THE ROLE OF METABOTROPIC GLUTAMATE RECEPTORS AND ASSOCIATED CELL SIGNALING PATHWAYS IN THE ACUTE SEDATIVE-HYPNOTIC EFFECTS OF ETHANOL

Amanda C. Sharko

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Approved by:

Regina Carelli, Ph.D.

T.Kendall Harden, Ph.D.

Clyde W. Hodge, Ph.D.

A.Leslie Morrow, Ph.D.

David Siderovski, Ph.D.

ABSTRACT

Amanda C. Sharko**:** The role of metabotropic glutamate receptors and associated cell signaling pathways in the acute sedative-hypnotic effects of ethanol

(Under the direction of Clyde W. Hodge)

Metabotropic glutamate receptors (mGluRs) mediate the slower, modulatory effects of glutamate neurotransmission. In this capacity, mGluRs can alter signaling at both NMDA and $GABA_A$ receptors, the receptors which are thought to mediate the majority of ethanol's sedative-hypnotic properties. The goal of the present study was to examine the role of mGluRs, and the intracellular signaling pathways with which they associate, in mediating the acute sedative-hypnotic properties of ethanol.

The first set of experiments examines the effects of three selective mGluR antagonists on the sedative and hypnotic effects of acutely administered high doses of ethanol. Inhibition of mGluR5 increased both ethanol-induced locomotor deficits and loss of righting reflex (LORR). Inhibition of mGluR5 also produced an increase in ketamine-induced LORR, but did not alter pentobarbital or midazolam-induced LORR. These data suggest that inhibition of mGluR5 selectively enhances the sedative-hypnotic effects of ethanol, and that this enhancement may be related to decreased mGluR5-NMDAR interactions.

The second set of experiments examines the effects of acute ethanol treatment on protein kinase C-gamma (PKCγ) phosphorylation over time. Acute ethanol treatment increased phosphorylated PKCγ (pPKCγ) immunoreactivity in the nucleus accumbens and the central amygdala and decreased pPKCγ immunoreactivity in the basolateral amygdala. Moreover, these

effects were time dependent. These data suggest that acute ethanol alters the abundance of the catalytically competent form of PKCγ and these changes may mediate some of the acute behavioral effects of ethanol.

The third set of experiments examines the effects of acute ethanol treatment and mGluR5 inhibition on extracellular-signal regulated kinase (ERK) phosphorylation. Acute ethanol treatment rapidly increased pERK immunoreactivity in the nucleus accumbens shell and the central amygdala and produced delayed increases in the basolateral amygdala, thalamus, and hypothalamus. pERK immunoreactivity was decreased in the nucleus accumbens core. Following mGluR5 inhibition, the effects of ethanol in the nucleus accumbens were reduced and the effects of ethanol in the basolateral amygdala and the hypothalamus were enhanced. Finally, inhibition of ERK phosphorylation reduced ethanol-induced LORR, suggesting that these changes in pERK may be related to the sedative-hypnotic effects of ethanol.

These studies identified signaling pathways that may mediate the sedative-hypnotic effects of ethanol.

To my parents, Paul and Janet Sharko, for dry ice and little nobody.

They got me where I am today.

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AMPA	α-amino-3-hydroxy-5-methyl-4-isoazolepropionic acid
ANOVA	analysis of variance
cm	centimeter
DMSO	dimethyl sulfoxide
ERK	extracellular-signal regulated kinase
g	gram
GABA	gamma amino-butyric acid
IC_{50}	dose required to induce 50% inhibition
IP	intraperitoneal
LORR	loss of righting reflex
MAPK	mitogen activated protein kinase
min	minute
mg	milligram
mGluR	metabotropic glutamate receptor
mm	millimeter
mM	millimolar
kg	kilogram
NAcc	nucleus accumbens
NMDA	N-methyl-D-aspartate
PKC	protein kinase C
PP1	protein phosphates 1
SEM	standard error of the mean

CHAPTER 1

GENERAL INTRODUCTION

Alcohol Use Disorders and the Treatment of Alcoholism

Humans have consumed alcohol for thousands of years. For the majority of the population, moderate consumption of alcoholic beverages is a safe practice. Indeed, emerging evidence suggests that moderate alcohol consumption has beneficial effects on cardiovascular health and may decrease risk for stroke and dementia (Pinder and Sandler 2004). However, excessive alcohol consumption can lead to alcohol abuse and the development of alcohol addiction. In the United States, more than 17 million people abuse alcohol every year, and almost half of these individuals (7.9 million) met the DSM-IV criteria for alcoholism (Grant et al. 2004).

Alcoholism is a chronic neurobiological and behavioral disorder. While genetics play a significant role in determining susceptibility for alcohol use disorders, the development of alcoholism follows a common pattern of behavioral and biological changes. Acute alcohol consumption produces positive subjective effects. As consumption increases, tolerance and dependence develop. Reduced sensitivity to alcohol, withdrawal symptoms, and craving can further increase alcohol consumption, which frequently results in negative social and health consequences. These negative consequences often push people to seek treatment and to attempt to abstain from drinking alcohol.

The difficulty in treating alcoholism is that it is a chronic relapsing disorder. Late stage alcoholism is characterized by periods of abstinence interspersed with bouts of relapse drinking. Preventing relapse is the goal of most treatment programs. However, standard psycho-social

therapy has shown poor clinical outcomes, with up to 70% of patients relapsing within one year (Finney et al. 1996). Recent efforts have been directed to developing pharmacological agents that will enhance the efficacy of psycho-social therapy. Disulfiram, acamprosate, and naltrexone are currently approved in the United States for the treatment of alcoholism. Disulfiram inhibits aldehyde dehydrogenase and prevents the metabolism of acetaldehyde, alcohol's primary metabolite. The accumulation of acetaldehyde causes unpleasant effects if alcohol is consumed, including headaches, sweating, nausea, and vomiting, and the association of these symptoms with alcohol consumption discourages further drinking (Johnson 2008). However, disulfiram does not alter alcohol craving and is only effective in highly motivated patients, since those who want to drink can simply stop taking the medication. Acamprosate is generally thought to antagonize NMDA receptors, ameliorating the disregulation of excitatory neurotransmission due to chronic alcohol consumption (De Witte et al. 2005). Although acamprosate reduces alcohol consumption in rodents (Czachowski et al. 2001), the effectiveness in humans is less certain. Initial studies suggested that acamprosate had a modest, but significant, effect at reducing drinking days and increasing the latency to resume drinking (Mann et al. 2004). However recent evidence indicates that acamprosate has limited therapeutic benefit in the general population and may be most effective in patients highly motivated to abstain from alcohol use (Anton et al. 2006). Naltrexone is a mu-opioid antagonist which may alter the expression of alcohol's reinforcing effects (Lee et al. 2005). Naltrexone reduces alcohol consumption in rodents and, although the effects are small, decreases heavy drinking days in humans (Volpicelli et al. 1992). Interestingly, naltrexone appears to be most effective in individuals with strong craving or a family history of alcohol dependence (Jaffe et al. 1996; Krishnan-Sarin et al. 2007). However, compliance must be high (80-90%) for naltrexone to be effective and side-effects, such as nausea, can reduce compliance below the necessary threshold (Croop et al. 1997; Volpicelli et al.

1997). Clearly, successful treatment of alcohol dependence is hampered by the limited efficacy of the current treatment options. Identification and characterization of the various neurobiological systems that respond to alcohol are key to designing new pharmacotherapies for alcohol dependence.

GABA and Glutamate: Ionotropic Receptors and Ethanol

The acute effects of ethanol are largely attributed to modulation of ionotropic gamma-aminobutyric acid (GABA) and glutamate neurotransmission. Over time, persistent modulation of these receptor systems by ethanol leads to neuroadaptations that are partly responsible for the development of alcohol dependence.

In the mammalian central nervous system, ionotropic GABA neurotransmission is mediated predominantly by $GABA_A$ receptors. $GABA_A$ receptors are a group of heteropentameric chloride ion channels that mediate fast inhibitory synaptic transmission. Modulation of these receptors produces a variety of behavioral effects. Activation by GABA induces anxiolysis and sedation, which are enhanced by positive allosteric modulators, such as barbiturates and benzodiazepines (Mehta and Ticku 1999). Inhibition of these receptors by bicuculine causes seizures (Devaud et al. 1995). Given that ethanol has anxiolytic and sedative-hypnotic properties, it is not surprising that ethanol enhances GABA mediated Cl^- influx (Allan and Harris 1987). Furthermore, the anxiolytic and sedative-hypnotic properties of ethanol are potentiated by $GABA_AR$ agonists and blocked by $GABA_AR$ antagonists (Grobin et al. 1998). Thus, it is generally understood that the acute behavioral effects of ethanol are mediated, in part, by positive modulation of $GABA_A$ receptors. Chronic ethanol exposure often results in withdrawal symptoms, such as anxiety and insomnia, which are characteristic effects of reduced inhibitory tone, indicating that ethanol also produces long term changes to GABAergic neurotransmission (Grobin et al. 1998)

Glutamate is the primary excitatory neurotransmitter in the mammalian central nervous system. Fast excitatory synaptic transmission is mediated by ionotropic glutamate receptors. These receptors are cation channels and are divided into three groups (NMDA, AMPA, and kainate receptors) based on agonist pharmacology. Although they differ in subunit number and composition, agonists of these receptors produce the same neurobiological and behavioral effects. Normal activation of ionotropic glutamate receptors stimulates arousal. Uncontrolled activation causes seizures and neurotoxicity (Battaglia et al. 2001; Mulle et al. 1998). Antagonists of these receptors have sedative-hypnotic properties and have been shown to protect against excitatoxicity (Sinner and Graf 2008). Acute ethanol exposure blocks NMDA, AMPA, and kainate-induced currents, suggesting that inhibition of ionotropic glutamate receptors contributes to ethanol's sedative-hypnotic effects (Dildy-Mayfield and Harris 1992). Persistent interruption of excitatory neurotransmission due to chronic ethanol exposure causes increases in synaptic glutamate receptor density (Chen et al. 1999). In the absence of ethanol, the resulting enhancement of excitatory tone triggers withdrawal symptoms, particularly seizures.

These two groups of receptors have been examined as possible therapeutic targets for a variety of neurobiological and behavioral disorders. $GABA_A$ receptor positive modulators, such as benzodiazepines and barbiturates, have been used to reduce withdrawal symptoms (Kosten and O'Connor 2003). However, these drugs have a high risk for dependency and produce fairly rapid tolerance, limiting their utility in the treatment of alcohol dependence (Stewart and Westra 2002). Ionotropic glutamate receptor antagonists are also being investigated as therapeutic agents for the treatment of alcohol dependence. NMDA receptor antagonists, such as acamprosate and memantine, reduce drinking behavior in animal models of ethanol dependence, but their efficacy in human alcoholics is less certain (Johnson 2008). Currently, the only ionotropic glutamate receptor antagonist that shows much promise in treating alcohol dependence is topiramate, a dual

AMPA/kainate receptor antagonist that is currently approved for the treatment of epilepsy. Topiramate reduces drinking and craving in human alcoholics, but limited effects in animal models of ethanol dependence have hindered identification of the mechanisms by which topiramate produces its effects (Johnson 2008).

GABA and Glutamate: Metabotropic Receptors and Ethanol

While modulation of the ionotropic GABA and glutamate receptors might represent the best treatment for withdrawal symptoms and establishing abstinence, reversing the persistent neuroadaptations produced by chronic ethanol exposure may require additional therapies. Both GABA and glutamate also signal through metabotropic, G-protein coupled receptors, which are responsible for slower, longer acting modulation of neurotransmission. Metabotropic GABA receptors, $GABA_B$ receptors, are coupled to G_i and G_o. Activation of these receptors inhibits cAMP formation and depolarizes the membrane by enhancing outward K^+ currents. Baclofen, a $GABA_BR$ agonist has been shown to reduce drinking in both dependent and non-dependent animals (Janak and Michael Gill 2003; Walker and Koob 2007). In humans, baclofen reduced withdrawal symptoms and improved abstinence (Addolorato et al. 2002). Although baclofen's effects on these measures were similar to diazepam, it produced no positive subjective effects, suggesting that it has less abuse potential than the $GABA_AR$ agonists (Addolorato et al. 2006).

Metabotropic glutamate receptors have also been considered as potential targets for therapies to treat alcohol dependence. The eight subtypes of metabotropic glutamate receptors are divided into three groups based on amino acid sequence, second messenger system coupling, and agonist pharmacology. Group 1 receptors (mGluR1 and 5) are coupled to Gq and stimulate diacylglycerol production and Ca^{2+} mobilization. Group 2 receptors (mGluR2 and 3) and Group 3 receptors (mGluR4, 6, 7, and 8) are coupled to $G_{i/o}$ and inhibit adenylyl cyclase. Much of the interest in these receptors as targets for pharmaceutical development has focused on the group 1

receptors, particularly mGluR5. Selective antagonists of mGluR5 (MPEP and MTEP) are neuroprotective and have anticonvulsant, anxiolytic and anti-depressant-like effects, suggesting that these receptors may be therapeutic targets for a variety of neurobiological disorders (Bao et al. 2001; Chapman et al. 2000; Cosford et al. 2003).

mGluR5 has also been shown to be of importance in mediating the effects of several drugs of abuse. Inhibition of mGluR5 decreases nicotine and cocaine self-administration and blocks the conditioned rewarding effects of cocaine and morphine (Kenny et al. 2003; McGeehan and Olive 2003; Popik and Wrobel 2002). In addition, emerging evidence suggests that mGluR5 mediates some of the rewarding properties of ethanol. Our laboratory has shown that selective inhibition of mGluR5 with MPEP decreases operant self administration in mice (Fig.1.1) MPEP also reduces relapse drinking and interferes with the discriminative stimulus properties of ethanol in rats (Backstrom et al. 2004; Besheer et al. 2006). Currently, the mechanisms by which mGluR5 inhibition alters the rewarding properties of ethanol are unclear. The goals of this set of experiments are to identify behaviors elicited by acute ethanol treatment that may be mediated by mGluR5 and determine whether acute ethanol acts on mGluR5 related signaling pathways.

Metabotropic Glutamate Receptor Subtype 5: Signaling

mGluR5 activity has been shown to modulate several cellular systems. mGluR5 agonists potentiate NMDA-mediated currents (Benquet et al. 2002; Pisani et al. 2001) and inhibit GABA-mediated currents (Drew and Vaughan 2004). In addition, mGluR5 activation facilitates both glutamate and GABA release (de Novellis et al. 2003; Diaz-Cabiale et al. 2002). While the increases in release are due to activation of presynaptic mGluR5 receptors, the effects on NMDA- and GABA-mediated currents are the result of postsynaptic mGluR5 activation and likely involve mGluR5 signaling pathway-mediated phosphorylation of NMDAR and $GABA_AR$

subunits (Krishek et al. 1994; Liu et al. 2006). At the membrane, mGluR5 is coupled to G_q and activates phospholipase C- β (PLC-β) to generate inositol triphosphate (IP_3) and diacylglycerol (DAG) (Fig 1.2a). IP_3 stimulates intracellular Ca^{2+} release from the endoplasmic reticulum and together, DAG and Ca^{2+} activate protein kinase C (PKC) (Fig1.2b). PKC has been shown to phosphorylate a variety of downstream targets, including NMDAR, $GABA_AR$, and mitogen-activated protein kinase (MAPK) cascades. One of the MAPK cascades known to be activated by PKC is the extracellular regulated kinase (ERK) cascade. PKC activates the ERK cascade through the indirect phosphorylation of Raf, which promotes the subsequent phosphorylations of MEK and ERK1/2 (Fig1.3c). Indeed, mGluR5 agonists have been shown to stimulate the phosphorylation of ERK1/2 in a PKC-dependent manner (Thandi et al. 2002). Phosphorylated ERK1/2 translocates to the nucleus and stimulates the activation of transcription factors, such as Elk-1 and CREB (Fig1.3d). Through ERK1/2, mGluR5 can modulate gene transcription and protein expression.

A number of these signaling molecules have been implicated in the biochemical and behavioral effects of ethanol. Acute ethanol inhibits mGluR5-mediated increases in Cl^- currents in *Xenopus* oocytes, indicating that mGluR5 activity is sensitive to ethanol (Minami et al. 1998). Acute ethanol also induces PKC translocation to the membrane where it can be activated (Virmani and Ahluwalia 1992). Finally, *in vitro* studies suggest that acute ethanol increases ERK1/2 phosphorylation in PKC dependent manner (Washington et al. 2003). Chronic ethanol exposure increases mGluR5 mRNA in the nucleus accumbens, a brain region known to mediate ethanol self-administration. Chronic ethanol also decreases membrane bound PKC, reduces overall PKC kinase activity, and decreases ERK phosphorylation (Pandey et al. 1993; Pascale et al. 1997; Sanna et al. 2002). Therefore, in an effort to more fully characterize the effects of

ethanol on the mGluR5 associated signaling pathways, the experiments presented here examine the effects of acute ethanol treatment on PKC and ERK phosphorylation *in vivo*.

Specific Aims

mGluR5 inhibition by MPEP reduces ethanol self-administration in rodents. One explanation for this effect is that MPEP enhances the pharmacological effects ethanol and a dose of ethanol produces greater subjective effects than would be expected. To determine if mGluR5 inhibition can enhance the behavioral effects of acute ethanol treatment, ethanol-induced hypo-locomotion and loss of righting reflex will be examined. Given that these methods focus on the sedative-hypnotic properties of ethanol, these experiments also will provide information as to whether the effects of MPEP on self-administration are related to motor deficits. If MPEP is enhancing the pharmacological effects of ethanol, ethanol-induced decreases in locomotor behavior should be enhanced and the duration of ethanol-induced loss of righting reflex should be increased.

Ethanol has been shown to inhibit mGluR5-mediated Cl^- currents *in vitro* (Minami et al. 1998), indicating that ethanol interrupts mGluR5 signaling. Phosphorylation events are central to most intracellular signaling and these events are regulated by kinases and phosphatases. To determine if ethanol alters mGluR5-mediated phosphorylation, the effects of acute ethanol treatment on the phosphorylation states of PKCγ and protein phosphatase 1α (PP1α) will be examined. The time course of ethanol induced changes across the brain will be examined. If ethanol inhibits mGluR5, PKCγ phosphorylation should be reduced. Although ethanol has been shown to inhibit mGluR5 signaling, there is no evidence that ethanol directly antagonizes the receptor. Another possibility is that ethanol alters phosphatase activity. PP1α phosphorylation will be examined at relevant time points, as determined by changes in PKCγ. A decrease in PP1α

phosphorylation would suggest that ethanol reduces kinase activity through enhanced phosphatase activity.

Finally, the relationship between mGluR5 inhibition, ethanol, and ERK activation will be examined. mGluR5 activation increases c-fos expression through ERK-dependent phosphorylation of transcription factors and both mGluR5 and ERK inhibitors reduce this mGluR5-mediated increase in c-fos (Mao et al. 2005; Yang et al. 2004). First, the effects of ERK inhibition on ethanol-induced loss of righting reflex will be determined. If mGluR5 inhibition enhances the sedative-hypnotic effects of ethanol, then inhibition of ERK phosphorylation with SL327 might have the same effect. Second, the effects of acute ethanol treatment on ERK phosphorylation will be examined. Again, if ethanol inhibits mGluR5, then ERK phosphorylation should be reduced in ethanol treated animals. Furthermore, selective mGluR5 inhibition with MPEP may decrease ERK phosphorylation and could enhance the effects of ethanol on ERK phosphorylation. These interactions will be examined at a time point determined from the time course of ethanol effects on ERK phosphorylation.

Figure 1.1 The mGluR5 antagonist MPEP significantly decreased the reinforcing function of ethanol. (a) Total number of ethanol- and water-reinforced responses plotted as a function of MPEP dosage. * significantly different from vehicle (veh) control, paired t test planned comparison ($p<0.05$). (b) Ethanol intake (grams per kilogram per 16 h) plotted as a function of MPEP dosage. * significantly different from veh control (Tukey test, $p<0.05$). All data are plotted as mean ± SEM.

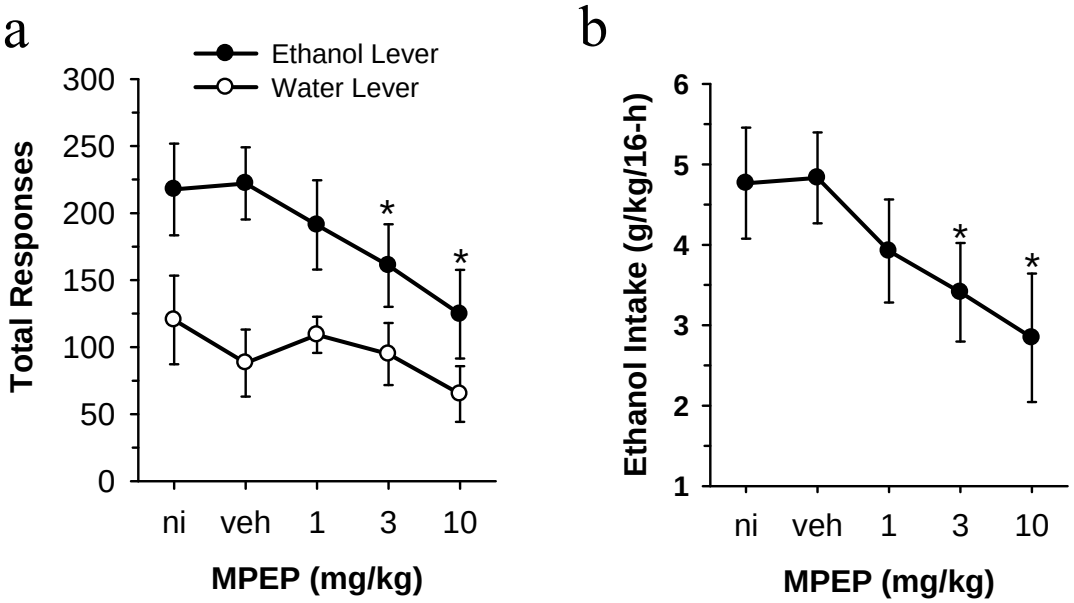
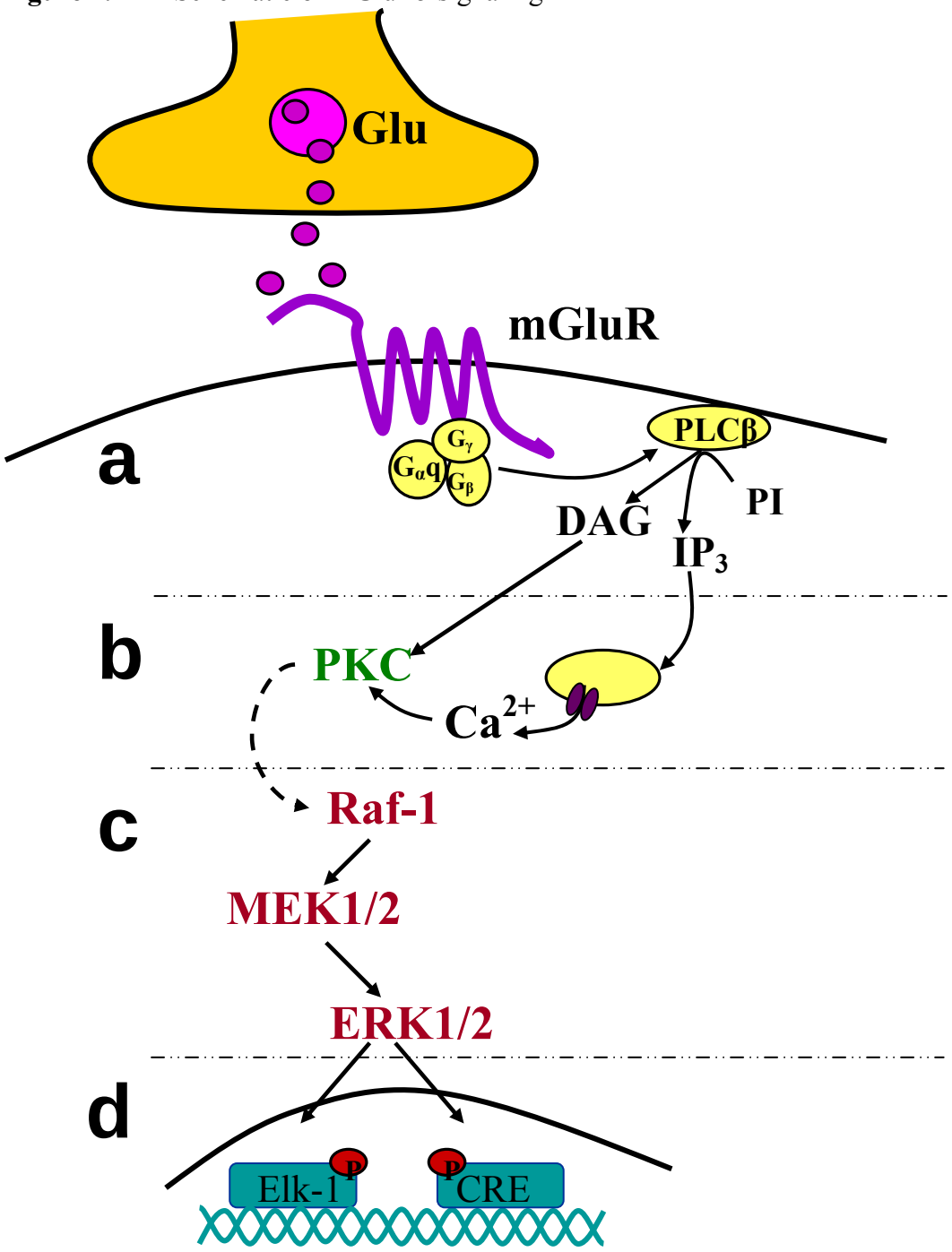


Figure 1.2 Schematic of mGluR5 signaling



CHAPTER 2

DIFFERENTIAL MODULATION OF ETHANOL-INDUCED SEDATION AND HYPNOSIS BY METABOTROPIC GLUTAMATE RECEPTOR ANTAGONISTS IN C57BL/6J MICE

INTRODUCTION

The amino-acid glutamate is the primary excitatory neurotransmitter in the mammalian central nervous system. Glutamate receptors are divided into two categories: ionotropic receptors (iGluRs), glutamate-gated cation channels including NMDA, AMPA, and kainate responsive receptors, and metabotropic receptors (mGluRs), a heterogeneous family of 7-transmembrane G-protein coupled receptors. Eight mGluR subtypes have been cloned. These receptors are divided into three broad groups based on amino acid sequence similarity, agonist pharmacology, and second messenger coupling. Group I mGlu receptors (mGluR1 and mGluR5) stimulate inositol phosphate metabolism and mobilization of intracellular Ca^{2+}, whereas group II (mGluR2 and mGluR3) and group III (mGluR4, -R6, -R7, and -R8) inhibit adenylyl cyclase and reduce synaptic transmission (Gereau and Conn 1995; Kew and Kemp 2005; Pin and Duvoisin 1995). In contrast to the iGluRs, which are responsible for the fast, excitatory responses to glutamate, mGluRs mediate the slower, modulatory responses to glutamate. In this capacity, mGluRs can modulate neurotransmission at both glutamatergic and non-glutamatergic synapses (Benquet et al. 2002; Diaz-Cabiale et al. 2002).

The availability of selective pharmacological agents (Table 2.1) has begun to reveal basic functional roles for the group I and II mGluR subtypes (Kew and Kemp 2005). Group I selective agonists have been shown to increase glutamate and GABA levels *in vivo* (de Novellis et al.

2003) and enhance glutamate-evoked depolarization (Doherty et al. 1997; Pisani et al. 2001) and GABA-gated Cl^- currents (Hoffpauir and Gleason 2002). Group II receptors are predominantly presynaptic autoreceptors (Cartmell and Schoepp 2000) and selective activation of these receptors has been shown to reduce glutamate-evoked excitatory postsynaptic currents through inhibition of glutamate release (Shen and Johnson 2003). mGluR selective ligands have also demonstrated behavioral activity in rodent models of anxiety and epilepsy. Group I antagonists and group II agonists have been shown to have anxiolytic (Klodzinska et al. 1999; Spooren et al. 2000) and anticonvulsant properties (Chapman et al. 2000; Moldrich et al. 2001).

Emerging evidence implicates mGluR function in ethanol's neurobiological and behavioral effects. Ethanol alters neuronal firing rates (Netzeband et al. 1997) and Ca^{2+} levels (Gruol et al. 1997) mediated by mGluRs *in vitro*. Chronic exposure to ethanol reduces mGluR1 mRNA levels in cerebellar Purkinje neurons of mice (Simonyi et al. 1996) and early withdrawal from ethanol leads to alterations in mGluR-evoked Ca^{2+} signaling in cerebellar neurons (Netzeband et al. 2002). In rats, chronic exposure to an ethanol containing liquid diet decreased mRNA levels for mGluR3 and mGluR5 in the dentate gyrus whereas mGluR1, mGluR5, and mGluR7 mRNA was decreased in the CA3 regions of the hippocampus (Simonyi et al. 2004). Neurobehaviorally, mGluR5 selective antagonists reduce ethanol self-administration in mice and rats (Cowen et al. 2005; Cowen et al. 2007; Hodge et al. 2006; Lominac et al. 2006), decrease relapse to ethanol self-administration in rats (Backstrom et al. 2004; Schroeder et al. 2005), and block the discriminative stimulus effects of ethanol (Besheer and Hodge 2005; Besheer et al. 2006).

The purpose of this study was to investigate the role of specific mGluRs in the acute sedative hypnotic effects of ethanol in mice. The three selective, systemically active mGluR antagonists MPEP (mGluR5), CPCCOEt (mGluR1), and LY341495 (mGluR2/3) were examined

for their ability to alter ethanol-induced inhibition of spontaneous locomotor activity (sedation) and ethanol-induced loss of righting reflex (hypnosis). To explore potential mechanisms by which mGluRs might modulate responses to ethanol, these antagonists were also tested with other sedative-hypnotics: the $GABA_A$ positive modulators pentobarbital and midazolam, and the NMDA antagonist ketamine. Blood-ethanol clearance studies were conducted to ensure that the observed effects were not due to alterations in ethanol clearance.

MATERIALS AND METHODS

Animals. Male adult C57BL/6J mice (Jackson Labs, Bar Harbor, ME; 10-24 weeks; 22-35g) were housed four per cage and maintained on a 12-hour light/dark cycle with food and water available ad libitum. Animal care and handling procedures were performed in accordance with approved institutional protocols and the National Institutes of Health Guide for Care and Use of Laboratory Animals (1996).

Drugs. Ethanol (95%w/v) was diluted in physiological saline (0.9%) to a concentration of 20% (v/v) and administered in various volumes to obtain the appropriate doses. The mGluR1 selective antagonist 7-(Hydroxyimino)cyclopropa[b]chromen-1a-carboxylate ethyl ester (CPCCOEt), mGluR5 selective antagonist 2-Methyl-6-(phenylethynyl)pyridine (MPEP), and the group II selective (mGluR2 and mGluR3) antagonist (2S)-2-Amino-2-[(1S,2S)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl) propanoic acid (LY 341495) were purchased from Tocris (Ellisville, MO, USA). The mGluR5 selective antagonist 3-[(2-methyl-1,3-thiazol-4-yl)-ethynyl]-pyridine (MTEP) was purchased from Ascent (Weston-super-Mare, UK). MPEP and MTEP were dissolved in physiological saline (0.9%). CPCCOEt and LY 341495 were suspended in (2-Hydroxypropyl)-β-cyclodextrin (45% w/v, Sigma, St. Louis, MO, USA) in distilled water. Pentobarbital, midazolam, and ketamine were purchased from Sigma-Aldritch (St. Louis, MO) and were dissolved in physiological saline (0.9%). Drug and vehicle solutions were administered to mice in a volume of 0.1 ml/10 g body weight and dose selections were made based on pilot experiments and published studies (Naveilhan et al. 2001; Quinlan et al. 1998).

Loss of Righting Reflex (LORR) Studies. Animals were treated with vehicle, MPEP (10-30mg/kg), CPCCOEt (10-30mg/kg), or LY341495 (10-30mg/kg) 10 minutes prior to ethanol

treatment (4.0 g/kg, IP) and the onset and duration of loss of righting reflex (sleep time) were measured. Onset was calculated as the time between ethanol injection and loss of righting reflex. LORR was defined as the inability of an animal to right itself within thirty seconds. Upon loss of righting reflex, mice were placed in V-shaped troughs (~90° angle) and the time to regain righting reflex was measured. Return of the righting reflex was defined as the ability of an animal to right itself three times in 30 seconds. Duration of LORR was calculated as the difference between loss and return of righting reflex.

The active antagonists (MPEP and LY341495) were then further tested with a series of ethanol doses (2.5g/kg-3.5g/kg) to examine ethanol dose-dependence. MTEP (10mg/kg) and MPEP (10mg/kg) were also tested with ethanol (3g/kg) to further confirm mGluR5 involvement. To examine possible mechanisms for the antagonist effects on ethanol-induced LORR, pentobarbital (50mg/kg)-, midazolam (60mg/kg)-, and ketamine (200mg/kg)-induced LORR were also examined following pretreatment with the active mGluR antagonists.

Locomotor Activity Studies. Spontaneous locomotor activity was measured in eight ENV 250 activity chambers (Med. Associates, St. Albans, VT). Infrared light sources and photodetectors were used to measure horizontal distance traveled during test sessions. All sessions were 60 minutes. Sessions were recorded in 5-minute time bins at 100ms resolution on a computer interfaced with the chambers. Following each session, chambers were cleaned with glacial acetic acid and rinsed with water.

All animals were chamber naïve prior to testing. Vehicle, MPEP (30mg/kg), CPCCOEt (30mg/kg), or LY341495 (30mg/kg) was administered by intraperitoneal injection (IP) 10 minutes prior to ethanol treatment (0 and 2.0g/kg, IP). Animals were placed in the activity chambers immediately after ethanol injection.

Blood Ethanol Clearance. Vehicle, MPEP (30mg/kg) or LY341495 (30mg/kg) was administered by IP injection 10 minutes prior to ethanol treatment (4.0g/kg). Blood (~30μl) was taken from the tail vein at various time points (10, 30, 60, 120, 180 minutes) after ethanol administration. Plasma from the samples was analyzed using an alcohol analyzer (Analox Instruments, Lunenburg, MA).

Analysis of Drug Effects. One-way ANOVA was used to determine the effects of the antagonists on single doses of sedative-hypnotic compounds. Two-way ANOVA was used to analyze the effects of the antagonists on total spontaneous locomotor activity and ethanol dose-dependent LORR. Three-way ANOVA with repeated measures was used to analyze the time course of antagonist effects on spontaneous locomotor activity. Two-way ANOVA with repeated measures and area under the curve (AUC) calculations were used to evaluate the blood-ethanol data. Upon identification of statistical significance, post-hoc comparisons were made with a Tukey's test where appropriate. In all cases, a value of $P<0.05$ was considered significant.

RESULTS

Ethanol-induced loss of righting reflex (LORR).

Systemic administration of the selective mGluR5 antagonist MPEP (0 or 30mg/kg) or the mGluR2/3 antagonist LY 341495 (0 or 30 mg/kg) produced differential effects on the time required for animals to regain their righting reflex following a high dose of ethanol (Fig 2.1). Pretreatment with the highest dose of MPEP (30mg/kg) increased the duration of LORR induced by ethanol (4g/kg) by 65% (Fig 2.1a; $F[2,25] = 13$; $P<0.001$). Follow up analysis shows that 30mg/kg MPEP was significantly different from saline and 10mg/kg MPEP (Tukey; $P<0.05$), indicating a dose dependent effect of MPEP. In contrast, pretreatment with the mGluR2/3 antagonist, LY341495, decreased the duration of ethanol-induced LORR (Fig 2.1b). LY341495 significantly reduced the duration of LORR produced by ethanol (4g/kg) ($F[2,31] = 11$; $P<0.001$), although responses to the 10mg/kg and 30mg/kg doses (54.2 ± 5.0 min and 38.8 ± 5.3 min, respectively) were not significantly different from each other. Neither dose of the mGluR1 antagonist CPCCOEt tested (10 and 30mg/kg) altered the duration of ethanol-induced LORR ($F[2,29] = 0.9$; $P = 0.415$) (Fig 2.1c).

Ethanol-induced locomotor inhibition.

mGluR5 and mGluR2/3 antagonists differentially modulated the ability of a sub-hypnotic dose of ethanol (2.0g/kg) to reduce spontaneous locomotor activity as measured by distance traveled in a novel environment. Two-way ANOVA revealed a main effect of ethanol on total ambulatory distance (Fig 2.2a; $F[1,27] = 112$; $P<0.001$). When administered prior to ethanol, MPEP (30mg/kg) further reduced exploratory locomotor activity compared to ethanol control. Although the analysis shows no main effect of MPEP, there was a significant MPEP x ethanol interaction ($F[1, 27] = 22$; $P<0.001$), indicating that the effect of MPEP on total motor activity

depended on the dose of ethanol (Fig. 2.2a). Analysis of the time course of MPEP's effects on ethanol-induced sedation by three-way RM ANOVA showed a significant interaction among MPEP, ethanol, and time (Fig. 2.2b, $F[11,297] = 2$; $P<0.01$) and a main effect of time ($F[11,297] = 43$; $P<0.001$), in addition to confirming the main effect of ethanol. Follow up analysis of these data showed that MPEP pretreatment significantly enhanced ethanol-induced motor impairment during the first 5 minutes (Tukey; $P<0.001$), as well as 25 and 55 minutes post injection (Tukey; $P<0.05$). Pretreatment with LY341495 (30mg/kg) reversed ethanol-induced locomotor inhibition, producing a main effect of LY341495 (Fig. 2.2c; $F[1,24] = 16$; $P<0.001$) but no LY341495 x ethanol interaction. However, time course analysis by three-way RM ANOVA showed an interaction among LY341495, ethanol, and time (Fig. 2.2d; $F[11,220] = 2$; $P<0.01$) and a main effect of time ($F[11,220] = 2$; $P<0.001$), while also confirming the main effect of LY341495. LY341495 pretreatment significantly diminished ethanol-induced motor impairment at 5, 10, 15, 20, 25, and 35 minutes after ethanol treatment (Tukey; $P<0.05$). Treatment with CPCCOEt (30mg/kg) had no effect on total locomotor activity when administered alone or prior to ethanol treatment (Fig. 2.2e; $F[1,24] = 0.05$; $P = 0.83$) and temporal analysis showed no interaction among CPCCOEt, ethanol, and time (Fig.2.2f).

Ethanol-induced LORR: ethanol dose-dependence.

To further characterize involvement of mGluR5 and mGluR2/3 receptors in ethanol-induced hypnosis, the highest effective dose of each antagonist was tested in combination with a range of ethanol doses. As shown in Figure 2.3a, the duration of LORR was dose-dependently increased by ethanol ($F[3, 53] = 103$; $P<0.001$). At a dose of 2.5g/kg, ethanol did not induce LORR. 3.0g/kg, 3.5g/kg, and 4.0g/kg doses of ethanol induced increasing durations of LORR. This dose-dependent effect of ethanol was enhanced by MPEP pretreatment. Two-way ANOVA showed a main effect of MPEP (30mg/kg) ($F[1, 53] = 80$; $P<0.001$). After MPEP pretreatment,

the time to regain the righting reflex was significantly increased compared to the corresponding saline pretreated controls at all doses of ethanol tested (Tukey; P's≤0.002). MPEP pretreatment also altered the sedative-hypnotic effects of the lowest dose of ethanol, resulting in a loss of righting reflex when combined with 2.5g/kg ethanol. However, there was no MPEP x ethanol interaction.

Figure 2.3b shows the ethanol dose response curve following vehicle and LY341495 pretreatment. Two-way ANOVA showed that both ethanol and LY341495 produced main effects (EtOH: ($F[2, 41] = 27$; $P<0.001$); LY341495: ($F[1, 41] = 12$; $P = 0.001$). Analysis also showed a significant LY341495 x ethanol interaction ($F[2, 41] = 5$; $P = 0.015$), indicating that LY341495 effects are dependent on ethanol dose. LY341495 (30mg/kg) significantly reduced the duration of ethanol-induced LORR for a 4.0g/kg ethanol dose (Tukey; $P<0.001$), but not for the 3.0 or 3.5 g/kg doses.

Examination of the time to onset of loss of righting reflex also demonstrated differences in the actions of MPEP and LY341495. As shown in Table 2.2, MPEP pretreatment resulted in a more rapid onset of loss of righting reflex for the 3.0 and 3.5g/kg doses of ethanol ($F[2, 39] = 20$; $P = 0.001$), while LY341495 pretreatment had no effect on onset time.

Ethanol-induced LORR: mGluR5 specificity

To further confirm mGluR5 involvement, a low dose of MPEP and the highly potent and selective mGluR5 antagonist MTEP were tested in parallel with a lower hypnotic dose of ethanol (3g/kg). As shown in Figure 2.4, both MPEP (10mg/kg) and MTEP (10mg/kg) significantly increased the duration of LORR induced by ethanol (3g/kg). The duration of ethanol-induced LORR was increased by 86% by MPEP and 133% by MTEP ($F[2,21]=13$; $P<0.001$). There was not a significant difference between the effects of MPEP and MTEP on ethanol-induced LORR

suggesting that the effects observed following MPEP (30mg/kg) are attributable to its actions on mGluR5.

Pentobarbital and Midazolam-induced LORR.

The $GABA_AR$ positive modulator pentobarbital (50mg/kg) induced an average duration of LORR comparable to that produced by a 4g/kg dose of ethanol. Pretreatment with MPEP (30mg/kg) did not alter the onset time or duration of LORR in mice treated with pentobarbital (Table 2.3). In contrast, LY341495 (30mg/kg) significantly increased onset time ($F[1,13] = 14$; $P=0.002$) and shortened the duration of pentobarbital-induced LORR by 67% ($F[1,13] = 39$; $P<0.001$). The $GABA_AR$ benzodiazepine positive modulator midazolam (60mg/kg) also produced an average duration of LORR similar to the highest dose of ethanol. Midazolam-induced LORR was unaffected by MPEP and completely reversed by LY 341495 (Table 2.3; Onset:$F[1,6] = 12$; $P=0.013$; Duration:$F[1,6] = 23$; $P=0.003$).

Ketamine-induced LORR.

Like ethanol, the NMDA receptor antagonist ketamine (200mg/kg)-induced hypnosis was differentially affected by pretreatment with MPEP and LY341495 (Table 2.3). MPEP (30mg/kg) increased the duration of LORR in mice treated with ketamine by 39% ($F[1,13] = 85$; $P<0.001$), while LY341495 (30mg/kg) reduced ketamine-induced LORR by 20% ($F[1,13] = 12$; $P=0.004$). Onset time was unaffected by either MPEP or LY341495 treatment.

Blood Ethanol Determination.

To address the possibility that MPEP and LY341495 are eliciting their effects by altering ethanol metabolism, blood-ethanol concentrations were measured following a 4g/kg dose of ethanol (Table 2.4). In vehicle treated animals, blood-ethanol concentrations decreased significantly over time (saline: $F[4,79] = 79$; $P<0.001$; cyclodextrin: $F[4,88] = 42$; $P<0.001$). Neither MPEP (30 mg/kg; $F[1,22] = 0.987$; $P=0.331$) nor LY341495 (30mg/kg; $F[1,14] = 1.3$;

P=0.274) pretreatment altered the blood-ethanol clearance time course. However, comparisons of the blood ethanol concentrations of saline and cyclodextrin treated animals revealed significant differences between the two vehicle groups ($F[1,100] = 10$; $P=0.003$; AUC: $F[1,22] = 11$; $P=0.003$), specifically at the 120 and 180 minute time points ($P<0.05$).

DISCUSSION

The purpose of this study was to investigate the role of specific mGluRs in the acute sedative-hypnotic effects of ethanol in mice by examining the effects of mGluR selective antagonists on ethanol induced sedation and hypnosis. One of the primary findings of the present study is that the mGluR5 antagonist MPEP increased ethanol-induced locomotor inhibition and loss of righting reflex. These findings indicate that inhibition of mGluR5 activity enhances the acute sedative-hypnotic effects of ethanol. Moreover, the more selective mGluR5 antagonist MTEP produced a comparable effect on ethanol-induced loss of righting reflex, further supporting the conclusion that mGluR5 activity influences the sedative-hypnotic properties of ethanol. Although the exact role of mGluR5 in ethanol's pharmacological effects has not been identified, these results suggest that ethanol produces some of its acute inhibitory effects through negative modulation of mGluR5 activity. This hypothesis is supported by evidence showing that pharmacologically relevant concentrations of ethanol inhibit glutamate-induced Ca^{2+}-dependent Cl^- currents in Xenopus oocytes expressing mGluR5 (Minami et al. 1998). Furthermore, this inhibition of mGluR5 is PKC dependent. Since acute ethanol activates PKCγ *in vivo* (Wilkie et al. 2007), which desensitizes mGluR5 through phosphorylation (Gereau and Heinemann 1998), it is possible that ethanol inhibits mGluR5 activity through PKC-dependent desensitization.

The other group I antagonist tested, the mGluR1 selective CPCCOEt, had no effect on ethanol-induced sedation or hypnosis. The absence of an effect for CPCCOEt is consistent with evidence suggesting that group I mGluR mediation of ethanol effects is specific to mGluR5. Acute ethanol treatment does not affect Cl^- currents in mGluR1-expressing oocytes (Minami et al. 1998), and our laboratory has shown no effect of CPCCOEt on ethanol consumption in either C57BL/6J mice or alcohol-preferring (P) Rats (Hodge et al. 2006; Schroeder et al. 2005).

However, our current results are inconsistent with a similar study (Lominac et al. 2006), which shows that pretreatment with CPCCOEt, but not MPEP, facilitates ethanol-induced motor impairment. While it is not entirely clear why these discrepancies exist, it may be due to differences in experimental procedure. We chose to use a higher dose of MPEP (30mg/kg) based on our initial LORR study that showed no effect of a 10mg/kg when paired with a 4g/kg dose of ethanol. Furthermore, we employ a shorter pretreatment time. The onset to LORR and temporal distribution analysis of locomotor behavior both suggest that MPEP's behavioral effects are rapidly induced, implying that a shorter pretreatment time is necessary to see MPEP effects. Furthermore, our data showing that both a lower dose of MPEP and another mGluR5 selective antagonist, MTEP, increase the duration of loss of righting reflex produced by a lower dose of ethanol (3 g/kg) support the conclusion that selective blockade of mGluR5 enhances ethanol's sedative-hypnotic effects. As for the differences in CPCCOEt effects, the longer pretreatment time and longer testing time used by Lominac et al. (2006) may be necessary for the expression of CPCCOEt enhancement of ethanol-induced motor impairment.

This study also shows that the mGluR2/3 antagonist, LY341495, decreased both the sedative and hypnotic effect of ethanol. Both doses of LY341495 (10 and 30mg/kg) reduced ethanol-induced LORR. LY341495 (30mg/kg) also reversed the sedative effects of ethanol, as measured by spontaneous locomotor activity. These results were not unexpected given that LY341495 has been shown to have locomotor activating properties (David and Abraini 2001). A growing body of evidence indicates that group II mGluRs are presynaptic autoreceptors and blockade of these receptors with LY341495 increases glutamate release (Xi et al. 2002). Increases in presynaptic glutamate release are known to promote increased locomotor activity (Vezina and Kim 1999), suggesting that reductions in ethanol-induced sedation and hypnosis by LY341495 are due to increased glutamatergic activity.

The ethanol dose response data highlight other differences between the mGluR5 and mGluR2/3 effects. The mGluR5 antagonist MPEP produced a significant increase in the duration of LORR regardless of the dose of ethanol administered. Furthermore, the increases produced by MPEP were the same for each dose of ethanol. Although MPEP alone produced no sedative-hypnotic effects, combination with a sub-hypnotic dose of ethanol (2.5 g/kg) produced full hypnosis and a duration of LORR similar to the increases seen when MPEP was combined with fully hypnotic doses of ethanol. In contrast, the mGluR2/3 antagonist LY341495 produced a significant decrease in the duration of LORR only when administered with the highest dose of ethanol. These results indicate that the effects of LY341495 are dependent on the dose of ethanol administered. The onset to LORR data also present a distinction between the actions of MPEP and LY341495. Pretreatment with MPEP resulted in a more rapid onset of LORR for the two lower doses of ethanol (3.0 and 3.5g/kg), providing further evidence that MPEP enhances the hypnotic properties of ethanol and does so rapidly. Pretreatment with LY341495 did not alter the onset of LORR at any of the ethanol doses tested.

Ethanol-induced hypnosis has been attributed largely to ethanol's ability to enhance inhibitory GABAergic responses and impair excitatory NMDA receptor activity (Beleslin et al. 1997). For an understanding of the mechanisms by which MPEP and LY341495 alter ethanol-induced hypnosis and a clarification as to whether these compounds selectively alter ethanol effects, MPEP and LY341495 were also tested with two $GABA_A$ receptor positive modulators, pentobarbital and midazolam, and an NMDA receptor antagonist, ketamine, that exhibit hypnotic properties. MPEP pretreatment increased the duration of LORR for ketamine but had no effect on the hypnotic properties of pentobarbital or midazolam. These data suggest that MPEP increases hypnosis selectively through modulation of NMDA receptors. This finding is consistent with reports that MPEP reduces NMDA-evoked responses (Attucci et al. 2001) and

antagonism of mGluR5 and NMDA receptors have additive detrimental effects on learning and memory (Homayoun et al. 2004). Our results for pentobarbital and midazolam appear to be at odds with reports that mGluR5 activation positively modulates $GABA_A$ receptor function *in vitro* (Hoffpauir and Gleason 2002), although this may be specific to benzodiazepine-sensitive $GABA_A$ receptors as MPEP inhibits the ethanol-like stimulus properties of diazepam, but not pentobarbital (Besheer and Hodge 2005). However, it has also been reported that the anxiolytic effects of MPEP do not involve benzodiazepine-sensitive $GABA_A$ receptors (Wieronska et al. 2004), indicating that further research must be done to determine the extent to which mGluR5 interacts with $GABA_A$ receptors in vivo. Overall, these results suggest that blockade of mGluR5 increases the hypnotic properties of ethanol by enhancing ethanol-induced inhibition of NMDA receptors without affecting ethanol's actions at $GABA_A$ receptors.

LY341495 reduced the duration of LORR for ketamine and pentobarbital and fully blocked induction of midazolam-induced LORR. These data are consistent with evidence showing that LY341495 has general stimulant effects (David and Abraini 2001). While LY341495 reversed the hypnotic effects of all three drugs, it did so to varying degrees. LY341495 reduced ketamine-induced LORR by 20%, pentobarbital-induced LORR by 67%, and midazolam-induced LORR by 100%. Interestingly, LY341495 appears to be least effective at reversing hypnosis induced by NMDA receptor inhibition. Based on our hypothesis that LY341495 reverses hypnosis by increasing glutamate release, these data suggest that the inhibition of mGluR2/3 results in enough glutamate release to counteract enhanced $GABA_AR$ activity, but not enough to prevent decreased glutamatergic activity due to NMDAR inhibition.

One limitation of the present study that merits discussion is the potential off target effects of the pharmacological compounds tested. High concentrations of MPEP (20μM and above) have been associated with NMDA receptor inhibition (O'Leary et al. 2000) and positive

allosteric modulation of mGlu4 receptors (Mathiesen et al. 2003). However, it has been reported that systemic administration of a 3mg/kg dose of MPEP produces submicromolar concentrations in the brain (0.83μM; (Cosford et al. 2003), making it unlikely that a 30mg/kg dose of MPEP would result in brain concentrations high enough to significantly effect NMDA or mGlu4 receptors. It has also been reported that MPEP can inhibit the norepinephrine transporter at nanomolar concentrations (Heidbreder et al. 2003), which may be related to the effects we report given that the norepinephrine transporter has been implicated in the differential ethanol sensitivities of the long-sleep and short-sleep mice (Haughey et al. 2005). However, given that a low dose of MPEP (10mg/kg) and the more selective mGluR5 antagonist MTEP (Varty et al. 2005) both enhanced the hypnotic effects of a low dose of ethanol, off target effects of MPEP do not appear to be a concern here.

Although there is currently no evidence that LY341495 has any off target effects, another mGluR2/3 antagonist has been shown to increase extracellular dopamine levels (Karasawa et al. 2006). Thus, additional research using gene knockout mice, RNA inhibition, or other more selective approaches are warranted to examine the selectivity of these compounds. The dose of ketamine used to induce LORR may also be having off target effects, namely at dopaminergic or nicotinic acetylcholine systems. Since ketamine has been shown to increase extracellular dopamine release (Aalto et al. 2005), it is unlikely that interactions with dopaminergic systems are contributing to ketamine's sedative-hypnotic profile. Ketamine has also been shown to inhibit α7-containing nicotinic acetylcholine receptors (nAChR) (Coates and Flood 2001). This interaction may play a role in ketamine-induced hypnosis, as α7-subunit null mutant mice are more sensitive to the sedative-hypnotic properties of ethanol (Bowers et al. 2005), and in the MPEP and LY341495 modulation of induced sedation, as recent evidence suggests a link

between mGluR function and α7-nAChR (Welsby et al. 2006). Again, further research is necessary to determine the roles of each of these systems.

Finally, neither MPEP nor LY341495 altered blood-ethanol clearance. However, cyclodextrin, the vehicle used for LY341495 and CPCCOEt, appears to slow the time course of ethanol elimination as compared to saline. While these differences do not invalidate results from individual experiments, it limits the comparisons that can be made between saline vehicle and cyclodextrin vehicle experiments. These differences may contribute to the variability seen for the three vehicle groups in the locomotor activity experiments.

In summary, the present data suggest that specific subtypes of metabotropic glutamate receptors differentially modulate ethanol-induced sedation and hypnosis without altering the pharmacokinetics of ethanol elimination. Inhibition of mGluR5 enhances the sedative-hypnotic effects of ethanol, whereas inhibition of mGluR2/3 reverses these effects of ethanol. Our results also suggest that mGluR5 and mGluR2/3 elicit these changes through differential modulation of $GABA_A$ and NMDA receptors.

Table 2.1 IC_{50} values (μM) of group 1 and 2 selective mGluR antagonists

		CPCCOEt (mGluR1)	MPEP (mGluR5)	LY341495 (mGluR2/3)
Group 1	**mGluR1**	7-23	>100	6.8
	mGluR5	>100	0.032	8.2
Group 2	**mGluR2**	>100	>100	0.021
	mGluR3	---	---	0.014

Table 2.2 Effects of mGluR antagonists on onset to ethanol-induced LORR

	MPEP Dose (mg/kg)		LY341495 Dose (mg/kg)	
Ethanol (g/kg)	**0**	**30**	**0**	**30**
3.0	2.4 ± 0.1	1.9 ± 0.1*	3.1 ± 0.2	3.2 ± 0.2
3.5	1.8 ± 0.2	1.2 ± 0.2*	2.6 ± 0.2	2.5 ± 0.2
4.0	1.5 ± 0.1	1.2 ± 0.1	1.9 ± 0.1	2.0 ± 0.2

Data expressed as mean ± SEM (n = 8-10);

*Significantly different from ethanol alone ($p<0.001$, Tukey's test).

Table 2.3 Effects of mGluR antagonists on LORR induced by GABAergic and glutamatergic sedative-hypnotics

	MPEP Dose (mg/kg)				LY341495 Dose (mg/kg)			
	0		30		0		30	
	Onset	**Duration**	**Onset**	**Duration**	**Onset**	**Duration**	**Onset**	**Duration**
Pentobarbital							12.3	
(50 mg/kg)	3.6	63.9	3.6	56.3	8.1	46.2	±	15.1
	± 0.4	± 3.5	± 0.5	± 2.1	± 0.7	± 11.5	0.9*	± 7.6*
Midazolam	10.6	47.3	6.2	47.5	25.5	39.6	0.0	0.0
(60 mg/kg)	± 2.5	± 3.2	± 1.2	± 3.6	± 7.3	±8.2	± 0.0*	±0.0*
Ketamine	1.4	41.0	1.4	56.8	2.7	40.2	2.1	32.3
(200 mg/kg)	± 0.1	± 1.3	± 0.1	± 1.1*	± 0.6	± 1.9	± 0.1	± 1.3*

Data expressed as mean ± SEM (n = 8-10); *Significantly different from pentobarbital, midazolam, or ketamine alone ($p<0.05$, Tukey's test).

Table 2.4 Effects of mGluR antagonists on blood-ethanol concentration

	Time Interval (min)					
	10	**30**	**60**	**120**	**180**	**AUC**
Saline	387.1 ± 18.6	381.6 ± 8.4	369.4 ± 6.9	314.7 ± 7.9	255.6 ± 8.8	57197.8 ± 1568.9
MPEP	391.2 ± 22.4	399.5 ± 14.6	387.1 ± 11.0	330.8 ± 11.3	275.5 ±12.7	60347.3 ± 2627.5
Cyclodextrin	390.7 ± 10.9	406.2 ± 5.6	385.4 ± 8.4	353.0 ± 5.9*	314.6 ± 6.1*	62026.1± 680.4*
LY341495	399.9 ± 17.3	381.5 ± 11.6	379.3 ± 14.3	333.7 ± 16.0	305.8 ±10.9	59802.8 ±2008.8

Data expressed as mean ± SEM mg/dl (n =7-16); AUC: mean area under the curve ± SEM;

*Significantly different from saline (Tukey; $p<0.05$)

Figure 2.1 Effects of mGluR antagonists on loss of righting reflex (LORR). Bars represent the mean (±SEM) duration of ethanol-induced LORR in minutes (n = 6-8) following pretreatment with MPEP (a), CPCCOEt (b), or LY341495 (c). *Significantly different from 4g/kg ethanol alone ($p<0.05$, Tukey).

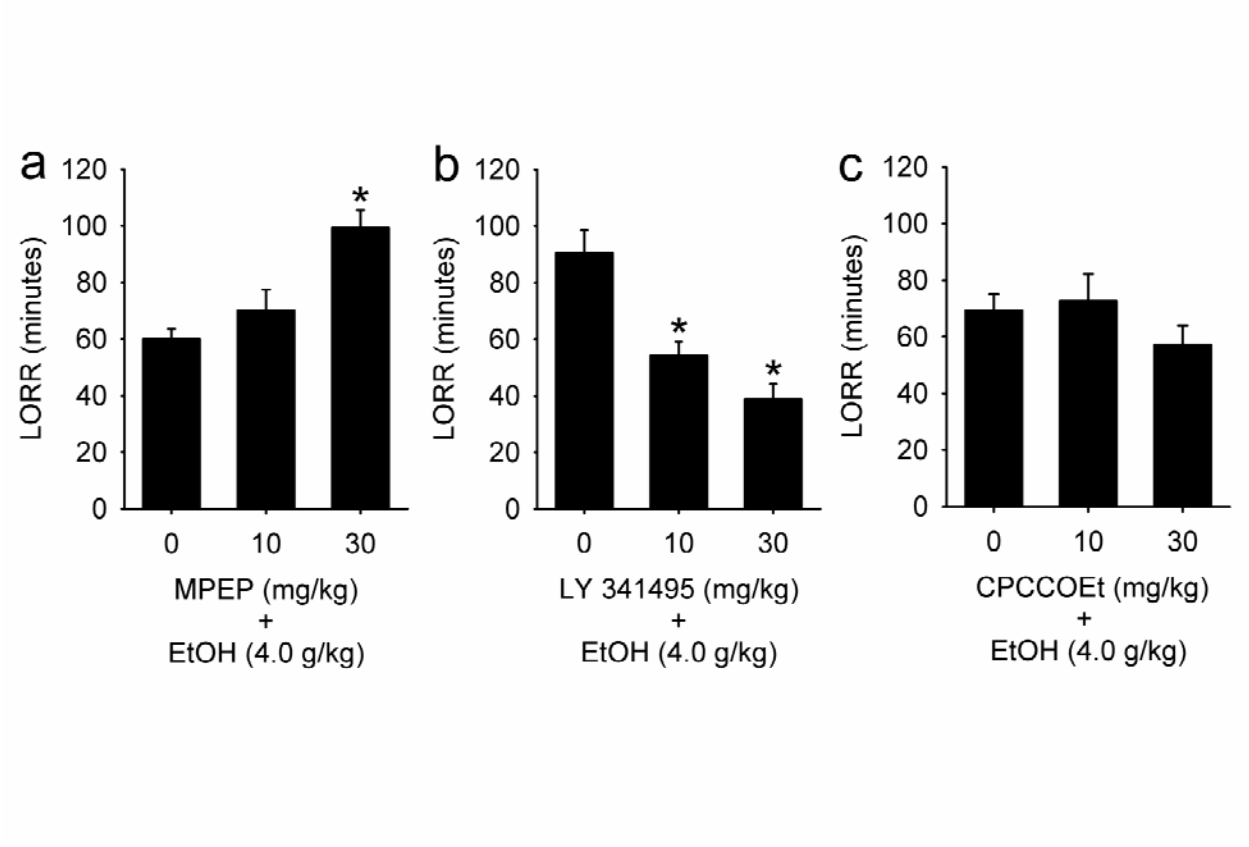


Figure 2.2 Effects of mGluR antagonists on total locomotor activity alone and in the presence of a sedative dose of ethanol (a, c, and e). Bars represent the mean (±SEM) horizontal distance traveled in 60 minutes (n = 6-8) following pre-treatment with vehicle, MPEP (30mg/kg) (a), LY341495 (30mg/kg) (c), or CPCCOEt (30 mg/kg) (e) with and without ethanol (2.0g/kg). * Significantly different from vehicle/vehicle ($p<0.05$, Tukey's test). ** Significantly different from vehicle/ethanol ($p<0.05$, Tukey's test). Temporal analysis of mean (±SEM) horizontal distance traveled in 5 minute time intervals (n = 6-8) following treatment with vehicle, MPEP (30mg/kg) (b), LY341495 (30mg/kg) (d), or CPCCOEt (30mg/kg) (f) with and without ethanol (2.0g/kg). * mGluR antagonist/ethanol treatment significantly different from vehicle/ethanol treatment at given time point ($p<0.05$, Tukey's test). † mGluR antagonist/vehicle treatment significantly different from vehicle/vehicle at given time point ($p<0.05$, Tukey's test).

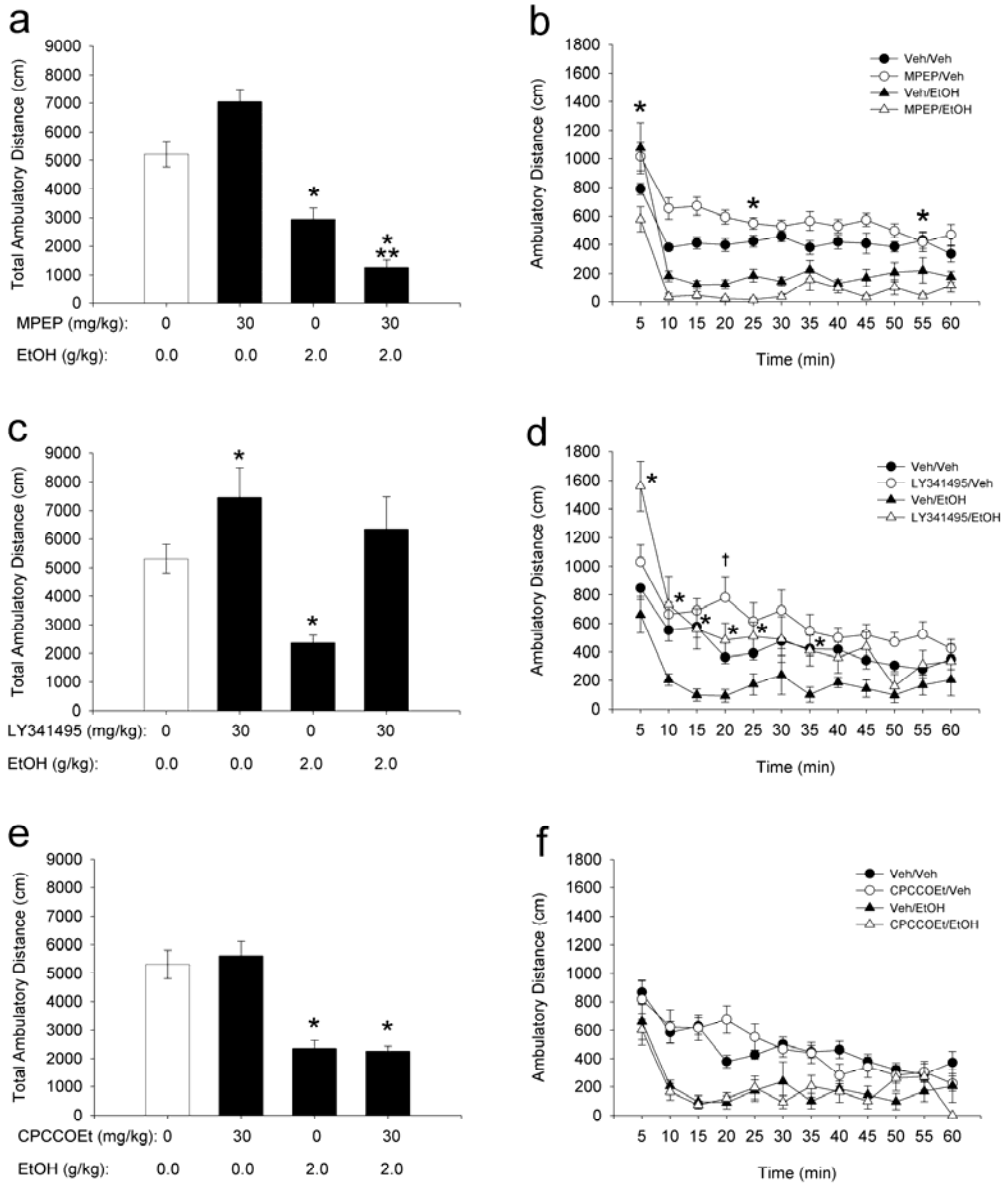

a
Total Ambulatory Distance (cm)
MPEP (mg/kg): 0 30 0 30
EtOH (g/kg): 0.0 0.0 2.0 2.0
b
Ambulatory Distance (cm)
Veh/Veh
MPEP/Veh
Veh/EtOH
MPEP/EtOH
Time (min)
c
Total Ambulatory Distance (cm)
LY341495 (mg/kg): 0 30 0 30
EtOH (g/kg): 0.0 0.0 2.0 2.0
d
Ambulatory Distance (cm)
Veh/Veh
LY341495/Veh
Veh/EtOH
LY341495/EtOH
Time (min)
e
Total Ambulatory Distance (cm)
CPCCOEt (mg/kg): 0 30 0 30
EtOH (g/kg): 0.0 0.0 2.0 2.0
f
Ambulatory Distance (cm)
Veh/Veh
CPCCOEt/Veh
Veh/EtOH
CPCCOEt/EtOH
Time (min)

Figure 2.3 Ethanol-induced Loss of Righting Reflex plotted as a function of ethanol dosage, symbols represent the mean (±SEM) duration of LORR in minutes (n = 8) following ethanol with saline pretreatment (open symbols) or in combination with MPEP (a) or LY341495 (b) pretreatment (closed symbols). *Significantly different from vehicle at corresponding dose of ethanol ($p<0.05$, Tukey's test).

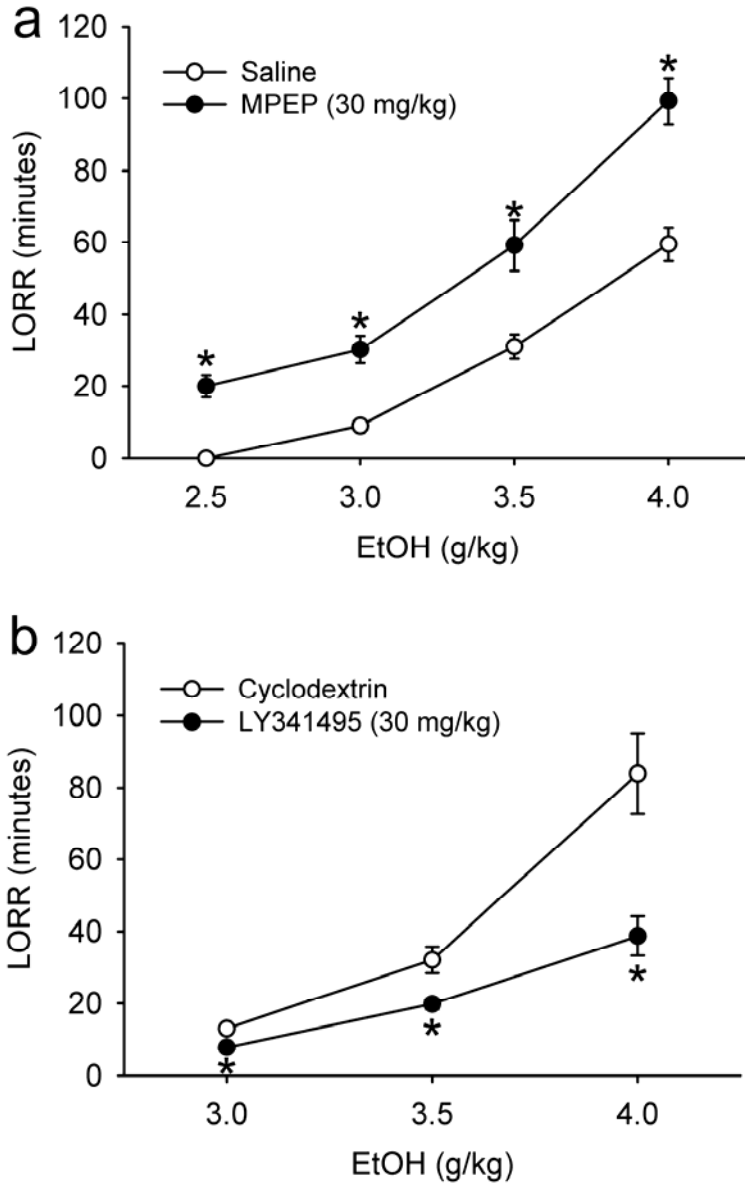
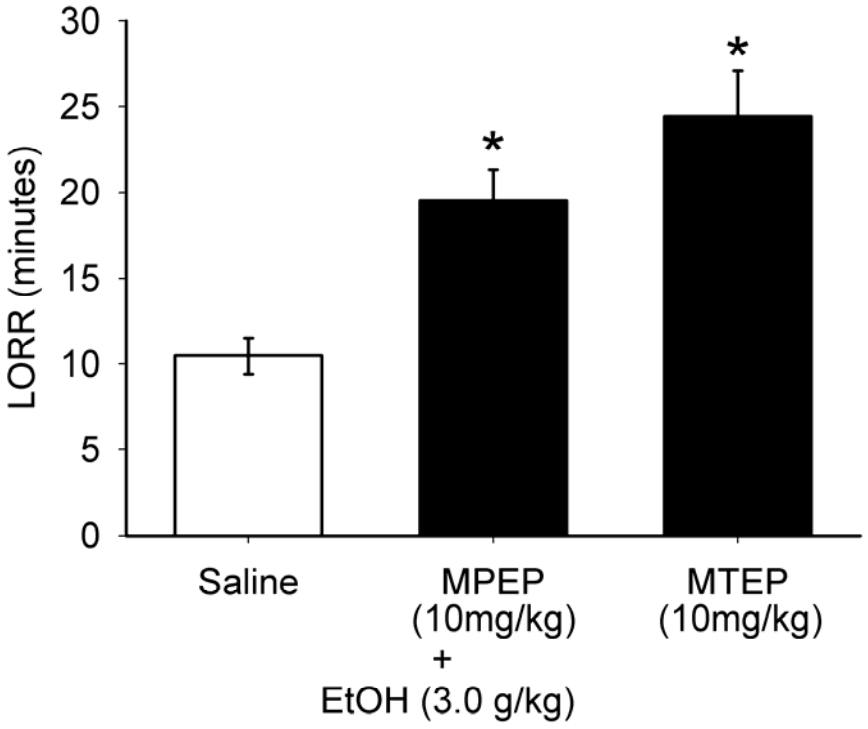


Figure 2.4 Confirmation of mGluR5 specificity in Loss of Righting Reflex.Bars represent mean (±SEM) duration of ethanol-induced LORR in minutes (n=8) following pretreatment with saline, MPEP, or MTEP. * Significantly different from saline pretreatment ($p<0.05$, Tukey's test).



CHAPTER 3

ACUTE ETHANOL ADMINISTRATION ALTERS PROTEIN KINASE C-GAMMA PHOSPHORYALTION IN A TIME AND BRAIN REGION DEPENDENT MANNER

INTRODUCTION

mGluR5 activation leads to DAG formation and release of intracellular Ca^{2+} stores, both of which enhance the translocation of the inactive form of protein kinase C (PKC) to the membrane. At the membrane, PKC undergoes a conformational transformation and is phosphorylated. Once PKC is fully phosphorylated, it is released into the cytosol as an active kinase, where it can activate various downstream targets such as mitogen-activated kinase cascades. In addition to mediating the downstream effects of mGluR5 activation, PKC has been shown to phosphorylate mGluR5. Phosphorylation of the c-terminus of mGluR5 inhibits inositol phosphate accumulation, suggesting that PKC also interrupts mGluR5 signaling (Francesconi and Duvoisin 2000).

PKC is a family of serine-threonine kinases that play a critical role in many cellular signal transduction pathways (Toker 1998). The PKC family is divided into three major groups based on structural and functional similarities. The conventional isozymes (α,βI,βII,γ) are calcium dependent and activated by both calcium and diacylglycerol (DAG); the novel isozymes (δ,ε,η,θ) are activated only by DAG, as they lack the calcium binding domain; and the atypical isozymes (λ,ζ) are insensitive to either calcium or DAG (Newton 2003). PKC is widely expressed in mammalian tissue; however, individual PKC isozymes show distinct patterns of tissue expression and cellular localization, suggesting selective functions for the various

isozymes (Akita 2002; Saito et al. 1988). While many of the PKC isozymes are found in the brain, PKCγ is expressed almost exclusively in neural tissue (Wetsel et al. 1992). In the brain, PKC regulates a variety of neural functions, including ion channel activity, neurotransmitter release, receptor desensitization, and differentiation (Tanaka and Nishizuka 1994).

In addition to these general functions, PKCγ has been shown to regulate the biochemical and behavioral effects of ethanol. Ethanol exposure has been shown to alter expression, localization and activity of PKCγ. In mice, PKCγ expression is decreased in the frontal cortex and increased in the limbic forebrain following chronic ethanol treatment (Narita et al 2001). Futhermore, acute ethanol increases the presence of phosphorylated PKCγ, the catalytically competent form of the kinase, across the rat brain (Wilkie et al. 2007). Ethanol has also been shown to alter subcellular localization of PKCγ. *In vitro*, ethanol induces the translocation of PKCγ from the nuclear/perinuclear region to the cytoplasm (Gordon et al. 1998). *In vivo* studies indicate that ethanol alters the presence of PKCγ at the membrane, as well. In rat cerebral cortex, membrane bound PKCγ expression is reduced 60 minutes after ethanol treatment (Kumar et al. 2006). Evidence from gene knockout studies indicates that PKCγ mediates both the acute and chronic effects of ethanol. PKCγ null mice are less sensitive to the hypnotic effects of ethanol (Harris et al. 1995), fail to develop tolerance to the ethanol hypnosis after liquid diet exposure (Bowers et al. 1999), and show increased ethanol consumption versus wild-type animals (Bowers and Wehner 2001).

The primary goal of the present study was to determine whether acute ethanol exposure alters the phosphorylation states of PKCγ in the mouse brain in a time and brain region specific manner. To accomplish this goal, mice were administered either an acute dose of ethanol or a corresponding volume of saline via intraperitoneal injection. Animals were sacrificed after 10,

30, or 90 minutes and the brains were prepared for analysis of phosphorylated PKCγ (phospho-T674) expression by immunohistochemical methods. This site was chosen for analysis because it is the hydrophobic motif phosphorylation site, and PKC is not fully activated until this site is phosphorylated. Newly synthesized PKC associates with the plasma membrane in an open conformation to allow binding of phophoinositide-dependent kinase-1 (PDK-1), which phosphorylates the activation loop (Cenni et al 2002). The turn motif and hydrophobic motif are then autophosphorylated (Parekh 2000). The triple phosphorylation locks PKC into a stable conformation which exposes the substrate binding pocket, resulting in "mature", active PKC. Total PKCγ immunoreactivity was analyzed to determine whether acute ethanol produced any changes in the overall expression of PKCγ.

In an effort to explore one potential mechanism by which ethanol could alter kinase phosphorylation, the phosphorylation state of protein phosphates-1α (PP1α) also was examined. PP1α is highly expressed in a number of brain regions implicated in the neurobiological and behavioral effects of ethanol (da Cruz e Silva et al. 1995)and, of the four protein phosphatase 1 subtypes, PP1α is most susceptible to phosphorylation by PKC (Takizawa et al. 1997). Furthermore, a variety of studies have also demonstrated roles for PP1 both upstream and downstream of the extracellular-signal regulated MAPK cascade, which is known to be coupled to mGluR5 (Mitsuhashi et al. 2003; Yi et al. 2008).

MATERIALS AND METHODS

Animals. Male adult C57/BL6J mice (Jackson Labs, Bar Harbor, ME; 10-24 weeks; 22-35g) were housed four per cage and maintained on a 12-hour light/dark cycle with food and water available ad libitum. Animal care and handling procedures were performed in accordance with approved institutional protocols and the National Institutes of Health Guide for Care and Use of Laboratory Animals.

Drugs. Ethanol (95%w/v) was diluted in physiological saline (0.9%) to a concentration of 20% (v/v) and administered in various volumes to obtain the appropriate doses.

Acute ethanol time course. Mice were allowed approximately 1 week to acclimate to the laboratory. Mice were habituated to handling (weighing) and intraperitoneal injection (saline, 0.1ml/10g) for three days prior to the start of the experiment. On the day of the experiment, mice received ethanol (0 or 3 g/kg, IP) and after 10, 30, or 90 minutes were deeply anesthetized with pentobarbital and brains were prepared as described below.

Immunohistochemical Analysis. Mice were transcardially perfused with 100mM phosphate buffer, followed by 4% formaldehyde (pH 7.4). The brains were post fixed for 48 hours at 4°C, rinsed with phosphate buffer, and removed from the skulls. The fixed brains were stored in phosphate buffer at 4°C until sectioning. Coronal brain sections (40μm) were cut on a vibratome (Leica, VT1000S) and stored in cryoprotectant at -20°C until processing for immunohistochemistry.

Sections were washed in PBS prior to free-floating immunohistochemical processing. Following preblocking, sections were agitated with one of the following antibodies for 16 hr at 4°C: phospho-PKCγ (1:2000; phospho-Thr 674, Abcam, Cambridge, MA), PKCγ (1:2,000; Santa Cruz Biotech, Santa Cruz,CA), or phospho-PP1α (1:150; Cell Signaling Tech, Danvers, MA). Sections were visualized with a biotinylated goat anti-rabbit IgG secondary antibody (1:2000

dilution) followed by Vectastain ABC (Vector, Burlingame, CA) and DAKO DAB chromagen (DAKOCytomation, Carpenteria, CA) or DAKO labeled polymer-HRP anti-rabbit secondary antibody followed by DAKO DAB+ chromagen. Sections were counterstained with toluidine blue. Sections were viewed at 20-40X magnification under brightfield illumination (Olympus CX41 light microscope, Olympus America, Center Valley, PA). Images were acquired using a digital camera (Regita OEM fast, QImaging, Burnaby, BC) and immunoreactivity was quantified using Bioquant Life Science software (R&M Biometric, Nashville, TN). Pixel density and cell count measurements were divided by the area of the region and expressed as pixels/mm^2.

Antibody Specificity. Specificity of the pPKCγ antibody for phosphorylated PKCγ was previously determined (Wilkie et al. 2007). Specificities of the PKCγ and pPKCγ antibodies for the gamma subtype were tested in PKCγ null mutant tissue. The PKCγ antibody produced no immunoreactivity. The pPKCγ antibody produced some staining and is known to cross-label PKCα and PKCβ, although it has lower affinity for these two subtypes (data not shown).

Data Collection and Statistics. Each treatment group consisted of 8 animals. Measurements were taken from the left and right side of each section and multiple sections were analyzed for each brain region, when possible. Measurements from individual brain regions for each animal were averaged to produce a single value for a given region in each animal. Data were analyzed within the area of interest, across time, by two-way ANOVA followed by the Tukey multiple comparison procedure (Graphpad Software, San Diego, CA).

Anatomical Coordinates. Anatomical coordinates used for analysis were taken from the Mouse Brain in Stereotaxic Coordinates (Paxinos and Watson, 2001). Analyses of the nucleus accumbens and proximal brain regions were in sections ranging from 0.86 to 1.34 mm anterior to Bregma. The amygdala, and proximal brain regions were evaluated in sections ranging from 1.58to -2.18 mm posterior to Bregma.

RESULTS

Distribution of PKCγ in Mouse Brain

PKCγ and pPKCγ immunoreactivties were detected in numerous brain regions (Table 1). The highest density of PKCγ immunoreactivity was observed in the prefrontal cortex, lateral septum, CA1 region of the hippocampus, and the basolateral amygdala. Moderate levels of immunostaining were observed in the piriform cortex, nucleus accumbens (core and shell), and the paraventricular nucleus of the thalamus. The cytological pattern of PKCγ immunoreactivity showed localization primarily at the plasma membrane, with diffuse staining in the stratum radiatum and oriens layer of the hippocampus. Upon analysis, no changes in total PKCγ immunoreactivity were detected following acute ethanol exposure (data not shown).

The highest levels of pPKCγ immunoreactivity were observed in the CA1 of the hippocampus with lower levels of immunostaining seen in the prefrontal and piriform cortex, nucleus accumbens (core and shell), lateral septum, paraventricular nucleus of the thalamus, and the amygdala (basolateral and central nuclei). pPKCγ immunoreactivity produced a punctuate pattern of staining in the prefrontal cortex, the nucleus accumbens, lateral septum, and the central amygdala, and membrane-like staining in the piriform cortex, CA1, basolateral amygdala and PVN thalamus. Diffuse staining was also observed in the stratum radiatum and oriens layer surrounding the CA1 in the hippocampus.

Effects of Acute Ethanol on pPKCγ Immunoreactivity

Nucleus Accumbens (NAcc). Acute ethanol (3.0g/kg, IP) induced a rapid increase in pPKCγ immunoreactivity in the nucleus accumbens (Fig 3.1). In the NAcc core, two-way ANOVA revealed a main effect of ethanol (Fig 3.1a; $[F(1,36)]= 1$; $p<0.005$), but no main effect of time or ethanol x time interaction. Analysis of the individual time points showed a significant

increase in pPKCγ immunoreactivity following ethanol treatment at the 30 minute time point (Tukey; $p<0.01$). The increase in pPKCγ immunoreactivity at 30 minutes was associated with a significant increase in pPKCγ-positive cell counts (Table 3.2; Tukey; $p<0.01$). In the NAcc core, two-way ANOVA revealed main effects of time and ethanol treatment, as well as an ethanol x time interaction (Fig 3.1b; $F[2,37]= 4$; $p<0.05$). As in the core, pPKCγ immunoreactivity in the shell was significantly increased at the 30 minute time point with a concomitant increase in pPKCγ-positive cell counts (Tukey; p's<0.001). Representative photomicrographs illustrating the cytological pattern of pPKCγ immunoreactivity in the nucleus accumbens at the 30 minute time point are shown in Figure 3.1c.

Amygdala. Acute ethanol (3g/kg, IP) induced temporally distinct increases in pPKCγ immunoreactivity in the central and basolateral nuclei of the amygdala (Fig 3.2). In the central amygdala, two-way ANOVA showed main effects of time and ethanol treatment, in addition to an ethanol x time interaction (Fig 3.2a; $F[2,40]= 8$; $p<0.005$). Ethanol significantly increased both pPKCγ immunoreactivity and pPKCγ positive cell counts at the 10 and 30 minute time points (Tukey; p's<0.05). In the basolateral amygdala, no interactions or main effects of treatment were seen, but ethanol significantly decreased pPKCγ immunoreactivity at the 90 minute time point (Fig 3.2b; Tukey; $p<0.05$). Cell count analyses were not done for the basolateral amygdala or the 90 minute time point as the cytological patterns of pPKCγ immunoreactivity were diffuse and membrane-like rather than punctuate in these samples. Representative photomicrographs illustrating the cytological pattern of pPKCγ immunoreactivity in the central amygdala at the 30 minute time point and the basolateral amygdala at the 90 minute time point are shown in Figure 3.2c. In addition, no changes were observed in total PKCγ in any brain region, suggesting that the ethanol-induced changes in pPKCγ are not related to changes in the over-all abundance of PKCγ (data not shown).

Effects of Acute Ethanol on pPP1α Immunoreactivity

pPP1α immunoreactivity was measured in several brain regions, including the core and shell of the nucleus accumbens, and the central and basolateral nuclei of the amygdala (Fig 3.3), as well as the medial prefrontal cortex, the medial thalamus, the CA1 and dentate gyrus of the hippocampus (data not shown). Acute ethanol treatment produced significant decreases in pPP1α immunoreactivity in the nucleus accumbens shell (Fig3.3a) and the basolateral amygdala (Fig3.3b) (Tukey; $p < 0.05$). pPP1α immunoreactivity was unchanged following ethanol treatment in the other regions examined.

DISCUSSION

The purpose of this study was to determine whether acute ethanol exposure alters the phosphorylation state of PKCγ in a time dependent manner. One of the primary finding of the present study is that acute ethanol treatment induces rapid increases in pPKCγ immunoreactivity in the core and shell of the nucleus accumbens and the central amygdala that return to baseline levels within 90 minutes of treatment. Furthermore, these increases in pPKCγ immunoreactivity were associated with concurrent increases in pPKCγ-positive cell counts. Interestingly, acute ethanol treatment also altered pPKCγ immunoreactivity in the basolateral amygdala. However, pPKCγ immunoreactivity was decreased in the basolateral amygdala after ethanol treatment and this effect was observed only at the 90 minute time point. These results suggest that ethanol alters PKCγ phosphorylation in a time and brain region dependent manner without altering the total PKCγ abundance.

Phosphorylation events regulate numerous cellular functions and changes in kinase activity are central to a variety of intracellular signaling pathways. Although kinases are responsible for intracellular phosphorylation, they are themselves regulated by phosphorylation. PKC requires phosphorylation of three sites to become an active kinase. Newly synthesized PKC associates with plasma membrane where the activation loop is phosphorylated by another kinase, PDK-1. The turn and hydrophobic motifs are autophosphorylated and PKC is then released into the cytosol in an inactive, "locked" conformation. Activation of phospholipase C and the resulting release of DAG and Ca^{2+} induce translocation of PKC from the cytosol to cell membranes. Association with the membrane provides the energy necessary to release the pseudo-substrate domain from the binding pocket, allowing binding of phosphorylation targets and rendering PKC an active, "mature" kinase.

Ethanol has previously been shown to positively modulate mechanisms leading to PKC activation. In cellular preparations, ethanol exposure activates phospholipase C and increases intracellular DAG and Ca^{2+} levels (Hoek and Rubin 1990). In mice, chronic exposure to ethanol-containing diet increases the abundance of PKCγ associated with the membrane (Narita 2001). Furthermore, recent *in vivo* evidence indicates that acute ethanol exposure specifically increases the abundance of phosphorylated PKCγ associated with the membrane (Wilkie 2007). In the present study, we show that acute ethanol increases the overall density of pPKCγ immunoreactivity and the number of pPKCγ-positive cells in the nucleus accumbens and central amygdala. Taken together, these data indicate that ethanol activates PKCγ in brain regions known to mediate the neurobiological and behavioral effects of ethanol and increases in PKCγ activity may mediate some of the effects of ethanol attributed to these regions.

Although there is no direct evidence that ethanol enhances the kinase activity of PKCγ, ethanol has been shown to enhance general PKC-mediated phosphorylation (Messing et al. 1991) and some of ethanol's biochemical effects have been shown to be PKC-dependent. Increases in GABA-mediated inhibitory post-synaptic currents (Weiner et al. 1994), glutamate transporter activity (Kim et al. 2003), and mitogen activated kinase activity (Washington et al. 2003) induced by acute ethanol exposure have all been shown to require PKC. These findings support the conclusion that the increases in PKCγ phosphorylation observed in this study result in increased PKCγ activity. Furthermore, the time dependent nature of the increases in PKCγ phosphorylation also suggests that PKCγ phosphorylation is mediating the rapid, transitory effects of acute ethanol. However, ethanol has been shown to modulate several of the PKC isozymes, therefore, additional studies examining the kinase activity of PKCγ following ethanol exposure would be necessary to confirm that these changes in pPKCγ are functionally relevant. Our results also suggest that the effects of chronic ethanol exposure on PKCγ phosphorylation

should be examined. Neurobiological systems that are up-regulated by acute ethanol exposure are commonly down-regulated after chronic ethanol exposure. These changes in intracellular signaling systems often lead to altered sensitivity to ethanol and are implicated in the development of ethanol tolerance and dependence.

Emerging evidence also points to PKCγ involvement in mediating the behavioral effects of ethanol that are consistent with the anatomical specificity observed in this study. For example, PKCγ null mutant mice are less sensitive to the acute sedative-hypnotic effects of ethanol than wild type littermates (Harris et al. 1995). Although it is not clear which brain regions regulate ethanol's sedative-hypnotic properties, microinjection of dopamine receptor agonists into the nucleus accumbens has been shown to increase locomotor behavior (Canales and Iversen 2000). Blood ethanol concentrations reach their peak 10 to 20 minutes following ethanol treatment (Quertemont et al. 2003), which corresponds well with the time course of the sedative-hypnotic effects of ethanol and our observed ethanol-induced increases in pPKCγ levels in the nucleus accumbens. These data suggest that ethanol-induced sedation may be mediated by PKCγ-dependent inhibition in the nucleus accumbens.

PKCγ null mutant mice also consume more ethanol than wild type littermates in two-bottle choice experiments (Bowers et al. 1999). While the mechanisms that control ethanol self-administration have not been fully characterized, both the nucleus accumbens and amygdala play a role in the development and maintenance of ethanol drinking behaviors. The nucleus accumbens shell and the central amygdala have been identified as components of the extended amygdala, an inter-regional circuit that influences ethanol related behaviors (McBride 2001). Microinjections of $GABA_AR$ antagonists into either the nucleus accumbens or the central amygdala reduce ethanol self-administration in dependent animals (Hodge et al. 1995; Hyytia and Koob 1995). Given that ethanol enhances GABAergic signaling in a PKC-dependent

manner, the observed increases in pPKCγ in the nucleus accumbens and central amygdala at the early time points may be responsible for increasing GABAergic tone and modulating ethanol self-administration. In mice lacking PKCγ, reduced ethanol-induced enhancements of GABAergic signaling may diminish the positive subjective effects of ethanol and could account for their increased ethanol consumption.

In contrast to the increases in pPKCγ found in the central amygdala, pPKCγ immunoreactivity was decreased in the basolateral amygdala. Moreover, the changes observed in the basolateral amygdala were temporally different from those found in the central amygdala, suggesting that these changes in pPKCγ regulate different aspects of the acute effects of ethanol. While changes at the 10 and 30 minute time points are likely related to the immediate, direct effects of ethanol, changes at the 90 minute time point may be related to acute withdrawal symptoms. Although more commonly associated with chronic ethanol exposure, a single, high dose of ethanol can produce withdrawal symptoms, such as anxiety, which are known to be regulated by the basolateral amygdala (Lack et al. 2007; Zhang et al. 2007). PKCγ null mutant mice exhibit less anxiety than wild type littermates (Bowers et al. 2000), suggesting that reduced pPKCγ would be anxiolytic. Therefore, decreased pPKCγ abundance in the basolateral amygdala may be a compensatory response to the anxiogenic effects of acute ethanol withdrawal.

Finally, PP1α phosphorylation was examined as a possible mechanism by which ethanol could alter kinase phosphorylation. Phosphatases are active in their unphosphorylated form and inactive in their phosphorylated form. Therefore, an increase in phosphatase activity would involve a decrease in phosphorylated phosphatase. Our data shows that pPP1α immunoreactivity is unchanged in most brain regions 10 minutes after ethanol exposure. Although pPP1α immunoreactivity was decreased in the nucleus accumbens shell and basolateral amygdala, these results do not correspond to reduced activity and suggest that the effects of acute ethanol on

pPP1α and pPKCγ are completely dissociated. However, an examination of total PP1α would be necessary to rule out the possibility that ethanol is decreasing the overall abundance of PP1α, which would contribute to less PP1α activity and more phosphorylation of PP1α target kinases.

In summary, the present data suggest that ethanol alters pPKCγ immunoreactivity in a time- and brain region-dependent manner and that these changes may mediate some of the acute effects of ethanol. Although PKCγ immunoreactivity was detected throughout the brain, ethanol-induced changes were limited to the nucleus accumbens and the amygdala. Interestingly, ethanol induced both increases and decreases in pPKCγ immunoreactivity which were observed in distinct brain regions and at different time points.

Table 3.1 Evaluation of the immunohistochemical distribution of phospho-PKCγ (pPKCγ) and total PKCγ. Immunoreactivity (IR) was ranked as minimal (--), low (+), or high (++).

Brain region	PKCγ IR	pPKCγ IR
Cerebral Cortex		
Prefrontal	++	+
Motor	--	--
Somatosensory	--	--
Piriform	+	+
Nucleus Accumbens		
Core	+	+
Shell	+	+
Lateral Septum	++	+
Hippocampus		
CA1	++	++
CA3	--	--
Dentate gyrus	--	--
Amygdala		
Basolateral	++	+
Central	+	+
PVN Thalamus	++	+
PVN Hypothalamus	--	--

Table 3.2 Effects of acute ethanol on pPKCγ-positive cells counts over time. Mean number (± SEM) of pPKCγ positive cells following saline or 3.0 g/kg ethanol treatment expressed as counts/mm^2. *Significantly different from saline control at the given time point ($p<0.05$; Tukey).

	10 minutes		30 minutes	
Brain region	**Saline**	**Ethanol**	**Saline**	**Ethanol**
Nucleus Accumbens				
Core	13.66 ± 4.05	29.96 ± 9.85	11.87 ± 3.75	48.39 ± 15.06*
Shell	27.91 ± 11.21	67.51 ± 18.95	36.52 ± 14.15	194.4 ± 37.69*
Central Amygdala	49.68 ± 12.28	178.8 ± 27.38*	33.51 ± 12.47	148.6 ± 37.79*

Figure 3.1 Effects of acute ethanol on PKCγ phosphorylation in the nucleus accumbensover time. **(a)** Mean (± SEM) immunoreactivity of the pPKCγ positive area in the nucleus accumbens core following 3.0 g/kg ethanol treatment expressed as relative change versus saline control. **(b)** Mean (± SEM) immunoreactivity of the pPKCγ positive area in the nucleus accumbens shell following 3.0 g/kg ethanol treatment expressed as relative change versus saline control. **(c)** Representative photomicrographs (20X) of the cytological pattern of pPKCγ immunoreactivity in the core and shell at 30 minutes. *Significantly different from saline control at the given time point ($p<0.05$; Tukey).

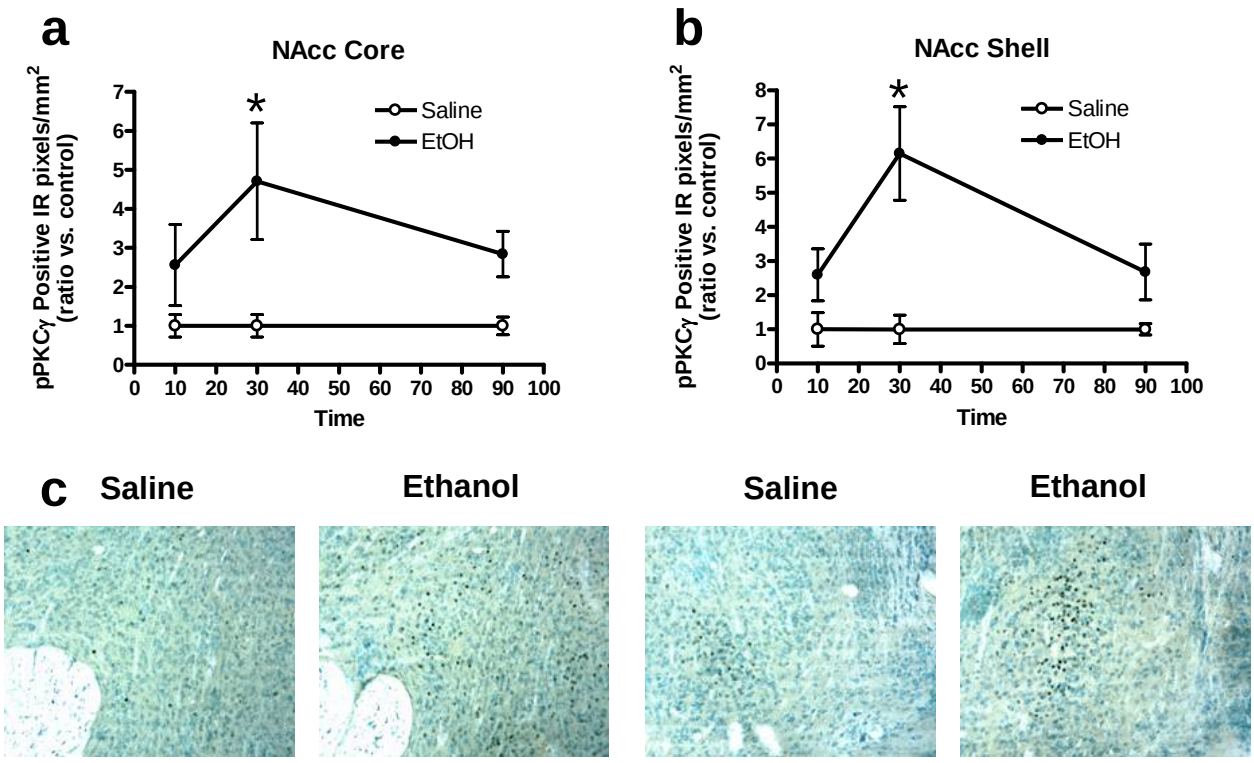


Figure 3.2 Effects of acute ethanol on PKCγ phosphorylation in the amygdala over time. **(a)** Mean (± SEM) immunoreactivity of the pPKCγ positive area in the central amygdala following 3.0 g/kg ethanol treatment expressed as relative change versus saline control. **(b)** Mean (± SEM) immunoreactivity of the pPKCγ positive area in the basolateral amygdala following 3.0 g/kg ethanol treatment expressed as relative change versus saline control. **(c)** Representative photomicrographs (20X) of the cytological pattern of pPKCγ immunoreactivity in the central amygdala at 30 minutes and basolateral amygdala at 90 minutes.*Significantly different from saline control at the given time point ($p<0.05$; Tukey).

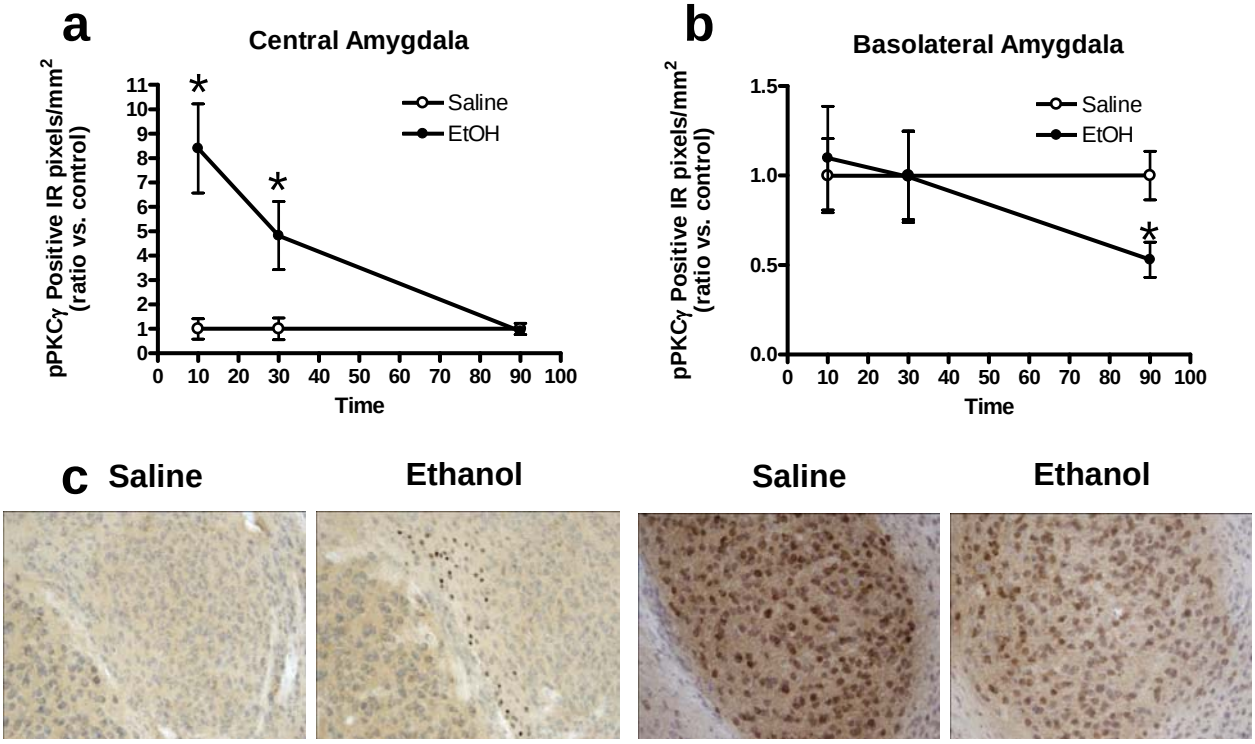
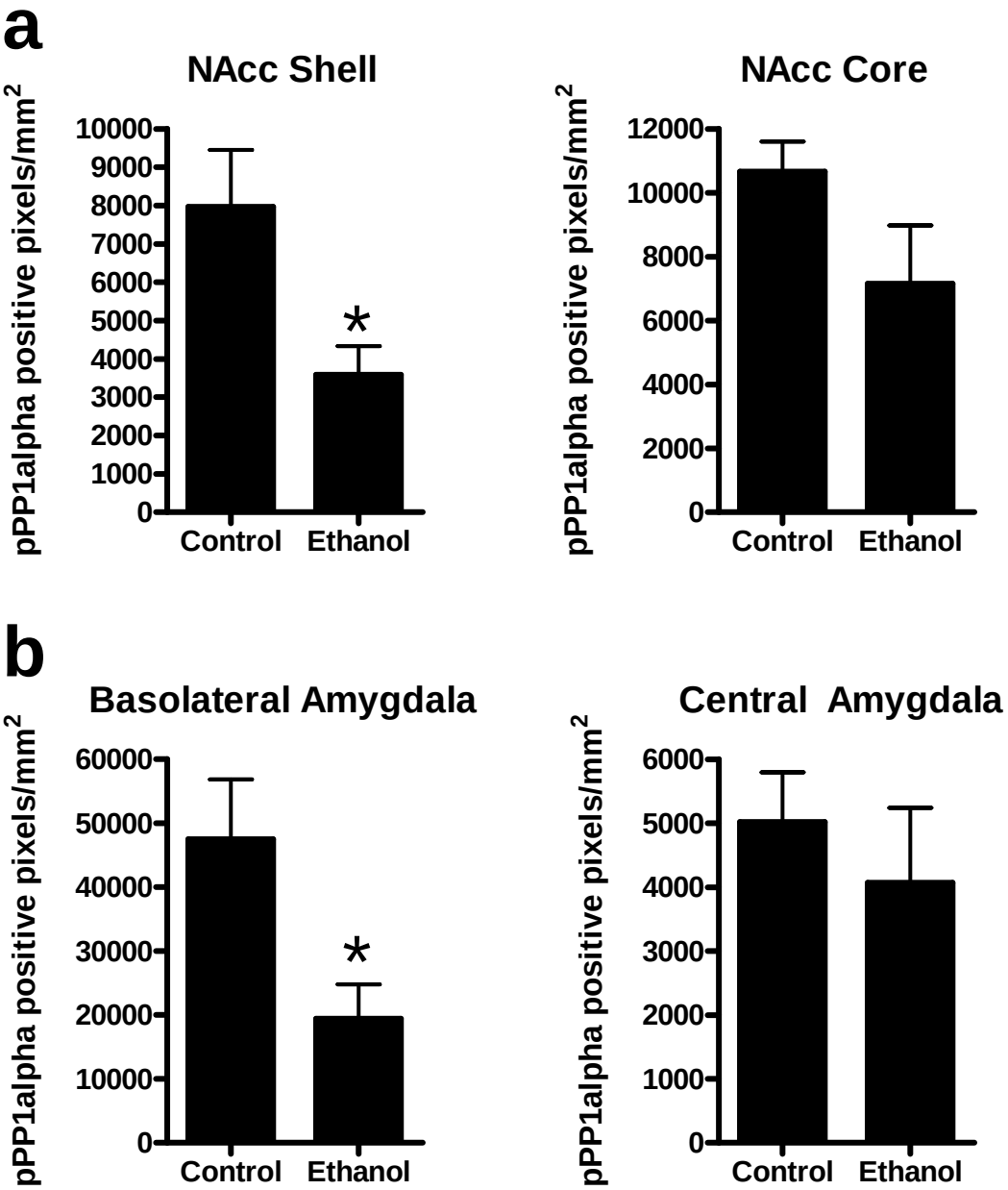


Figure 3.3 Effects of acute ethanol on PP1α phosphorylation in the nucleus accumbens and amygdala at 10 minutes. Mean (± SEM) immunoreactivity of the pPP1α-positive area (pixels/mm^2) in the nucleus accumbens shell and core **(a)**, and the central and basolateral nuclei of the amygdala **(b)** following treatment with vehicle (saline) or 3.0g/kg ethanol. *Significantly different from saline control ($p < 0.05$; Tukey).



CHAPTER 4

ACUTE ETHANOL ADMINISTRATION ALTERS EXTRACELLULAR-SIGNAL REGULATED KINASE PHOSPHORYALTION IN A TIME AND BRAIN REGION DEPENDENT MANNER

INTRODUCTION

Mitogen-activated protein kinases (MAPK) are a family of serine/threonine kinases, including p38, c-Jun amino-terminal kinase (JNK), and extracellular-signal regulated kinases 1 and 2 (ERK), which are involved in the transmittance of extracellular signals to the nucleus (Roux and Blenis 2004). Extracellular signals are relayed to the nucleus though a series of sequential kinase phosphorylations, known as MAPK cascades. These cascades play key roles in gene expression and cytoplasmic activities, including development, differentiation, apoptosis, and stress responses (Schaeffer and Weber 1999).

The ERK cascade responds to a variety of extracellular stimuli, including growth factors, receptor tyrosine kinases, and G-protein coupled receptor activation (Grewal et al. 1999). These extracellular signals initiate the serial phosphorylation of Raf-1, MEK1/2, and ERK. Upon phosphorylation, ERK accumulates in the nucleus and activates a number of nuclear substrates, such as Elk-1 and CREB, to alter gene expression (Sgambato et al. 1998). Through these phosphorylation events, ERK has been shown to regulate an assortment of neuronal functions, including cell survival (Basu and Sivaprasad 2007), differentiation (Eriksson et al. 2007), synaptic plasticity (Kelleher et al. 2004), and learning and memory (Bozon et al. 2003).

Indeed, MAPK cascades are responsive to many drugs of abuse and have been implicated in the development of drug dependence (Rubino et al. 2005; Valjent et al. 2006). Recent evidence suggests that ERK activity is modulated by ethanol and that the ERK cascade may mediate some of ethanol's neurobiological and behavioral effects (Ku et al. 2007; Radwanska et al. 2007). ERK has been shown to be differentially responsive to both acute and chronic ethanol challenges depending on tissues examined and treatment time. Acute ethanol treatment has been shown to decrease pERK in cultured cortical neurons (Kalluri and Ticku 2003), mouse cortex (Kalluri and Ticku 2002), and rat cortex (Chandler and Sutton 2005), and to increase pERK in the Edinger-Westphal nuclei of mice (Bachtell et al. 2002). Chronic ethanol treatment increases pERK in cultured cortical neurons (Kalluri and Ticku 2003) but decreases pERK in rat brain tissue (Sanna et al. 2002). These discrepancies indicate that a comprehensive examination of ethanol's brain-regional and time-dependent effects on ERK phosphorylation is necessary to better clarify ethanol's effect on the ERK pathway.

One purpose of the present study was to determine whether acute ethanol exposure alters ERK phosphorylation in the mouse brain in a time and brain region specific manner. To accomplish this goal, mice were administered either an acute dose of ethanol or a corresponding volume of saline via intraperitoneal injection. Animals were sacrificed 10, 30, or 90 minutes after ethanol injection and the brains were prepared for analysis of phosphorylated ERK (phospho-Thr202/Tyr204) expression by immunohistochemical methods.

A second purpose of the study was to examine the relationship between mGluR5 and ERK in the neurochemical and behavioral effects of ethanol. mGluR5 has been shown to signal through ERK, with mGluR5 selective agonists increasing the abundance of phosphorylated ERK (pERK) (Choe and Wang 2001). mGluR5 mediated increases in pERK are associated with increases in Elk-1 and CREB phosphorylation and expression of the immediate early gene c-fos

(Mao et al. 2008), demonstrating that mGluR5 mediated increases in pERK also enhance ERK activity. However, the functional relevance of this signaling pathway is not well defined. As both mGluR5 and ERK have been independently shown to respond to ethanol, two experiments were performed to examine the relationship between mGluR5 and ERK during acute ethanol exposure. First, mice were pretreated with MPEP, the selective mGluR5 antagonist, or saline and then given an acute dose of ethanol or a corresponding volume of saline via intraperitoneal injection. Animals were sacrificed 10 minutes after ethanol injection and the brains were prepared for analysis of phosphorylated ERK (phospho-Thr202/Tyr204) expression by immunohistochemical methods. Second, to compare the behavioral effects of mGluR5 inhibition and ERK inhibition, loss of righting reflex experiments were done to determine the effect of ERK inhibition on the sedative/hypnotic effects of ethanol.

MATERIALS AND METHODS

Animals. Male adult C57/BL6J mice (Jackson Labs, Bar Harbor, ME; 10-24 weeks; 22-35g) were housed four per cage and maintained on a 12-hour light/dark cycle with food and water available ad libitum. Animal care and handling procedures were performed in accordance with approved institutional protocols and the National Institutes of Health Guide for Care and Use of Laboratory Animals. Mice were allowed at least one week to acclimate to the laboratory prior to experiments.

Drugs. Ethanol (95%w/v) was diluted in physiological saline (0.9%) to a concentration of 20% (v/v) and administered in various volumes to obtain the appropriate dose. The mGluR5 selective antagonist 2-Methyl-6-(phenylethynyl)pyridine (MPEP) was purchased from Sigma (St. Louis, MO), and the MEK1/2 selective inhibitor (SL327) was purchased from Tocris (Ellisville, MO, USA). MPEP was dissolved in physiological saline (0.9%). SL327 was dissolved in 15% DMSO.

Loss of Righting Reflex (LORR) Studies. Animals were treated with DMSO vehicle or SL327 (30mg/kg; IP) 30 minutes prior to ethanol treatment (3.0 g/kg, IP) and the onset and duration of loss of righting reflex (sleep time) were measured. Onset was calculated as the time between ethanol injection and loss of righting reflex. LORR was defined as the inability of an animal to right itself within thirty seconds. Upon loss of righting reflex, mice were placed in V-shaped troughs (~90° angle) and the time to regain righting reflex was measured. Return of the righting reflex was defined as the ability of an animal to right itself three times in 30 seconds. Duration of LORR was calculated as the difference between loss and return of righting reflex.

Acute ethanol time course. Mice were habituated to handling (weighing) and intraperitoneal injection (saline, 0.1ml/10g) for three days prior to the start of the experiment. On the day of the experiment, mice received ethanol (0 or 3 g/kg, IP) and after 10, 30, or 90

minutes were deeply anesthetized with pentobarbital and brains were prepared as described below.

Acute ethanol with MPEP. Mice were habituated to handling (weighing) and intraperitoneal injection (saline, 0.1ml/10g) for three days prior to the start of the experiment. On the day of the experiment, mice received a 10 minute pretreatment with MPEP (0 or 30m/kg, IP) followed by a single dose of ethanol (0 or 3 g/kg). 10 minutes after ethanol treatment, the mice were deeply anesthetized with pentobarbital and brains were prepared as described below.

Tissue Preparation and Immunohistochemical Analysis. Mice were transcardially perfused with 100mM phosphate buffer, followed by 4% formaldehyde (pH 7.4). The brains were post fixed for 48 hours at 4°C, rinsed with phosphate buffer, and removed from the skulls. The fixed brains were stored in phosphate buffer at 4°C until sectioning. Coronal brain sections (40μm) were cut on a vibratome (Leica, VT1000S) and stored in cryoprotectant at -20°C until processing for immunohistochemistry.

Sections were washed in PBS prior to free-floating immunohistochemical processing. Following preblocking, sections were agitated with either phospho-ERK1/2, ERK1/2 (1:400; Cell Signaling Tech, Danvers, MA), or phospho-PP1α (1:150; Cell Signaling Tech, Danvers, MA) for 16 hr at 4°C. Sections were washed and visualized with DAKO labeled polymer-HRP anti-rabbit secondary antibody (full strength) followed by DAKO DAB+ chromagen (DAKOCytomation, Carpinteria, CA). Sections were counterstained with toluidine blue and coverslipped with Cytoseal. Sections were viewed at 20-40X magnification under brightfield illumination (Olympus CX41 light microscope, Olympus America, Center Valley, PA). Images were acquired using a digital camera (Regita OEM fast, QImaging, Burnaby, BC) and immunoreactivity was quantified using Bioquant Life Science software (R&M Biometric,

Nashville, TN). Cell counts and pixel density measurements were divided by the area of the region and expressed as cell counts/mm^2 or pixels/mm^2.

Data Collection and Statistics. Each treatment group consisted of 8 animals. Measurements were taken from the left and right side of each section and multiple sections were analyzed for each brain region, when possible. Measurements from individual brain regions for each animal were averaged to produce a single value for a given region in each animal. Data were analyzed within the area of interest, across time, by two-way ANOVA followed by the Tukey multiple comparison procedure (Graphpad Software, San Diego, CA).

Anatomical Coordinates. Anatomical coordinates used for analysis were taken from the Mouse Brain in Stereotaxic coordinates (Paxinos and Watson, 2001). Analyses of the nucleus accumbens and proximal brain regions were evaluated in coronal sections ranging from 0.86 to 1.34 mm anterior to Bregma. The amygdala and proximal brain regions were evaluated in coronal section ranging from -1.58 to -2.18 mm posterior to Bregma.

RESULTS

Distribution of ERK in Mouse Brain

nERK and pERK immunoreactivties were detected in numerous brain regions (Table 4.1). The highest densities of nERK immunoreactivity were observed in the prefrontal cortex, nucleus accumbens (core and shell), bed stria terminalis, and the central amygdala. Moderate levels of immunostaining were observed in the cingulate, insular, and piriform cortices, the granular layer of the hippocampus (dentate gyrus and CA3 region), the basolateral amygdala, and the ventral tegmental area, as well as in various thalamic and hypothalamic nuclei. The cytological pattern of nERK immunoreactivity showed localization primarily at the plasma membrane, with diffuse staining in the stratum radiatum of the hippocampus. Upon analysis, no changes in total ERK immunoreactivity were detected following acute ethanol exposure (data not shown).

The highest levels of pERK immunoreactivity were observed in the prefrontal cortex, nucleus accumbens (core and shell), paraventricular nuclei (PVN) of the thalamus and hypothalamus, and the central amygdala, with lower levels of immunostaining seen in the cingulate, insular, and piriform cortices, the basolateral amygdala, and the ventral tegmental area. pERK immunoreactivity produced a punctuate pattern of staining in all brain regions examined. Diffuse staining was also observed in the stratum radiatum surrounding the CA3 and the polymorph layer of the dentate gyrus in the hippocampus. No pERK immunoreactivity was detected in either the caudate putamen or the granular layer of the hippocampus.

Effect of Acute Ethanol on pERK Immunoreactivity

Nucleus Accumbens. The effects of acute ethanol (3g/kg) administration on pERK immunoreactivity in nucleus accumbens were subregionally specific. In the core of the nucleus accumbens, acute ethanol reduced pERK immunoreactivity in a rapid and time dependent

manner (Fig 4.1a; $F[1,36] = 41$; $p<0.001$) and two-way ANOVA analysis showed only a main effect of ethanol. pERK immunoreactivity was significantly lower at 10 and 30 minutes (Tukey; p's <0.05) following ethanol treatment as compared to vehicle controls. In contrast, acute ethanol increased pERK immunoreactivity in the shell of the nucleus accumbens (Fig 4.1b; $F[1,33] = 38$; $p<0.05$), with two-way ANOVA showing only a main effect of ethanol. pERK immunoreactivity was significantly higher in ethanol treated tissue at the 30 minute time point (Tukey; $p<0.01$). The decrease in pERK immunoreactivity in the nucleus accumbens core was associated with a corresponding decrease in the number of pERK positive cells. In the core, pERK positive cell counts were reduced by 86% at the 10 minute time point and 45% at the 30 minute time point (Table 4.2). In the shell, pERK positive cell counts were increased by 143% at the 10 minute time point, but this was temporally dissociated from the increase in pERK immunoreactivity. Representative photomicrographs illustrating the cytological pattern of pERK immunoreactivity in the nucleus accumbens are shown in Figure 4.1c.

Amygdala. Acute ethanol (3g/kg) administration increased pERK immunoreactivity in both the central ($F[1,39] = 44$; $p=0.001$) and basolateral ($F[1,37] = 42$; $p<0.05$) nuclei of the amygdala. Two-way ANOVA analyses showed main effects of ethanol for both regions, but no main effects of time or ethanol x time interactions. In the central amygdala, the ethanol-induced increase in ERK phosphorylation was rapid, with a significant increase in pERK immunoreactivity observed 30 minutes (Tukey; $p<0.005$) after ethanol treatment (Fig 4.2a). In contrast, ethanol effects in the basolateral amygdala were delayed, with significant increases in pERK immunoreactivity observed 90 minutes (Tukey; $p<0.005$) after ethanol treatment (Fig 4.2c). The increases in pERK immunoreactivity in the amygdala were associated with significant increases in the number of pERK positive cells (Table 4.2). In the central amygdala, ethanol increased the number of pERK positive cells by 82% at the 10 minute time point and 156% at the 30 minutes

time point. In the basolateral amygdala, the number of pERK positive cell counts was increased by 374%, at the 90 minute time point. Representative photomicrographs illustrating the cytological pattern of pERK immunoreactivity in the central amygdala (30 minutes) and basolateral amygdala (90 minutes) are shown in Figure 4.1, panels b and d.

Cortex. Acute ethanol (3g/kg) treatment increased pERK immunoreactivity in the medial prefrontal ($F[1,33] = 11$; $p<0.01$), cingulate ($F[1,39] = 13$; $p<0.001$), insular ($F[2,38] = 3$; $p<0.05$), and piriform ($F[1,36] = 5$; $p<0.05$), regions of the cortex in a time dependent manner (Table 4.2). Two-way ANOVA analyses revealed main effects of ethanol and time, and ethanol x time interactions in all four of these cortical regions. Ethanol significantly increased pERK immunoreactivity in the medial prefrontal, cingulate, and insular regions of the cortex at 30 minutes (Tukey; p's<0.01). In the piriform cortex, ethanol significantly increased pERK immunoreactivity at the 10 minute time point (Tukey; $p<0.001$). Increases in pERK immunoreactivity in the medial prefrontal, cingulate, and piriform regions were associated with concurrent increases in pERK-positive cell counts. No increases in pERK-positive cell counts were observed in the insular cortex. Furthermore, no pERK immunoreactivity was detected in the motor or somatosensory regions of the cortex.

Other Regions. Other brain regions showed time-dependent increases in pERK immunoreactivity following acute ethanol treatment, including the bed stria terminalis ($F[1,32] = 6$; $p<0.05$) and the PVN of the thalamus ($F[1,34] = 17$; $p<0.001$) and hypothalamus ($F[1,35] = 13$; $p<0.05$). In the hypothalamus, two-way ANOVA analysis showed main effects of ethanol and time, and an ethanol x time interaction. In the bed stria terminalis and the thalamus, two-way ANOVA analyses only revealed main effects of ethanol. In the PVN thalamus (Fig 4.3b), pERK immunoreactivity was significantly elevated at 10 and 90 minutes after ethanol treatment (Tukey; $p<0.01$). pERK positive cell counts were only increased in the PVN thalamus at the 90

minute time point. The greatest effects of acute ethanol treatment were seen in the bed stria terminalis and the PVN hypothalamus. In the bed stria terminalis (Fig 4.3a), acute ethanol significantly increased pERK immunoreactivity at the 90 minute time point (Tukey; $p<0.01$) and pERK positive cell counts were increased by 951%. In the PVN hypothalamus (Fig 4.3c), pERK immunoreactivity was also elevated at 90 minutes after ethanol treatment (Tukey; $p<0.005$), with a 1,982% increase in pERK positive cell counts. Representative photomicrographs illustrating the cytological pattern of pERK immunoreactivity are shown in Figure 4.3d.

Effect of MPEP and Acute Ethanol on pERK Immunoreactivity

Two-way ANOVA analyses revealed main effects of ethanol in both the core and shell of the nucleus accumbens ($F[1,31] = 11$; $p<0.005$, $F[1,33] = 7$; $p<0.05$), the central amygdala ($F[1,29] = 13$; $p<0.001$), and the bed stria terminalis ($F[1,28] = 110$; $p<0.005$). No region showed a main effect of time or an ethanol x time interaction.

Nucleus Accumbens. In the nucleus accumbens core (Fig 4.4a), pretreatment with MPEP (30mg/kg, IP) did not significantly alter pERK immunoreactivity, although there was a trend to increased pERK immunoreactivity, and pretreatment with saline did not alter ethanol-induced (3g/kg, IP) decreases in pERK immunoreactivity (Tukey; $p<0.05$). In animals treated with ethanol following a 10 minute pretreatment with MPEP, pERK immunoreactivity abundance was not significantly different from ethanol alone, but it was also not significantly different from saline control. In the nucleus accumbens shell (Fig 4.4b), pretreatment with MPEP did not significantly alter pERK immunoreactivity and pretreatment with saline did not alter ethanol-induced increases in pERK immunoreactivity (Tukey; $p<0.05$). Again, MPEP pretreatment did not significantly reduce ethanol-induced increases in pERK immunoreactivity, but the MPEP-ethanol group was also not significantly different from the saline control group. Although MPEP pretreatment did not significantly alter ethanol-induced changes in pERK immunoreactivity in

the core and the shell, MPEP did produce a trend towards reducing the effects of ethanol in both regions.

Amygdala. In the central amygdala (Fig 4.4c), pretreatment with MPEP alone significantly increased pERK immunoreactivity (Tukey; $p<0.05$). However, neither pretreatment condition (saline nor MPEP) affected ethanol-induced increases in pERK immunoreactivity (Tukey; p's<0.05). No changes were observed in the basolateral amygdala.

Other Regions. In the previous experiment, we showed that pERK immunoreactivity was increased 90 minutes after ethanol treatment in the bed stria terminalis and the PVN of the hypothalamus. No significant effect of MPEP or ethanol alone was found in either of these brain regions. However, 10 minutes of ethanol treatment following a 10 minute MPEP pretreatment significantly increased pERK immunoreactivity in both of these brain regions (Fig 4.4, panels d and e; Tukey; p's<0.05). No changes were observed in the PVN of the thalamus. Finally, acute ethanol treatment had previously been shown to rapidly increase pERK immunoreactivity in the Edinger-Westphal nuclei (Bachtell et al. 2002). However, no changes in pERK immunoreactivity were found under any of the treatment conditions (Fig 4.4f).

Ethanol-induced LORR: Effects of SL327

Although there are no selective antagonists of ERK currently available, SL327, a selective inhibitor of MEK1/2, effectively prevents ERK activation by blocking MEK1/2 mediated phosphorylation of ERK. Systemic administration of SL327 (0 or 30mg/kg, IP) significantly increased the time necessary to regain the righting reflex following a high dose of ethanol (3g/kg, IP) (Fig 4.5). A 30 minute pretreatment with SL327 increase the duration of ethanol-induced LORR by 61% (Tukey, $p<0.05$).

DISCUSSION

The present study was conducted to determine whether acute ethanol exposure alters phosphorylation of ERK in vivo. We found that acute IP administration of ethanol produced time dependent changes in ERK phosphorylation in a number of brain regions without altering total ERK abundance. At the 10 and 30 minute time points, ethanol (3g/kg) increased pERK immunoreactivity in several regions of the cortex, nucleus accumbens core, and the central amygdala. At 90 minutes after ethanol treatment, pERK immunoreactivity was elevated in the bed stria terminalis, basolateral amygdala, and the paraventricular nuclei of the thalamus and hypothalamus. The only brain region in which ethanol decreased pERK immunoreactivity was the nucleus accumbens core (10 and 30 minutes). These results suggest that ethanol alters ERK activity in a time and brain region dependent manner.

Our results are consistent with recent studies showing that acute ethanol exposure can both enhance and diminish ERK phosphorylation. In the majority of the brain regions we examined, pERK immunoreactivity was elevated after an acute ethanol challenge. These results complement previous findings showing that ethanol (2.4g/kg) increased pERK immunoreactivity in the Edinger-Westphal nucleus (EW) 15 minutes after treatment (Bachtell et al. 2002). Acute ethanol exposure has also been shown to increase pERK levels in mouse hippocampal neurons (100 mM; 1, 3, or 6 hours of exposure) (Ku et al. 2007), human mast cells (100 mM; 30 minutes) (Jeong, 2005), rat vascular muscle cells (8 mM; 5 minutes) (Washington et al. 2003), and pancreatic stellate cells (50 mM; 15 minutes) (McCarroll, 2003). Furthermore, acute ethanol-induced increases in c-fos expression, a down-stream effector of ERK, in the EW can be blocked by SL327, a MEK/ERK-signaling pathway inhibitor (Bachtell et al. 2002), suggesting that increases in pERK levels are associated with increased ERK activity.

Our finding that acute ethanol exposure decreases pERK immunoreactivity in the nucleus accumbens shell is consistent with evidence that acute ethanol also reduces ERK phosphorylation. In mice, acute exposure to ethanol (3.5 g/kg; 10 minutes) decreased pERK levels in the cerebral cortex (Kalluri and Ticku 2002). In rats, acute ethanol treatment (3.5 g/kg, 60 minutes) decreased pERK levels in the cerebral cortex, as well as in the hippocampus and cerebellum (Chandler and Sutton 2005). Acute ethanol exposure (100-150 mM; 10-60 minutes) also reduced pERK levels in cultured cortical neurons (Kalluri and Ticku 2003), NG108-15 cells (Constantinescu, 2004), and SH-SY5Y cells (Seiler et al. 2001). It must be noted that most *in vivo* studies assessing the effects of acute ethanol treatment have found only decreases in ERK phosphorylation, which is in direct contrast to the majority of our findings. However, the scope of previous experiments has been limited. Most of these studies have focused on a single time point and examined the cortex as a whole, using western blot methods to measure changes in pERK. By using immunohistochemical methods, we can survey the entire brain and evaluate changes in ERK phosphorylation in specific subnuclei. Our experiments also incorporate a time element which allows us to examine changes over time and to differentiate between immediate and gradual responses to acute ethanol exposure. Furthermore, the time course makes it possible to consider how the observed changes are related to inter-regional connectivity.

Discrepancies in the literature and the relative paucity of information concerning the functional relationship between ethanol and ERK contribute to the difficulty in interpreting the observed ethanol-induced changes in ERK phosphorylation. Current findings suggest that ERK mediates some of the neurobiological and behavioral effects of ethanol. A recent study using immunolabeling techniques showed that acute ethanol increases pERK in the central amygdala of rats, and this increase was associated with increased Elk-1 and CREB phosphorylation and increased expression of the immediate early gene, arc (Pandey et al. 2008). Acute ethanol

exposure has also been shown to increase c-fos expression in an ERK-dependent manner (Bachtell et al. 2002). These data suggest that ethanol-induced increases in ERK phosphorylation result in enhanced ERK activity, and support the conclusion that ERK mediates some of the neurobiological effects of ethanol. Chronic ethanol vapor exposure reduces ERK phosphorylation and withdrawal in these same animals increases ERK phosphorylation (Sanna et al. 2002). These changes were found in several brain regions known to mediate ethanol self-administration, but there is currently no evidence linking ERK and ethanol drinking behaviors. However, ERK has been implicated in anxiety. Anxious rats had elevated levels of pERK and concurrent increases in c-fos expression were blocked by ERK inhibition (Ailing et al. 2008). These data suggest that ethanol withdrawal-induced anxiety may be related to elevated levels of pERK. Interestingly, all of the brain regions in which pERK immunoreactivity was elevated at the 90 minute time point are known to mediate stress and anxiety responses (Bremner et al. 1996). Therefore, the increases in pERK observed in these regions may be mediating acute withdrawal symptoms.

Many drugs of abuse influence common signaling cascades and some behavioral aspects of tolerance and dependence are mediated by the same pathways, regardless of the drug. Although there is little information about the role of ERK in the development of ethanol dependence, ERK has been shown to be important in cocaine seeking and reward. Inhibition of ERK activation prevents both locomotor sensitization to cocaine (Valjent et al. 2006) and induction of conditioned place preference by cocaine (Valjent et al. 2000). ERK has also been implicated in conditioned behavioral responses to cocaine. In self-administering animals, cocaine-associated cues increase pERK and inhibition of ERK activation prevents cocaine seeking (Lu et al. 2005). Based on these findings, the ethanol-induced increases in pERK may be mediating the positive subjective effects of ethanol or driving ethanol craving. The introduction

of the systemically active inhibitor of ERK activity, SL327, will make it easier to test these hypotheses in behavioral assays.

Another goal of this study was to examine the relationship between mGluR5 and ERK in mediating the acute effects of ethanol. In the nucleus accumbens, mGluR5 inhibition with MPEP did not significantly alter pERK immunoreactivity in the core or the shell, nor did MPEP alter ethanol-induced changes in pERK immunoreactivity in these brain regions. In the central amygdala, MPEP significantly increased pERK levels when administered alone, but had no effect on ethanol-induced increases. In the Edinger-Westphal nuclei, which was previously reported to show increases in pERK immunoreactivity after acute ethanol exposure, no changes were observed in any treatment group. These findings suggest that inhibition of mGluR5 does not enhance the acute subjective properties of ethanol by increasing ethanol-induced changes in pERK immunoreactivity in these brain regions. However, 3g/kg is a high dose of ethanol and the lack of effect of MPEP may represent a ceiling effect at this time point. Therefore similar experiments performed with a higher dose of MPEP or a lower dose of ethanol, or with examination of a different time point may reveal changes in these brain regions.

Interestingly, mGluR5 inhibition increased pERK immunoreactivity in the BST and the PVN of the hypothalamus when paired with ethanol. These were two of the regions that responded to acute ethanol exposure at the 90 minute time point. In these regions, MPEP appears to be hastening the effects of ethanol on pERK. However, blood ethanol concentrations peak around 10 minutes and are steadily diminishing at 90 minutes (Ferraro et al. 1990), suggesting that the increases observed at 10 minutes with MPEP are functionally distinct from the increases observed at 90 following ethanol alone. The effects at the 10 minute time point closely coincide with the sedative-hypnotic effects of ethanol, which are enhanced by MPEP pretreatment. Furthermore, the BST and PVN of the hypothalamus are known to mediate arousal and the sleep-

wake cycle (Aston-Jones et al. 2001; Peyron et al. 1998), suggesting that MPEP-ethanol induced increases in pERK immunoreactivity in these brain regions may modulate MPEP's enhancement of ethanol-induced loss or righting reflex. To explore this possibility, we examined the effects of SL327, the MEK1/2 inhibitor, on ethanol-induced loss of righting reflex. Like MPEP, SL327 increased the duration of loss of righting reflex. These results appear disparate, as blockade of ERK phosphorylation increases ethanol-induced loss of righting reflex, and MPEP, which also increases ethanol-induced loss of righting reflex, enhances ERK phosphorylation. The answer may lie in MPEP's effects in the BST and PVN of the hypothalamus being related to recovery from ethanol-induced loss of righting reflex, rather than enhancement of ethanol sedative-hypnotic effects.

In summary, ethanol-induced changes in pERK may play a role in a variety of the acute effects of ethanol, and likely contribute to long-term changes seen in ethanol tolerance and dependence. However, further study will be necessary to confirm these hypotheses. MPEP's effects on ethanol-induced increases in pERK were limited to the BST and the PVN of the hypothalamus. These effects may be related to MPEP enhancement of ethanol-induced loss of righting reflex, but further investigation is needed to determine the role of ERK phosphorylation in the sedative hypnotic effects of ethanol.

Table 4.1 Evaluation of the immunohistochemical distribution of phospho-ERK (pERK) and total ERK. Immunoreactivity (IR) was ranked as minimal (--), low (+), or high (++).

Brain region	ERK IR	pERK IR
Cerebral Cortex		
Prefrontal	++	++
Cingulate	+	+
Motor	--	--
Somatosensory	--	--
Piriform	+	+
Insular	+	+
Nucleus Accumbens		
Core	++	++
Shell	++	++
Lateral Septum	++	++
Caudate Putamen	+	--
Bed Stria Terminalis	++	++
Hippocampus		
CA1	--	--
CA3	+	--
Dentate gyrus	+	--
Amygdala		
Basolateral	+	+
Central	++	++
Thalamus		
PVN	++	++
Medial	+	+
Hypothalamus		
PVN	+	++
Medial	+	+
Lateral	+	+
Ventral Tegmental Area	+	+

Table 4.2 Effects of acute ethanol on pERK-positive cells counts over time. Mean number (± SEM) of pERK positive cells following saline or 3.0 g/kg ethanol treatment expressed as counts/mm^2. *Significantly different from saline control at the given time point ($p<0.01$; Tukey).

	10 minutes		30 minutes		90 minutes	
Brain region	**Saline**	**Ethanol**	**Saline**	**Ethanol**	**Saline**	**Ethanol**
Prefrontal Cortex	282.0 ± 57.38	466.9 ± 26.67*	109.5 ± 64.56	446.9 ± 39.31*	127.3 ± 41.57	136.2 ± 33.47
Cingulate Cortex	109.0 ± 16.23	143.7 ± 19.71	54.02 ± 24.45	172.2 ± 19.66*	39.69 ± 12.12	49.17 ± 15.42
Insular Cortex	125.5 ± 10.74	135.4 ± 11.33	29.42 ± 17.29	64.67 ± 11.95	36.46 ± 11.27	42.75 ± 12.40
Piriform Cortex	80.85 ± 18.21	245.7 ± 43.06*	109.1 ± 34.26	78.47 ± 25.55	11.61 ± 5.962	14.46 ± 8.162
Nucleus Accumbens						
Core	298.5 ± 75.30	42.13 ± 7.182*	82.67 ± 20.22	37.35 ± 6.211*	156.8 ± 34.71	89.58 ± 13.23
Shell	117.1 ± 17.52	284.8 ± 48.32*	91.84 ± 25.58	158.3 ± 40.16	456.0 ± 84.43	574.6 ± 209.5
Bed Stria Terminalis	249.1 ± 62.44	731.9 ± 128.7	245.2 ± 109.4	649.9 ± 153.8	20.31 ± 6.484	213.5 ± 98.19*
Amygdala						
Basolateral	146.3 ± 19.82	136.9 ± 30.49	28.05 ± 9.167	42.79 ± 10.47	9.394 ± 2.630	44.57 ± 15.49*
Central	652.9 ± 128.8	1188 ± 108.3*	276.2 ± 59.35	708.3 ± 104.5*	167.0 ± 55.46	240.7 ± 68.06
PVN Thalamus	248.0 ± 26.62	437.3 ± 64.46*	87.19 ± 50.12	187.2 ± 53.07	42.02 ± 12.41	175.9 ± 41.05*
PVN Hypothalamus	450.8 ± 159.8	509.7 ± 127.7	388.1 ± 160.8	954.8 ± 363.0	45.63 ± 31.34	950.1 ± 382.4*

Table 4.3 Effects of acute ethanol on pERK immunoreactivity in the anterior cortex. Mean number (± SEM) Mean (± SEM) immunoreactivity of the pERK positive area in four regions of the anterior cortex following 3.0 g/kg ethanol treatment expressed as pixels/mm^2. *Significantly different from saline control at the given time point ($p<0.05$; Tukey).

	10 minutes		30 minutes		90 minutes	
Brain region	**Saline**	**Ethanol**	**Saline**	**Ethanol**	**Saline**	**Ethanol**
Prefrontal Cortex	5230 ± 1935	10586 ± 676.2	2107 ± 1437	10086 ± 1789*	1731 ± 792.2	1994 ± 687.3
Cingulate Cortex	1522 ± 346.0	2575 ± 427.9	722.5 ± 449.2	3708 ± 683.2*	442.3 ± 152.7	719.3 ± 306.0
Insular Cortex	2139 ± 273.2	2669 ± 396.0	92.65 ± 48.07	1155 ± 302.4*	482.0 ± 161.8	684.7 ± 244.8
Piriform Cortex	1239 ± 400.2	5925 ± 1315*	1568 ± 669.1	1369 ± 584.9	129.7 ± 71.11	73.79 ± 33.95

Figure 4.1 Effects of acute ethanol on ERK phosphorylation in the nucleus accumbens over time. **(a)** Mean (± SEM) immunoreactivity of the pERK positive area in the nucleus accumbens core following 3.0 g/kg ethanol treatment expressed as relative change versus saline control. **(b)** Mean (± SEM) immunoreactivity of the pERK positive area in the nucleus accumbens shell following 3.0 g/kg ethanol treatment expressed as relative change versus saline control. **(c)** Representative photomicrographs (20X) of the cytological pattern of pERK immunoreactivity in the core and shell at 30 minutes. *Significantly different from saline control at the given time point ($p<0.05$; Tukey).

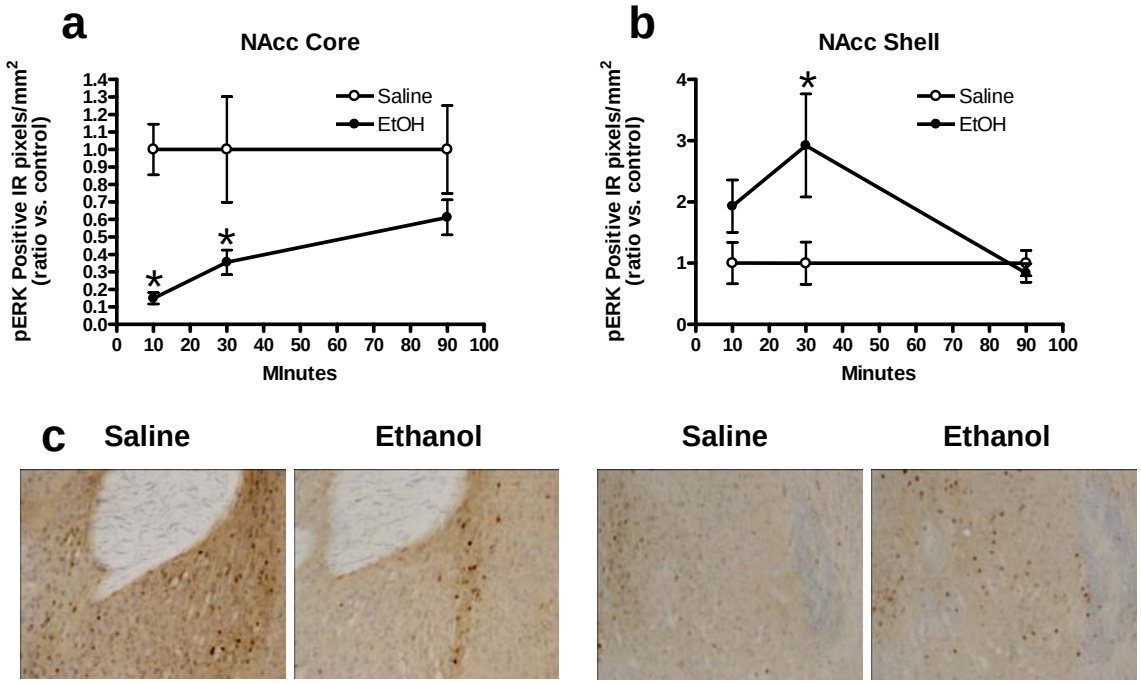


Figure 4.2 Effects of acute ethanol on ERK phosphorylation in the amygdala overtime. **(a)** Mean (± SEM) immunoreactivity of the pERK positive area in the central amygdala following 3.0 g/kg ethanol treatment expressed as relative change versus saline control. **(b)** Representative photomicrographs of the cytological pattern of pERK immunoreactivity in the central amygdala at 30 minutes. **(c)** Mean (± SEM) immunoreactivity of the pERK positive area in the basolateral amygdala following 3.0 g/kg ethanol treatment expressed as relative change versus saline control. **(d)** Representative photomicrographs of the cytological pattern of pERK immunoreactivity in the basolateral amygdala at 90 minutes. *Significantly different from saline control at the given time point ($p<0.05$; Tukey).

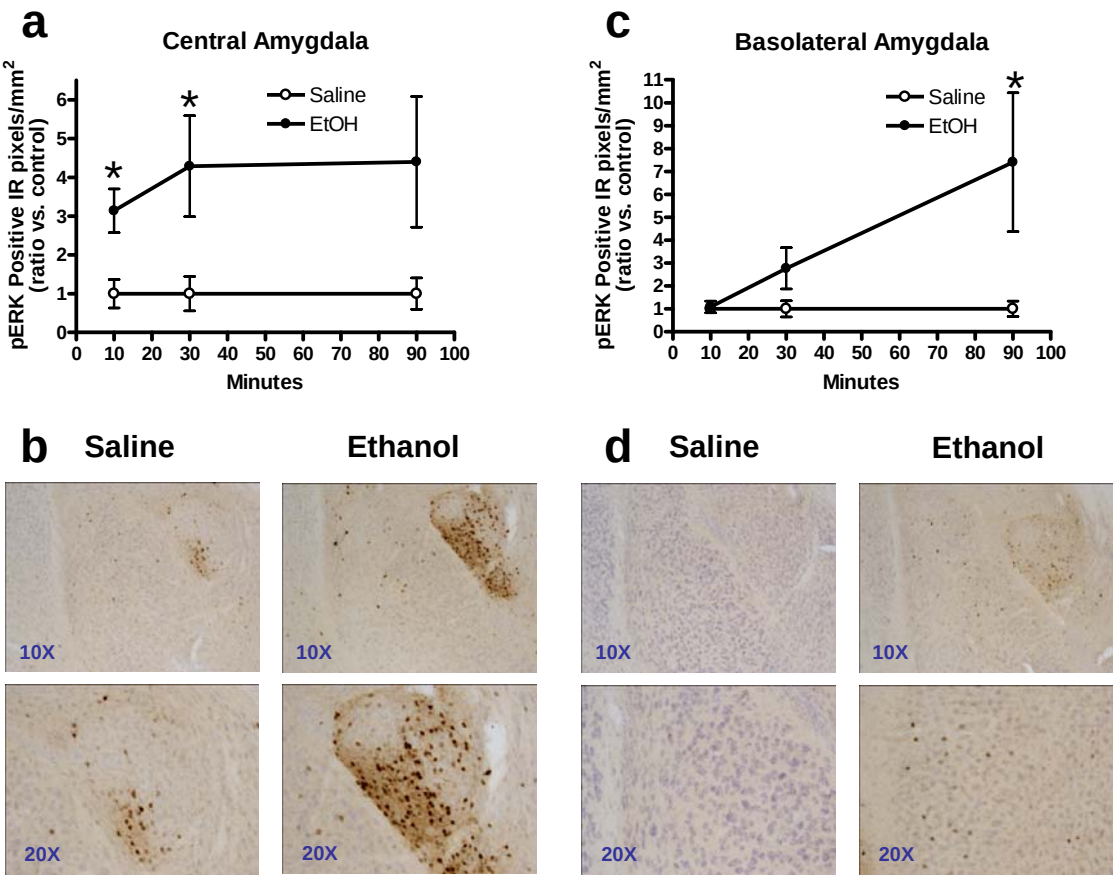


Figure 4.3 Effects of acute ethanol on ERK1/2 phosphorylation in **(a)** the bed striaterminalis (BST), **(b)** paraventricular nuclei of the thalamus (PVN thal), and **(c)** paraventricular nuclei of the hypothalamus (PVN hypo) over time. **(a-c)** Mean (± SEM) immunoreactivity of the pERK positive area following 3.0 g/kg ethanol treatment expressed as relative change versus saline control. **(d)** Representative photomicrographs (10-20X) of the cytological pattern of pERK immunoreactivity in the BST, PVN thal, and PVN hypo at 90 minutes. *Significantly different from saline control at the given time point ($p<0.05$; Tukey).

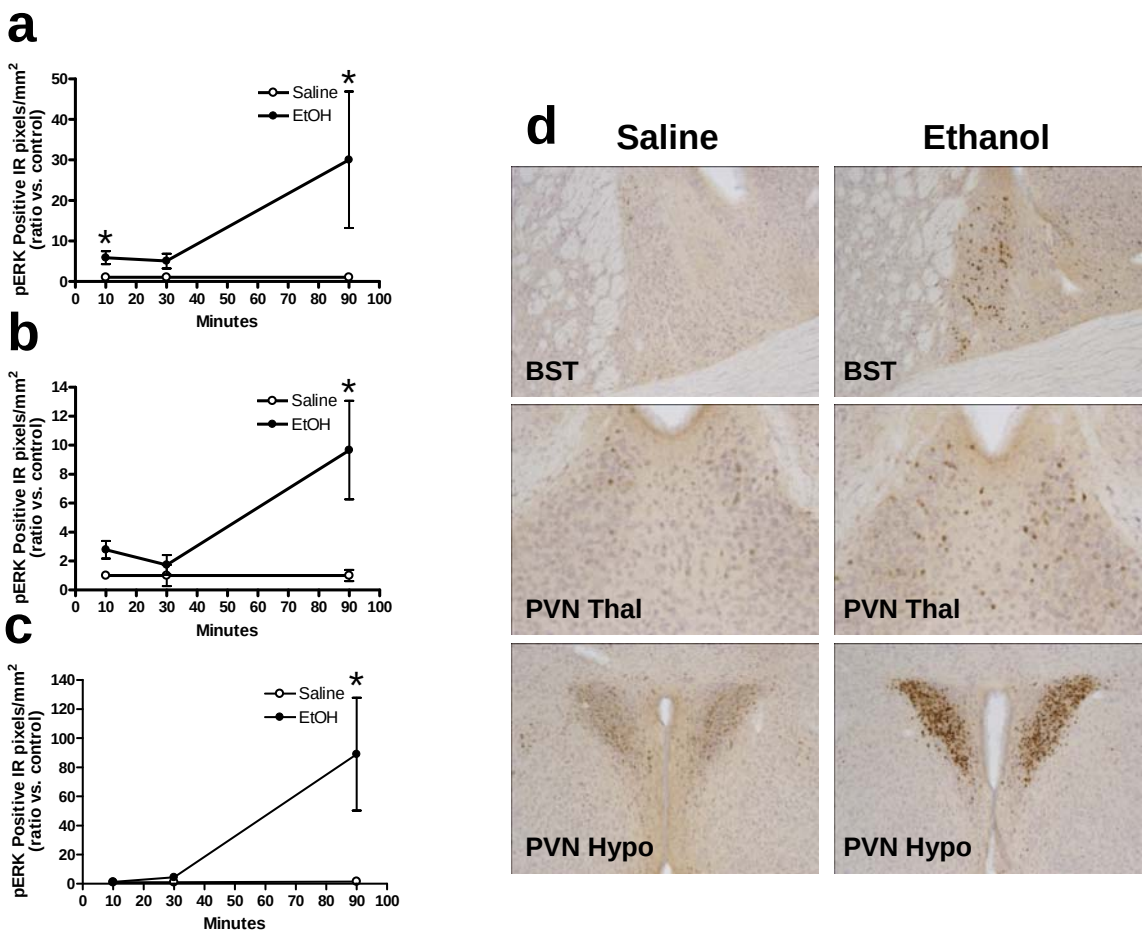
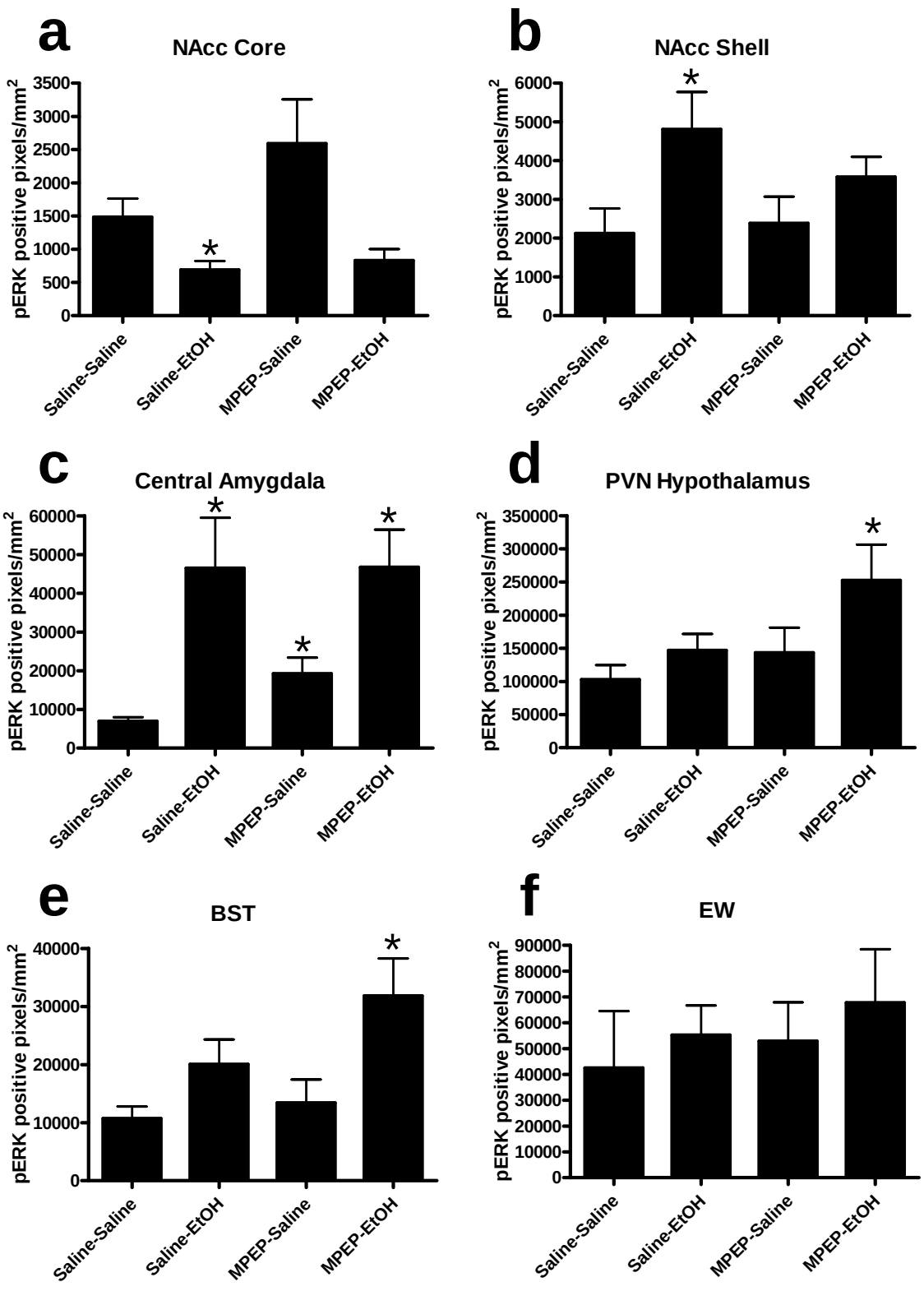


Figure 4.4 Effects of MPEP (30mg/kg) and acute ethanol (3 g/kg) on ERK phosphorylation across the brain, expressed as pixels/mm^2. (a) Mean (± SEM) immunoreactivity of the pERK positive area in the nucleus accumbens core. (b) Mean (± SEM) immunoreactivity of the pERK positive area in the nucleus accumbens shell. (c) Mean (± SEM) immunoreactivity of the pERK positive area in the central amygdala. (d) Mean (± SEM) immunoreactivity of the pERK positive area in the PVN hypothalamus. (e) Mean (± SEM) immunoreactivity of the pERK positive area in the bed stria terminalis (BST). (f) Mean (± SEM) immunoreactivity of the pERK positive area in the Edinger-Westphal (EW) nuclei. *Significantly different from saline-saline control ($p<0.05$; Tukey)


a
NAcc Core
pERK positive pixels/mm2
3500
3000
2500
2000
1500
1000
500
0
*
Saline-Saline
Saline-EtOH
MPEP-Saline
MPEP-EtOH
b
NAcc Shell
pERK positive pixels/mm2
6000
5000
4000
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1000
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Saline-EtOH
MPEP-Saline
MPEP-EtOH
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Central Amygdala
pERK positive pixels/mm2
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30000
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Saline-EtOH
MPEP-Saline
MPEP-EtOH
d
PVN Hypothalamus
pERK positive pixels/mm2
350000
300000
250000
200000
150000
100000
50000
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Saline-EtOH
MPEP-Saline
MPEP-EtOH
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20000
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Saline-EtOH
MPEP-Saline
MPEP-EtOH
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pERK positive pixels/mm2
90000
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60000
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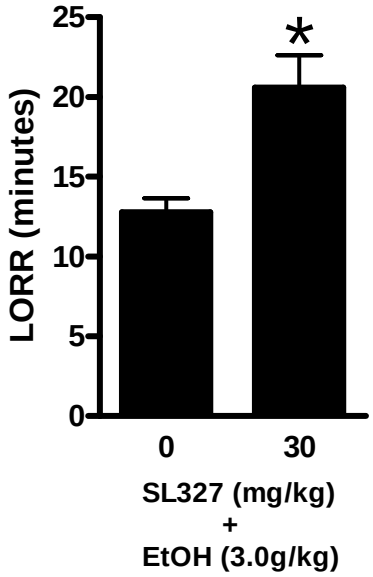
Saline-Saline
Saline-EtOH
MPEP-Saline
MPEP-EtOH

Figure 4.5 Effect of MEK1/2 inhibition on ethanol-induced loss of righting reflex (LORR). Bars represent the mean (± SEM) duration of ethanol-induced LORR in minutes (n = 4) following pretreatment with SL327. *Significantly different from 3g/kg ethanol alone ($p<0.05$, Tukey).



CHAPTER 5

GENERAL DISCUSSION

Experimental Results

The present studies examined the role of mGluR5 and its associated signaling pathways in the acute sedative-hypnotic effects of ethanol. The first set of experiments assessed the effects of three selective metabotropic glutamate receptor antagonists on ethanol-induced locomotor deficits and loss of righting reflex. The mGluR5 specific antagonist, MPEP, selectively reduces operant ethanol self-administration (Hodge et al. 2006). One purpose of these experiments was to determine if MPEP reduced ethanol consumption by enhancing ethanol-induced locomotor deficits. MPEP selectively enhanced the sedative-hypnotic properties of ethanol, but only at a dose higher than the one shown be effective in reducing ethanol drinking. These data suggest that MPEP effects on operant ethanol self-administration are not due to decreased locomotor behavior. Although mGluR2/3 inhibition with LY341495 had no effect on operant ethanol self-administration, LY341495 did reverse the sedative-hypnotic effects of ethanol. This is attributed to blockade of presynaptic autoreceptors and increased presynaptic glutamate release.

mGluR5 modulates both $GABA_AR$ and NMDAR activity, receptors known to mediate the sedative-hypnotic properties of ethanol. To determine if mGluR5 interactions with either of these receptors contributed to MPEP effects on hypnosis, the effects of

MPEP on pentobarbital, midazolam, and ketamine-induced loss of righting reflex were examined. MPEP enhanced ketamine induced loss of righting reflex with altering pentobarbital or midazolam-induced hypnosis. These data indicate that interactions with NMDAR, and not $GABA_AR$, may influence the effects of MPEP on ethanol-induce sedation. The effects of LY341495 were also tested to examine selectivity for effects on $GABA_AR$ and NMADR specific loss of righting reflex. Blockade of mGluR2/3 reversed pentobarbital, midazolam, and ketamine-induced hypnosis, further supporting the conclusion that LY341495 reduces ethanol-induced loss of righting reflex by enhancing excitatory glutamatergic transmission.

The second set of experiments assessed the effects of *in vivo* acute ethanol exposure on the phosphorylation state of PKCγ in the brain. Ethanol rapidly increased pPKCγ immunoreactivity in the core and shell of the nucleus accumbens and the central amygdala, with a return to baseline levels within 90 minutes of ethanol treatment. Ethanol decreased pPKCγ in the basolateral amygdala 90 minutes after ethanol treatment. These data suggest PKCγ mediates different effects of acute ethanol in different brain regions. PP1α phosphorylation was also examined as increased phosphatase activity could explain ethanol-induced changes in kinase phosphorylation. Ethanol decreased pPP1α in the nucleus accumbens shell and basolateral amygdala 10 minutes after ethanol treatment. Based on evidence that PP1α is phosphorylated by PKCγ and that acute ethanol treatment enhances the phosphorylation of PP1α target kinases, the data presented here indicate that ethanol-induced decreases in PP1α are completely dissociated from ethanol-induced increases in pPKCγ and pERK.

The third set of experiments assessed the role of ERK in mGluR5 modulation of acute ethanol effects. Although the acute effects of ethanol on ERK phosphorylation have previously been examined, discrepancies in the literature prompted a time course experiment to examine the effects of acute ethanol treatment across the brain. Ethanol rapidly increased pERK immunoreactivity in the shell of the nucleus accumbens, the central amygdala, and several regions of the anterior cortex. In contrast, ethanol decreased pERK immunoreactivity in the core of the nucleus accumbens. The effects of ethanol in ERK phosphorylation were also distributed across time, with increases in pERK immunoreactivity observed in the basolateral amygdala**,** the BST, and the PVN of the thalamus and hypothalamus at 90 minutes following ethanol treatment. These data suggest that some of the acute effects of ethanol are mediated by ERK activation in a time and brain region-dependent manner. A second set of immunohistochemical experiments were performed to examine the effects of mGluR5 inhibition with MPEP on ethanol-induced changes in pERK. MPEP alone enhanced pERK immunoreactivity in the central amygdala. In combination with ethanol, MPEP increased pERK immunoreactivity in the BST and PVN of the hypothalamus. Finally, a loss of righting reflex experiment was done to determine the effects of inhibiting ERK activation with SL327 on ethanol-induce hypnosis. Like MPEP, SL327 increased the duration of ethanol-induced loss of righting reflex. These data indicate that ERK influences ethanol-induced hypnosis, although it is still unclear whether ERK mediates MPEP-induced enhancements of ethanol's acute sedative-hypnotic effects.

The roles of mGluR5, PKCγ, and ERK in the acute effects of ethanol

In experiment 1, we determined that inhibition mGluR5 and mGluR2/3 had differential effects on the acute sedative-hypnotic properties of ethanol. While the effects of the mGluR2/3 antagonist are easily explained, the effects of MPEP are more complex. mGluR5 inhibition could be enhancing ethanol-induced sedation and hypnosis in several ways. First, inhibition of any glutamate receptor generally reduces arousal, and the MPEP effect may be attributable to a reduction in overall glutamatergic signaling. Second, as our study with glutamatergic and GABAergic sedatives shows, mGluR5 may modulate the sedative-hypnotic effects of ethanol by altering activity at the NMDA receptors. Since mGluR5 is known to positively modulate NMDAR, MPEP may prevent a compensatory action that is mediated by mGluR5. Finally, ethanol may induce locomotor deficits by inhibiting mGluR5, either directly or indirectly, and MPEP enhances ethanol's effects by further inhibition of mGluR5. It may be a combination of these activities, as well.

At the doses tested in these experiments, MPEP did not produce locomotor deficits on its own. Indeed, exploratory behavior was increased in animals receiving MPEP alone and this is likely due to MPEP's anxiolytic properties and the novelty of the locomotor chamber. MPEP has been shown to produce no effects on spontaneous locomotor behavior up to 100 mg/kg (Spooren et al. 2000), suggesting that blockade of mGluR5 is not sedating. Although this does not rule out separate, but complementary actions of MPEP and ethanol on sedation and hypnosis, the literature and our results from experiment 2 suggest that ethanol indirectly inhibits mGluR5.

In *Xenopus* oocytes, acute ethanol exposure selectively inhibited mGluR5 mediated Cl^- currents (Minami et al. 1998), indicating that ethanol can interrupt mGluR5

mediating signaling, even if it doesn't directly inhibit the receptor. Our initial hypothesis was that if ethanol inhibits mGluR5 and PKCγ is positively coupled to mGluR5, then PKCγ phosphorylation would be decreased following ethanol treatment. However, we found that ethanol rapidly increased pPKCγ immunoreactivity in several brain regions. PKC has been shown to phosphorylate the c-terminus of mGluR5, desensitizing the receptor (Peavy et al. 2002). This evidence supports the conclusion that ethanol-indirectly inhibits mGluR5 by increasing pPKCγ mediated receptor desensitization and that the MPEP effect on the sedative-hypnotic properties of ethanol is related to additive inhibition at the mGluR5 receptor. Our data also supports this conclusion, in that a 30 mg/kg dose of MPEP increased the duration of loss of righting reflex by the same interval of time, regardless of the dose of ethanol administered (Fig 2.3a).

The time course of changes in PKCγ phosphorylation suggests a different role for PKCγ. A 3g/kg dose will produce an average "sleep time" of 10-12 minutes, and pretreatment with MPEP extends this time to 25-30 minutes. Therefore, most of the observed changes in PKCγ phosphorylation occur after the animals have already regained their righting reflex. This suggests that the increases in pPKCγ immunoreactivity are not related to the hypnotic effects of ethanol, and any resulting inhibition of mGluR5 does not affect hypnosis. Evidence that PKCγ null mutant mice are less sensitive the sedative-hypnotic effects of ethanol (Bowers et al. 1999) suggests that there is a role for PKCγ in the response to hypnotic doses of ethanol, but further experimentation is needed to define this role.

For the third set of experiments, we hypothesized that if ethanol inhibits mGluR5 receptors, ERK phosphorylation would be decreased following ethanol treatment.

However, the majority of brain regions examined showed increases in pERK following ethanol treatment. It has previously been reported that activation of mGluR5 increases ERK phosphorylation, and inhibition of mGluR5 blocks amphetamine–induced increases in pERK (Choe et al. 2002), indicating that ERK is positively coupled to mGluR5. However, MPEP pretreatment, alone and in combination with ethanol, produced increases in pERK, suggesting that inhibition of mGluR5 may also be positively coupled to ERK. This would indicate that increases in pERK may mediate the acute effects of ethanol. Our finding that inhibition of ERK phosphorylation enhances the hypnotic properties of ethanol clearly suggests a role for ERK in mediating the sedative-hypnotic of ethanol. However, these results conflict with our immunohistochemical data that suggest that increased pERK contributes to ethanol-induced sedation and hypnosis. An alternative hypothesis would be that ERK has a role in recovery from the acute sedative-hypnotic effects of ethanol. Regardless, further studies to examine the effects of SL327 on ethanol-related behaviors are necessary

mGluR5 as a target for pharmacotherapy development

One of the primary goals in researching a disease state is to identify the mechanisms by which the disease develops, progresses, and persists, often in the hope of finding new targets for treatment. mGluR5 has been implicated in a variety of diseases and conditions, including Parkinson's, seizure disorders, anxiety, and pain, in addition to drug and alcohol dependence (Bordi and Ugolini 1999). The mGluR5 antagonist, MPEP, has been shown to have anxiolytic, neuroprotective and anticonvulsant properties (Chapman et al. 2000; O'Leary et al. 2000; Spooren et al. 2000).

Our laboratory has previously reported that MPEP reduced ethanol self-administration and decreased motivation to self-administer (Besheer et al. 2008; Hodge et al. 2006). The present study shows that MPEP enhances the sedative-hypnotic effects of ethanol. These data suggest that MPEP may reduce ethanol consumption by enhancing the subjective effects of ethanol. However, our laboratory has also shown that MPEP does not substitute for ethanol and blocks the discriminative stimulus properties of ethanol (Besheer and Hodge 2005), suggesting that MPEP does not enhance all of the effects of ethanol. If inhibition of mGluR5 can reduce craving (motivation to self-administer) in humans without substituting for ethanol, then mGluR5 could be a target for pharmacotherapies to prevent relapse. The ability of MPEP to enhance some of the subjective effects of ethanol without substituting for ethanol suggest that mGluR5 inhibitors might also be used in harm-reduction therapy. We present here that MPEP increases sensitivity to the sedative-hypnotic effects of ethanol. If inhibition of mGluR5 can increase an individual's sensitivity to ethanol (reversing tolerance), then less ethanol would be necessary to produce the same subjective effects. Several GABAergic drugs, including diazepam and pentobarbital, have been studied as possible treatments for various aspects of ethanol dependence because they have behavioral profiles similar to that of MPEP. They have anxiolytic and anticonvulsant properties and have been shown to reduce ethanol consumption and enhance the acute effects of ethanol. However, the use of these drugs is limited predominantly to acute treatment of severe withdrawal symptoms because they are sedative-hypnotics, they are reinforcing on their own and produce dependence, and chronic ethanol exposure produces cross tolerance to them. In contrast, MPEP does not produce sedation or hypnosis on it own (Spooren et al. 2000)

and it blocks the rewarding effects of stimulants and depressants without being rewarding or aversive on its own (McGeehan and Olive 2003; Popik and Wrobel 2002). Although there is currently no evidence that ethanol does not produce cross tolerance to MPEP, the fact that MPEP does not substitute for ethanol, as many GABAergic drugs do, suggests that MPEP tolerance would not develop following chronic ethanol exposure.

Future Directions

Although these studies provide valuable information about the relationship between mGluR5 and its associated signaling pathways in the acute effects of ethanol, further investigation is necessary to fully elucidate the roles of mGluR5, PKC, and ERK in mediating the acute effects of ethanol. First, to explore the role of ERK in MPEP enhanced ethanol-induced sedation and hypnosis, locomotor behavior and loss of righting reflex experiments could be done with a combined MPEP and SL327 pretreatment to determine if ERK inhibition alters MPEP effects on ethanol's acute sedative-hypnotic properties. Second, cell-based assays could provide a more flexible way to examine the mGluR5 signaling pathway. Cell culture eliminates issues of blood brain barrier permeation and low potency, which can be limitations for *in vivo* experiments. Activation of mGluR5 has been shown to increase ERK phosphorylation in a variety of cellular preparations. The effects of ethanol, selective mGluR activation and inhibition, selective and non-selective PKC inhibition, and ERK inhibition could all be tested in a cell-based assay using western blot analysis to measure ERK phosphorylation.

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