	18.5 ± 0.6

Treatment	Body weight (kg)		
	Pre-treatment	During treatment	Post treatment
Vehicle (10)	0.386 ± 0.017	0.390 ± 0.018	0.393 ± 0.019
0.3 mg/kg (10)	0.392 ± 0.018	0.394 ± 0.020	0.397 ± 0.020
1.0 mg/kg (9)	0.387 ± 0.020	0.387 ± 0.021	0.389 ± 0.021
3.0 mg/kg (10)	0.386 ± 0.017	0.382 ± 0.016 *†	0.383 ± 0.016

* indicates a significant difference from pre-treatment baseline, $P < 0.05$ (Tukey/Kramer).

† indicates a significant difference from vehicle treatment, $P < 0.05$ (Tukey/Kramer).

Number of rats is indicated in parentheses.

EFFECTS OF MEMANTINE AT A DOSE OF 10.0 mg/kg ON ETHANOL CONSUMPTION

Treatment	Ethanol consumed (g/kg/day)		
	Pre-treatment	During treatment	Post treatment
Vehicle (7)	6.94 ± 0.39	6.60 ± 0.41	6.45 ± 0.66
10.0 mg/kg (7)	7.11 ± 0.48	3.69 ± 0.51 *†	5.88 ± 0.28

Treatment	Proportion of ethanol consumed (ethanol (ml)/[ethanol (ml) + water (ml)])		
	Pre-treatment	During treatment	Post treatment
Vehicle (7)	0.62 ± 0.05	0.61 ± 0.05	0.58 ± 0.02
10.0 mg/kg (7)	0.62 ± 0.04	0.47 ± 0.06 *†	0.58 ± 0.05

Treatment	Total fluids consumed (ml/day)		
	Pre-treatment	During treatment	Post treatment
Vehicle (7)	37.7 ± 2.0	37.3 ± 2.8	37.5 ± 3.8
10.0 mg/kg (7)	38.9 ± 2.1	26.1 ± 1.7 *†	34.8 ± 2.0

Treatment	Food consumed (g/day)		
	Pre-treatment	During treatment	Post treatment
Vehicle (7)	18.6 ± 0.4	16.7 ± 0.6 *	17.1 ± 0.5
10.0 mg/kg (7)	17.4 ± 0.9	15.9 ± 0.8	17.4 ± 0.7

Treatment	Body weight (kg)		
	Pre-treatment	During treatment	Post treatment
Vehicle (7)	0.336 ± 0.009	0.343 ± 0.010 *	0.350 ± 0.010
10.0 mg/kg (7)	0.338 ± 0.008	0.334 ± 0.007 †	0.340 ± 0.007

* indicates a significant difference from pre-treatment baseline, $P < 0.05$ (Tukey/Kramer).

† indicates a significant difference from vehicle treatment, $P < 0.05$ (Tukey/Kramer).

Number of rats is indicated in parentheses.

ACTIVITY MONITOR RESULTS

	Distance Traveled (Centimeters \pm S.E.M.)			
	1 st Epoch	2 nd Epoch	3 rd Epoch	4 th Epoch
Group I ($n = 7$)				
Ctl/Ctl	4015 \pm 522	2535 \pm 257	1044 \pm 228	1530 \pm 383
Ctl/EtOH (1.0 g/kg)	4389 \pm 490	2619 \pm 407	1071 \pm 216	458 \pm 124*
Group II ($n = 8$)				
Ctl/Ctl	3625 \pm 462	1923 \pm 436	1128 \pm 497	1737 \pm 561
Ctl/EtOH (2.5 g/kg)	3876 \pm 680	2338 \pm 462	412 \pm 70	166 \pm 38*
Group III ($n = 8$)				
Ctl/Ctl	4144 \pm 235	2436 \pm 236	1282 \pm 336	1456 \pm 365
Mem/Ctl	2583 \pm 459*	1588 \pm 407*	1374 \pm 270	2041 \pm 471
Group IV ($n = 8$)				
Ctl/Ctl	4771 \pm 293	2114 \pm 379	1921 \pm 449	1713 \pm 227
Mem/EtOH (1.0 g/kg)	1884 \pm 254* [†]	2238 \pm 369	1130 \pm 152	994 \pm 161 [†]
Group V ($n = 7$)				
Ctl/Ctl	4724 \pm 468	2365 \pm 362	1933 \pm 431	1415 \pm 298
Mem/EtOH (2.5 g/kg)	2347 \pm 228*	1971 \pm 435	670 \pm 170*	873 \pm 174 ^Δ
	Resting Time (Seconds \pm S.E.M.)			
	1 st Epoch	2 nd Epoch	3 rd Epoch	4 th Epoch
Group I ($n = 7$)				
Ctl/Ctl	415 \pm 35	529 \pm 33	686 \pm 32	662 \pm 48
Ctl/EtOH (1.0 g/kg)	386 \pm 37	519 \pm 33	698 \pm 28	790 \pm 27*
Group II ($n = 8$)				
Ctl/Ctl	421 \pm 36	580 \pm 51	701 \pm 43	635 \pm 47
Ctl/EtOH (2.5 g/kg)	409 \pm 45	559 \pm 39	805 \pm 15*	855 \pm 9*
Group III ($n = 8$)				
Ctl/Ctl	397 \pm 23	519 \pm 17	686 \pm 37	661 \pm 45
Mem/Ctl	523 \pm 49*	624 \pm 38*	613 \pm 36	560 \pm 52
Group IV ($n = 8$)				
Ctl/Ctl	341 \pm 20	581 \pm 54	608 \pm 49	634 \pm 31
Mem/EtOH (1.0 g/kg)	606 \pm 35* [†]	487 \pm 42	642 \pm 31	664 \pm 35 [†]
Group V ($n = 7$)				
Ctl/Ctl	349 \pm 32	527 \pm 47	601 \pm 54	668 \pm 39
Mem/EtOH (2.5 g/kg)	513 \pm 32*	525 \pm 62	736 \pm 33	721 \pm 38 ^Δ

* indicates a significant difference from "Control/Control", $P < 0.05$ (paired t-test).

[†] indicates a significant difference from "Ctl/EtOH (1.0 g/kg)", $P < 0.05$ (pooled two-sample t-test).

^Δ indicates a significant difference from "Ctl/EtOH (2.5 g/kg)", $P < 0.05$ (pooled two-sample t-test).

n = Number of rats.

Ctl = control (0.9% saline); EtOH = ethanol; Mem = memantine (10.0 mg/kg)

EFFECTS OF MEMANTINE AND ETHANOL ON RECTAL TEMPERATURE

	Baseline Rectal Temperature (°C) ± S.E.M.	Change in Rectal Temperature (°C) ± S.E.M. (from baseline temperature)	
		30' Post-1 st Tx	30' Post-2 nd Tx
Group I (<i>n</i> = 8)			
Ctl/Ctl	37.19 ±0.19	0.63 ±0.25	0.71 ±0.36
Ctl/EtOH (1.0 g/kg)	37.24 ±0.13	0.68 ±0.22	0.03 ±0.34
Group II (<i>n</i> = 8)			
Ctl/Ctl	37.45 ±0.23	0.49 ±0.31	0.56 ±0.30
Ctl/EtOH (2.5 g/kg)	37.40 ±0.28	0.76 ±0.21	-1.61 ±0.20 *
Group III (<i>n</i> = 8)			
Ctl/Ctl	37.40 ±0.09	0.54 ±0.24	0.45 ±0.22
Mem/Ctl	37.01 ±0.22	-0.41 ±0.45	-0.20 ±0.44
Group IV (<i>n</i> = 8)			
Ctl/Ctl	37.46 ±0.25	0.20 ±0.26	0.41 ±0.27
Mem/EtOH (1.0 g/kg)	37.35 ±0.27	-0.05 ±0.48	-1.06 ±0.34 *†
Group V (<i>n</i> = 7)			
Ctl/Ctl	37.67 ±0.17	-0.16 ±0.23	-0.43 ±0.25
Mem/EtOH (2.5 g/kg)	37.31 ±0.16	-0.79 ±0.33 ^Δ	-2.39 ±0.25 * ^Δ

* indicates a significant difference from "Control/Control", *P* < 0.05 (paired t-test).

† indicates a significant difference from "CTL/EtOH (1.0 g/kg)", *P* < 0.05 (pooled two-sample t-Test).

‡ indicates a significant difference from "CTL/EtOH (1.0 g/kg)", *P* < 0.05 (pooled two-sample t-Test).

n = Number of rats.

Ctl = control (0.9% saline); EtOH = ethanol; Mem = memantine (10.0 mg/kg)

EFFECTS OF DOPAMINERGIC D1 AND D2 DRUGS ON ETHANOL CONSUMPTION

Treatment	Ethanol consumed (g/kg/day)		
	Pre-treatment	During treatment	Post treatment
Vehicle (7)	6.70 ± 1.09	5.21 ± 0.80 *	5.88 ± 0.88
10.0 (+)SULP (10)	5.67 ± 0.59	4.45 ± 0.49 *	4.93 ± 0.48
3.0 (-)SULP (8)	6.11 ± 0.52	5.36 ± 0.52	5.88 ± 0.66
10.0 (-)SULP (7)	6.05 ± 0.52	5.18 ± 0.72	5.10 ± 0.90
0.1 SCH (8)	6.57 ± 0.91	4.46 ± 0.79 *	5.99 ± 1.03
0.3 SCH (8)	5.65 ± 0.52	2.65 ± 0.47 *	4.99 ± 0.79
3.0 (-)SULP & 0.3 SCH (7)	4.65 ± 0.58	3.06 ± 0.57 *	5.10 ± 0.69
10.0 (-)SULP & 0.3 SCH (8)	5.66 ± 0.82	3.20 ± 0.41 *	5.08 ± 0.70

Treatment	Proportion of ethanol consumed (ethanol (ml)/[ethanol (ml) + water (ml)])		
	Pre-treatment	During treatment	Post treatment
Vehicle (7)	0.56 ± 0.03	0.54 ± 0.05	0.60 ± 0.05
10.0 (+)SULP (10)	0.58 ± 0.03	0.52 ± 0.03	0.56 ± 0.03
3.0 (-)SULP (8)	0.59 ± 0.03	0.55 ± 0.03	0.59 ± 0.05
10.0 (-)SULP (7)	0.59 ± 0.02	0.54 ± 0.05	0.54 ± 0.05
0.1 SCH (8)	0.67 ± 0.03	0.58 ± 0.06	0.56 ± 0.04
0.3 SCH (8)	0.61 ± 0.04	0.45 ± 0.05	0.50 ± 0.05
3.0 (-)SULP & 0.3 SCH (7)	0.49 ± 0.02	0.45 ± 0.03	0.51 ± 0.03
10.0 (-)SULP & 0.3 SCH (8)	0.59 ± 0.05	0.52 ± 0.05	0.50 ± 0.02

Treatment	Total fluids consumed (ml/day)		
	Pre-treatment	During treatment	Post treatment
Vehicle (7)	36.2 ± 1.3	30.4 ± 1.1 *	31.0 ± 1.2
10.0 (+)SULP (10)	35.2 ± 2.4	31.3 ± 2.0 *	32.3 ± 2.1
3.0 (-)SULP (8)	33.6 ± 2.4	31.1 ± 1.9	32.1 ± 2.2
10.0 (-)SULP (7)	32.9 ± 1.3	30.1 ± 1.7	29.2 ± 1.0
0.1 SCH (8)	31.1 ± 1.6	23.9 ± 1.0 *	32.9 ± 2.2
0.3 SCH (8)	34.7 ± 1.6	21.6 ± 1.2 *	35.2 ± 2.2
3.0 (-)SULP & 0.3 SCH (7)	33.9 ± 2.2	22.9 ± 0.8 *	35.0 ± 2.7
10.0 (-)SULP & 0.3 SCH (8)	31.3 ± 1.4	20.4 ± 0.9 *	32.6 ± 1.3

Treatment	Food consumed (g/day)		
	Pre-treatment	During treatment	Post treatment
Vehicle (7)	16.7 ± 0.7	16.8 ± 0.8	17.0 ± 1.1
10.0 (+)SULP (10)	17.0 ± 0.9	17.0 ± 0.9	16.9 ± 0.7
3.0 (-)SULP (8)	17.7 ± 0.8	17.2 ± 0.7	16.7 ± 1.0
10.0 (-)SULP (7)	17.3 ± 0.6	17.7 ± 1.0	19.2 ± 0.9
0.1 SCH (8)	16.2 ± 0.6	17.5 ± 0.7	18.1 ± 0.8
0.3 SCH (8)	18.0 ± 1.0	18.1 ± 1.0	17.2 ± 1.0
3.0 (-)SULP & 0.3 SCH (7)	17.6 ± 1.4	16.1 ± 0.8	16.5 ± 0.9
10.0 (-)SULP & 0.3 SCH (8)	18.4 ± 0.8	15.8 ± 0.4	16.5 ± 0.7

Treatment	Body weight (kg)		
	Pre-treatment	During treatment	Post treatment
Vehicle (7)	0.388 ± 0.012	0.394 ± 0.014	0.395 ± 0.013
10.0 (+)SULP (10)	0.363 ± 0.013	0.369 ± 0.013 *	0.369 ± 0.013
3.0 (-)SULP (8)	0.347 ± 0.013	0.353 ± 0.014 *	0.353 ± 0.013
10.0 (-)SULP (7)	0.383 ± 0.012	0.389 ± 0.012	0.386 ± 0.011
0.1 SCH (8)	0.375 ± 0.014	0.380 ± 0.013	0.380 ± 0.013
0.3 SCH (8)	0.360 ± 0.016	0.364 ± 0.016 *	0.365 ± 0.016
3.0 (-)SULP & 0.3 SCH (7)	0.357 ± 0.012	0.361 ± 0.012 *	0.357 ± 0.012
10.0 (-)SULP & 0.3 SCH (8)	0.390 ± 0.013	0.392 ± 0.012	0.387 ± 0.012

* indicates a significant difference from pre-treatment baseline, $P < 0.05$ (Tukey/Kramer).

Number of rats is indicated in parentheses.

SULP = Sulpiride; SCH = SCH23390; Number before the drug name indicates dose (mg/kg).

EFFECTS OF SKF38393 ON ETHANOL CONSUMPTION

Treatment	Ethanol consumed (g/kg/day)		
	Pre-treatment	During treatment	Post treatment
Vehicle (7)	6.33 ± 0.57	5.18 ± 0.46 *	6.16 ± 0.37
1.25 mg/kg (7)	5.99 ± 0.38	4.13 ± 0.27 *	5.60 ± 0.32
2.5 mg/kg (6)	5.80 ± 1.02	4.07 ± 0.99 *†	6.52 ± 1.18
5.0 mg/kg (7)	6.08 ± 0.96	2.74 ± 0.42 *†	5.94 ± 0.72
10.0 mg/kg (7)	6.51 ± 0.99	2.64 ± 0.29 *†	5.76 ± 0.57

Treatment	Proportion of ethanol consumed (ethanol (ml)/[ethanol (ml) + water (ml)])		
	Pre-treatment	During treatment	Post treatment
Vehicle (7)	0.68 ± 0.06	0.65 ± 0.06	0.69 ± 0.05
1.25 mg/kg (7)	0.66 ± 0.05	0.54 ± 0.03 *	0.64 ± 0.05
2.5 mg/kg (6)	0.61 ± 0.04	0.48 ± 0.08 †	0.62 ± 0.05
5.0 mg/kg (7)	0.65 ± 0.05	0.41 ± 0.05 *†	0.66 ± 0.03
10.0 mg/kg (7)	0.62 ± 0.04	0.38 ± 0.05 *†	0.69 ± 0.05

Treatment	Total fluids consumed (ml/day)		
	Pre-treatment	During treatment	Post treatment
Vehicle (7)	36.0 ± 3.7	30.6 ± 2.8 *	35.0 ± 2.8
1.25 mg/kg (7)	35.1 ± 3.1	29.8 ± 2.4 *	34.4 ± 2.4
2.5 mg/kg (6)	33.3 ± 2.7	29.5 ± 3.2	38.6 ± 3.5
5.0 mg/kg (7)	33.7 ± 2.9	25.1 ± 2.1 *	32.8 ± 2.9
10.0 mg/kg (7)	37.9 ± 3.5	27.0 ± 2.0 *	31.8 ± 3.2

Treatment	Food consumed (g/day)		
	Pre-treatment	During treatment	Post treatment
Vehicle (7)	17.1 ± 1.3	16.9 ± 0.7	16.3 ± 0.8
1.25 mg/kg (7)	17.4 ± 0.7	17.6 ± 0.9	18.6 ± 0.8
2.5 mg/kg (6)	17.3 ± 0.7	18.0 ± 1.0	17.2 ± 0.7
5.0 mg/kg (7)	17.0 ± 0.7	16.4 ± 1.3	18.2 ± 1.1
10.0 mg/kg (7)	16.6 ± 0.7	15.8 ± 0.5	18.9 ± 0.9

Treatment	Body weight (kg)		
	Pre-treatment	During treatment	Post treatment
Vehicle (7)	0.394 ± 0.014	0.397 ± 0.013 *	0.399 ± 0.014
1.25 mg/kg (7)	0.408 ± 0.019	0.411 ± 0.020 †	0.411 ± 0.019
2.5 mg/kg (6)	0.394 ± 0.014	0.397 ± 0.014	0.398 ± 0.014
5.0 mg/kg (7)	0.387 ± 0.013	0.388 ± 0.014 †	0.388 ± 0.013
10.0 mg/kg (7)	0.387 ± 0.014	0.394 ± 0.014 *	0.392 ± 0.015

* indicates a significant difference from pre-treatment baseline, $P < 0.05$ (Tukey/Kramer).

† indicates a significant difference from vehicle treatment, $P < 0.05$ (Tukey/Kramer).

Number of rats is indicated in parentheses.

EFFECTS OF CO-ADMINISTERED SKF38393 AND SCH23390 ON ETHANOL CONSUMPTION

Treatment	Ethanol consumed (g/kg/day)		
	Pre-treatment	During treatment	Post treatment
Vehicle (6)	6.79 ± 0.15	5.43 ± 0.58 *	5.17 ± 0.60
SKF + SCH (6)	6.65 ± 0.49	3.68 ± 0.75 *†	5.87 ± 0.90

Treatment	Proportion of ethanol consumed (ethanol (ml)/[ethanol (ml) + water (ml)])		
	Pre-treatment	During treatment	Post treatment
Vehicle (6)	0.54 ± 0.01	0.48 ± 0.04	0.46 ± 0.03
SKF + SCH (6)	0.51 ± 0.02	0.44 ± 0.08	0.48 ± 0.07

Treatment	Total fluids consumed (ml/day)		
	Pre-treatment	During treatment	Post treatment
Vehicle (6)	40.9 ± 2.4	36.9 ± 3.0	36.7 ± 3.0
SKF + SCH (6)	41.4 ± 3.6	26.7 ± 1.3 *†	39.4 ± 2.0

Treatment	Food consumed (g/day)		
	Pre-treatment	During treatment	Post treatment
Vehicle (6)	18.2 ± 0.8	17.7 ± 0.4	18.6 ± 0.5
SKF + SCH (6)	18.7 ± 0.7	16.8 ± 1.1	16.9 ± 1.1

Treatment	Body weight (kg)		
	Pre-treatment	During treatment	Post treatment
Vehicle (6)	0.343 ± 0.016	0.347 ± 0.015 *	0.354 ± 0.015
SKF + SCH (6)	0.342 ± 0.009	0.347 ± 0.009 *	0.345 ± 0.010

* indicates a significant difference from pre-treatment baseline, $P < 0.05$ (Tukey/Kramer).

† indicates a significant difference from vehicle treatment, $P < 0.05$ (Tukey/Kramer).

Number of rats is indicated in parentheses.

SKF = SKF38393 (5.0 mg/kg); SCH = SCH23390 (0.3 mg/kg)

EFFECTS OF CO-ADMINISTERED SKF38393 AND SCH23990 ON ETHANOL CONSUMPTION

Treatment	Ethanol consumed
	% Change
5.0 SKF (7)	-52.5 ± 7.0
0.3 mg/kg SCH (8)	-53.9 ± 5.7
5.0 SKF + 0.3 SCH (6)	-46.8 ± 6.3

Treatment	Proportion of ethanol consumed
	% Change
5.0 SKF (7)	-38.4 ± 4.3
0.3 mg/kg SCH (8)	-24.3 ± 9.9
5.0 SKF + 0.3 SCH (6)	-16.2 ± 13.7

Treatment	Total fluids consumed
	% Change
5.0 SKF (7)	-23.1 ± 7.6
0.3 mg/kg SCH (8)	-37.7 ± 2.5
5.0 SKF + 0.3 SCH (6)	-34.5 ± 2.7

Treatment	Food consumed
	% Change
5.0 SKF (7)	-3.5 ± 6.5
0.3 mg/kg SCH (8)	1.0 ± 4.1
5.0 SKF + 0.3 SCH (6)	-10.3 ± 3.6

Treatment	Body weight
	% Change
5.0 SKF (7)	0.4 ± 0.8
0.3 mg/kg SCH (8)	1.2 ± 0.2
5.0 SKF + 0.3 SCH (6)	1.6 ± 0.4

Data are reported as percent change from pre-treatment levels.
 No significant difference between treatments was observed ($P < 0.05$, Kruskal-Wallis).
 Number of rats is indicated in parentheses.
 5.0 SKF = SKF38393 (5.0 mg/kg); 0.3 SCH = SCH23390 (0.3 mg/kg)

EFFECTS OF CO-ADMINISTERED MEMANTINE AND SCH23390 ON ETHANOL CONSUMPTION

Treatment	Ethanol consumed (g/kg/day)		
	Pre-treatment	During treatment	Post treatment
Vehicle (11)	6.94 ± 0.24	6.15 ± 0.43	6.12 ± 0.46
MEM + SCH (11)	6.61 ± 0.49	3.90 ± 0.41 *†	5.94 ± 0.43

Treatment	Proportion of ethanol consumed (ethanol (ml)/[ethanol (ml) + water (ml)])		
	Pre-treatment	During treatment	Post treatment
Vehicle (11)	0.57 ± 0.02	0.52 ± 0.03	0.52 ± 0.03
MEM + SCH (11)	0.54 ± 0.04	0.51 ± 0.05	0.48 ± 0.04

Treatment	Total fluids consumed (ml/day)		
	Pre-treatment	During treatment	Post treatment
Vehicle (11)	39.0 ± 1.6	38.1 ± 2.3	38.7 ± 2.2
MEM + SCH (11)	41.6 ± 1.9	26.1 ± 1.2 *†	42.5 ± 1.6

Treatment	Food consumed (g/day)		
	Pre-treatment	During treatment	Post treatment
Vehicle (11)	18.3 ± 0.5	17.4 ± 0.5	17.9 ± 0.5
MEM + SCH (11)	17.1 ± 0.8	17.9 ± 0.8	17.6 ± 0.6

Treatment	Body weight (kg)		
	Pre-treatment	During treatment	Post treatment
Vehicle (11)	0.340 ± 0.011	0.346 ± 0.011	0.353 ± 0.011
MEM + SCH (11)	0.359 ± 0.008	0.367 ± 0.010 *†	0.367 ± 0.009

* indicates a significant difference from pre-treatment baseline, $P < 0.05$ (Tukey/Kramer).

† indicates a significant difference from vehicle treatment, $P < 0.05$ (Tukey/Kramer).

Number of rats is indicated in parentheses.

Mem = Memantine (1.0 mg/kg); SCH = SCH23390 (0.3 mg/kg)

EFFECTS OF LY379268 ON ETHANOL CONSUMPTION

Treatment	Ethanol consumed (g/kg/day)		
	Pre-treatment	During treatment	Post treatment
Vehicle (7)	6.23 ± 0.49	4.60 ± 0.36	5.70 ± 0.49
0.3 mg/kg (7)	7.02 ± 0.60	5.70 ± 0.48	6.45 ± 0.83
1.0 mg/kg (7)	7.25 ± 0.59	5.02 ± 0.41 *	6.39 ± 0.85
3.0 mg/kg (7)	6.08 ± 0.36	4.13 ± 0.58 *	5.32 ± 0.54

Treatment	Proportion of ethanol consumed (ethanol (ml)/[ethanol (ml) + water (ml)])		
	Pre-treatment	During treatment	Post treatment
Vehicle (7)	0.62 ± 0.06	0.52 ± 0.04	0.57 ± 0.05
0.3 mg/kg (7)	0.62 ± 0.04	0.56 ± 0.08	0.63 ± 0.06
1.0 mg/kg (7)	0.61 ± 0.05	0.52 ± 0.05	0.59 ± 0.08
3.0 mg/kg (7)	0.63 ± 0.05	0.51 ± 0.06	0.58 ± 0.08

Treatment	Total fluids consumed (ml/day)		
	Pre-treatment	During treatment	Post treatment
Vehicle (7)	36.0 ± 3.9	31.9 ± 3.2	36.4 ± 2.4
0.3 mg/kg (7)	38.5 ± 4.6	36.5 ± 4.9	33.5 ± 2.8
1.0 mg/kg (7)	38.4 ± 3.9	29.6 ± 2.2 *	35.0 ± 3.8
3.0 mg/kg (7)	34.1 ± 2.2	26.4 ± 2.0 *	32.5 ± 2.5

Treatment	Food consumed (g/day)		
	Pre-treatment	During treatment	Post treatment
Vehicle (7)	19.1 ± 1.3	18.7 ± 1.4	17.9 ± 1.2
0.3 mg/kg (7)	16.6 ± 0.7	13.6 ± 0.8 *†	18.1 ± 0.5
1.0 mg/kg (7)	17.2 ± 1.0	13.6 ± 1.0 *†	19.2 ± 1.0
3.0 mg/kg (7)	18.1 ± 1.1	12.2 ± 1.0 *†	20.5 ± 1.0

Treatment	Body weight (kg)		
	Pre-treatment	During treatment	Post treatment
Vehicle (7)	0.327 ± 0.020	0.332 ± 0.021 *	0.337 ± 0.021
0.3 mg/kg (7)	0.316 ± 0.021	0.312 ± 0.021 †	0.318 ± 0.020
1.0 mg/kg (7)	0.326 ± 0.017	0.319 ± 0.017 *†	0.328 ± 0.017
3.0 mg/kg (7)	0.328 ± 0.019	0.317 ± 0.018 *†	0.331 ± 0.020

* indicates a significant difference from pre-treatment baseline, $P < 0.05$ (Tukey/Kramer).

† indicates a significant difference from vehicle treatment, $P < 0.05$ (Tukey/Kramer).

Number of rats is indicated in parentheses.

EFFECTS OF LY341495 ON ETHANOL CONSUMPTION

Treatment	Ethanol consumed (g/kg/day)		
	Pre-treatment	During treatment	Post treatment
Vehicle (8)	6.55 ± 0.46	5.79 ± 0.52 *	6.32 ± 0.47
0.3 mg/kg (7)	7.61 ± 0.40	4.87 ± 0.56 *	6.12 ± 0.63
1.0 mg/kg (8)	6.42 ± 0.44	5.10 ± 0.61	7.14 ± 0.49
3.0 mg/kg (8)	6.48 ± 0.39	3.64 ± 0.50 *†	6.25 ± 0.60

Treatment	Proportion of ethanol consumed (ethanol (ml)/[ethanol (ml) + water (ml)])		
	Pre-treatment	During treatment	Post treatment
Vehicle (8)	0.52 ± 0.04	0.48 ± 0.04	0.50 ± 0.04
0.3 mg/kg (7)	0.55 ± 0.04	0.40 ± 0.04 *	0.48 ± 0.05
1.0 mg/kg (8)	0.53 ± 0.03	0.43 ± 0.03	0.56 ± 0.05
3.0 mg/kg (8)	0.50 ± 0.03	0.40 ± 0.07	0.49 ± 0.05

Treatment	Total fluids consumed (ml/day)		
	Pre-treatment	During treatment	Post treatment
Vehicle (8)	41.9 ± 2.2	40.9 ± 2.4	44.6 ± 3.6
0.3 mg/kg (7)	43.0 ± 2.9	36.5 ± 2.1 *	40.0 ± 2.6
1.0 mg/kg (8)	38.9 ± 2.3	30.6 ± 1.6 *†	40.7 ± 2.3
3.0 mg/kg (8)	43.5 ± 3.4	29.7 ± 2.1 *†	44.3 ± 5.5

Treatment	Food consumed (g/day)		
	Pre-treatment	During treatment	Post treatment
Vehicle (8)	16.8 ± 1.0	17.0 ± 0.7	16.8 ± 0.9
0.3 mg/kg (7)	16.7 ± 0.4	18.1 ± 0.5	18.2 ± 0.7
1.0 mg/kg (8)	16.4 ± 0.8	17.1 ± 0.7	16.5 ± 1.0
3.0 mg/kg (8)	16.8 ± 0.8	17.6 ± 0.6	17.1 ± 0.9

Treatment	Body weight (kg)		
	Pre-treatment	During treatment	Post treatment
Vehicle (8)	0.332 ± 0.016	0.337 ± 0.016 *	0.341 ± 0.016
0.3 mg/kg (7)	0.323 ± 0.014	0.327 ± 0.014 *†	0.330 ± 0.014
1.0 mg/kg (8)	0.318 ± 0.014	0.318 ± 0.015 †	0.322 ± 0.015
3.0 mg/kg (8)	0.334 ± 0.016	0.332 ± 0.015 †	0.339 ± 0.016

* indicates a significant difference from pre-treatment baseline, $P < 0.05$ (Tukey/Kramer).

† indicates a significant difference from vehicle treatment, $P < 0.05$ (Tukey/Kramer).

Number of rats is indicated in parentheses.

EFFECTS OF CO-ADMINISTERED LY341495 AND LY379268 ON ETHANOL CONSUMPTION

Treatment	Ethanol consumed (g/kg/day)		
	Pre-treatment	During treatment	Post treatment
LY341495 + LY379268 (7)	5.06 ± 0.40	2.01 ± 0.38 *	4.16 ± 0.26

Treatment	Proportion of ethanol consumed (ethanol (ml)/[ethanol (ml) + water (ml)])		
	Pre-treatment	During treatment	Post treatment
LY341495 + LY379268 (7)	0.51 ± 0.04	0.30 ± 0.04 *	0.50 ± 0.04

Treatment	Total fluids consumed (ml/day)		
	Pre-treatment	During treatment	Post treatment
LY341495 + LY379268 (7)	36.5 ± 5.0	20.5 ± 1.1 *	29.3 ± 3.7

Treatment	Food consumed (g/day)		
	Pre-treatment	During treatment	Post treatment
LY341495 + LY379268 (7)	19.3 ± 1.1	14.1 ± 0.8 *	19.0 ± 1.1

Treatment	Body weight (kg)		
	Pre-treatment	During treatment	Post treatment
LY341495 + LY379268 (7)	0.386 ± 0.022	0.375 ± 0.021 *	0.375 ± 0.021

* indicates a significant difference from pre-treatment baseline, $P < 0.05$ (Tukey/Kramer).
 Number of rats is indicated in parentheses.
 Each drug was administered at a dose of 3.0 mg/kg.

APPENDIX C

MOBILE PHASE, STANDARDS, AND SAMPLES FOR HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

MOBILE PHASE FOR HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

1. Combine 0.10 M KH_2PO_4 (3.4g) and 250 ml deionized H_2O . Mix. Adjust pH to approximately 3.75 with 0.40 M citric acid or 2 N KOH.
2. Combine 0.10 mM Na_2EDTA (8.4 mg) and 1.12 mM SOS (0.0650 g) and q.s. to 250 ml with phosphate solution from step 1. Stir until dissolved.
3. Measure out 22.5 ml MeOH and q.s. to 250 ml with solution from step 2. Stir. Adjust final pH to 3.90 with 0.40 M citric acid or 2 N KOH.
4. Filter buffer and de-gas. Transfer to a HPLC reservoir.

STANDARDS AND SAMPLES FOR HIGH PERFORMANCE LIQUID CHROMATOGRAPHY
(HPLC)

1. Prepare "stock solution" standards:

10 μ l of 0.5 mg/ml DA + 0.99 ml of 0.10 M H_3PO_4 = 5 μ g/ml DA

10 μ l of 0.5 mg/ml DOPAC + 0.99 ml of 0.10 M H_3PO_4 = 5 μ g/ml DOPAC

10 μ l of 0.5 mg/ml DHBA + 0.99 ml of 0.10 M H_3PO_4 = 5 μ g/ml DHBA

(*The 0.5 mg/ml DA, 0.5 mg/ml DOPAC, and 0.5 mg/ml DHBA stock solutions are prepared in 0.01 N HCl and stored at 4°C.)

2. Prepare "non-extracted" standard:

10 μ l of 5 μ g/ml DHBA

10 μ l of 5 μ g/ml DA

10 μ l of 5 μ g/ml DOPAC

970 μ l of 0.10 M H_3PO_4

3. Label homogenate tubes. Put tubes in ice with pestle. Add 800 μ l of 0.4 N HClO_4 to each homogenate tube. Add 10 μ l of 5 μ g/ml DHBA to each homogenate tube.

4. Prepare "extracted" standards in triplicate:

"50 ng @":

10 μ l of 5 μ g/ml DHBA

10 μ l of 5 μ g/ml DA

10 μ l of 5 μ g/ml DOPAC

770 μ l of 0.4 N HClO_4

"50/250":

10 μ l of 5 μ g/ml DHBA

50 μ l of 5 μ g/ml DA

50 μ l of 5 μ g/ml DOPAC

690 μ l of 0.4 N HClO_4

5. Weigh tissue, place in homogenate tube containing 800 μl of 0.4 N HClO_4 and 10 μl of 5 $\mu\text{g/ml}$ DHBA, and homogenize. (If storing homogenate overnight, freeze along with extracted standards at -20°C .)
6. Perform an alumina extraction for each tissue sample and "extracted" standard:
 - Microcentrifuge homogenate for 2.5 minutes.
 - Add 40-50 mg alumina to a fresh labeled microcentrifuge tube for each homogenate and "extracted" standard.
 - Add 0.5 ml supernate or 0.5 ml "extracted" standard.
 - Add 0.5 ml of 0.5 M Tris.
 - Shake for 7 minutes.
 - Microcentrifuge for 2 minutes.
 - Aspirate supernate.
 - Add 1.0 ml of 0.005 M Tris.
 - Shake for 7 minutes.
 - Microcentrifuge for 2 minutes.
 - Aspirate.
 - Add 1.0 ml deionized H_2O .
 - Shake for 7 minutes.
 - Microcentrifuge for 2 minutes.
 - Aspirate.
 - Add 0.5 ml of 0.10 M H_3PO_4 .
 - Shake for 7 minutes.
 - Microcentrifuge for 2 minutes.
 - Transfer supernate into a labeled 13 X 100 mm borosilicate glass tube. This sample or "extracted" standard is analyzed on the HPLC.

APPENDIX D

STOCK AND WORKING SOLUTIONS (BUFFERS) FOR WESTERN BLOTTING

STOCK SOLUTIONS10% Sodium Dodecyl Sulfate (SDS)

2.5 g Sodium Dodecyl Sulfate (SDS)
25 ml deionized H₂O

10X Running Buffer

30.3 g Tris-Base
144.1 g Glycine
10.00 g Sodium Dodecyl Sulfate (SDS)
Adjust volume to 1L with deionized H₂O, pH to 8.6

10X Tris/Glycine Buffer

30.3 g Tris-Base
144.1 g Glycine
Adjust volume to 1L with deionized H₂O

10X Phosphate Buffered Saline (PBS) [pH 7.4]

80.00 g NaCl
2.00 g KCl
14.40 g Na₂HPO₄
2.40 g KH₂PO₄
Adjust volume to 1L with deionized H₂O, pH to 7.4

WORKING SOLUTIONS

Lysis Buffer

Phosphate Buffered Saline (PBS) [pH 7.4]	22.5 ml
1% Sodium Dodecyl Sulfate (SDS)	2.5 ml 10% SDS
2 mM ethylenediamine tetraacetate (EDTA)	16.8 mg
5 mM ethylene glycol tetraacetic acid (EGTA)	47.6 mg
25 mM Sodium Fluoride (NaF)	26.2 mg
2 mM Sodium Orthovanadate (Na ₃ VO ₄)	9.2 mg

Running Buffer

100 ml 10X Running Buffer
Adjust volume to 1L with deionized H₂O

Sample Loading Buffer

950 µl Bio-Rad Laemmli Sample Buffer
50 µl β-mercaptoethanol (β-ME)

Transfer Buffer

200 ml 10X Tris/Glycine Buffer
400 ml Methanol (MeOH)
Adjust volume to 2L with deionized H₂O

Phosphate Buffered Saline (PBS)

100 ml 10X Phosphate Buffered Saline (PBS)
Adjust volume to 1L with deionized H₂O

Phosphate Buffered Saline (PBS) [pH 7.4]

10 ml 10X Phosphate Buffered Saline (PBS)
Adjust volume to 100 ml with deionized H₂O, pH to 7.4

5% Non-Fat Dry Milk Blocker

2.0 g Blotting Grade Blocker Non-Fat Dry Milk (Bio-Rad)
40 ml 1X Phosphate Buffered Saline (PBS)
Filter

Phosphate Buffered Saline-0.1% Tween-20 (PBS-T)

100 ml 10X Phosphate Buffered Saline (PBS)

1.0 ml Tween-20

Adjust volume to 1L with deionized H₂O

APPENDIX E

PROTEIN CONCENTRATION DETERMINATION WITH BCA REAGENT

This protein assay requires a Pierce Bicinchoninic Acid (BCA) Protein Assay Kit (Pierce Thermo Scientific, Rockford, IL).

1. Prepare homogenate, or if using a homogenate already prepared and frozen, thaw sample on ice.
2. Microcentrifuge homogenates at 14,000 rpm for 1-2 minutes at 4°C.
3. Prepare 25 μ l of diluted sample if measuring duplicate samples. Prepare 40 μ l of diluted sample if measuring triplicate samples. Dilute all samples in deionized water. The concentration (generally, 1:5 or 1:10) will depend upon the density of the homogenate.
4. Prepare serial dilutions of the Bovine Serum Albumin (BSA) standard (2 mg/ml) at the following concentrations: 1.0, 0.5, 0.25, and 0.125 mg/ml. Dilute each standard in deionized water. Also prepare a "blank" standard using deionized water alone. Prepare adequate amounts of each standard to allow for 10 μ l of each to be loaded in duplicate.
5. Add 10 μ l of the standards, beginning with the "blank" and sequentially using higher concentrations, in duplicate to the top row of the 96-well plate.
6. Add 10 μ l of the diluted protein samples, in duplicate or triplicate to the second row (and additional rows, if needed) of a 96-well plate.
7. Calculate the total volume of detection solution needed by multiplying the [number of wells (N) + 2 extra samples] x 0.2 ml. Round the results up to the nearest whole number to allow for extra solution. This is the total volume of detection solution to be prepared.

8. Calculate the volume of Reagent B needed. Since Reagent B is at a 50X concentration, divide the total volume of detection solution needed by 50. This is the volume of Reagent B needed.
9. Calculate the volume of Reagent A needed by subtracting the volume of Reagent B needed from the total volume of detection solution being prepared.
10. Add the appropriate volumes of Reagent A and Reagent B to a clean tube and mix well.
11. Add 200 μ l of the Reagent A+B mixture to each well of the 96-well plate containing a standard or sample.
12. Cover the plate and incubate at 37°C for 30 minutes.
13. At least 15 minutes prior to the end of the 30-minute incubation period, turn on the Bio Tek Synergy HT Multi-Mode Microplate Reader (Bio Tek, Winooski, VT).
14. To read the 96-well plate, run program KC4 and set wavelength at 562 nm.
15. When generating the standard curve, R^2 should be at least 0.99. If samples were measured in duplicate, average the readings. If samples were measured in triplicate, average the two readings that are closest.

APPENDIX F

IMMUNOBLOTTING PROCEDURE

1. Prior to beginning the Western blot, a cooling unit for transfer must be prepared. Fill a Bio-Rad Bio-Ice Cooling Unit (Bio-Rad, Hercules, CA) with tap water. Freeze at -20°C .
2. Prepare working sample buffer solution.
3. Dilute homogenate in sample buffer. Use concentrations of protein based upon previous optimization studies. Dilute each homogenate based upon results from the BCA protein assay previously performed.
4. Boil for ten minutes.
5. Prepare electrophoresis apparatus:
 - a. Assemble unit with two gels or one gel and a dam. Use "Bio-Rad Ready Gels, 10% Tris-HCl, 15-well, 15 μl " (precast polyacrylamide).
 - b. Dilute 10X running buffer to 1X working solution.
 - c. Using 1X working solution, fill the area between the gels and the bottom of the electrophoresis unit to cover the electrodes at the bottom of the apparatus.
6. Load pre-stained standard (Bio-Rad Precision Plus Kaleidoscope) as follows: 5 μl in Lane 1 and 1 μl in Lanes 2 and 15.
7. Depending on the concentration of protein to be added per well, load 10 μl or 15 μl of each sample in duplicate wells.
8. Run the gel(s) at 110 volts until the dye front is near the bottom of the gel.
9. Prepare transfer buffer.
10. Chill 0.5 L 1X working buffer at -20°C .
11. For each gel, soak the following in transfer buffer:
 - a. Bio-Rad Mini Gel Holder Cassette

- b. Two Bio-Rad Mini Trans-Blot fiber pads
 - c. Two sheets of Bio-Rad Mini Trans-Blot filter paper
 - d. Nitrocellulose membrane (0.45 μm pore size)
12. Equilibrate the gel(s) in transfer buffer for 20 minutes.
13. Load the transfer cassette(s) as follows:
- a. Place a fiber pad on the dark side of the cassette.
 - b. Add filter paper.
 - c. Add gel.
 - d. Add nitrocellulose.
 - e. Add filter paper.
 - f. Add fiber pad.
 - g. Close and latch the cassette.
 - h. Insert vertically into electrophoresis blotting unit.
 - i. Insert into buffer tank.
 - j. Add Bio-Ice cooling unit.
 - k. Add stir bar.
 - l. Place assembled unit in a pan filled with ice.
 - m. Place assembly on stir plate in cold room.
 - n. Fill buffer tank with chilled transfer buffer.
 - o. Add lid.
14. Transfer for 1 hour at 350 mA.
15. Remove filter paper and gel.
16. Block non-specific binding by incubating the nitrocellulose with 5% blotting grade blocker non-fat dry milk (Bio-Rad) for 1 hour at room temperature.

17. Rinse 5 times with PBS-T.
18. Add the primary antibodies diluted in PBS-T and incubate overnight in the cold room.
Use concentrations of primary antibodies previously determined by optimization studies.
19. Wash 3 times with PBS-T for 5 minutes per wash.
20. Add the secondary antibodies diluted in PBS-T and incubate for 1 hour in the cold room. Use concentrations of secondary antibodies previously determined by optimization studies. Keep in the dark.
21. Wash 3 times with PBS-T for 5 minutes per wash. Keep in the dark.
22. Scan the membrane on the LiCor Odyssey imaging system. Allowing the membranes to dry prior to scanning may enhance the image. Scan with the following settings: Preset = Membrane; Quality = Medium; Focus Offset = 0.0 mm. For all membranes in each set of experiments, i.e. combination of brain region and primary antibody being studied, use the same settings for intensity. Scan images to be quantitated at Resolution = 84 μm .

APPENDIX G

DATA TABLES FROM BIOCHEMICAL EXPERIMENTS

HPLC RESULTS

Prefrontal Cortex			
Treatment Group	<i>n</i>	Dopamine	DOPAC
Ctl/Ctl	7	0.13 ±0.02	0.12 ±0.03
Ctl/EtOH (1.0 g/kg)	7	0.12 ±0.02	0.10 ±0.02
Ctl/EtOH (2.5 g/kg)	7	0.15 ±0.03	0.12 ±0.02
Mem/Ctl	7	0.12 ±0.03	0.10 ±0.01
Mem/EtOH (1.0 g/kg)	6	0.18 ±0.04	0.14 ±0.03
Mem/EtOH (2.5 g/kg)	8	0.13 ±0.01	0.11 ±0.01

Nucleus Accumbens			
Treatment Group	<i>n</i>	Dopamine	DOPAC
Ctl/Ctl	8	8.64 ±1.38	8.31 ±0.98
Ctl/EtOH (1.0 g/kg)	7	5.61 ±0.26	6.34 ±0.65
Ctl/EtOH (2.5 g/kg)	7	9.13 ±1.30	8.18 ±0.67
Mem/Ctl	7	6.02 ±0.56	6.52 ±0.72
Mem/EtOH (1.0 g/kg)	8	7.34 ±0.90	8.34 ±0.81
Mem/EtOH (2.5 g/kg)	8	6.94 ±0.63	8.50 ±0.69

Striatum			
Treatment Group	<i>n</i>	Dopamine	DOPAC
Ctl/Ctl	8	11.79 ±1.22	7.82 ±1.14
Ctl/EtOH (1.0 g/kg)	8	10.68 ±1.25	8.34 ±0.82
Ctl/EtOH (2.5 g/kg)	7	11.67 ±1.19	11.42 ±1.42
Mem/Ctl	7	11.69 ±0.82	9.13 ±0.48
Mem/EtOH (1.0 g/kg)	8	10.82 ±1.00	10.70 ±0.65
Mem/EtOH (2.5 g/kg)	9	9.71 ±0.92	11.89 ±1.02

Data are reported as μg per g wet weight of tissue (Mean \pm S.E.M.).

No significant treatment effects were observed ($P < 0.05$, Tukey/Kramer).

n = Number of samples.

Ctl = control (0.9% saline); EtOH = ethanol; Mem = memantine (10.0 mg/kg)

WESTERN BLOT RESULTS

Prefrontal Cortex			
Treatment Group	DARPP-32	DARPP-32(Thr34)	DARPP-32(Thr75)
Ctl/Ctl	100.0 (6)	n/a	100.0 (6)
Ctl/EtOH (1.0 g/kg)	116.7 ± 20.1 (6)	n/a	96.6 ± 11.9 (6)
Ctl/EtOH (2.5 g/kg)	127.3 ± 21.6 (6)	n/a	108.9 ± 5.2 (5)
Mem/Ctl	146.2 ± 39.2 (5)	n/a	94.5 ± 13.5 (6)
Mem/EtOH (1.0 g/kg)	135.9 ± 25.2 (6)	n/a	83.5 ± 21.7 (6)
Mem/EtOH (2.5 g/kg)	95.4 ± 16.2 (6)	n/a	93.7 ± 12.9 (6)

Nucleus Accumbens			
Treatment Group	DARPP-32	DARPP-32(Thr34)	DARPP-32(Thr75)
Ctl/Ctl	100.0 (6)	100.0 (6)	100.0 (6)
Ctl/EtOH (1.0 g/kg)	107.5 ± 6.8 (6)	122.6 ± 8.7 (5)	142.0 ± 15.9 (5)
Ctl/EtOH (2.5 g/kg)	129.3 ± 18.2 (6)	145.0 ± 17.5 (6)	164.1 ± 26.5 (5)
Mem/Ctl	114.8 ± 16.7 (6)	143.2 ± 24.6 (6)	137.9 ± 22.0 (6)
Mem/EtOH (1.0 g/kg)	112.4 ± 13.4 (6)	135.9 ± 20.0 (6)	138.9 ± 16.9 (5)
Mem/EtOH (2.5 g/kg)	109.0 ± 20.5 (6)	104.0 ± 18.8 (6)	152.3 ± 32.7 (5)

Striatum			
Treatment Group	DARPP-32	DARPP-32(Thr34)	DARPP-32(Thr75)
Ctl/Ctl	100.0 (6)	100.0 (5)	100.0 (6)
Ctl/EtOH (1.0 g/kg)	110.7 ± 27.8 (5)	76.2 ± 15.0 (5)	107.1 ± 17.1 (6)
Ctl/EtOH (2.5 g/kg)	86.7 ± 11.7 (6)	127.3 ± 41.9 (5)	126.8 ± 33.3 (6)
Mem/Ctl	116.8 ± 39.3 (5)	102.8 ± 17.4 (5)	120.7 ± 36.2 (6)
Mem/EtOH (1.0 g/kg)	65.8 ± 13.7 (6)	77.7 ± 14.2 (5)	78.6 ± 18.1 (6)
Mem/EtOH (2.5 g/kg)	66.1 ± 10.4 (5)	79.2 ± 10.2 (5)	75.4 ± 10.2 (6)

Data are reported as percentage of controls (control/control) (Mean ± S.E.M.).

No significant treatment effects were observed ($P < 0.05$, Tukey/Kramer).

Number of samples is indicated in parentheses. Each sample for NAc contained pooled tissue from 2 to 4 rats. Samples for mPFC and STR were not pooled.

Ctl = control (0.9% saline); EtOH = ethanol; Mem = memantine (10.0 mg/kg)

APPENDIX H

ALTERNATE ANALYSIS FOR WESTERN BLOT RESULTS

ALTERNATE ANALYSIS FOR WESTERN BLOT RESULTS

Prefrontal Cortex			
Treatment Group	DARPP-32(Thr34)/DARPP-32		DARPP-32(Thr75)/DARPP-32
Ctl/Ctl	n/a		100.0 (6)
Ctl/EtOH (1.0 g/kg)	n/a		93.1 ± 16.5 (6)
Ctl/EtOH (2.5 g/kg)	n/a		144.6 ± 36.3 (5)
Mem/Ctl	n/a		93.6 ± 32.4 (5)
Mem/EtOH (1.0 g/kg)	n/a		69.8 ± 16.1 (6)
Mem/EtOH (2.5 g/kg)	n/a		111.9 ± 17.6 (6)
Nucleus Accumbens			
Treatment Group	DARPP-32(Thr34)/DARPP-32		DARPP-32(Thr75)/DARPP-32
Ctl/Ctl	100.0	(5)	100.0 (6)
Ctl/EtOH (1.0 g/kg)	115.7 ± 8.7	(5)	141.9 ± 21.1 (5)
Ctl/EtOH (2.5 g/kg)	200.4 ± 84.5	(4)	125.2 ± 14.9 (5)
Mem/Ctl	148.7 ± 37.7	(4)	120.9 ± 7.9 (6)
Mem/EtOH (1.0 g/kg)	139.9 ± 24.0	(4)	121.9 ± 14.7 (6)
Mem/EtOH (2.5 g/kg)	108.6 ± 16.1	(4)	145.3 ± 26.9 (6)
Striatum			
Treatment Group	DARPP-32(Thr34)/DARPP-32		DARPP-32(Thr75)/DARPP-32
Ctl/Ctl	100.0	(5)	100.0 (5)
Ctl/EtOH (1.0 g/kg)	75.3 ± 5.1	(4)	110.3 ± 22.1 (4)
Ctl/EtOH (2.5 g/kg)	132.0 ± 26.7	(4)	151.6 ± 30.3 (5)
Mem/Ctl	108.4 ± 20.9	(4)	140.4 ± 39.0 (4)
Mem/EtOH (1.0 g/kg)	142.6 ± 21.9	(4)	159.7 ± 35.3 (5)
Mem/EtOH (2.5 g/kg)	134.7 ± 25.9	(4)	136.0 ± 44.2 (5)

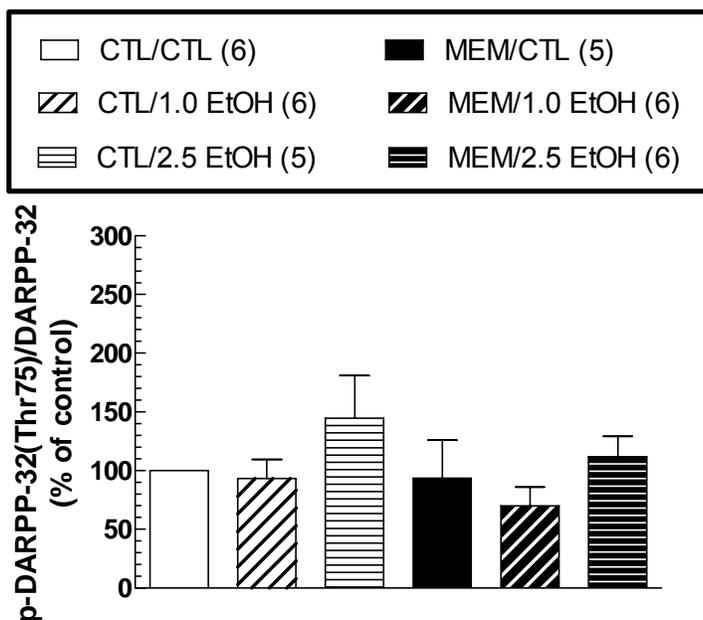
Data are reported as percentage of controls (control/control) (Mean ± S.E.M.).

No significant treatment effects were observed ($P < 0.05$, Tukey/Kramer).

Number of samples is indicated in parentheses. Each sample for NAc contained pooled tissue from 2 to 4 rats. Samples for mPFC and STR were not pooled.

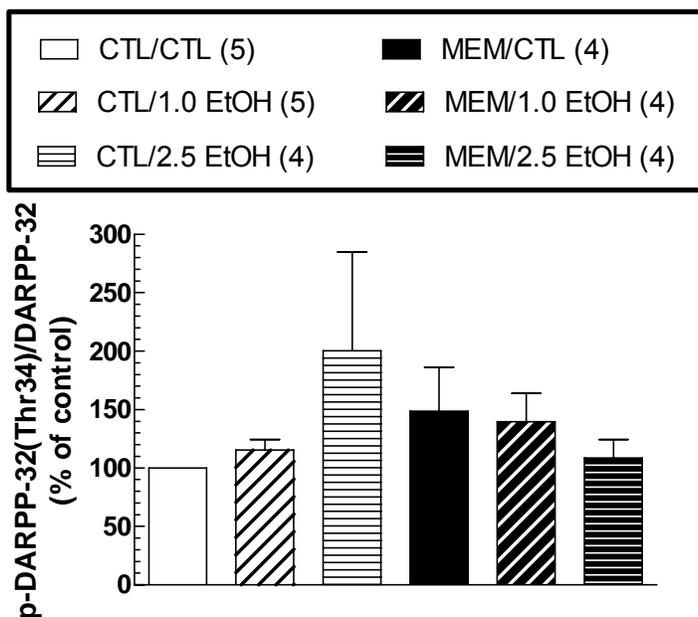
Ctl = control (0.9% saline); EtOH = ethanol; Mem = memantine (10.0 mg/kg)

MEDIAL PREFRONTAL CORTEX



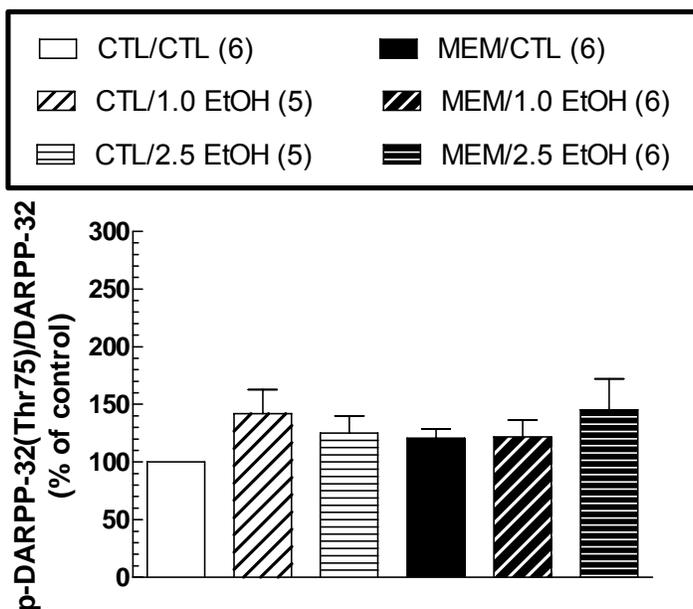
Effects of ethanol (1.0 or 2.5 g/kg, i.p.) and/or memantine (10.0 mg/kg, i.p.) on the abundance of phospho-DARPP-32 (Thr75) in the medial prefrontal cortex (mPFC). The number of samples for each treatment group is indicated in parentheses. Each sample contained tissue obtained from a single male mHEP rat. Data are presented as the ratio of phospho-DARPP-32 (Thr75)/GAPDH to DARPP-32/GAPDH, expressed as a percentage of controls (control/control). No significant differences were observed between treatment groups for the protein and brain region studied. (CTL = control; EtOH = ethanol; MEM = memantine).

NUCLEUS ACCUMBENS



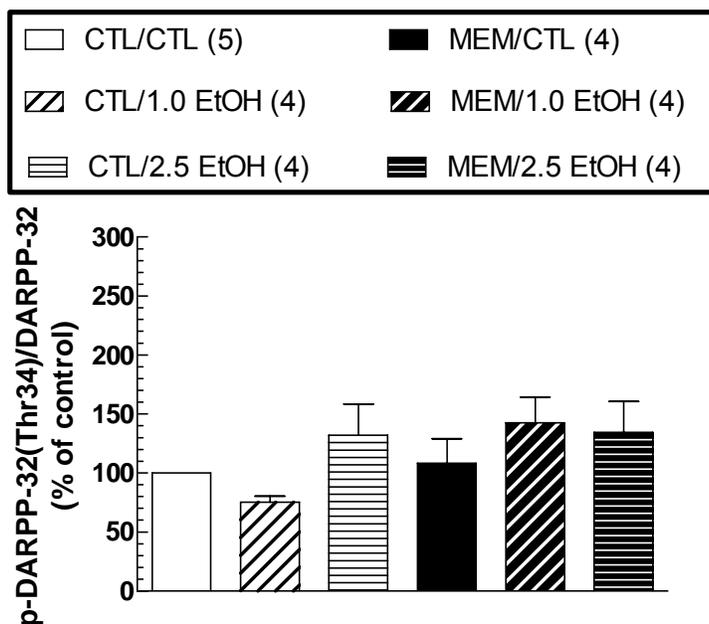
Effects of ethanol (1.0 or 2.5 g/kg, i.p.) and/or memantine (10.0 mg/kg, i.p.) on the abundance of phospho-DARPP-32 (Thr34) in the nucleus accumbens (NAc). The number of samples for each treatment group is indicated in parentheses. Each sample contained tissue obtained from 2 to 4 male mHEP rats. Data are presented as the ratio of phospho-DARPP-32 (Thr34)/GAPDH to DARPP-32/GAPDH, expressed as a percentage of controls (control/control). No significant differences were observed between treatment groups for the protein and brain region studied. (CTL = control; EtOH = ethanol; MEM = memantine).

NUCLEUS ACCUMBENS



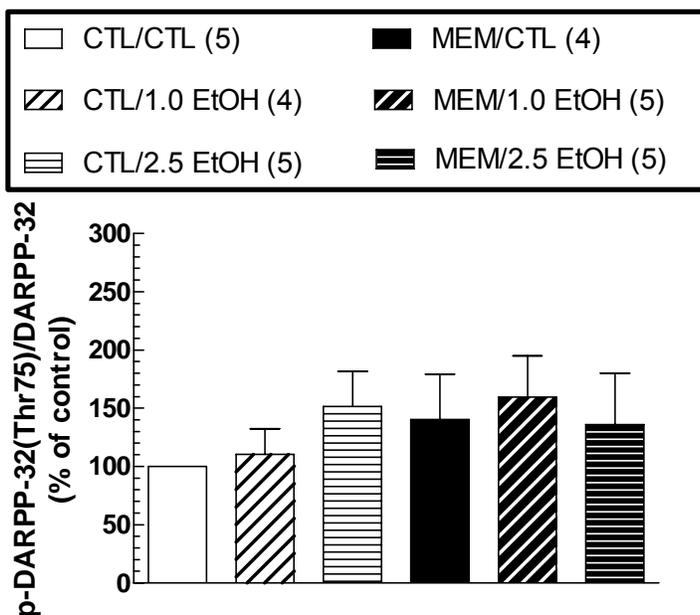
Effects of ethanol (1.0 or 2.5 g/kg, i.p.) and/or memantine (10.0 mg/kg, i.p.) on the abundance of phospho-DARPP-32 (Thr75) in the nucleus accumbens (NAc). The number of samples for each treatment group is indicated in parentheses. Each sample contained tissue obtained from 2 to 4 male mHEP rats. Data are presented as the ratio of phospho-DARPP-32 (Thr75)/GAPDH to DARPP-32/GAPDH, expressed as a percentage of controls (control/control). No significant differences were observed between treatment groups for the protein and brain region studied. (CTL = control; EtOH = ethanol; MEM = memantine).

STRIATUM



Effects of ethanol (1.0 or 2.5 g/kg, i.p.) and/or memantine (10.0 mg/kg, i.p.) on the abundance of phospho-DARPP-32 (Thr34) in the striatum (STR). The number of samples for each treatment group is indicated in parentheses. Each sample contained tissue obtained from a single male mHEP rat. Data are presented as the ratio of phospho-DARPP-32 (Thr34)/GAPDH to DARPP-32/GAPDH, expressed as a percentage of controls (control/control). No significant differences were observed between treatment groups for the protein and brain region studied. (CTL = control; EtOH = ethanol; MEM = memantine).

STRIATUM



Effects of ethanol (1.0 or 2.5 g/kg, i.p.) and/or memantine (10.0 mg/kg, i.p.) on the abundance of phospho-DARPP-32 (Thr75) in the striatum (STR). The number of samples for each treatment group is indicated in parentheses. Each sample contained tissue obtained from a single male mHEP rat. Data are presented as the ratio of phospho-DARPP-32 (Thr75)/GAPDH to DARPP-32/GAPDH, expressed as a percentage of controls (control/control). No significant differences were observed between treatment groups for the protein and brain region studied. (CTL = control; EtOH = ethanol; MEM = memantine).