Investigating the Mechanism of Ethanol-enhanced GABA Release

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ABSTRACT

MARY KATHERINE KELM: Investigating the Mechanism of Ethanol-enhanced GABA Release
(Under the direction of George Breese)

Historically, while research on the actions of ethanol at the GABAergic synapse has focused on postsynaptic mechanisms, recent data have demonstrated that ethanol also increases both evoked and spontaneous GABA release in many brain regions. However, the mechanism through which ethanol acts to enhance GABA release is unknown. The purpose of this dissertation project was to study the mechanism responsible for ethanol-enhanced GABA release at the interneuron-Purkinje cell synapse. First, the ability of ethanol to increase GABA release was characterized with whole-cell voltage clamp recordings. Ethanol increased miniature inhibitory postsynaptic current frequency and decreased the paired-pulse ratio, which suggests that ethanol increases spontaneous and evoked GABA release, respectively.

I found that calcium release from inositol-1,4,5-trisphosphate receptors (IP_3Rs) and ryanodine receptors, adenylate cyclase, protein kinase A, phospholipase C and protein kinase C all play a role in the ability of ethanol to increase spontaneous GABA release, while influx of extracellular calcium into the neuron was not involved in this mechanism. Because of the questionable selectively of the IP_3R antagonist, electron microscopy was used

to show that IP_3Rs are located in the presynaptic terminals at this synapse. Activation of cannabinoid receptors or $GABA_B$ receptors inhibited ethanol-enhanced spontaneous GABA release, but this ethanol mechanism was unaffected by tonic activation of these receptors. It was also determined that both protein kinase A and protein kinase C contribute to the generation of spontaneous GABA release and cross-talk is not occurring between these two intracellular messengers.

Overall, the large majority of the intracellular messengers investigated were involved in ethanol-enhanced GABA release. This result is not surprising considering the promiscuous nature of ethanol and the fact that these intracellular messengers can contribute to the generation of spontaneous GABA release. The ability of ethanol to increase GABA release contributes to the GABAergic profile of ethanol, and modulation of the GABAergic system contributes to alcohol intoxication. A person who is less sensitive to the intoxicating effects of alcohol is prone to developing alcoholism; therefore, understanding the molecular mechanisms contributing to alcohol intoxication will further our understanding of this disease.

DEDICATION

First and foremost, I dedicate this dissertation to Matthew Jonathan Kelm. I thank him for being the husband every wife deserves and for being one of the first people to tell me I should be a scientist. He should be a coauthor for the amount of support he has provided me throughout this process. This work is also dedicated to my parents because they made me the person that I am today. They continuously love me, scientist or not, and knowledge of that love and the security it brings gives me the strength to always aim higher. And of course I must dedicate this work to my wonderful dog Hattie. She greets me every day when I come home from work like I am royalty, which is particularly important on days when I feel like a joker. While I was at home writing this monstrosity of a document, she remained faithfully at my side (quite literally, actually) throughout the entire process. I love you all very much.

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LIST OF ABBREVIATIONS

2-APB: 2-Aminoethoxydiphenylborane

ACSF: artificial cerebrospinal fluid

AMPA: alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

ANOVA: analysis of variance

AP5: D-2-amino-5-phosphonopentanoate

ATP: adenosine-5'-triphosphate

BAPTA: 1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid

BAPTA-AM: 1,2-Bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis(acetoxymethyl ester)

Ca^{2+}_{ext} : calcium external

cAMP: 3'-5'-cyclic adenosine monophosphate

$CdCl_2$: cadmium chloride

CNQX: 6-cyano-7-nitroquinoxaline-2,3-dione

CRF: corticotropin-releasing factor

DAG: diacylglycerol

DDA: 2′,3′-dideoxyadenosine

DMSO: dimethyl sulfoxide

EGTA: ethylene glycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid

eIPSC: evoked inhibitory postsynaptic current

GABA: gamma-aminobutyric Acid

GPCR: G protein-coupled receptors

HEPES: 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid

IP_3R: inositol 1,4,5-trisphosphate receptor

K^+_{ext} -potassium external

MDN: mechanically dissociated neuron preparation

mEPSC: miniature excitatory postsynaptic current

mGluR: metabotropic glutamate receptors

mIPSC: miniature inhibitory postsynaptic current

NMDA: N-methyl-D-aspartic acid

NPY: neuropeptide Y

PI: phosphoinositol

PKA: protein kinase A

PKC: protein kinase C

PKC(19-36): protein kinase C inhibitor Peptide [19-36]

PKI: protein kinase inhibitor-(6-22)-amide

PLC: phospholipase C

PMA: Phorbol 12-myristate 13-acetate

PP: paired-pulse

Rp-cAMP: Rp-Adenosine 3′,5′-cyclic monophosphorothioate triethylammonium salt hydrate

RyR: ryanodine receptor

SEM: standard error of the mean

SERCA: sarco(endo)plasmic reticulum calcium ATPase

SOC: store-operated channel

SQ 22536: 9-(Tetrahydro-2-furanyl)-9H-purin-6-amine

τ-$_{fast}$: fast decay time

τ-$_{slow}$: slow decay time

TRP: transient receptor potential

TTX: tetrodotoxin

VTA: ventral tegmental area

WIN: WIN 55,212-2 mesylate

Chapter I: General Introduction

Alcoholism: the disease

Alcoholism is a chronic, progressive disease that drives someone to drink regardless of the negative consequences. The entire body is involved in the development of the physical dependence and tolerance associated with chronic alcohol use, while the actual development of alcoholism is due to a number of neuroadaptations that guide a person towards a relapsing drinking cycle that incapacitates their ability to stop drinking. Obviously a person can drink throughout a lifetime and not develop alcoholism. The difference between this group of people and alcoholics is a complex interaction between genetic and environmental factors that makes a person more susceptible to developing alcoholism (Schuckit, 2009).

Interestingly, alcohol is the only drug of abuse to have a separate section at the National Institutes of Health, the National Institute on Alcohol Abuse and Alcoholism. This is probably due to the fact that the effects of alcoholism on the population are widespread: it is estimated that approximately 10% of Americans will suffer from alcoholism at some point in their lives, with men more affected than women (Grant, 1994). Excessive alcohol consumption is responsible for 76,000 deaths annually and is the third leading preventable cause of death (Midanik, 2004). Considering the number of people affected, it is not surprising

that the estimated cost placed on society due to alcohol misuse is 185 billion dollars yearly (Harwood, 2000). Most alarming, though, is that alcoholism is often left untreated with only roughly 30% of alcoholics receiving treatment (Cohen et al., 2007), suggesting that more people are suffering from alcoholism than these statistics reflect. Even when an individual undergoes treatment to become abstinent, 45-75% of people relapse at least once in their lifetime (Boothby and Doering, 2005). Therefore, development of more effective treatment options is essential if there is going to be an improvement in these bleak statistics.

Neuropharmacology of alcoholism and addiction theory

About 10% of alcoholics receiving treatment use pharmacotherapy to treat their disease (Mark et al., 2003). Because only 30% of alcoholics receive treatment (Cohen et al., 2007), approximately 3% of alcoholics are taking advantage of available medication. There are drugs to block craving or reduce alcohol intake, induce aversion to alcohol, treat alcohol withdrawal, induce sobriety in intoxicated individuals, and, if necessary, treat concomitant psychiatric disorders and/or associated drug abuse (Gatch and Lal, 1998). Unfortunately, there is no “magic bullet” to cure alcoholism, which is one of the factors contributing to the low prescribing rate (Mark et al., 2003). Currently there are three medications approved by the U.S. Food and Drug Administration for alcoholism: disulfiram (Antabuse), acamprosate (Campral), and naltrexone (ReVia, Vivitrol).

Disulfiram induces aversion to alcohol by blocking alcohol metabolism. Specifically, disulfiram inhibits the aldehyde dehydrogenase enzyme, which prevents acetaldehyde from converting to acetate. Increased acetaldehyde levels following alcohol consumption causes adverse physiological effects (ex: flushing, chest pain, vomiting, etc.) that deter someone from further alcohol drinking (Swift, 1999). Disulfiram has demonstrated questionable efficacy in multiple clinical trials but is more effective in treatment programs that involve monitoring medicine consumption (Garbutt, 2009). Naltrexone and acamprosate are different from disulfiram in that they target the neurobiological processes thought to be involved in the development of alcoholism. Naltrexone, an opioid receptor antagonist, reduces alcohol intake and craving by blocking the rewarding effects of alcohol (Volpicelli et al., 1995). Acamprosate normalizes glutamatergic neurotransmission, which is one of the many neurotransmitter systems that is dysregulated during chronic alcohol use, but the exact mechanism is unknown (Heilig and Egli, 2006). One study suggests that acamprosate acts as a glutamate stabilizer specifically during alcohol withdrawal, (Mann et al., 2008), thereby preventing withdrawal from becoming a motivating factor to drink again.

Even these drugs are limited- in a clinical trial testing the effectiveness of treatment options for alcoholics, a combination of naltrexone and acamprosate had a comparable effect to intense behavioral therapy (Anton et al., 2006). While the most successful treatment of alcoholism might require both pharmacological and psychological treatment, there is obviously still room for improvement

regarding the pharmacotherapy options. Advances in this field will require a better understanding of the cellular and molecular effects of alcohol, which have been quite difficult to study due to the promiscuous effects of alcohol on multiple neurobiological pathways and the multiple stages involved in the development of alcoholism.

There are three stages to addiction: preoccupation/anticipation (i.e. craving), binge intoxication, and withdrawal. These stages are interconnected and feed off of each other, contributing to a cycle that gets more intense over time, which can eventually lead to addiction (Koob and Le Moal, 1997). The desire to drink comes from both positive and negative reinforcing effects (Koob and Le Moal, 1997). Positive reinforcing effects involve feeling aroused/tense before taking a drink and euphoric during the act of drinking. Negative reinforcing effects involve feeling anxious/stressed before taking a drink and relieved during the act of drinking. During the addiction cycle, the positive reinforcing effects become less motivating and the negative reinforcing effects predominate. Because addiction is a complex disease, there are a number of neuropeptide and neurotransmitter systems that are affected either directly or indirectly by alcohol.

The role of stress-related peptides in the development of alcoholism

Neuropeptide Y (NPY) and corticotropin-releasing factor (CRF) are neuropeptides that play opposite roles in regulating anxiety: NPY is anxiolytic while CRF is anxiogenic (Valdez and Koob, 2004; Heilig and Egli, 2006). NPY

null mice consume more alcohol and are less sensitive to the sedative/hypnotic effects of alcohol compared to wild-type controls (Thiele et al., 1998). In accordance with these data, transgenic mice that overexpress NPY consume less alcohol and are more sensitive to the sedative/hypnotic effects of alcohol (Thiele et al., 1998). A number of studies have confirmed these results (for review see Heilig and Egli, 2006). These data suggest that NPY contributes to the rewarding/intoxicating effects of alcohol.

In contrast to NPY, CRF contributes to the anxiety experienced during alcohol withdrawal, which is a negative reinforcing effect for future alcohol consumption. Because a CRF type 1 (CRF1) receptor antagonist blocks the anxiety/stress response, the CRF1 receptor is thought to mediate the CRF effects linked to stress and anxiety (Britton et al., 1986). Administration of a CRF1 receptor antagonist decreases alcohol withdrawal-induced anxiety (Rassnick et al., 1993b; Breese et al., 2004; Knapp et al., 2004; Overstreet et al., 2004), and alcohol withdrawal increases levels of CRF in the amygdala (Merlo Pich et al., 1995). A CRF1 receptor antagonist reduces alcohol intake in alcohol-dependent animals, but has no effect in nondependent animals (Sabino et al., 2006). It is hypothesized that during alcohol intoxication CRF levels decrease and NPY levels increase and return to baseline levels afterwards. After a period of chronic alcohol abuse, CRF and NPY do not return to baseline levels. This “altered baseline” is one of the neuroadaptations that can contribute to a person’s inability to stop drinking as they attempt to “fix” this imbalance with more alcohol consumption (Valdez and Koob, 2004).

The reward pathway and the development of alcoholism

The mesolimbic pathway, or "reward pathway" involves dopamine neurons originating in the ventral tegmental area (VTA) releasing dopamine into the nucleus accumbens in response to rewarding experiences (Wise, 1998). Alcohol increases dopamine release in the nucleus accumbens (Imperato and Di Chiara, 1986), and certain acute behavioral effects of alcohol are attenuated in the presence of a dopamine receptor antagonist (Liljequist et al., 1981; Risinger et al., 1992). There is a direct relationship between the amount of dopamine signaling in the mesolimbic pathway and the amount of alcohol consumption (Hodge et al., 1997; Cohen et al., 1998; Gonzales et al., 2004; Rodd et al., 2008). During alcohol withdrawal, the functionality of this dopaminergic system is reduced, which could contribute to relapse because the alcoholic is trying to "overcome" the low dopamine state (Diana et al., 1993; Weiss et al., 1996; Shen et al., 2007; Volkow et al., 2007).

Desensitization of the mesolimbic dopamine system and its related behaviors is thought to be a critical component of the development of drug addiction (Robinson and Berridge, 1993). However, while behavioral sensitization to psychostimulants and opiates involves sensitization of drug-evoked dopamine levels, this is not the case for development of alcohol behavioral sensitization (Zapata et al., 2006). Therefore, while dopamine has a role in the development of alcoholism, caution must be taken when attempting to group alcoholism into drug addiction in general.

Opioids are widely known as the neuropeptides that lead to a “feel good” response once they bind to three potential opioid receptors; moreover, opioid receptor agonists are used in pain management, such as morphine (McQuay, 1989). In addition to pain management, the opioid system plays a role in reward and reinforcement through activation of mu- and delta- opioid receptors that increase dopamine release in the mesolimbic pathway (Spanagel et al., 1990). Naltrexone, one of the three drugs approved for the treatment of alcoholism, is a nonspecific competitive opioid receptor antagonist that inhibits the reward felt from drinking alcohol, thereby reducing the amount of alcohol consumed (Volpicelli et al., 1995). Naltrexone can reverse the alcohol-induced increase in dopamine signaling in the mesolimbic pathway, suggesting that opioids are a mediator in the alcohol-dopamine interaction (Benjamin et al., 1993). Alcohol increases opioid neurotransmission (Gianoulakis, 2001), which could in turn increase dopamine neurotransmission, leading to the rewarding properties of alcohol consumption. Data suggest that opioids do not affect the VTA dopamine neurons directly, but instead hyperpolarize the GABAergic interneurons (i.e. disinhibition) that provide inhibitory signals to the dopamine VTA neurons (Johnson and North, 1992). Overall, these data suggest that the opioid and dopamine systems play an important role in the rewarding effects (i.e. positive reinforcing effects) associated with alcohol consumption.

Glutamate neurotransmission and the development of alcoholism

Glutamate, the major excitatory neurotransmitter in the brain, can activate

both metabotropic glutamate receptors (mGluRs) and ionotropic glutamate receptors (Ozawa et al., 1998). There are three ionotropic glutamate receptors: N-methyl-D-aspartic acid (NMDA) receptors, alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors and kainate receptors, which are all affected by alcohol. At physiologically relevant concentrations, ethanol inhibits kainate receptor-mediated excitatory neurotransmission (Weiner et al., 1999; Carta et al., 2003; Lack et al., 2008). Because activation of kainate receptors causes an anxiety response, the anxiolytic properties of acute alcohol exposure could be mediated through inhibition of kainate receptors (Lack et al., 2008). Ethanol also inhibits AMPA receptors (Martin et al., 1995; Wang et al., 1999; Frye and Fincher, 2000; Wirkner et al., 2000; Jones et al., 2008). At the behavioral level, increasing AMPA receptor-mediated neurotransmission can reverse some of the acute alcohol effects in the rat (Jones et al., 2008). Additionally, AMPA receptor-mediated neurotransmission contributes to alcohol-seeking behavior and relapse (Sanchis-Segura et al., 2006).

The interaction between alcohol and NMDA receptors has been more extensively studied. Like the other ionotropic receptors, acute ethanol inhibits NMDA receptors, and different NMDA receptor subunit combinations have different sensitivities to ethanol (for review see Allgaier, 2002). Behaviorally, NMDA receptors have been implicated in alcohol withdrawal, craving, relapse, tolerance and dependence (Khanna et al., 1993; Riaz and Faingold, 1994; Ripley and Little, 1995; Holter et al., 2000; Ron, 2004). Chronic alcohol exposure results in an upregulation of NMDA receptors, which is consistent with alcohol

withdrawal-induced hyperactivation of the glutamate system (Spanagel and Zieglgansberger, 1997). Despite this role for ionotropic glutamate receptors in alcohol related behaviors, developing pharmacotherapies specifically targeting these sites is problematic due to the essential role these receptors play in brain functioning and their ubiquitous expression throughout the brain (Heilig and Egli, 2006).

The mGluRs offer a unique advantage over the ionotropic glutamate receptors because of differences in presynaptic versus postsynaptic localization of the different mGluR subtypes (Heilig and Egli, 2006). There are eight different mGluR subtypes, which are divided into three groups: group 1 (mGluR1, mGluR5), group 2 (mGluR2, mGluR3), and group 3 (mGluR4, mGluR6, mGluR7 and mGluR8). The group 1 mGluRs are primarily located postsynaptically, while group 2 and 3 mGluRs are primarily located presynaptically (Raiteri, 2008). A mGluR5 antagonist reduces alcohol drinking and craving in animal studies (Backstrom et al., 2004; Cowen et al., 2005; Schroeder et al., 2005; Hodge et al., 2006), possibly through a reduction in the subjective effects of alcohol (Besheer and Hodge, 2005). On the molecular level, a mGluR5 antagonist reduces glutamatergic signaling through NMDA receptors by preventing PKC phosphorylation of the NMDA receptor (Hermans and Challiss, 2001; Kotecha et al., 2003); moreover, this could be the mechanism responsible for the effectiveness of the mGluR5 antagonist on reducing alcohol consumption (Heilig and Egli, 2006). While the majority of studies have investigated the mGluR5s, an agonist at the group 2 mGluRs reduces excessive alcohol drinking and craving in

alcohol preferring rats (McKinzie et al., 2005). This agonist reduces anxiety and stress in preclinical and clinical studies, which offers one potential mechanism for the reduced alcohol drinking (Marek, 2004). Overall, the mGluRs are a promising target for the development of new pharmacotherapies to treat alcoholism.

GABA neurotransmission and the development of alcoholism

Gamma-aminobutyric Acid (GABA) is the most abundant inhibitory neurotransmitter in the brain and can bind to two ionotropic GABA receptors ($GABA_A$ and $GABA_C$) and one metabotropic GABA receptor ($GABA_B$). $GABA_B$ receptors are G protein-coupled receptors (GPCRs) that are located at both presynaptic and postsynaptic sites (Raiteri, 2008). The presynaptic $GABA_B$ receptors are negative autoreceptors that decrease GABA release from the presynaptic terminal upon GABA binding to the $GABA_B$ receptor (Raiteri, 2008). Baclofen, a $GABA_B$ receptor agonist, suppresses alcohol drinking in alcohol naïve rats and alcohol dependent rats (Daoust et al., 1987; Colombo et al., 2000; Colombo et al., 2002; Anstrom et al., 2003; Colombo et al., 2003a; Janak and Michael Gill, 2003). It also suppresses the motivational properties of alcohol (Colombo et al., 2003b; Maccioni et al., 2005), the development of tolerance to behavioral effects (Cott et al., 1976; Zaleski et al., 2001), and alcohol withdrawal symptoms (Colombo et al., 2000; Knapp et al., 2007). In clinical trials baclofen promotes alcohol abstinence, prevents alcohol relapse and reduces alcohol withdrawal in alcoholics (Addolorato et al., 2006). It is also effective in alcoholics

with cirrhosis of the liver, which is caused by extensive alcohol use (Addolorato et al., 2007). The current drugs used to treat alcoholism undergo extensive liver metabolism, rendering them useless in severe alcoholics with cirrhosis of the liver. Therefore, baclofen could potentially offer an untreated population of alcoholics a pharmacotherapy option.

At the GABAergic synapse, GABA is released from the presynaptic GABAergic neuron and binds to GABA binding sites on the postsynaptic ionotropic GABA receptors. The $GABA_A$ and $GABA_C$ receptors have a pentameric structure, with the five subunits coming together to form a chloride channel that allows chloride to flow into the neuron after GABA binding, which results in hyperpolarization of the neuron (Baumann et al., 2001). There are many types of subunits that can contribute to the formation of the ionotropic GABA receptors: α1-6, β1-3, γ1-3, δ, ε, θ, π, and ρ1-3. While the number of possible GABA subunit combinations is enormous, only a small number of combinations have been found in brain (McKernan and Whiting, 1996). The $GABA_C$ receptors contain one or more ρ subunits and are only found in retina, thalamus, hippocampus, pituitary and gut (Chebib, 2004). The interaction between $GABA_C$ receptors and alcohol has not been thoroughly studied, but there is one study suggesting that ethanol potentiates the response of $GABA_C$ receptors (Yeh and Kolb, 1997). The most common GABA receptor in the brain is the $GABA_A$ receptor, with the majority of $GABA_A$ receptors consisting of two α subunits, two β subunits and one γ subunit (Farrar et al., 1999). In addition to GABA, there are binding sites on the $GABA_A$ receptor for benzodiazepines,

barbiturates and neurosteroids (Mohler and Okada, 1977; Briley and Langer, 1978; Majewska et al., 1986).

There are a number of behavioral studies suggesting there is an interaction between alcohol and the $GABA_A$ receptor**.** Specifically, the effects of alcohol on motor function (Frye et al., 1980; Frye and Breese, 1982; Liljequist and Engel, 1982), sleep time (Liljequist and Engel, 1982; Givens and Breese, 1990), temperature (Liljequist and Engel, 1982), convulsions (Liljequist and Engel, 1982), anxiety (Frye et al., 1980) and learning and memory (White et al., 1997) were similar to those seen with drugs that act directly on the $GABA_A$ receptor, the benzodiazepines and barbiturates (Mohler and Okada, 1977; Briley and Langer, 1978). Additionally, benzodiazepines and barbiturates enhance the alcohol effect on motor impairment (Martz et al., 1983). Consistent with these data, $GABA_A$ receptor antagonists and a benzodiazepine inverse agonist block the behavioral effects of alcohol (Liljequist and Engel, 1984; Koob et al., 1986; Suzdak et al., 1986a; Koob et al., 1988; Ticku and Kulkarni, 1988; McCown and Breese, 1989) and reduce alcohol consumption (Rassnick et al., 1993a; Petry, 1995). Benzodiazepines and barbiturates substitute for alcohol in drug discrimination studies, suggesting that the drugs have similar subjective effects (Grant et al., 2000; Shannon et al., 2004). Additionally, GABA agonists, benzodiazepines and barbiturates can attenuate some of the negative effects associated with alcohol withdrawal (Cooper et al., 1979; Frye et al., 1983; McCown et al., 1986; Criswell and Breese, 1989).

Overall, these data provide evidence from pre-clinical and clinical studies that alcohol affects GABAergic neurotransmission at multiple sites to manifest certain behaviors. To complement the behavioral data, there is evidence that ethanol affects GABAergic neuronal activity, which occurs in a brain region-specific manner (Nestoros, 1980; Mereu and Gessa, 1985; Bloom and Siggins, 1987; Givens and Breese, 1990; Simson et al., 1991; Criswell et al., 1993; Criswell et al., 1995). The ability of ethanol to alter GABAergic neurotransmission in a brain region-specific manner is consistent with alcohol-induced behaviors that are linked to certain brain regions (McCown et al. 1986). Therefore, the GABAergic synapse has the potential to be a useful target when developing new treatment options. The effects of ethanol at the GABAergic synapse on the cellular and molecular level will be discussed in more detail below.

Ethanol and postsynaptic $GABA_A$ receptor interactions

As described above, ethanol has similar behavioral effects to the benzodiazepines and barbiturates, which bind to postsynaptic $GABA_A$ receptors. Therefore, the initial hypothesis was that ethanol binds directly to $GABA_A$ receptors. This idea was supported by a number of studies that found ethanol enhances GABA-induced Cl^- currents (Ticku et al., 1983; Suzdak et al., 1986b; Aguayo, 1990; Allan et al., 1991; Reynolds et al., 1992; Sigel et al., 1993; Harris et al., 1995; Mori et al., 2000); however, these results were not always reproducible (Siggins et al., 1987; Palmer and Hoffer, 1990; White et al., 1990;

Frye et al., 1994; Marszalec et al., 1998; Peoples and Weight, 1999; Ming et al., 2001). One possible explanation for these inconsistent results is differences in the various preparations used, which includes cell lines, oocytes, and primary neuronal cultures (Siggins et al., 2005; Lovinger and Homanics, 2007).

Another explanation is that ethanol interacts with select $GABA_A$ receptor subunits. This hypothesis is supported by data showing that ethanol affects GABA-mediated neuronal activity and alcohol-related behaviors in a brain region-specific manner (see above). Additionally, experiments investigating the effect of ethanol on $GABA_A$ receptor-mediated synaptic currents in the slice have seen a brain region-specific effect (Carlen et al., 1982; Siggins et al., 1987; Proctor et al., 1992; Weiner et al., 1994; Wan et al., 1996; Ariwodola and Weiner, 2004; Nie et al., 2004). Therefore, it was proposed that $GABA_A$ receptor subunit composition dictates the effectiveness of ethanol at a $GABA_A$ receptor and contributes to the brain region-specific ethanol effects. The most abundant $GABA_A$ receptor in the brain is the $2\alpha_1 2\beta_2\gamma_2$ $GABA_A$ receptor, and zolpidem is a selective agonist at α_1-containing $GABA_A$ receptors (McKernan and Whiting, 1996). Early work supported that the effect of zolpidem on a $GABA_A$ receptor could predict the effect of ethanol on that $GABA_A$ receptor, implying that ethanol selectively acts at α_1-containing $GABA_A$ receptors (Criswell et al., 1993; Criswell et al., 1995); however, later work disproved this hypothesis (Criswell et al., 2003). Despite this setback, focus still remained on ethanol interacting with specific $GABA_A$ receptor subunits, but the focus shifted to extrasynaptic $GABA_A$ receptors.

Ethanol and extrasynaptic $GABA_A$ receptor interactions

Extrasynaptic $GABA_A$ receptors are located outside of the GABAergic synapse and exhibit different properties than synaptic $GABA_A$ receptors. Extrasynaptic $GABA_A$ receptors can be activated from the reversal of GABA transporters, GABA release from astrocytes, and GABA spillover from presynaptic GABA release (for review see Glykys and Mody, 2007). Synaptic $GABA_A$ receptors respond to high concentrations of GABA (in the mM range) and are activated for only a few milliseconds. This "phasic conductance" of synaptic $GABA_A$ receptors is in contrast to the "tonic conductance" of extrasynaptic $GABA_A$ receptors, which are sensitive to lower concentrations of GABA and remain activated for a longer period of time (Saxena and Macdonald, 1994; Mody, 2001). Consequently, since GABA is almost always present in the extracellular space, most extrasynaptic $GABA_A$ receptors are continuously activated (Glykys and Mody, 2007; Lovinger and Homanics, 2007). This tonic conductance is seen in the cerebellum, cortex, hippocampus, thalamus and spinal cord, which are areas that are also sensitive to ethanol (Glykys and Mody, 2007).

Extrasynaptic $GABA_A$ receptors are more sensitive to the effects of ethanol compared to the synaptic $GABA_A$ receptors. Studies in oocytes found that ethanol acts at $GABA_A$ receptors containing δ and β3 subunits associated with α4 or α6 subunits at concentrations as low as 1 mM (Sundstrom-Poromaa et al., 2002; Wallner et al., 2003), which was confirmed in the slice (Wei et al., 2004; Hanchar et al., 2005; Glykys et al., 2007). However, studies in another

laboratory attempting to replicate the oocyte studies have been unsuccessful (Borghese et al., 2006). Interestingly, ethanol-induced GABA release onto cerebellar granule neurons is proposed to activate a tonic current mediated by $\alpha_6\beta_x\delta$ $GABA_A$ receptors via GABA spillover, which suggests that at least in one brain region ethanol can affect extrasynaptic $GABA_A$ receptors indirectly (Carta et al., 2004). Site-directed mutagenesis studies using this same subunit combination concluded that ethanol directly affects the $\alpha_6\beta_x\delta$ $GABA_A$ receptors (Hanchar et al., 2005), but this result could not be reproduced (Botta et al., 2007). Therefore, while the majority of evidence supports there being an ethanol-extrasynaptic $GABA_A$ receptor interaction, more studies are necessary to determine the mechanism. Overall, while ethanol most likely interacts with $GABA_A$ receptors in a subunit-specific manner, the GABAergic profile of ethanol is more convoluted than previously thought (Weiner and Valenzuela, 2006).

The tipsy terminal: ethanol actions at the GABA terminal

In the presynaptic terminal, GABA is synthesized from glutamate by glutamate decarboxylase and loaded into the synaptic vesicles through a vesicular GABA transporter (Martin, 1993; McIntire et al., 1997; Sagne et al., 1997). The vesicles dock at the plasma membrane and are primed for vesicle fusion. Once the vesicles fuse with the membrane, GABA is released from the presynaptic terminal into the synaptic cleft and binds to the postsynaptic $GABA_A$ receptors (Sudhof, 1995). GABA transporters located on the presynaptic GABAergic neurons as well as surrounding astrocytes remove GABA from the

synaptic cleft and extracellular space. At this point GABA is inactivated by GABA-transaminase or is recycled as a neurotransmitter (Madsen et al., 2008).

GABA was not recognized as a neurotransmitter until the late sixties (Otsuka et al., 1966; Roberts and Kuriyama, 1968), and around this same time data surfaced showing that ethanol changes the concentration of GABA in the brain (Gordon, 1967). However, because researchers within the alcohol field were focused on the direct effects of ethanol on the $GABA_A$ receptor, there were no studies investigating the potential presynaptic effects of ethanol. Many years later, intracellular recordings were used to study the GABAergic effect of ethanol. Carlen et al. (1982) found that ethanol enhances evoked inhibitory postsynaptic currents (eIPSCs) in the hippocampus, and while analysis of eIPSCs cannot indicate whether a presynaptic or postsynaptic GABAergic effect was responsible for the change, the authors postulated that the eIPSC enhancement was mediated by a presynaptic effect of ethanol.

However, a few years later a study found that ethanol inhibits eIPSCs at this same synapse (Siggins et al., 1987). Additionally, Siggins and colleagues had data suggesting that the change in eIPSC amplitude was primarily due to a presynaptic mechanism. However, they concluded that ethanol was *decreasing* GABA release, which was consistent with biochemical studies showing that ethanol decreases GABA release in cortical regions (Carmichael and Israel, 1975; Howerton and Collins, 1984; Strong and Wood, 1984). Similar studies investigating the effect of ethanol on eIPSCs in the hippocampus continued to report inconsistencies (Proctor et al., 1992; Weiner et al., 1994), which led to the

Siggins group repeating this study (Wan et al., 1996). The new study used glutamate antagonists because of a potential source of glutamate neurotransmission in their preparation (Davies and Collingridge, 1989), and this time they found no ethanol effect on eIPSC amplitude. However, in the presence of a $GABA_B$ receptor antagonist, ethanol consistently increased eIPSC amplitude (Wan et al., 1996). The Siggins group postulated again that the change in eIPSC amplitude was due a presynaptic effect, but they discounted this possibility because their new data was consistent with ethanol *increasing* GABA release, which was not in agreement with the biochemical data (Carmichael and Israel, 1975; Howerton and Collins, 1984; Strong and Wood, 1984). However, studies in the nucleus accumbens slice provided additional evidence for ethanol having presynaptic GABAergic effects, and as a result, the Siggins group came back to the hypothesis that ethanol acts presynaptically to increase GABA release (see Siggins et al., 2005).

In response to these findings, Dr. Dennis Twombly organized a National Institute on Alcohol Abuse and Alcoholism workshop in 2002 to discuss the deficiency in studies exploring the presynaptic effects of ethanol (Roberto et al., 2006). Shortly thereafter, a number of studies came out supporting the idea that ethanol increases GABA release. An injection of ethanol into mice one day before electrophysiology recordings caused an increase in GABA release in the VTA (Melis et al., 2002). The Siggins group published a study showing that ethanol increases both spontaneous and evoked GABA release in the central nucleus of the amygdala (Roberto et al., 2003). Two studies came out

simultaneously showing that ethanol increases GABA release in motor neurons, with one of the papers showing that ethanol actually inhibited postsynaptic $GABA_A$ receptors (Sebe et al., 2003; Ziskind-Conhaim et al., 2003).

Currently, ethanol has been shown to increase GABA release in the following brain regions: central nucleus of the amygdala (Roberto et al., 2003; Nie et al., 2004), basolateral amygdala (Zhu and Lovinger, 2006), hippocampus (Ariwodola and Weiner, 2004; Sanna et al., 2004; Li et al., 2006), VTA (Melis et al. 2002, Theile et al. 2008), cerebellum (Carta et al., 2004; Ming et al., 2006; Criswell et al., 2008) and brainstem/spinal cord (Sebe et al., 2003; Ziskind-Conhaim et al., 2003). Ethanol has no effect on GABA release in the cortex, thalamus, and lateral septum, which is consistent with the brain region-specific effects of ethanol on GABA neurotransmission *in vivo* (Criswell et al., 2008; Jia et al., 2008; Mameli et al., 2008). Due to the novelty of the concept that ethanol acts presynaptically, the mechanism through which ethanol acts to increase GABA release has yet to be elucidated. *The focus of this dissertation is to determine the mechanism responsible for ethanol-enhanced GABA release.*

Description of model system: interneuron-Purkinje cell synapse

Multiple studies have shown that ethanol exposure impairs the development of the cerebellum and causes volume loss in a developed cerebellum (Bauer-Moffett and Altman, 1977; Servais et al., 2007). The cerebellum plays a role in controlling coordination, sensory perception, motor control, motor learning and reflex adaptation (Robinson, 1976; McCormick and

Thompson, 1984; Baillieux et al., 2008); these cerebellar functions are consistent with the acute effects of alcohol involving alterations in balance, speech, and motor coordination. Ethanol depresses $GABA_A$ receptor-mediated Purkinje cell firing (Siggins and French, 1979; Sorensen et al., 1980; George and Chu, 1984), which is consistent with ethanol increasing the amount of GABA released from the interneurons (i.e. stellate/basket cells) onto the Purkinje cells (Ming et al., 2006; Criswell et al., 2008; Mameli et al., 2008).

The cerebellum has four main types of neurons: Purkinje cells, granule cells, stellate/basket cells, and Golgi cells (Voogd and Glickstein, 1998; see Fig. 1.1). The Purkinje cells are large, GABAergic neurons that project solely to the deep cerebellar nuclei. Purkinje cells are oriented in a single row, which serves as the border between the molecular cell layer and the granule cell layer. In addition to the Purkinje cell dendritic trees, the molecular layer houses the stellate and basket cells, both of which are GABAergic and provide inhibitory input to the Purkinje cells. The basket cells synapse around the Purkinje cell soma and main dendrites, while the stellate cells synapse with more distal dendrites (Vincent et al., 1992).

The other inhibitory interneurons in the cerebellum are the Golgi cells, which synapse onto the granule cells. Granule cells are small glutamatergic neurons that are abundantly expressed throughout the granule cell layer. The granule cell axons (i.e. parallel fibers) ascend to the molecular cell layer and synapse onto Purkinje cell spines. The Golgi cells receive input from the parallel fibers and provide inhibitory feedback to the granule cells. The granule cells

receive excitatory input from the mossy fibers, and the Golgi cells act as a filter at this synapse. Climbing fibers, which originate from the inferior olive, are excitatory and synapse onto the Purkinje cells.

For these experiments no distinction was made between the basket cells and stellate cells. One difference between the two cell types is expression of parvalbumin, which is absent in stellate cells up to postnatal day 12 but present in basket cells throughout development (Collin et al., 2005a). Because parvalbumin plays a role in presynaptic calcium signaling, these experiments do not use rats under postnatal day 12. Otherwise, the basket and stellate cells have very similar characteristics (Sultan and Bower, 1998).

Ethanol-enhanced spontaneous GABA release and intracellular messengers

Previous studies investigating ethanol-enhanced GABA release have focused on GPCRs, specifically the $GABA_B$ receptor, δ opioid receptor, nociceptin/orphanin FQ peptide receptor, and the CRF1 receptor (Wan et al., 1996; Ariwodola and Weiner, 2004; Nie et al., 2004; Roberto and Siggins, 2006; Kang-Park et al., 2007). The following work will focus on the signal transduction mechanisms that are downstream of the GPCRs. When GPCRs are activated by an external stimulus, there is a conformational change in the receptor that causes an allosteric change in the nearby G protein. The Gα subunit substitutes guanosine 5'-diphosphate for guanosine-5'-triphosphate and dissociates from the

βγ subunit, freeing both the Gα subunit and the βγ subunit to interact with their respective downstream messengers (for review see Hepler and Gilman, 1992).

These experiments will place focus on the signal transduction pathways affected by 3 G protein subunits: $G\alpha_i$, $G\alpha_s$, and $G\alpha_q$. The $G\alpha_i$- and $G\alpha_s$-linked pathways affect (in opposite directions) levels of adenylate cyclase and protein kinase A (Hanoune and Defer, 2001), while the $G\alpha_q$–linked pathway activates phospholipase C and leads to calcium release from the internal stores and activation of protein kinase C (Rhee, 2001). The purpose of the following studies was to determine if these intracellular messengers are playing a role in the ability of ethanol to increase spontaneous GABA release at the interneuron-Purkinje cell synapse.

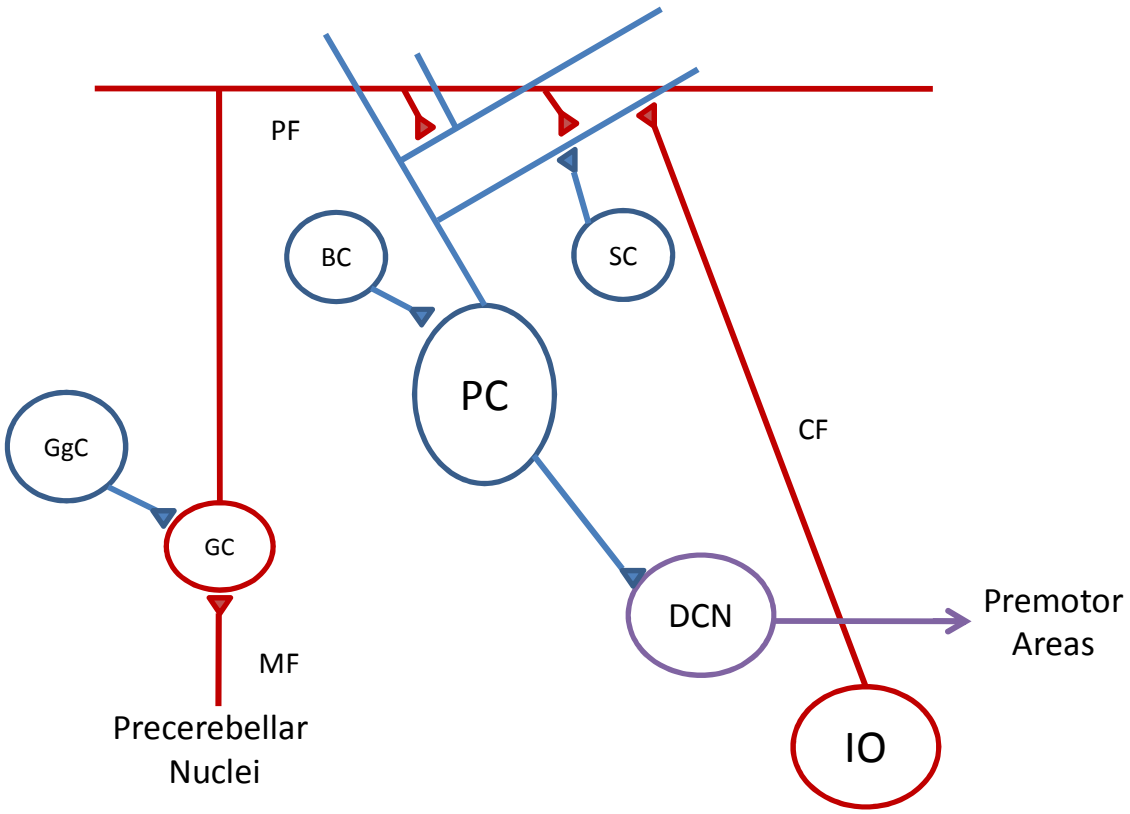


Figure 1.1. Cerebellum circuitry. This diagram includes the circuitry discussed in the text and does not depict all synapses in the cerebellum. Excitatory neurotransmission is represented by red, inhibitory neurotransmission is represented by blue, and a mixture of both is represented by purple. MF: mossy fiber; DCN: deep cerebellar nuclei; IO: inferior olive; CF: climbing fiber; GC: granule cell; PF: parallel fiber; PC: Purkinje cell; GgC: Golgi cell; SC: stellate cell; BC: basket cell. Adapted from: http://en.wikipedia.org/wiki/Cerebellum.

Chapter II: General Methods

Slice preparation for electrophysiology. Sprague-Dawley rats, 13-20 days old, were anesthetized with an intraperitoneal injection of 75% urethane (Sigma, St. Louis, MO) and decapitated after disappearance of the plantar reflex. The brain was rapidly removed and placed in a 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) buffered solution of the following composition (in mM): 145 NaCl, 5 KCl, 10 HEPES, 2 $CaCl_2$, 1 $MgCl_2$, 10 glucose and 5 sucrose (pH to 7.4 with NaOH). The cerebella were isolated and parasagittal slices, 350 µm thick, were cut with a vibrating microtome (Leica VT1000S, Vashaw Scientific, Norcross, GA) in a low sodium solution of the following composition (in mM): 112.5 sucrose, 63 NaCl, 3 KCl, 1.25 NaH_2PO_4, 24 $NaHCO_3$, 6 $MgSO_4$, 0.5 $CaCl_2$, 10 glucose, and gassed with 95% O_2/5% CO_2. The slices were placed in a chamber containing oxygenated artificial cerebrospinal fluid (ACSF) of the following composition (in mM): 124 NaCl, 3.25 KCl, 1.25 KH_2PO_4, 10 glucose, 2 $MgSO_4$, 20 $NaHCO_3$, 2 $CaCl_2$, and gassed with 95% O_2/5% CO_2. The slices were equilibrated at least one hour at room temperature before starting experiments.

Whole-cell voltage clamp recordings. A slice was placed at the bottom of a chamber that was attached to the stage of a microscope (BX5OWI, Olympus,

Japan) and was perfused with oxygenated ACSF (21-24℃) at a flow rate of 0.5 ml/min. The cells were visualized using infrared illumination under differential interference contrast optics with a 40X LUMPlanFl water-immersion objective (Olympus) and displayed on a monitor via a video camera (C2400, Hamamatsu, Japan). Recording electrodes were pulled from borosilicate glass (Drummond Scientific Company, Broomall, PA) and had a resistance of 2.5-3 MΩ when filled with internal solution. The composition of the internal solution was the following (in mM):150 KCl, 3.1 $MgCl_2$, 15 HEPES, 5 K-ATP, 5 EGTA, 15 phosphocreatine. The internal solution pH was adjusted to 7.4 with KOH and the osmolarity was approximately 310 mOsms. For the 1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid (BAPTA) experiments, the composition of the internal solution was the following (in mM):105 KCl, 3.1 $MgCl_2$, 15 HEPES, 5 K-ATP, 30 BAPTA tetrapotassium salt, 15 phosphocreatine (pH to 7.4 with KOH). For the paired-pulse studies, 5 mM N-(2,6-Dimethylphenylcarbamoylmethyl) triethylammonium bromide (QX-314) was added to the internal solution to block the generation of action potentials.

Data were displayed on an oscilloscope (V-212, Hitachi, Japan), digitized at 5 kHz, and stored on a personal computer. The membrane potential was held at -70 mV using a patch-clamp amplifier (Axopatch 200B, Axon Instruments, Union City, CA), and data were collected with Clampex 8.1 software (Axon Instruments). The capacitance and access resistance were monitored continuously throughout the recordings and a change of 10% or more was

sufficient to exclude the recording from analysis. Only one protocol/recording was conducted per slice to avoid contamination.

Drug preparation and drug delivery system. Each drug was made up as a concentrated stock solution, which was diluted in aCSF and inserted into sealed syringes on the day of use. CNQX (10 µM) and AP5 (50 µM) were added to all solutions inserted into the sealed syringes. Table 2.1 includes a description of each drug, the supplier, the stock solution concentration and the storage conditions. The final concentration of dimethyl sulfoxide (DMSO) used in the experiments was less that 0.1%, which does not alter the miniature inhibitory postsynaptic current (mIPSC) properties (n = 4, data not shown). When BAPTA-AM, SQ 22,536, DDA, edelfosine, bafilomycin A_1 and H-89 were used in an experiment, there was a 30 minute to 2 hour drug pre-incubation period with the slice before starting the experiment. Each sealed syringe was attached to Teflon tubing that was connected to a multi-barrel perfusion pencil (Automate Scientific, Inc.; Sarasota, FL; 250 µm tip diameter), which was positioned 150-250 µm from the cell being tested.

Protocol and analysis for paired-pulse experiments. Platinum-iridium stimulating electrodes were lowered into the molecular layer approximately 150 µm from the experimental Purkinje cell. The membrane of the cell was ruptured, and the control solution was delivered through the multi-barrel perfusion pencil. After allowing the cell to stabilize, two stimuli were delivered (0.2 ms duration and

50 ms inter-stimulus interval) at 0.1 Hz, which generated a paired-pulse (PP) record of two closely spaced evoked inhibitory postsynaptic currents (eIPSCs). A maximum stimulation was applied to determine the maximum current response. The stimulus strength was reduced to a level that generated half the maximum current response, and this stimulus strength was used for the experiment. Following a minimum of 6 PP records obtained at 10 sec intervals for the pre-control value, the ethanol solution was delivered through the multi-barrel perfusion pencil and 5 minutes later a second series of PP records was collected. Ethanol was washed out for at least 5 minutes and a final series of PP records was collected. The miniAnalysis software (version 5.6.4; Synaptosoft, Decatur, GA) was used to generate averaged PP traces from the PP records collected for each cell. The averaged PP trace was used to calculate the paired-pulse ratio (PPR), which is the ratio of the second eIPSC amplitude to the first ($eIPSC_2/eIPSC_1$). The PPR value for each cell in a given group (pre-control, ethanol, washout) were averaged together and represented as the mean ± standard error of the mean (SEM). The "averaged control" values were calculated from the pre-control and washout ((pre-control + washout)/2).

Mechanically dissociated neuron preparation. A slice was transferred to a recording chamber containing the HEPES-buffered solution. A 0.3 mm probe touched the surface of the cell layer of the submerged slice and was vibrated (~0.2 mm amplitude at 10 Hz) for 3 minutes. When the resulting mechanical forces break a neuron free from the matrix, most of the dendritic tree is sheared

off as well as the distal parts of axon; however, the presynaptic terminals remain attached to the soma (Akaike and Moorhouse, 2003). After the mechanical dissociation the slice was removed from the chamber and the mechanically dissociated neurons were allowed to settle to the bottom. The same protocol described for the whole-cell voltage clamp recordings in the slice was used with the mechanically dissociated neurons. The nominally calcium free solution used in the mechanically dissociated neuron experiments consisted of the following composition: 145 mM NaCl, 5 mM KCl, 10 mM HEPES, 2 mM $MgCl_2$ and 10 mM glucose (pH to 7.4 with NaOH).

Protocol and analysis for mIPSC experiments. After the membrane of the cell was ruptured, the control solution, which included 1 µM TTX in addition to the CNQX and AP5, was delivered through the multi-barrel perfusion pencil. Once a steady state mIPSC frequency was obtained (determined from the average of at least two repetitive recordings), a pre-control recording was collected for at least 60 seconds. For the ethanol experiments, in addition to the pre-control recording, an ethanol recording and washout recording were collected. The ethanol recoding in the slice was collected five minutes after the start of the ethanol perfusion while the ethanol recording in the mechanically dissociated neuron preparation was collected 30 seconds after the start of the ethanol perfusion. The percent (%) change in mIPSC frequency, decay time and amplitude was calculated as follows: 100 x (“ethanol response”/((“pre-control”+“washout”)/2))-100. The “control” ethanol data points in the data

chapters went through the same protocol (pre-control, ethanol, washout) but were never exposed to ethanol.

For the experiments that do not involve ethanol, there was a control recording and a drug recording with no washout. The % change in mIPSC frequency, decay time and amplitude for these experiments was calculated as follows: 100 x (|“drug response” – “control”|) /”control”). When different antagonists were tested against a drug effect on mIPSC frequency, a stable baseline mIPSC rate was established in the presence of the antagonist before exposure to the drug to avoid a summation of effects. Therefore, for these experiments the baseline mIPSC frequency value in the presence of the antagonist served as the pre-control or control value.

For the ethanol experiments that were conducted in the presence of a drug, all experiments initially used 100 mM ethanol, and if a drug treatment was sufficient to inhibit the ethanol effect on mIPSC frequency, this was the only ethanol concentration tested. However, if 100 mM ethanol still had an effect in the presence of a drug treatment, additional experiments were carried out with 50 mM ethanol, which was the lowest ethanol concentration that significantly increased mIPSC frequency, to determine if the antagonist was having an effect on lower ethanol concentrations.

All data were expressed as the mean ± SEM. If the control and pre-control baseline mIPSC frequency were lower than 0.5 Hz (except for experiments with the pre-control value including exposure to WIN or baclofen), the experiment was excluded from analysis. Addition of 50 µM bicuculline

methochloride, a $GABA_A$ antagonist, abolished the mIPSCs (n = 4, data not shown), which confirmed that the mIPSCs were GABAergic. Data were analyzed with miniAnalysis software. The mIPSC decay time was determined from a bi-exponential fit using miniAnalysis software. Fast decay time (τ-$_{fast}$) and slow decay time (τ-$_{slow}$) were analyzed separately.

Tissue preparation for electron microscopy. Male Sprague-Dawley rats were anesthetized with sodium pentobarbital (60 mg/kg, intraperitoneal). After inducing deep anesthesia, rats were intracardially perfused with heparinized saline followed by 500 ml of fixative. The fixative was a mixture of 2% paraformaldehyde and 2% glutaraldeyhde in phosphate buffer (0.1 M, pH 7.4). The brains were removed and postfixed with the same fixative for 2 hours at 4ºC. Brains were sectioned with a vibratome (40-60 µM) and collected in cold phosphate buffer.

Tissue staining for electron microscopy. Cerebellar areas of interest were excised, cryoprotected in 30% glycerol, quick frozen, freeze-substituted in a Leica Electron Microscopy Automatic Freeze Substitution System, and embedded in Lowicryl HM-20. Sections were cut at ~90 nm with an ultramicrotome and collected on nickel grids. The sections were incubated with normal rabbit serum for 20 minutes to suppress nonspecific binding and then incubated overnight on a shaker at room temperature with the IP_3R primary antibody (goat, 1 : 30K), which has been characterized previously (Sharp et al.,

1993a). Following overnight incubation, the sections were rinsed and incubated for 20 minutes in normal rabbit serum. The sections were incubated with rabbit anti-goat Immunoglobulin G conjugated to 10 nm gold (Ted Pella, 1:15) for two hours at room temperature. After rinsing, the sections were counterstained with 1% uranyl acetate followed by Sato's lead and examined with a Philips Tecnai electron microscope (Hillsboro, OR) at 80 kV. Images were collected with a Gatan 12-bit 1024 x 1024 CCD camera (Pleasanton, CA).

Quantitative analysis for electron microscopy. Attention was restricted to Purkinje cell and molecular layers of the cerebellum. Once a suitable area was found, pictures of the field were taken at x6500 magnification and saved for later analysis. For analysis, profiles representing GABAergic presynaptic terminals, glutamatergic presynaptic terminals, parallel fibers, and Purkinje cell dendrites were identified and gold particles coding for IP_3R were counted for the identified profiles. Using Image J software, the area of the profile was determined. The number of particles per square µM was determined and data were expressed as the mean ± SEM. IP_3R staining associated with mitochondria or the plasma membrane was excluded from analysis because of potential nonspecific staining. Parallel fibers were analyzed as bundles, as opposed to individual fibers, and underwent additional analysis to account for the plasma membrane around each fiber. Specifically, after the area of the parallel fiber bundle was calculated, the number of individual fibers within the group was counted and multiplied by the

calculated average area of the membrane surrounding the individual fiber. This value was subtracted from the area of the parallel fiber bundle.

In experiments that did not include a GABA antibody, visual assessments were made to differentiate between GABAergic terminals and glutamatergic terminals. The glutamatergic synapse has an asymmetric synapse due to a thick postsynaptic density (Palay and Chan-Palay, 1974). GABAergic synapses have a symmetric synapse that is less pronounced compared to the glutamatergic synapse. GABA terminals contain a mixture of flattened, smaller vesicles and spherical vesicles compared to the glutamate terminals that contain only spherical vesicles (Palay and Chan-Palay, 1974). I found that the GABAergic terminals were generally larger than the glutamatergic terminals. In the region examined, GABA presynaptic terminals are believed to originate from either basket cells or stellate cells. Golgi cells are also GABAergic, but are only found in the granule cell layer. The glutamatergic synapses were either parallel fibers or climbing fibers.

Statistics. Paired Student's t-test, Student's t-test, one-way analysis of variance (ANOVA) and Dunnett's post hoc test were performed as indicated. A two-tailed *p* value less than 0.05 was accepted as statistically significant.

Drug Name	Mechanism	Supplier	Solvent	[Stock]	Storage
1,2-Bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid tetrapotassium salt (BAPTA)	membrane impermeable calcium chelator	Anapec	internal solution		-20℃
1,2-Bis(2-aminophenoxy) ethane-N,N, N′,N'-tetraacetic acid tetrakis (acetoxymethyl ester) (BAPTA-AM)	membrane permeable calcium chelator	Sigma	DMSO	1000x	-20℃
2′,3′-Dideoxyadenosine (DDA)	adenylate cyclase antagonist	Sigma	DMSO	1000x	-20℃
2-Aminoethoxy diphenylborane (2-APB)	IP_3R antagonist	Tocris	DMSO	1000x	-20℃
9-(Tetrahydro-2-furanyl)-9H-purin-6-amine (SQ 22536)	adenylate cyclase antagonist	Sigma	dd H_20	500x	-20℃
AM-251	cannabinoid 1 receptor antagonist	Tocris	DMSO	5000x	-20℃
Baclofen	$GABA_B$ receptor agonist	Tocris	dd H_20	1000x	-20℃
Bafilomycin A_1	proton pump inhibitor	Alexis	DMSO	1000x	-20℃
Bicuculline methochloride	$GABA_A$ receptor antagonist	Tocris	dd H_20	1000x	-20℃
Cadmium chloride ($CdCl_2$)	voltage-dependent Ca^{2+} channel inhibitor	Sigma	dd H_20	1000x	RT
CGP 52432	$GABA_B$ receptor antagonist	Tocris	dd H_20	1000x	-20℃
Chelerythrine chloride	PKC antagonist	Tocris	dd H_20	1000x	-20℃
Cyano-7-nitroquinoxaline-2,3-dione (CNQX)	ionotropic glutamate receptor antagonist	Sigma	dd H_20	1000x	-20℃
D-2-amino-5-phosphonopentanoate (AP5)	NMDA receptor antagonist	Sigma	dd H_20	1000x	-20℃
Dibutyryl-cAMP sodium salt (dBcAMP)	PKA agonist	Tocris	dd H_20	1000x	-20℃
Edelfosine	PLC antagonist	Tocris	dd H_20	1000x	-20℃
Epsilon-V1-2	PKCε antagonist	Anaspec	dd H_20	1000x	-20℃
H-89	PKA antagonist	Sigma	dd H_20	1000x	-20℃
JNJ 16259685	mGluR1 antagonist	Tocris	DMSO	1000X	-20℃

Drug Name	Mechanism	Supplier	Solvent	[Stock]	Storage
N-(2,6-Dimethylphenyl carbamoylmethyl)triethyl ammonium bromide (QX-314)	voltage dependent sodium channel inhibitor	Sigma	internal solution		-20℃
Noradrenaline (+)-bitartrate salt (Norepinephrine)	adrenergic receptor agonist	Sigma	dd H_20	1000x	Day of use
Phorbol 12-myristate 13-acetate (PMA)	PKC agonist	Tocris	DMSO	1000x	-20℃
Protein kinase C inhibitor Peptide [19-36] (PKC(19-36))	membrane impermeable PKC antagonist	Calbio-chem	dd H_20	1000x	-20℃
Protein kinase inhibitor-(6-22)-amide (PKI)	membrane impermeable PKA antagonist	Tocris	dd H_20	1000x	-20℃
Rp-Adenosine 3′,5′-cyclic monophosphorothioate triethylammonium salt hydrate (Rp-cAMP)	PKA antagonist	Sigma	dd H_20	1000x	-20℃
Ryanodine	RyR antagonist	Calbio-chem	aCSF	10x	-20℃
SC9	PKC agonist	Tocris	DMSO	1000x	-20℃
Tetrodotoxin (TTX)	voltage-dependent Na^+ channel antagonist	Sigma	dd H_20	1000x	4℃
Thapsigargin	SERCA pump inhibitor	Tocris	DMSO	1000x	-20℃
WIN 55,212-2 mesylate (WIN)	cannabinoid 1 receptor agonist	Tocris	DMSO	5000x	-20℃

Table 2.1. Information for each drug used in the experiments.

Chapter III: The Effect of Ethanol on GABA Release

INTRODUCTION

The effect of ethanol on spontaneous and evoked neurotransmitter release can be measured in an *in vitro* slice preparation. Spontaneous and evoked neurotransmitter release differ in their dependence on action potentials: spontaneous neurotransmitter release occurs in the absence of action potentials, while action potentials are necessary for evoked neurotransmitter release. To study the mechanism of ethanol-enhanced GABA release, I determined if ethanol could increase GABA release in the chosen model system, the interneuron-Purkinje cell synapse. When GABA is released from the interneuron presynaptic terminals, it binds to and opens the $GABA_A$ receptors on the cerebellar Purkinje neuron. The whole-cell voltage clamp technique measures the flow of chloride through the $GABA_A$ receptor in response to GABA binding and records this $GABA_A$ receptor-mediated chloride flux. When TTX is applied to block action potentials, this $GABA_A$ receptor-mediated chloride flux response is known as a miniature inhibitory postsynaptic current (mIPSC). An increase in the frequency of the mIPSC events is interpreted as an increase in spontaneous GABA release.

When measuring evoked GABA release, a stimulator artificially creates an action potential to stimulate GABA release and this recorded chloride flux response is known as an evoked inhibitory postsynaptic current (eIPSC).

However, unlike spontaneous release, there is no direct readout for changes in presynaptic activity with analysis of eIPSCs. Therefore, a paired pulse ratio (PPR) is used to make this assessment, which determines if there is a change in the ratio of the amplitude of two closely-spaced eIPSCs ($eIPSC_2/eIPSC_1$) that are the result of two stimuli applied to the brain slice 20-200 milliseconds apart (for review see Zucker, 1989). If there is a change in the amplitude of the first eIPSC, it reflects a change in neurotransmitter release probability. If there is a change in the amplitude of the second eIPSC, it could reflect a change in residual calcium, postsynaptic receptor desensitization or a change in action potential duration. The drug effect on the PPR is inversely related to the drug effect on evoked neurotransmitter release (Siggins et al., 2005). Hence, if ethanol increases evoked GABA release, there would be a decrease in the PPR. At the interneuron-Purkinje cell synapse, I determined the effect of ethanol on mIPSC frequency and the PPR to measure ethanol-induced changes in spontaneous and evoked GABA release, respectively.

RESULTS

Ethanol selectively increases mIPSC frequency in the slice. Compared to control (-0.6 ± 2.8%, n = 8), the mIPSC frequency of cerebellar Purkinje neurons was significantly increased by 50 mM (10.9 ± 2.0%, n = 9), 75 mM (18.6 ± 3.5%, n = 9) and 100 mM (22.9 ± 3.8%, n = 12) ethanol, but not by 25 mM (2.2 ± 3.1%, n = 12) ethanol, as illustrated in Figure 3.1A. There was a significant linear trend across concentrations for the ethanol effect on mIPSC frequency ($r = .69$, $p<.05$), which shows that ethanol dose-dependently increased mIPSC frequency. In contrast, none of the ethanol concentrations changed the mIPSC decay time and amplitude (data not shown, see Ming et al., 2006). A trace and a cumulative probability curve from a representative neuron are shown in Figure 3.1B and 3.1C, respectively. In Figure 3.1C, ethanol shifts the distribution of the interevent interval curve to the left, which is interpreted as ethanol increasing mIPSC frequency. The lack of ethanol effect on mIPSC decay time and amplitude from the same representative neuron is shown in Figure 3.1D. These results suggest that ethanol is increasing spontaneous GABA release in the slice preparation.

Ethanol selectively increases mIPSC frequency in the mechanically dissociated neuron preparation. Mechanically dissociated neurons were also used to determine if ethanol increases spontaneous GABA release. The mechanically dissociated neuron preparation consists of the Purkinje cell soma and the attached presynaptic terminals (Akaike and Moorhouse, 2003). Compared to mIPSCs that were recorded in the slice, baseline mIPSC decay

time, rise time, and amplitude were all different in the mechanically dissociated neuron preparation (see Table 3.1). Similar to data collected in the slice, 50 mM (36.4 ± 9.1%, n = 9) and 100 mM (22.0 ± 11.4%, n = 10) ethanol significantly increased the mIPSC frequency of mechanically dissociated cerebellar Purkinje neurons compared to control (-10.8 ± 6.2%, n = 9, Fig. 3.2A), while having no effect on mIPSC decay time and amplitude (data not shown). A representative neuron showing that 100 mM ethanol increases mIPSC frequency in a mechanically dissociated neuron preparation is shown in Figure 3.2B. It should be noted that 50 mM ethanol significantly increased mIPSC frequency to a greater extent in the mechanically dissociated neuron preparation compared to the slice ($p<.05$, Student's t-test). Overall, these results suggest that ethanol increases spontaneous GABA release in the mechanically dissociated neuron preparation.

Ethanol does not increase mIPSC frequency in the presence of bafilomycin A_1. To confirm that the ethanol-induced increase in mIPSC frequency is the result of an increase in vesicular GABA release, a vacuolar-type proton pump inhibitor, bafilomycin A_1, was used (Bowman et al., 1988). Inhibition of the proton pump eliminates the pH and electrical gradients that are necessary for GABA transporters to fill the vesicles with GABA (Maycox et al., 1990; Drose and Altendorf, 1997). Following at least a two hour incubation of the slice with 2 μM bafilomycin A_1, 100 mM ethanol did not increase mIPSC frequency (0.022 ± 0.01 Hz, n = 5) compared to control (0.022 ± 0.008 Hz, Fig. 3.2C). These results

suggest that the ethanol-induced increase in mIPSC frequency is due to the release of GABA-filled vesicles.

Ethanol decreases the PPR. The PPR was significantly decreased by 50 mM ethanol (16.2 ± 6.3%, n = 10), 75 mM ethanol (19.8 ± 3.5%, n = 9), and 100 mM ethanol (22.5 ± 7%, n = 9), but not by 25 mM ethanol (4.2 ± 4.3%, n = 10) and 0 mM ethanol (3.9 ± 2.7%, n = 10), as illustrated in Figure 3.3A. There was a significant linear trend across concentrations for the ethanol effect on the PPR ($r = -.45$, $p<.05$), which shows that ethanol dose-dependently decreased the PPR. In Figure 3.3B, averaged PP traces from a representative neuron show that 100 mM ethanol decreases the ratio of the amplitude of the second eIPSCs to the first compared to the pre-control and washout. These results suggest that ethanol increases evoked GABA release at the interneuron-Purkinje cell synapse.

DISCUSSION

I found that ethanol increased mIPSC frequency at the interneuron-Purkinje cell synapse while having no effect on mIPSC decay time or amplitude in both the slice and mechanically dissociated neuron preparation. These findings are consistent with many studies that have shown ethanol increases mIPSC frequency while having no effect on mIPSC decay time (Sebe et al., 2003; Li et al., 2006; Ming et al., 2006; Zhu and Lovinger, 2006). The lack of ethanol effect on mIPSC decay time indicates that ethanol did not alter postsynaptic $GABA_A$ receptor function in these recording conditions. In addition, ethanol did not alter mIPSC amplitude, which is another indication that ethanol does not affect postsynaptic $GABA_A$ receptors. While there was no postsynaptic ethanol effect in the current study, the possibility cannot be ruled out that ethanol is having a postsynaptic effect under physiological conditions. The intracellular milieu of the postsynaptic neuron is altered during whole-cell voltage clamp recordings (for review see Sarantopoulos et al., 2004); moreover, the presence of high intracellular chloride, as was used in the present study, reduces the ethanol effect on $GABA_A$ receptors in the hippocampus (Silberman et al., 2005).

In addition to the slice, I utilized a mechanically dissociated neuron preparation in this study. This preparation consists of a soma and the attached presynaptic terminals (Akaike and Moorhouse, 2003); therefore, communication from nearby glia and neurons is eliminated. Because ethanol increased spontaneous GABA release in the mechanically dissociated neuron preparation, the GABAergic presynaptic terminals are the likely source of the GABA released

onto the Purkinje neurons. Additionally, in the presence of bafilomycin A_1, a drug that blocks vesicles from filling with GABA, ethanol had no effect on mIPSC frequency. These results provide further evidence that ethanol is acting at the interneuron presynaptic terminal to increase GABA release. Overall, this work and the work of others suggest that ethanol increases spontaneous GABA release in multiple brain regions through a presynaptic mechanism.

There were some differences between the slice and mechanically dissociated neuron preparation. Specifically, I found differences in baseline mIPSC decay time, rise time, and amplitude, and it is thought that the mechanical dissociation procedure alters presynaptic terminal excitability (Akaike and Moorhouse, 2003). However, ethanol increased mIPSC frequency in both preparations. It is important to note that 50 mM ethanol increased mIPSC frequency to a higher degree in the mechanically dissociated neuron preparation. Previously, ethanol was shown to increase GABA release using this same preparation with basolateral amygdala neurons (Zhu and Lovinger, 2006). These investigators also saw a larger effect of ethanol on GABA release in the mechanically dissociated neuron preparation compared to the slice. Because the mechanically dissociated neuron preparation allows for instantaneous access of ethanol to the neuron, the effect of ethanol can be seen on a seconds timescale compared to the minutes required in the slice. Therefore, Zhu and Lovinger (2006) hypothesized that the additional time it takes to see an ethanol effect in the slice allows for tolerance to develop, resulting in an overall smaller ethanol effect in the slice compared to the mechanically dissociated neuron preparation.

In addition to increasing mIPSC frequency, ethanol decreased the PPR, which is interpreted as ethanol increasing evoked GABA release. Ethanol decreases the PPR in central nucleus of the amygdala neurons (Roberto et al., 2003), hippocampal CA1 pyramidal neurons (Weiner et al., 1997; Ariwodola and Weiner, 2004), VTA neurons (Theile et al., 2008) and cerebellar Purkinje neurons (Criswell et al., 2008). Additionally, if there is no ethanol effect on mIPSC frequency, there is also no effect on the PPR (Criswell et al., 2008; Jia et al., 2008).

Interestingly, Mameli and colleagues (2008) found that at the interneuron-Purkinje cell synapse ethanol increases spontaneous GABA release but increases evoked GABA release in a location-dependent manner. When the stimulator is in the outer third of the molecular layer (i.e. distal stimulation), which is the location of the interneuron stellate cells, ethanol decreases the PPR. However, if the stimulator is moved to the inner third of the molecular layer (i.e. proximal stimulation), which is the location of the interneuron basket cells, ethanol has no effect on the PPR. A similar phenomenon is observed in the basolateral amygdala and hippocampus, except that the proximal stimulation elicits an ethanol effect while the distal stimulation is without effect (Wu et al., 2005; Silberman et al., 2008). In the present protocol, the stimulator was placed in the middle of the molecular layer, which most likely resulted in stimulation of both the stellate and the basket cells.

Overall, I found that ethanol increases mIPSC frequency and decreases the PPR at the interneuron-Purkinje cell synapse, which suggests that ethanol

increases both spontaneous and evoked GABA release, respectively. The mechanically dissociated neuron study and the bafilomycin A_1 study provided evidence that the ethanol effect on mIPSC frequency involves an increase in the vesicular release of GABA. I will primarily utilize the mIPSC technique to investigate the mechanism of ethanol-enhanced GABA release because analysis of mIPSCs provides a direct way to assess changes in neurotransmitter release, and I have more thoroughly characterized the source of the GABA release in this technique.

	Frequency (Hz)	τ-fast (ms)	τ-slow (ms)	Rise Time (ms)	Amplitude (pA)
Slice	2.2 ± 0.4	11.9 ± 0.6	11.9 ± 0.6	2.6 ± 0.1	15.9 ± 1.6
MDN	1.4 ± 0.4	5.6 ± 0.5*	16.9 ± 2.0*	1.0 ± 0.1*	29. 4 ± 3.9*

Table 3.1. A comparison of mIPSC properties between the mechanically dissociated neuron (MDN) preparation and the slice at the interneuron-Purkinje cell synapse. There was no significant effect on mIPSC frequency between the two groups. There was a significant decrease in the mIPSC fast decay time (τ-fast) and rise time and an increase in the mIPSC slow decay time (τ-slow) and amplitude in the MDN preparation compared to the slice (*, $p<.05$, Student's t test).

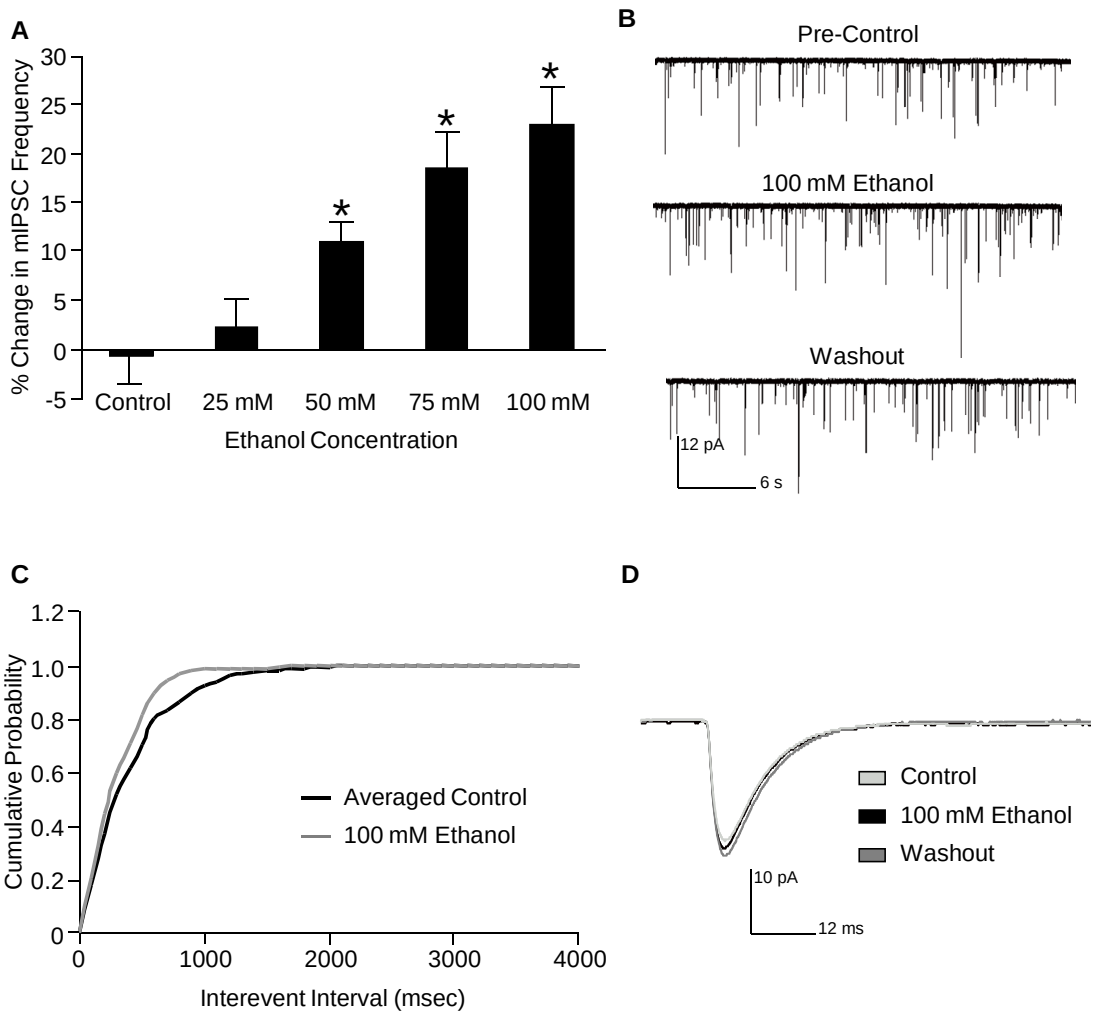
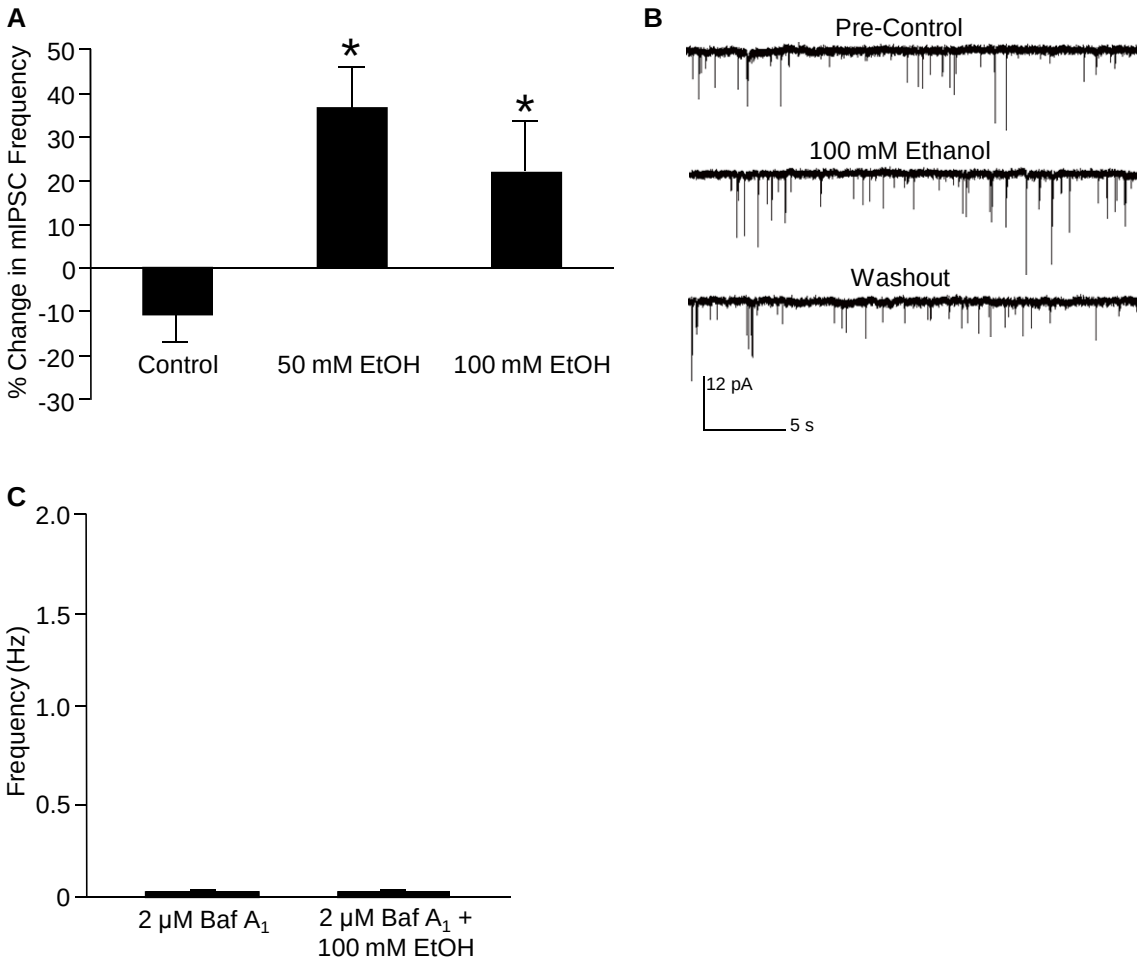


Figure 3.1. In the slice ethanol increased mIPSC frequency and had no effect on mIPSC amplitude and decay time. A, there was an increase in mIPSC frequency at 50, 75, and 100 mM ethanol (*, $p<.05$, one-way ANOVA, Dunnett's post hoc test). B, a trace from a representative neuron showing the effect of 100 mM ethanol on mIPSC frequency. C, a cumulative frequency histogram from the same representative neuron demonstrating the effect of 100 mM ethanol on the interevent interval curve. D, a trace from the same representative neuron showing the effect of 100 mM ethanol on mIPSC decay time and amplitude.



<u>Figure 3.2.</u> Ethanol increased mIPSC frequency in the mechanically dissociated neuron preparation and had no effect in the presence of bafilomycin A_1. A, there was an increase in mIPSC frequency at 50 and 100 mM ethanol (EtOH) in the mechanically dissociated neuron preparation (*, $p<.05$, one-way ANOVA, Dunnett's post hoc test). B, a trace from a representative neuron demonstrating the effect of 100 mM ethanol on mIPSC frequency in the mechanically dissociated neuron preparation. C, incubating the slice with bafilomycin A_1 (Baf A_1) prevented 100 mM ethanol from increasing mIPSC frequency.

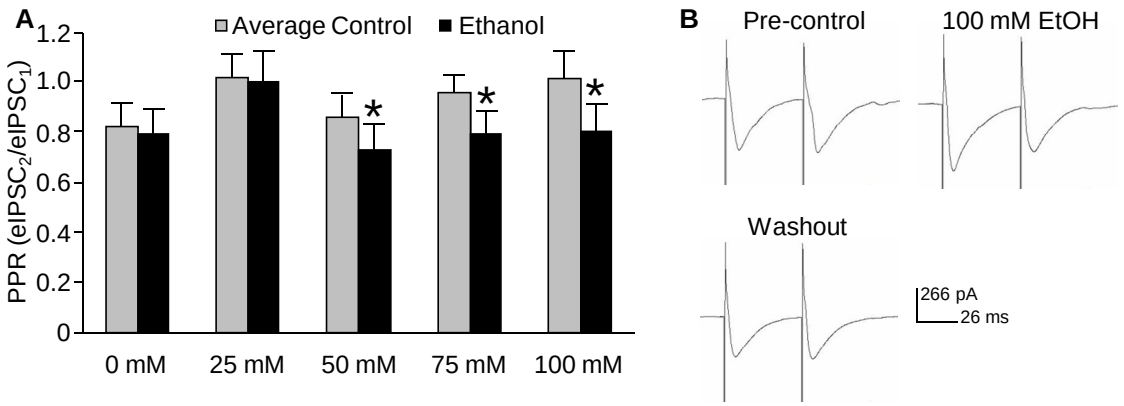


Figure 3.3. Ethanol decreased the paired-pulse ratio. A, the paired-pulse ratio (PPR) was decreased at 50, 75 and 100 mM ethanol (*, $p<.05$, paired Student's t test). B, traces from a representative neuron demonstrating the effect of 100 mM ethanol on the ratio of the amplitude of the second evoked inhibitory postsynaptic current (eIPSC) to the amplitude of the first eIPSC.

Chapter IV: Calcium Signaling and Ethanol-enhanced GABA release

INTRODUCTION

I provided evidence that ethanol increases GABA release at the interneuron-Purkinje cell synapse, but the intracellular messengers mediating this effect are unknown. One possibility is calcium because of the imperative role it plays in a number of neuronal processes, including all neurotransmitter release. It is generally accepted that physiologically relevant ethanol concentrations increase levels of intracellular calcium (Daniell and Harris, 1989; Mironov and Hermann, 1996; Xiao et al., 2005). Moreover, changes in presynaptic intracellular calcium levels can alter spontaneous and evoked GABA release (Bardo et al., 2002; Bardo et al., 2006; Yamasaki et al., 2006; Glitsch, 2008). Therefore, it seems plausible that ethanol increases spontaneous GABA release through a mechanism that involves an increase in intracellular calcium.

This increase in intracellular calcium can occur through an increase in calcium influx via voltage-dependent calcium channels, receptor-operated channels, and store-operated channels (SOCs) or through an increase in calcium release from the inositol 1,4,5-trisphosphate receptors (IP_3Rs) and ryanodine receptors (RyRs) located on the internal stores (i.e. endoplasmic reticulum). The endoplasmic reticulum is a single, continuous intracellular organelle that extends throughout the neuron- from the dendrites to the presynaptic terminals (for review

see Verkhratsky, 2005). I tested the hypothesis that ethanol enhances GABA release at the interneuron-Purkinje cell synapse through a presynaptic calcium-dependent mechanism. I then determined the origin of the calcium that regulated the increase in GABA release.

RESULTS

The ability of ethanol to increase mIPSC frequency is occurring through a presynaptic, calcium-dependent mechanism. The slices were incubated with 100 μM BAPTA-AM, a membrane permeable calcium-chelator, and it was determined if ethanol could increase mIPSC frequency in these conditions. The ability of ethanol to increase mIPSC frequency was blocked in the presence of BAPTA-AM compared to control (7.4 ± 2.7%, n = 8, Fig. 4.1A). These results suggest that ethanol is increasing spontaneous GABA release through a calcium-dependent mechanism.

Because BAPTA-AM was applied to the bath, it could be acting at the postsynaptic neuron instead of the presynaptic neuron. This could alter the release of a calcium-dependent retrograde messenger, which could be responsible for the presynaptic effect of ethanol in lieu of ethanol acting directly on the presynaptic site. Therefore, BAPTA tetrapotassium salt (30 mM), which is membrane impermeable, was included in the internal solution to limit BAPTA exposure to the postsynaptic neuron. Compared to control (2.4 ± 2.8%, n = 7, Fig. 4.1B), 50 mM (20.0 ± 5.6%, n = 8) and 100 mM (23.9 ± 4.3%, n = 6) ethanol were still able to significantly increase mIPSC frequency with 30 mM BAPTA in the internal solution. In addition, inclusion of BAPTA in the internal solution significantly increased baseline mIPSC decay time (control: 11.8 ± 0.3 ms vs. BAPTA: 14.1 ± 1.1 ms, Fig. 4.1C) and significantly decreased baseline mIPSC amplitude (control: 15.4 ± 0.7 pA vs. BAPTA: 12.2 ± 1.1 pA, Fig. 4.1C) and frequency (control: 2.5 ± 0.3 Hz vs. BAPTA: 1.8 ± 0.3 Hz, Fig. 4.1C). These data

suggest that BAPTA was reaching the postsynaptic site but did not inhibit ethanol-enhanced spontaneous GABA release. Therefore, the ability of BAPTA-AM to inhibit ethanol-enhanced spontaneous GABA release is occurring through a presynaptic mechanism.

Influx of extracellular calcium is not required for ethanol to increase mIPSC frequency. A nominally calcium free extracellular (0 mM Ca^{2+}_{ext}) solution was used to eliminate all sources of extracellular calcium that could contribute to the ethanol-induced increase in mIPSC frequency. When conducting this experiment in a slice, extended exposure to the 0 mM Ca^{2+}_{ext} solution is necessary to ensure that the solution is reaching the neuron; however, extended exposure to the 0 mM Ca^{2+}_{ext} solution can also reduce intracellular calcium levels, which is not desirable because the contribution of extracellular calcium needs to be investigated independently. Therefore, the mechanically dissociated neuron preparation was used because it allows for almost instantaneous access of the solution to the neuron and dramatically reduces the possibility of the 0 mM Ca^{2+}_{ext} solution affecting levels of intracellular calcium. In the presence of the 0 mM Ca^{2+}_{ext} solution, 50 mM (68.7 ± 11.7%, n = 9) and 100 mM (65.8 ± 13.7%, n = 8) ethanol significantly increased mIPSC frequency compared to control (-6.2 ± 3.8%, n = 7, Fig. 4.2A). Interestingly, the effect of ethanol on mIPSC frequency was significantly enhanced in the presence of the 0 mM Ca^{2+}_{ext} solution compared to the effect of ethanol on mIPSC frequency in control conditions (see Fig. 3.2A, $p<.05$, Student's t test). Compared to neurons exposed to control

conditions (1.7 ± 0.4 Hz, n = 11), the 0 mM Ca^{2+}_{ext} solution did not significantly decrease baseline mIPSC frequency (0.9 ± 0.2 Hz, n = 7), although there was a trend towards a decrease. To confirm that the solution around the neuron was free of calcium, the ability of the 0 mM Ca^{2+}_{ext} solution to block calcium-dependent GABA release was tested. Compared to control (0.5 ± 0.2 Hz, n = 4), addition of a high potassium (K^+, 15 mM) HEPES buffered solution in the absence of TTX increased the frequency of spontaneous IPSCs (5.2 ± 1.3 Hz, n = 4) by depolarizing the presynaptic terminal and activating voltage-dependent calcium channels, while a 0 mM Ca^{2+}_{ext} solution with the same high K^+ concentration returned the spontaneous IPSC frequency to baseline values (0.3 ± 0.03 Hz, n = 4).

Similar experiments were conducted with cadmium chloride ($CdCl_2$), a nonspecific voltage-dependent calcium channel inhibitor, to confirm the lack of involvement of extracellular calcium influx in the mechanism of ethanol-enhanced GABA release. As expected, 50 mM (40.6 ± 11.6%, n = 10) and 100 mM (50.3 ± 10.3%, n = 9) ethanol still significantly increased mIPSC frequency in the presence of 50 µM $CdCl_2$ compared to control (-4.8 ± 8.5%, n = 11, Fig. 4.2A). Interestingly, in the presence of 50 µM $CdCl_2$ the effect of ethanol did not always wash out, as demonstrated with a representative neuron in Figure 4.2B and C. Because a lack of washout could affect the calculated percent change in mIPSC frequency (see general methods), all of the $CdCl_2$ mechanically dissociated neuron data were reanalyzed without the washout included (data not shown). This additional analysis resulted in the same conclusion made from the data with

the washout included. Compared to neurons exposed to control conditions (1.4 ± 0.4 Hz, n = 9), 50 µM $CdCl_2$ did not significantly decrease baseline mIPSC frequency (0.9 ± 0.2 Hz, n = 11), although there was a trend towards a decrease. The same control with the high K^+ HEPES buffered solution was conducted in the presence of 50 µM $CdCl_2$. Compared to control (1.0 ± 0.6 Hz, n = 3), addition of a high K^+ HEPES buffered solution increased spontaneous IPSC frequency (14.1 ± 2.8 Hz, n = 3, $p<.05$, paired Student's t test), while addition of 50 µM $CdCl_2$ to the high K^+ HEPES buffered solution returned the frequency to baseline values (1.5 ± 0.7 Hz, n = 3). These data collectively suggest that influx of extracellular calcium is not required for ethanol-enhanced spontaneous GABA release.

Inhibition of calcium release from internal stores prevents the ethanol-induced increase in mIPSC frequency. To determine the involvement of internal calcium stores in ethanol-enhanced spontaneous GABA release, a sarco/endoplasmic-reticulum calcium ATPase (SERCA) pump inhibitor, thapsigargin, was used to prevent calcium reuptake into internal stores. Preventing calcium reuptake will eventually lead to depletion of the internal stores because the IP_3Rs and RyRs are continually releasing calcium from the internal stores; however, this process can take an extended period of time because of slow calcium release from the IP_3Rs and RyRs (Simkus and Stricker, 2002). Therefore, to diminish internal calcium stores at a faster rate I used a high potassium extracellular (K^+_{ext}) solution (15 mM) to depolarize the presynaptic terminals, which increases the rate of calcium release from the IP_3Rs

and RyRs (Simkus and Stricker, 2002). Any effect of the high K^+_{ext} solution on the mIPSCs disappeared within 2 minutes (n = 4, data not shown), and exposing the slice to the high K^+_{ext} solution had no effect on the ability of ethanol to increase mIPSC frequency after the high K^+_{ext} solution had been washed out (27.8 ± 5.0%, n = 3). After completing the high K^+_{ext} solution protocol in the presence of thapsigargin, ethanol was not able to increase mIPSC frequency (5.2 ± 4.7, n = 11, Fig. 4.3A). A representative neuron demonstrating the lack of ethanol effect on mIPSC frequency in the presence of thapsigargin is shown in Figure 4.3B. Compared to neurons exposed to control conditions (2.2 ± 0.4 Hz, n = 9), depleting internal stores did not have an effect on baseline mIPSC frequency (2.1 ± 0.4 Hz, n = 11).

Because depleting internal calcium stores prevented ethanol from increasing spontaneous GABA release, I next determined whether inhibition of the IP_3Rs and RyRs would prevent ethanol from increasing spontaneous GABA release. The IP_3R antagonist, 2-APB (14 µM), significantly blocked the ability of 100 mM ethanol to increase mIPSC frequency (5.1 ± 2.7%, n = 10, Fig. 4.3A). 2-APB (14 µM) did not have a significant effect on baseline mIPSC frequency (1.8 ± 0.3 Hz, n = 10) compared to control (2.2 ± 0.4 Hz, n = 9). Subsequently, the effect of ethanol on mIPSC frequency in the presence of a RyR antagonist was determined. At a concentration of 100 µM, ryanodine is an open-channel blocker of the RyR; however, it can take up to two hours to block the RyRs because of slow channel opening (Simkus and Stricker, 2002). To avoid this difficulty that is sometimes overlooked (Llano et al., 2000; Bardo et al., 2002), cells were

exposed to the high K^+_{ext} solution (Simkus and Stricker, 2002) to open the presynaptic RyRs and allow access of ryanodine to the channel. Inhibition of the RyRs was sufficient to block the effect of ethanol on mIPSC frequency (8.7 ± 4.6%, n = 13, Fig. 4.3A). Compared to neurons exposed to control conditions (2.2 ± 0.4 Hz, n=9), 100 µM ryanodine did not have an effect on baseline mIPSC frequency (2.2 ± 0.2 Hz, n = 13). These results suggest that calcium release from internal stores plays an important role in ethanol-enhanced spontaneous GABA release.

The cerebellar interneuron presynaptic terminals are immunopositive for IP_3R. To add further support to the conclusion that calcium release from internal stores is necessary for ethanol to increase spontaneous GABA release from the cerebellar interneurons, I used double-label immunogold electron microscopy to determine if IP_3Rs are expressed in the GABAergic interneuron presynaptic terminals. IP_3R and GABA immunogold labeling was colocalized in 18 out of the 30 (60%) presynaptic GABAergic terminals. In the absence of the IP_3R antibody, IP_3R and GABA immunogold labeling was colocalized in 4 out of the 30 (13%) presynaptic GABAergic terminals. This semi-quantitative analysis demonstrates that at least 45% of the presynaptic GABAergic terminals in the molecular layer are immunopositive for IP_3R and that there is minimal nonspecific binding with the IP_3R primary antibody.

In sections stained with just the IP_3R antibody, the amount of IP_3R staining in the GABA terminals, glutamate terminals, parallel fibers and Purkinje cell

dendrites was quantified. The densest staining was found in Purkinje cell dendrites (49.5 ± 6.4 particles/μm^2, n = 47, Figure 4.4A). GABA terminals (10.9 ± 2 particles/μm^2, n = 34), glutamate terminals (9.8 ± 1.8 particles/μm^2, n = 39), and parallel fibers (4.5 ± 1 particles/μm^2, n = 29, Fig. 4.4A) also contained IP_3R. Compared to the parallel fibers, taken as a control for background staining, IP_3R expression was significantly greater in the Purkinje cell dendrites, GABA terminals and glutamate terminals. Micrographs of a glutamatergic presynaptic terminal and a GABAergic presynaptic terminal immunopositive for IP_3R are shown in Figure 4.4B and 4.4C, respectively. Results from an experiment conducted with serial sections were also consistent with IP_3R staining being present in the GABAergic presynaptic terminals (data not shown). Additionally, every section for a profile was not immunopositive for IP_3R. Overall, these results suggest that there is IP_3R expression in the presynaptic terminals of the cerebellar interneurons.

DISCUSSION

The purpose of this study was to determine the role of calcium signaling in ethanol-enhanced spontaneous GABA release. Because incubating the slices with BAPTA-AM prevented ethanol from increasing spontaneous GABA release, the mechanism of ethanol-enhanced GABA release is dependent on calcium signaling. Ethanol was still able to increase spontaneous GABA release with BAPTA in the internal solution, which suggests that neither postsynaptic calcium nor calcium-dependent retrograde messengers are responsible for the effect of ethanol on spontaneous GABA release. This finding is consistent with previous results (Zhu and Lovinger, 2006). Therefore, ethanol is acting through a presynaptic, calcium-dependent mechanism to increase spontaneous GABA release.

Voltage-dependent calcium channels, receptor-operated channels and SOCs increase intracellular calcium levels by allowing extracellular calcium to flow into the neuron. A 0 mM Ca^{2+}_{ext} solution was used to eliminate the functionality of these channels to determine their involvement in the mechanism of ethanol-enhanced spontaneous GABA release. The mechanically dissociated neuron preparation was used for this experiment because it allows for instantaneous access of the 0 mM Ca^{2+}_{ext} solution to the neuron, which dramatically reduces the possibility of concomitantly reducing levels of intracellular calcium. Ethanol continued to increase spontaneous GABA release in the presence of the 0 mM Ca^{2+}_{ext} solution, which suggests that extracellular calcium influx does not play a role in this ethanol mechanism. Surprisingly, the

effect of ethanol on spontaneous GABA release in the presence of the 0 mM Ca^{2+}_{ext} solution was actually enhanced compared to control conditions. In the presence of the 0 mM Ca^{2+}_{ext} solution, only "extracellular-calcium insensitive" GABA release is left, which I predict is the specific type of GABA release that ethanol affects. Therefore, when the extracellular calcium sensitive mIPSCs were eliminated in the presence of the 0 mM Ca^{2+}_{ext} solution, ethanol had a larger effect on the % change in mIPSC frequency.

Because influx of extracellular calcium was not required for ethanol to increase spontaneous GABA release, the focus shifted to calcium release from internal stores. When internal stores were depleted of calcium with the thapsigargin protocol, ethanol was not able to increase spontaneous GABA release. Additionally, there was no significant change in spontaneous GABA release after using the thapsigargin protocol compared to a group of control neurons. In the absence of the high K^{+}_{ext} solution protocol, there is an increase in baseline mIPSC frequency in the presence of thapsigargin (Bardo et al., 2002; Li et al., 2004), which is consistent with calcium still being released from internal stores while thapsigargin is blocking calcium reuptake through the SERCA pump and/or activation of SOCs. There is evidence that the effect of thapsigargin on mIPSC frequency subsides after a period of time when even more calcium is depleted from the internal stores (Li et al., 2004). Therefore, because we used the high K^{+}_{ext} solution to deplete the stores at a faster rate, it is likely that any increase in mIPSC frequency due to incomplete emptying of the internal stores and/or activation of the SOCs occurred during exposure to the high K^{+}_{ext} solution.

After determining that calcium release from internal stores plays an imperative role in ethanol-enhanced spontaneous GABA release, I wanted to investigate whether the IP_3Rs and RyRs were involved in this ethanol mechanism. The IP_3R antagonist, 2-APB, significantly blocked the ethanol-induced increase in mIPSC frequency. Even though 2-APB is the most widely used membrane permeable IP_3R antagonist, there are questions regarding its selectivity with respect to intracellular calcium signaling that need to be discussed. When 2-APB concentrations higher than 90 µM are used, there is a nonspecific calcium leak from internal stores and slight inhibition of the SERCA pump (Missiaen et al., 2001). This nonspecific effect of 2-APB offers an explanation for the large increase in miniature excitatory postsynaptic current frequency (Simkus and Stricker, 2002) and mIPSC frequency (data not shown) seen with 2-APB concentrations higher than 80 µM. To circumvent these nonspecific effects of 2-APB, a low 2-APB concentration (14 µM) was used that did not increase baseline mIPSC frequency, which is consistent with the work of others (Simkus and Stricker, 2002; Glitsch, 2006).

There are additional 2-APB nonspecific effects that could occur at any concentration, including inhibition of SOCs as well as transient receptor potential (TRP) channels (Lievremont et al., 2005). Calcium entry through SOCs is activated when internal stores are depleted of calcium (Parekh and Penner, 1997); therefore, because inhibition of IP_3Rs prevents the depletion of internal calcium stores, the normal functioning of the SOCs is reduced. In addition, TRP channels are involved in this store-operated calcium entry mechanism (Zhu et al.,

1996). Therefore, because IP_3Rs, SOCs, and TRP channels are mechanistically linked, defining the selectivity of 2-APB has been controversial (Boulay et al., 1999; Lievremont et al., 2005). However, 2-APB was not acting through SOCs and TRP channels to inhibit ethanol-enhanced spontaneous GABA release because removal of extracellular calcium did not prevent ethanol from increasing spontaneous GABA release. Therefore, these data suggest that calcium release from the IP_3Rs is playing a role in ethanol-enhanced spontaneous GABA release.

Inhibition of RyRs also prevented ethanol from increasing spontaneous GABA release. The fact that both an IP_3R antagonist and a RyR antagonist can inhibit ethanol-enhanced spontaneous GABA release suggests that either both the IP_3R and RyR are necessary for this ethanol mechanism or that inhibition of calcium release from the IP_3R affects calcium release from the RyR, and vice versa. For the latter to be true, the IP_3R and RyR would have to exist on one internal store, but there is controversy surrounding whether separate IP_3R and RyR stores exist. Studies suggesting that separate stores exist were primarily conducted in cell lines and embryonic cultures, while studies supporting one internal store were conducted in primary cultures and brain slices (for review see Verkhratsky, 2005), which is similar to the slice preparation used in the present experiments. At this time I cannot distinguish between the relative importance of the IP_3Rs and RyRs in this ethanol mechanism; however, it is overwhelmingly evident that internal calcium stores play an imperative role in ethanol-enhanced spontaneous GABA release.

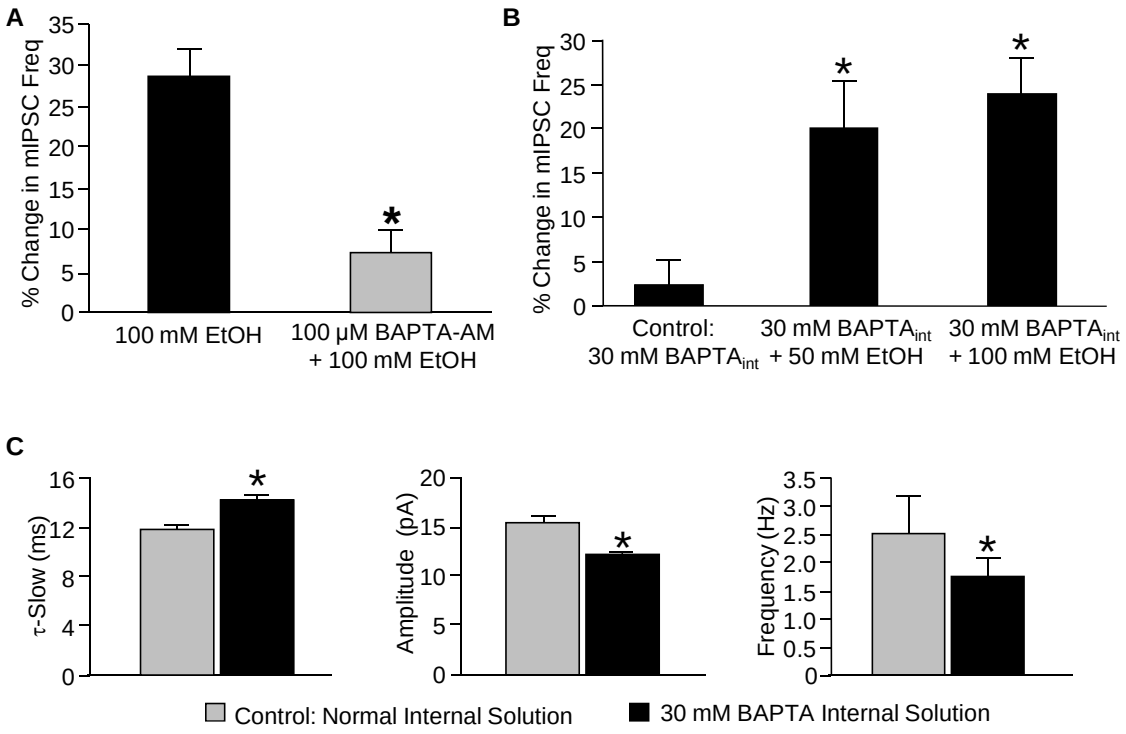
Because I was not able to definitively determine the involvement of the IP_3Rs and RyRs in ethanol-enhanced spontaneous GABA release, I wanted to determine if IP_3Rs and RyRs are expressed in the presynaptic terminals of the cerebellar interneurons. An elegant study by Llano and colleagues (2000) provided evidence from electrophysiology, calcium imaging and immunohistochemistry studies that RyRs play a functional role in the generation of spontaneous GABA release at the interneuron-Purkinje cell synapse. Since this report, there have been a number of studies confirming the role of RyRs in spontaneous neurotransmitter release as well as defining a role for RyRs in evoked transmitter release and long-term synaptic plasticity (for review see Collin et al., 2005b). For these reasons, I considered electron microscopy studies examining RyR expression at this synapse to be unnecessary.

Unlike the RyRs, few studies have examined whether IP_3Rs are expressed in presynaptic terminals. In rat barrel cortex, inhibition of IP_3Rs reduces spontaneous glutamate release, suggesting that IP_3Rs are present in the presynaptic terminals (Simkus and Stricker, 2002). However, like the current study, this study is limited due to the potential nonspecific effects seen with the IP_3R inhibitors (Boulay et al., 1999; Missiaen et al., 2001; Lievremont et al., 2005). In retinal amacrine cells, which release transmitter from dendrites, immunohistochemistry, electrophysiology and calcium imaging studies found that only the IP_3Rs contribute to the release of transmitter, despite the presence of both the IP_3R and RyR in the amacrine cell dendrites (Peng et al., 1991; Warrier et al., 2005). Electron microscopy studies in the bed nucleus of the stria

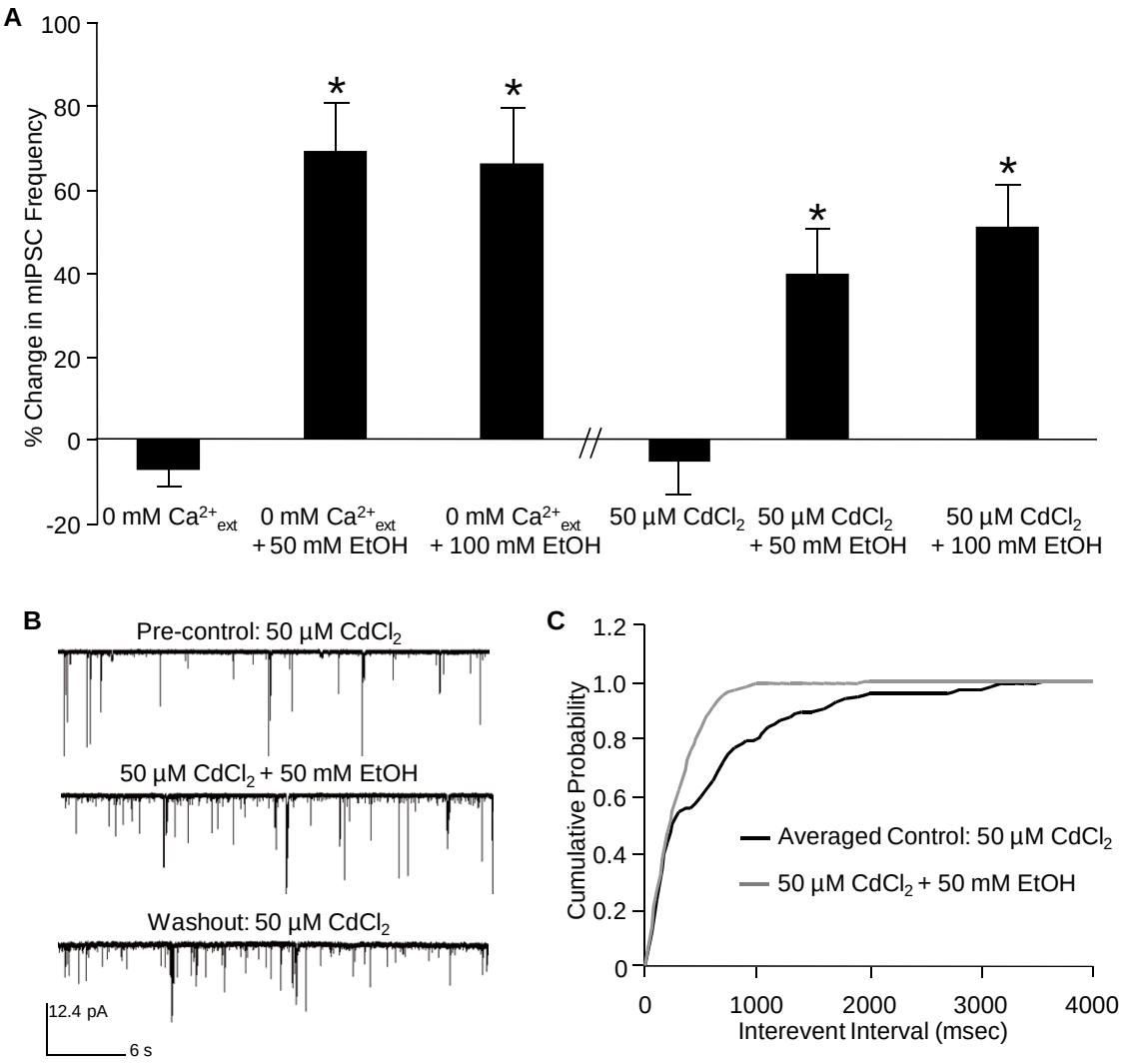
terminalis, deep cerebellar nuclei and substantia nigra show some IP_3R staining in synaptic terminals, but no quantification was done to further characterize this observation (Sharp et al., 1993b; Sharp et al., 1999). Light microscopy studies found IP_3R staining in the molecular layer of the cerebellum, which is described as “fine granular staining” suggestive of neuropil distribution (Sharp et al., 1999). Because of the lack of data on the presynaptic localization of IP_3Rs, I conducted extensive experiments to determine if IP_3Rs are located in the presynaptic terminals of cerebellar interneurons.

IP_3R staining was found in the presynaptic terminals of cerebellar interneurons using electron microscopy. The IP_3R antibody had been characterized previously (Sharp et al., 1993a), and I have shown that removal of the primary antibody resulted in minimal nonspecific staining. The cerebellar Purkinje cells had a much higher level of IP_3R staining compared to the presynaptic terminals and parallel fibers, which is consistent with IP_3R expression in Purkinje cells being the highest in the brain (Worley et al., 1989). Unfortunately, because there was IP_3R staining in all three layers (molecular, granule cell, and Purkinje) of the cerebellum (Sharp et al., 1999), there was not a straightforward negative control. However, IP_3R expression was significantly higher in the Purkinje cell dendrites, GABA terminals and glutamate terminals compared to the parallel fibers. If one assumes that the parallel fiber staining represented the maximum possible level of nonspecific staining, these results suggest there was significant IP_3R expression in the presynaptic GABAergic terminals. Therefore, the large preponderance of evidence argues in favor of the

IP_3R staining in the GABAergic presynaptic terminals reflecting actual IP_3R expression. While not every GABAergic terminal was immunopositive for IP_3R, it is not known if this variability is due something biological or experimental since there is an inherit randomness in the labeling process.



<u>Figure 4.1.</u> A presynaptic, calcium-dependent mechanism was responsible for the ethanol-induced increase in mIPSC frequency. A, incubation of the slice with BAPTA-AM (100 µM) prevented 100 mM ethanol (EtOH) from increasing mIPSC frequency (*, $p<.05$, Student's t test). B, ethanol (50 and 100 mM) still increased mIPSC frequency with 30 mM BAPTA in the internal solution ($BAPTA_{int}$; *, $p<.05$, one-way ANOVA, Dunnett's post hoc test). C, in the presence of 30 mM $BAPTA_{int}$, there was an increase in baseline mIPSC slow decay time (τ-Slow) and a decrease in baseline mIPSC amplitude and frequency (*, $p<.05$, Student's t test).



<u>Figure 4.2.</u> A presynaptic, calcium-dependent mechanism was responsible for the ethanol-induced increase in mIPSC frequency. A, incubation of the slice with BAPTA-AM (100 µM) prevented 100 mM ethanol (EtOH) from increasing mIPSC frequency (*, $p<.05$, Student's t test). B, ethanol (50 and 100 mM) still increased mIPSC frequency with 30 mM BAPTA in the internal solution ($BAPTA_{int}$; *, $p<.05$, one-way ANOVA, Dunnett's post hoc test). C, in the presence of 30 mM $BAPTA_{int}$, there was an increase in baseline mIPSC slow decay time (τ-Slow) and a decrease in baseline mIPSC amplitude and frequency (*, $p<.05$, Student's t test).

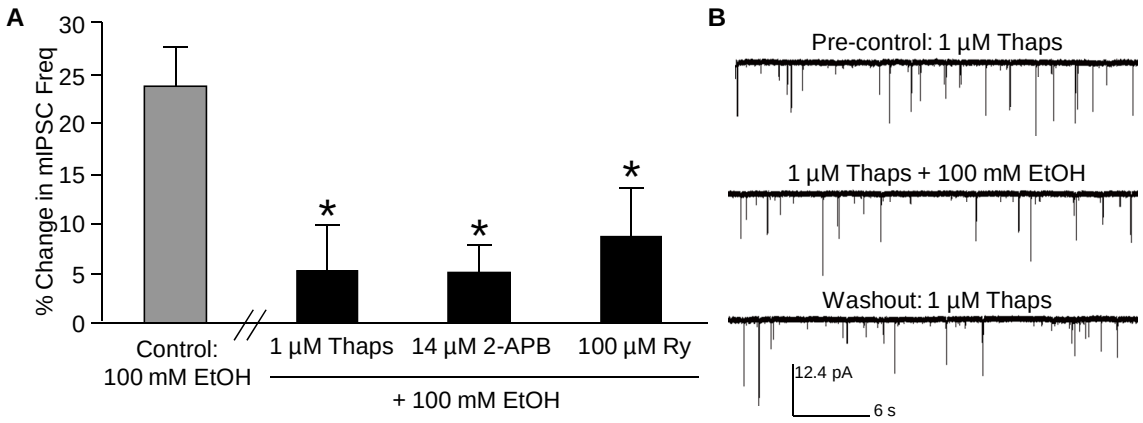


Figure 4.3. Inhibition of calcium release from internal stores prevented ethanol from increasing mIPSC frequency. A, the ability of 100 mM ethanol (EtOH) to increase mIPSC frequency was prevented by 1 µM thapsigargin (thaps), 100 µM ryanodine (Ry), and 14 µM 2-APB (*, $p<.05$, one-way ANOVA, Dunnett's post hoc test). B, a trace from a representative neuron showing the effect of 100 mM ethanol on mIPSC frequency in the presence of 1 µM thapsigargin.

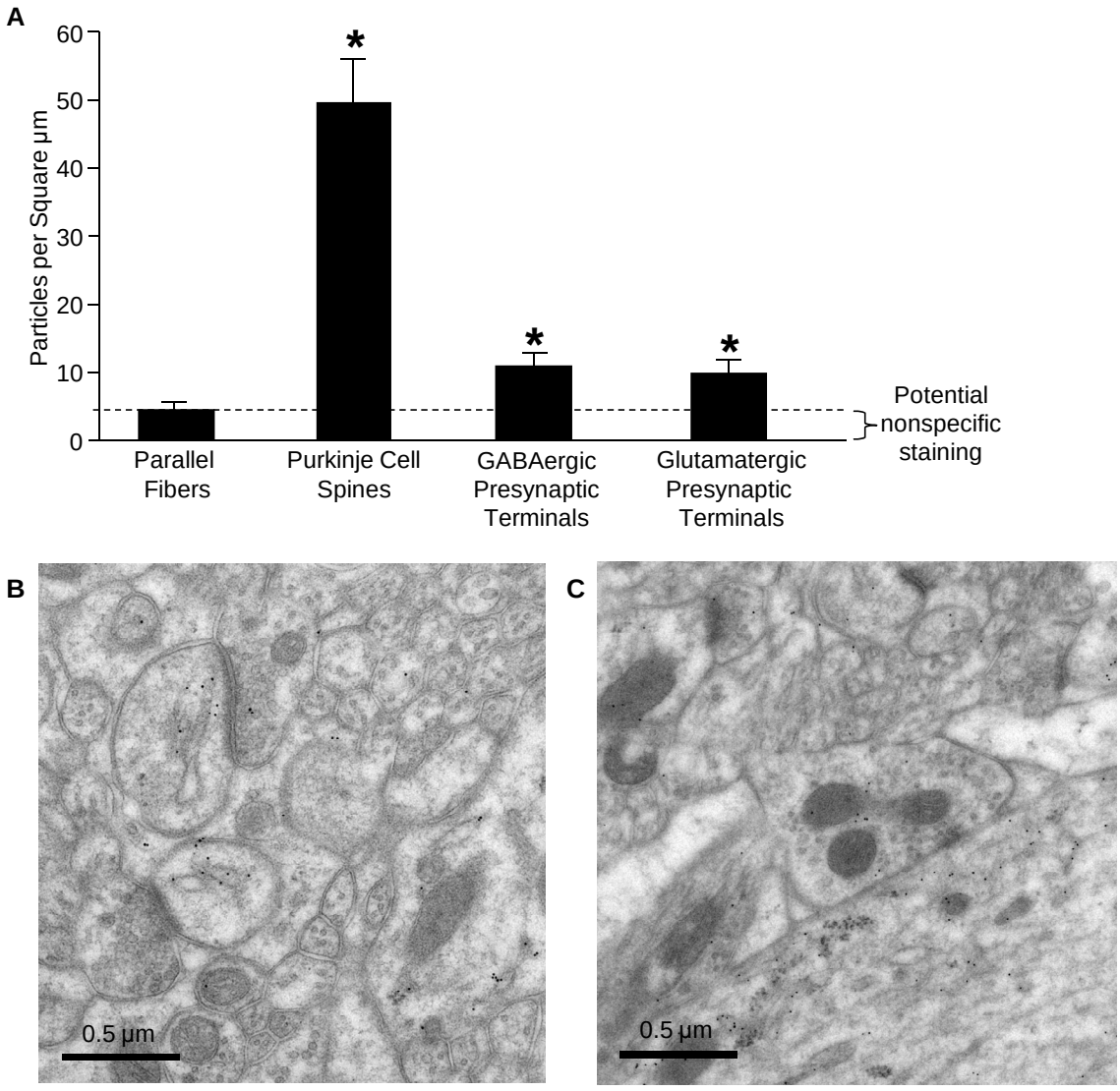


Figure 4.4. GABAergic presynaptic terminals were immunopositive for IP_3R. A, presynaptic terminals likely (because of their morphology) to be GABAergic (*, $p<.05$, Student's t test), probable glutamatergic presynaptic terminals (*, $p<.05$, Student's t test) and Purkinje cell spines (*, $p<.05$, Student's t test) had significantly more IP_3R staining compared to parallel fibers. B, a micrograph of a glutamatergic presynaptic terminal that is immunopositive for IP_3R (top). An immunonegative glutamatergic terminal is visible below it. C, a micrograph of an immunopositive GABAergic terminal that synapses onto a Purkinje cell dendrite.

Chapter V: The Role of the Adenylate Cyclase/PKA pathway in Ethanol-enhanced GABA Release

INTRODUCTION

I provided evidence that calcium release from internal stores plays an essential role in ethanol-enhanced spontaneous GABA release at the cerebellar interneuron-Purkinje cell synapse; moreover, this ethanol action is not dependent on the influx of extracellular calcium. However, the manner in which ethanol interacts with internal calcium stores is uncertain. While internal stores release calcium through activation of the IP_3Rs and RyRs, there is no current evidence suggesting that ethanol interacts directly with these receptors. The amount of calcium released from the IP_3Rs and RyRs is regulated by a number of factors, including calcium itself, nucleotides and protein kinases (Patterson et al., 2004; Bardo et al., 2006). Therefore, one hypothesis is that ethanol indirectly modulates calcium release from internal stores to influence GABA release.

In addition to internal calcium stores, ethanol-enhanced GABA release is altered by activation of GPCRs that are linked to $G\alpha_i$ and $G\alpha_s$ G proteins. Nociceptin, which is the endogenous ligand of a $G\alpha_i$-coupled GPCR (nociceptin/orphanin FQ peptide receptor), blocks ethanol from enhancing GABA release in the central nucleus of the amygdala (Roberto and Siggins, 2006). Antagonists for the δ-opioid receptor and the $GABA_B$ receptor, both of which are $G\alpha_i$-linked GPCRs, augment the ability of ethanol to increase GABA release in

the amygdala and hippocampus (Ariwodola and Weiner, 2004; Zhu and Lovinger, 2006; Kang-Park et al., 2007). Consistent with these results, activation of the CRF1 receptor, a GPCR coupled to $G\alpha_s$, enhances the effect of ethanol on GABA release in the central nucleus of the amygdala (Nie et al., 2004). These results suggest that a variety of $G\alpha_{i/s}$-coupled GPCRs can regulate ethanol-enhanced spontaneous GABA release.

Both the $G\alpha_i$ and $G\alpha_s$ subunits modulate adenylate cyclase with $G\alpha_s$ activating adenylate cyclase and $G\alpha_i$ inhibiting it. When adenylate cyclase is activated, it converts adenosine-5'-triphosphate (ATP) into 3'-5'-cyclic adenosine monophosphate (cAMP), which can bind to protein kinase A (PKA) regulatory subunits (Hanoune and Defer, 2001). The binding of cAMP to PKA frees the PKA catalytic subunits from the regulatory subunits, allowing the catalytic subunits to phosphorylate nearby targets. There are PKA phosphorylation sites on both the IP_3R (Mignery et al., 1990; Patterson et al., 2004) and RyR (Sobie et al., 2006), and phosphorylation of these receptors leads to increased calcium release (Bugrim, 1999; Bardo et al., 2006). In addition, PKA acts at the neurotransmitter release machinery to regulate synaptic transmission (Trudeau et al., 1996; Chheda et al., 2001; Seino and Shibasaki, 2005). Therefore, activation of adenylate cyclase and PKA could be playing a role in the ethanol-mediated increase in GABA release. The present study will investigate the role of the adenylate cyclase/PKA pathway in ethanol-enhanced spontaneous GABA release as well as the role of PKA in GABAergic neurotransmission.

RESULTS

Activation of $G\alpha_i$-coupled GPCRs prevents ethanol from increasing mIPSC frequency. Because of previous research that demonstrated a link between $G\alpha_{i/s}$-coupled GPCRs and ethanol-enhanced GABA release in other brain regions (Ariwodola and Weiner, 2004; Nie et al., 2004; Roberto and Siggins, 2006; Zhu and Lovinger, 2006; Kang-Park et al., 2007), it was determined whether this observation was also true at the interneuron-Purkinje cell synapse. Both $GABA_B$ receptors and cannabinoid 1 receptors are $G\alpha_i$-coupled GPCRs located in the molecular layer of the cerebellum, and activation of either receptor inhibits baseline mIPSC frequency at this synapse (Takahashi and Linden, 2000; Harvey and Stephens, 2004; Yamasaki et al., 2006). Similar to these results, application of a $GABA_B$ receptor agonist, baclofen (5 µM), or a cannabinoid receptor agonist, WIN 55,212-2 (WIN, 5 µM), caused a significant reduction in baseline mIPSC frequency (control: 2.3 ± 0.6 Hz, baclofen: 0.6 ± 0.2 Hz, n = 4; control: 2.9 ± 0.8 Hz, WIN: 1.1 ± 0.3 Hz, n = 13; Fig. 5.1A) with no significant effect on decay time or amplitude (data not shown). A cumulative probability curve from a representative neuron demonstrating that 5 µM WIN shifts the interevent interval curve to the right, which is interpreted as WIN decreasing mIPSC frequency, is shown in Figure 5.1B. In the presence of baclofen or WIN, the ability of ethanol to increase mIPSC frequency was significantly blocked (baclofen: 3.9 ± 7.1%, n = 5; WIN: -1.2 ± 3.4%, n = 8; Fig. 5.1C) compared to control (28.7 ± 3.4%, n = 12). Shown in Figure 5.1D is a cumulative probability curve from a representative neuron showing that 5 µM WIN blocks 100 mM

ethanol from shifting the curve to the left. Collectively, these data demonstrate that activation of the $G\alpha_i$-coupled GPCR pathway reduces baseline spontaneous GABA release and prevents ethanol-enhanced spontaneous GABA release at the interneuron-Purkinje cell synapse.

Tonic activation of $GABA_B$ receptors does not alter the ability of ethanol to increase mIPSC frequency and decrease the PPR. Because activation of $GABA_B$ receptors with baclofen inhibited ethanol-enhanced spontaneous GABA release from the interneurons, I wanted to determine if tonic activation of $GABA_B$ receptors was masking the extent of the ethanol effect on both spontaneous and evoked GABA release. First, it was determined if ethanol still increased mIPSC frequency to a similar extent in the presence of a $GABA_B$ receptor antagonist, CGP 52432 (10 µM). Compared to control (-1.8 ± 0.8%, n = 6, Fig. 5.2A), 50 mM (7.1 ± 1.9%, n = 6) and 100 mM (22.7 ± 2.1%, n = 6) ethanol significantly increased mIPSC frequency in the presence of CGP 52432 to a similar extent to that seen in the absence of CGP 52432.

To see if an effective concentration of CGP 52432 was used, the ability of CGP 52432 to antagonize the effect of baclofen on mIPSC frequency was determined. The baclofen (5 µM) effect on mIPSC frequency (control: 2.5 ± 0.6 Hz; baclofen: 1.2 ± 0.4 Hz, n = 6) was reversed in the presence of the $GABA_B$ receptor antagonist (CGP 52432 + baclofen: 2.4 ± 0.6 Hz, n = 6, Fig. 5.2B). A trace from a representative neuron showing the effect of baclofen on mIPSC frequency and the ability of CGP 52432 to reverse this effect is shown in Figure

5.2C. CGP 52432 (10 µM) significantly increased baseline mIPSC frequency (control: 1.6 ± 0.2 Hz; CGP 52432: 1.8 ± 0.3 Hz, n = 14, Fig. 5.2D), suggesting that there is tonic activation of $GABA_B$ receptors that affects spontaneous GABA release. In the presence of CGP 52432, 100 mM ethanol significantly decreased the PPR (20.7 ± 3.1%, n = 10, Fig. 5.2E) to a similar extent to that seen in the absence of CGP 52432. Overall, these results suggest that, despite tonic activation of $GABA_B$ receptors, antagonism of $GABA_B$ receptors does not alter the ability of ethanol to increase evoked and spontaneous GABA release.

A cannabinoid receptor antagonist has opposite effects on the ethanol-induced increase in mIPSC frequency and decrease in PPR. Activation of cannabinoid receptors with WIN inhibited ethanol-enhanced spontaneous GABA release (Fig. 5.1C); therefore, I wanted to determine if tonic activation of cannabinoid receptors was masking the extent of the ethanol effect on both spontaneous and evoked GABA release. First, it was determined if ethanol still increased mIPSC frequency to a similar extent in the presence of a cannabinoid receptor antagonist, AM-251 (5 µM). In the presence of AM-251, 50 mM (15.3 ± 3.4%, n = 7) and 100 mM (29.4 ± 7.1%, n = 7) ethanol significantly increased mIPSC frequency compared to control (0.9 ± 3.6%, n = 7, Fig. 5.3A) to a similar extent to that seen in the absence of AM-251.

To document that this was an effective concentration of AM-251 (5 µM), the ability of AM-251 to block the cannabinoid agonist-induced decrease in mIPSC frequency was tested. WIN (5 µM), significantly reduced (0.9 ± 0.2 Hz, n

= 6) mIPSC frequency compared to control (1.7 ± 0.2 Hz, n = 6), and 5 µM AM-251 reversed this WIN effect (1.6 ± 0.3 Hz, n = 6, Fig. 5.3B). AM-251 had no effect on baseline mIPSC frequency (control: 1.8 ± 0.2 Hz; AM-251: 2.0 ± 0.2 Hz, n = 13, Fig. 5.3C), suggesting that there is not tonic activation of cannabinoid receptors that affects spontaneous GABA release. These results suggest that a cannabinoid receptor antagonist has no effect on spontaneous GABA release or on ethanol-enhanced spontaneous GABA release. Interestingly, in the presence of AM-251, the ability of 100 mM ethanol to decrease the PPR was blocked (1.1 ± 4.3%, n = 8, Fig. 5.3E) and AM-251 significantly decreased the baseline PPR (44.4 ± 12.9%, n = 6, Fig. 5.3D). These results demonstrate that there is tonic activation of cannabinoid receptors that affects evoked GABA release and that inhibition of cannabinoid receptors blocks ethanol-enhanced evoked GABA release.

Inhibition of adenylate cyclase or PKA blocks ethanol from increasing mIPSC frequency. Next it was determined if inhibiting adenylate cyclase and PKA, the intracellular messengers downstream of the $G\alpha_i$-coupled GPCRs, could prevent ethanol from increasing spontaneous GABA release. To assess the role of adenylate cyclase in this ethanol mechanism, two different purine site inhibitors (SQ 22,536 and DDA) were used that inhibit all isoforms of adenylate cyclase (Dessauer et al., 1999). As shown in Figure 5.4A, the ability of ethanol to increase mIPSC frequency was significantly reduced in the presence of 300 µM SQ 22,536 (15.0 ± 3.6%, n = 10) and 10 µM DDA (12.4 ± 2.3%, n = 9)

compared to control. SQ 22,536 inhibits the norepinephrine-induced increase in mIPSC frequency at the interneuron-Purkinje cell synapse (Harvey and Stephens, 2004), which is an effect mediated by activation of $G\alpha_s$-coupled GPCRs. Similar to these results, 10 µM norepinephrine caused a 103 ± 11.8% (n = 5) increase in mIPSC frequency, while in the presence of 300 µM SQ 22,536 the norepinephrine effect was significantly reduced to 15.0 ± 5.8% (n = 3, $p<05$, Student's t-test). Therefore, an effective concentration of SQ 22,536 was used in these experiments. These results suggest that adenylate cyclase is involved in ethanol-enhanced spontaneous GABA release.

To determine the role of PKA in ethanol-enhanced GABA release, two PKA antagonists, H-89 and Rp-cAMP, were used that have different mechanisms of action. H-89 acts at the PKA ATP-binding site, while Rp-cAMP binds to the cAMP binding sites to prevent the regulatory subunits from dissociating from the catalytic subunits (Lochner and Moolman, 2006). Both 10 µM H-89 and 10 µM Rp-cAMP significantly reduced the ability of ethanol to increase mIPSC frequency (8.2 ± 2.1%, n = 7 and 1.4 ± 2.5%, n = 10, respectively, Fig. 5.4A) compared to control. A trace from a representative neuron showing the effect of ethanol on mIPSC frequency in the presence of 10 µM Rp-cAMP is shown in Figure 5.4B. A lower Rp-cAMP concentration (1 µM) did not prevent ethanol from increasing mIPSC frequency (22.5 ± 8.2%, n = 3). A higher concentration of H-89 was not tested because of known nonspecific effects that can start to occur at even 10 µM (Lochner and Moolman, 2006). Overall, these results suggest that PKA plays an important role in ethanol-enhanced spontaneous GABA release.

To determine if the PKA antagonists were acting at the presynaptic terminal and not the postsynaptic neuron, the membrane impermeable PKA antagonist, PKI, was included in the pipette internal solution, which limits the PKA antagonist to the postsynaptic neuron. With 5 µM PKI in the pipette internal solution, both 50 (14.6 ± 2.8%, n = 8) and 100 mM (27.1 ± 9.2%, n = 6) ethanol significantly increased mIPSC frequency compared to control (-1.3 ± 1.6%, n = 8, Fig. 5.4C). A cumulative probability curve from a representative neuron shows that 100 mM ethanol still shifts the distribution of the interevent interval curve to the left with 5 µM PKI in the pipette internal solution (Fig. 5.4D). Because there was no PKI effect, there was concern that PKI was not reaching the postsynaptic neuron; however, PKI blocked the effect of a PKA agonist in a separate experiment (see Fig. 5.6A). These results suggest that the PKA antagonist is acting presynaptically to inhibit ethanol-enhanced spontaneous GABA release.

Cannabinoids and ethanol act through similar downstream messengers to alter mIPSC frequency. A CB1 receptor agonist decreases spontaneous GABA release at the interneuron-Purkinje cell synapse (Fig. 5.1A), and this effect involves calcium release from RyRs (Yamasaki et al., 2006). Therefore, it was determined if the downstream messengers shown to play a role in the ethanol-induced increase in spontaneous GABA release also play a role in the cannabinoid-induced decrease in spontaneous GABA release. To confirm the involvement of internal calcium stores, the thapsigargin protocol described in Chapter 4 was used. Compared to control (58.4 ± 3.0%, n = 13), 1 µM

thapsigargin significantly prevented WIN from decreasing mIPSC frequency (21.7 ± 7.7%, n = 7, Fig. 5.5A). The high potassium (15 mM) extracellular solution in the absence of thapsigargin had no effect on the ability of WIN to decrease mIPSC frequency (55.8 ± 5.5%, n = 5, Fig. 5A). The IP_3R antagonist, 2-APB (14 µM), significantly reduced the ability of WIN to decrease mIPSC frequency (31.5 ± 6.3%, n = 8, Fig. 5.5A). As mentioned above, a similar effect has been shown with a RyR antagonist (Yamasaki et al., 2006). The voltage dependent calcium channel inhibitor, $CdCl_2$ (50 µM), did not significantly prevent WIN from decreasing mIPSC frequency (43.3 ± 6.9%, n = 8, Fig. 5.5A). In addition, inclusion of 30 mM BAPTA in the pipette internal solution, which limits BAPTA to the postsynaptic neuron, was ineffective at blocking WIN (46.4 ± 3.8%, n = 8, Fig. 5A). Overall, these results suggest that the mechanism of the cannabinoid agonist-mediated decrease in mIPSC frequency is a presynaptic, calcium-dependent process that most likely involves calcium release from internal stores with minimal involvement (if any at all) from the voltage dependent calcium channels.

Next the role of PKA in the cannabinoid-induced decrease in mIPSC frequency was determined. The ability of WIN to decrease mIPSC frequency was significantly reduced in the presence of the PKA agonist dBcAMP (100 µM: 37.1 ± 4.4%, n = 7; 300 µM: 31 ± 9.3%, n = 7; Fig. 5.5B) compared to control, but there was no effect at 30 µM dBcAMP (48.3 ± 8.0%, n = 6). There was a significant linear trend across concentrations for the effect of the PKA agonist on the WIN-induced decrease in mIPSC frequency (n = 7, r = -0.53, $p<.05$), which

suggests that dBcAMP inhibits WIN from decreasing spontaneous GABA release in a dose-dependent manner.

Activation of PKA affects baseline mIPSC properties. At 30 and 100 µM dBcAMP, there was a significant decrease in baseline mIPSC amplitude (30 µM: 21.6 ± 4.5%, n = 7; 100 µM: 18.0 ± 7.4%, n = 7) with no change in baseline mIPSC τ-$_{slow}$ (Fig. 5.6A). At 300 µM dBcAMP, there was a significant decrease in baseline mIPSC amplitude (32 ± 2.9%, n = 6. Fig. 5.6A) and increase in baseline mIPSC slow decay time (33.7 ± 9.4%, n = 6). A trace from representative neuron showing that 300 µM dBcAMP increases mIPSC slow decay time and decreases mIPSC amplitude is shown in Figure 5.6Ba. There was no effect on baseline mIPSC frequency or on baseline mIPSC fast decay time at any dBcAMP concentration (data not shown).

The change in mIPSC slow decay time and amplitude appears to be due to a postsynaptic PKA mechanism because inclusion of 5 µM PKI in the internal solution blocked 300 µM dBcAMP from increasing mIPSC slow decay time (-1.0 ± 4.4%, n = 7) and decreasing mIPSC amplitude (2.3 ± 4.9%, n = 7, Fig. 5.6A). A trace from a representative neuron showing this PKI effect is in Figure 5.6Bb. These results suggest that the PKA agonist is having a PKA-dependent, postsynaptic effect that is manifested through a change in mIPSC slow decay time and amplitude.

Based on the above results, it appeared as if a PKA agonist did not increase spontaneous GABA release. While I was initially hesitant to try higher

concentrations of the PKA agonist, dBcAMP, there are reports that higher concentrations of the PKA agonist could increase mIPSC frequency (Kano and Konnerth, 1992; Kondo and Marty, 1997); therefore, a higher concentration of dBcAMP was tested. At 1 mM dBcAMP, there was a significant increase in mIPSC frequency (control: 1.3 ± 0.1 Hz, dBcAMP: 2.0 ± 0.2 Hz, n = 8, Fig. 5.6C). Consistent with the results seen at lower dBcAMP concentrations, there was also an increase in mIPSC slow decay time (control: 13.2 ± 0.8 ms, dBcAMP: 24.1 ± 4.8 ms, Fig. 5.6C) and a decrease in mIPSC amplitude (control: 24 ± 1.2 pA, dBcAMP: 19.8 ± 1.3 pA, Fig. 5.6C). These results suggest that a PKA agonist has both presynaptic and postsynaptic effects at the GABAergic synapse.

Buffering presynaptic calcium prevents a PKA antagonist from decreasing baseline mIPSC frequency. Because of the established role of PKA in neurotransmitter release (Trudeau et al., 1996; Chheda et al., 2001; Seino and Shibasaki, 2005), the effect of a PKA antagonist on baseline mIPSC frequency was tested. Both 10 µM and 25 µM Rp-cAMP significantly decreased mIPSC frequency (by 23.3 ± 6.8%, n = 7 and 31.2 ± 3.6%, n = 11, respectively), while 1 µM Rp-cAMP was without effect (0.6 ± 4.8%, n = 4, Fig. 5.7A). At these concentrations of Rp-cAMP, no changes in mIPSC decay time or amplitude were observed (data not shown). In Figure 5.7B a cumulative probability curve from a representative neuron demonstrates that 25 µM Rp-cAMP shifts the distribution of the interevent interval curve to the right, which is interpreted as Rp-cAMP decreasing mIPSC frequency. At 100 µM Rp-cAMP there was a significant

decrease in mIPSC frequency (78.9 ± 8.3%, n = 3), but there was also a significant decrease in mIPSC amplitude (29.3 ± 3.9%), making it difficult to conclude whether a presynaptic and/or postsynaptic mechanism was responsible for this change. These results suggest that presynaptic activation of PKA plays a role in the generation of spontaneous GABA release.

Because of the role of calcium and PKA in the ethanol and cannabinoid-induced change in spontaneous GABA release, I determined whether there is a link between PKA, calcium, and spontaneous GABA release. In the presence of BAPTA-AM, a membrane permeable calcium chelator, the ability of 25 µM Rp-cAMP to decrease mIPSC frequency was significantly reduced (5.9 ± 2.5%, n = 8, Fig. 5.7C) compared to control. A cumulative probability curve from a representative neuron shows that 100 µM BAPTA-AM prevents 25 µM Rp-cAMP from shifting the curve (Fig. 5.7D). Addition of 30 mM BAPTA to the pipette internal solution did not prevent Rp-cAMP from decreasing mIPSC frequency (31.8 ± 3.8%, n = 7, Fig. 5.7C). These results suggest that changes in presynaptic calcium are required for a PKA antagonist to decrease mIPSC frequency. Thapsigargin was also able to significantly block 25 µM Rp-cAMP from decreasing mIPSC frequency (10.7 ± 5.6%, n = 6), suggesting that this Rp-cAMP mechanism involves calcium release from internal stores. However, the high potassium solution protocol also blocked the PKA antagonist effect (-2.1 ± 2.6%, n = 3). Therefore, the role of internal calcium stores in the PKA antagonist-mediated suppression of spontaneous GABA release is inconclusive.

DISCUSSION

The $G\alpha_i$-coupled GPCR agonists, WIN and baclofen, blocked ethanol from increasing spontaneous GABA release at the interneuron-Purkinje cell synapse. Consistent with these results, it has been presented recently that WIN inhibits ethanol-enhanced GABA release in the basolateral and central nucleus of the amygdala (Roberto et al., 2008; Talani and Lovinger, 2008). Similarly, baclofen prevents ethanol from increasing action potential-dependent GABA release in the hippocampus (Ariwodola and Weiner, 2004). However, in the VTA baclofen does not inhibit ethanol from increasing action potential-dependent GABA release, despite the fact that baclofen affects action potential-dependent GABA release (Theile et al., 2008).

Because activation of $G\alpha_i$-linked GPCRs blocked ethanol from increasing spontaneous GABA release onto cerebellar Purkinje cells, I tested if tonic activation of $G\alpha_i$-coupled GPCRs was preventing ethanol from fully eliciting GABA release at the interneuron-Purkinje cell synapse. Despite tonic activation of the $GABA_B$ receptors, a $GABA_B$ receptor antagonist did not enhance the ability of ethanol to increase mIPSC frequency or decrease the PPR at the interneuron-Purkinje cell synapse. Similar results are seen in the VTA (Theile et al., 2008); however, a $GABA_B$ receptor antagonist enhances the ability of ethanol to increase GABA release onto basolateral amygdala neurons and CA1 hippocampal neurons (Ariwodola and Weiner, 2004; Zhu and Lovinger, 2006). Overall, these variable results suggest that the ability of the $GABA_B$ receptor

agonist and antagonist to affect ethanol-enhanced spontaneous GABA release is brain region-specific.

There was not tonic activation of the cannabinoid receptors that affected spontaneous GABA release, and consistent with this, a cannabinoid receptor antagonist had no effect on the ethanol-induced increase in spontaneous GABA release. There was tonic activation of cannabinoid receptors that decreased evoked GABA release, and a cannabinoid receptor *antagonist* actually blocked the ability of ethanol to increase *evoked* GABA release. This result was unexpected considering that a cannabinoid receptor *agonist* blocks ethanol-enhanced *spontaneous* GABA release. Because mGluR1 activation on Purkinje cells can induce endocannabinoid release from the Purkinje cell (for review see Hashimotodani et al., 2007), I hypothesized that the lack of ethanol effect in the presence of the CB receptor antagonist was due to a mGluR1-mediated effect. However, in the presence of a mGluR1 antagonist (JNJ 16259685, 20 μM) and a cannabinoid receptor antagonist, ethanol still did not increase evoked GABA release (n = 3, data not shown). Another hypothesis is the cannabinoid receptor antagonist increases the amount of evoked glutamate release (in addition to GABA release), which could activate presynaptic group II mGluRs that are not normally activated in the absence of the cannabinoid receptor antagonist. Like the $GABA_B$ receptors and cannabinoid receptors, the mGluRs are $G\alpha_i$-linked GPCRs that inhibit GABA release at the interneuron-Purkinje cell synapse (Raiteri, 2008). Therefore, I predict that activation of group II mGluRs due to the presence of the cannabinoid receptor antagonist inhibits ethanol-enhanced

evoked GABA release. This possibility will be tested in the future by combining a group II mGluR antagonist with the cannabinoid receptor antagonist and determining if ethanol can increase evoked GABA release in these conditions.

Adenylate cyclase and PKA, which are downstream messengers of the $G\alpha_{i/s}$–coupled GPCRs, contribute an essential part to ethanol-enhanced spontaneous GABA release. Because a membrane impermeable PKA antagonist in the pipette internal solution did not prevent ethanol from increasing spontaneous GABA release, this PKA effect is occurring presynaptically. There is considerable evidence connecting the adenylate cyclase/PKA pathway to the effects of ethanol (Pandey, 1998; Newton and Messing, 2006). Adenylate cyclase isoforms 1,7 and 8 have all been linked to ethanol with biochemical, electrophysiological and behavioral studies in transgenic mice (Hanoune and Defer, 2001; Maas et al., 2005). Through a PKA dependent mechanism, an *in vivo* ethanol exposure induces a long-lasting potentiation of GABAergic synapses in the VTA (Melis et al., 2002). The adenosine A2 receptor, which leads to increased activation of the adenylate cyclase/PKA pathway, mediates important ethanol effects (Mailliard and Diamond, 2004). At the behavioral level, a reduction in PKA signaling affects alcohol consumption and the sensitivity to the sedative effects of alcohol (Thiele et al., 2000; Wand et al., 2001; Fee et al., 2006; Misra and Pandey, 2006; Lai et al., 2007). Therefore, the adenylate cyclase/PKA pathway plays an important role in multiple alcohol actions extending from molecular to behavioral.

To learn more about the mechanism behind the ethanol-induced *increase* in GABA release, I studied the mechanism responsible for the WIN-induced *decrease* in GABA release. Previously it was shown that calcium release from RyRs plays a role in this cannabinoid mechanism (Yamasaki et al., 2006). The present work confirmed the involvement of internal calcium stores in the cannabinoid-induced decrease in GABA release and illustrated that calcium release from IP_3Rs is involved. Voltage dependent calcium channels did not play a significant role in the cannabinoid-mediated suppression of spontaneous GABA release at this synapse; a similar conclusion was made previously based on data showing that $CdCl_2$ has no significant effect on baseline mIPSC frequency (Takahashi and Linden, 2000). Additionally, including BAPTA in the internal solution did not significantly block the cannabinoid-induced decrease in spontaneous GABA release, which supports the idea that the calcium-dependent portion of this cannabinoid mechanism is occurring presynaptically.

A PKA agonist was used to determine the role of PKA in the cannabinoid-mediated suppression of spontaneous GABA release. The PKA agonist, dBcAMP, dose-dependently reduced the ability of WIN to decrease mIPSC frequency, which suggests that the ability of the cannabinoid agonist to decrease spontaneous GABA release involves inhibition of PKA. This result is consistent with activation of $G\alpha_i$-coupled GPCRs leading to reduced activation of PKA, and data showing that a PKA antagonist decreases spontaneous GABA release (Fig. 5.7A; Jeong et al., 2003; Lee et al., 2008). Overall, these results suggest that internal calcium stores and PKA are playing an important role in the CB-mediated

decrease in spontaneous GABA release, as has been shown for the mechanism of ethanol-enhanced spontaneous GABA release.

I determined if a PKA agonist had an effect on GABAergic neurotransmission. Interestingly, during the application of 300 µM dBcAMP baseline mIPSC slow decay time and amplitude were increased and decreased, respectively. Both deceases and increases in mIPSC amplitude have been reported after application of PKA and PKA agonists (Kano and Konnerth, 1992; Nusser et al., 1999; Poisbeau et al., 1999). A possible reason for this discrepancy is differences in $GABA_A$ receptor subunit composition and in $GABA_A$ receptor associated proteins (Nusser et al., 1999). Regardless, this effect appears to be a postsynaptic, PKA specific action because the dBcAMP effect was blocked when a membrane impermeable PKA antagonist was included in the pipette internal solution. At a higher concentration of the PKA agonist (1 mM), there was the same increase and decrease in mIPSC slow decay time and amplitude, respectively. However, there also was an increase in mIPSC frequency, which is consistent with other studies using this high concentration (Kano and Konnerth, 1992; Kondo Marty, 1997). Possible explanations for the necessity of a high dBcAMP concentration to increase spontaneous GABA release include low cell permeability of dBcAMP at the presynaptic terminal and/or degradation of dBcAMP by phosphodiesterases.

The PKA antagonist, Rp-cAMP (10 µM and 25 µM), inhibited baseline spontaneous GABA release, which is consistent with similar studies conducted in the hippocampus and hypothalamus (Jeong et al., 2003; Lee et al., 2008). There

was no PKA antagonist effect in the tuberomammillary nucleus, but this could be due to the low PKA antagonist concentration used in this study (Yum et al., 2008). At 1 µM Rp-cAMP, there was no effect on baseline spontaneous GABA release or on ethanol-enhanced spontaneous GABA release. These results suggest that the concentration of the PKA antagonist must be high enough to decrease baseline spontaneous GABA release if the antagonist is going to be effective at reducing ethanol-enhanced spontaneous GABA release.

Incubating slices with BAPTA-AM blocked Rp-cAMP from decreasing mIPSC frequency, suggesting that the ability of a PKA antagonist to decrease spontaneous GABA release involves a calcium-dependent mechanism. When BAPTA was limited to the postsynaptic neuron, Rp-cAMP still decreased GABA release. These results suggest that a presynaptic, calcium-dependent mechanism is responsible for the PKA antagonist-mediated decrease in spontaneous GABA release. Two possible calcium-dependent mechanisms related to PKA involve PKA phosphorylating the IP_3R and RyR to increase calcium release from internal stores (Mignery et al., 1990; Bugrim, 1999; Patterson et al., 2004; Bardo et al., 2006; Sobie et al., 2006) and/or PKA phosphorylating a protein in the neurotransmitter release machinery that is involved in calcium dependent exocytosis (Trudeau et al., 1996; Chheda et al., 2001).

I attempted to determine if internal calcium stores are involved in the Rp-cAMP-mediated suppression of spontaneous GABA release. Even though the thapsigargin protocol was successful at blocking the PKA antagonist effect, the

results are not interpretable because the high potassium solution protocol had the same effect. One possible explanation is that the presynaptic depolarization induced by the high potassium solution altered the phosphorylation state of proteins that are normally affected by the PKA antagonist. Therefore, the role of internal calcium stores in the PKA antagonist-induced decrease in spontaneous GABA release is unknown. However, cAMP-dependent GABA release occurs in the absence of extracellular calcium (Kondo and Marty, 1997), which suggests the possibility that PKA-mediated changes in spontaneous GABA release are not dependent on the influx of extracellular calcium.

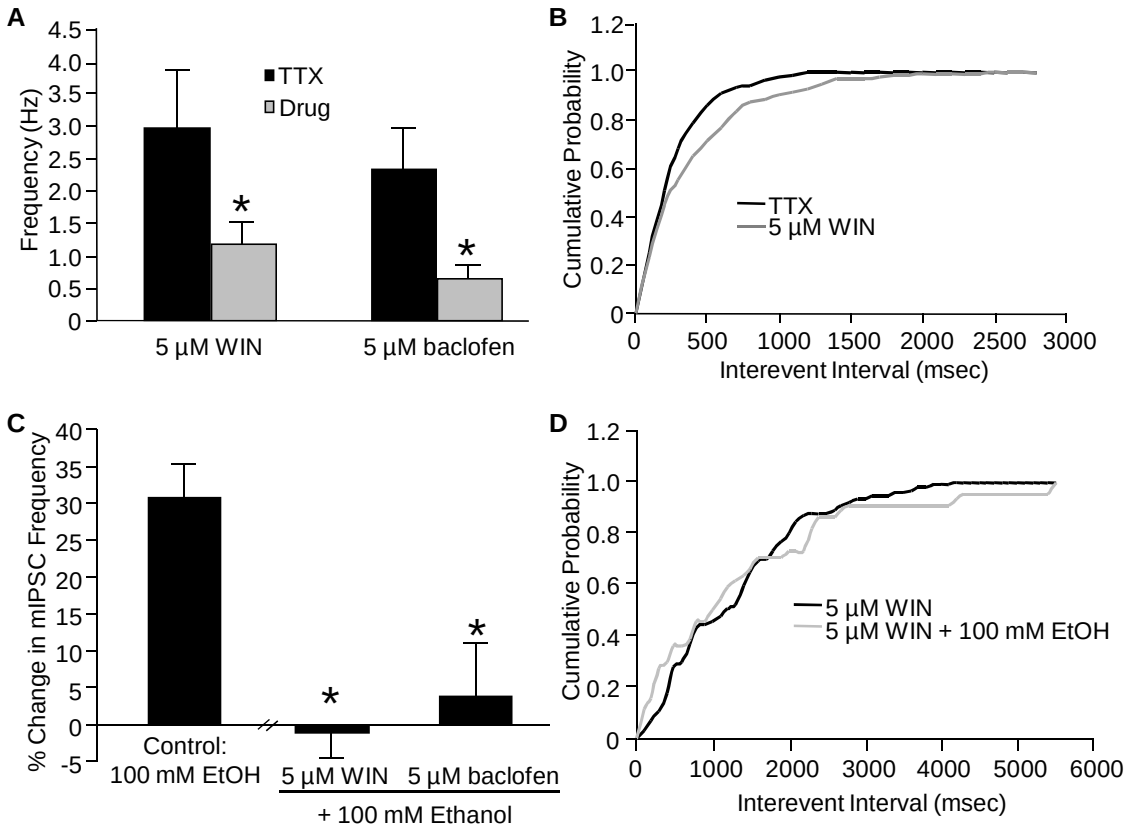


Figure 5.1. WIN 55,212-2 and baclofen decreased baseline mIPSC frequency and prevented ethanol from increasing mIPSC frequency. A, WIN 55,212-2 (WIN, 5 µM) and baclofen (5 µM) reduced baseline mIPSC frequency (*, p<.05, paired Student's t test). B, a cumulative frequency histogram showing the effect of 5 µM WIN on the interevent interval curve. C, WIN and baclofen prevented 100 mM ethanol (EtOH) from increasing mIPSC frequency (*, p<.05, one-way ANOVA, Dunnett's post hoc test). D, a cumulative frequency histogram from a representative neuron demonstrating the effect of 100 mM ethanol on the interevent interval curve in the presence of WIN.

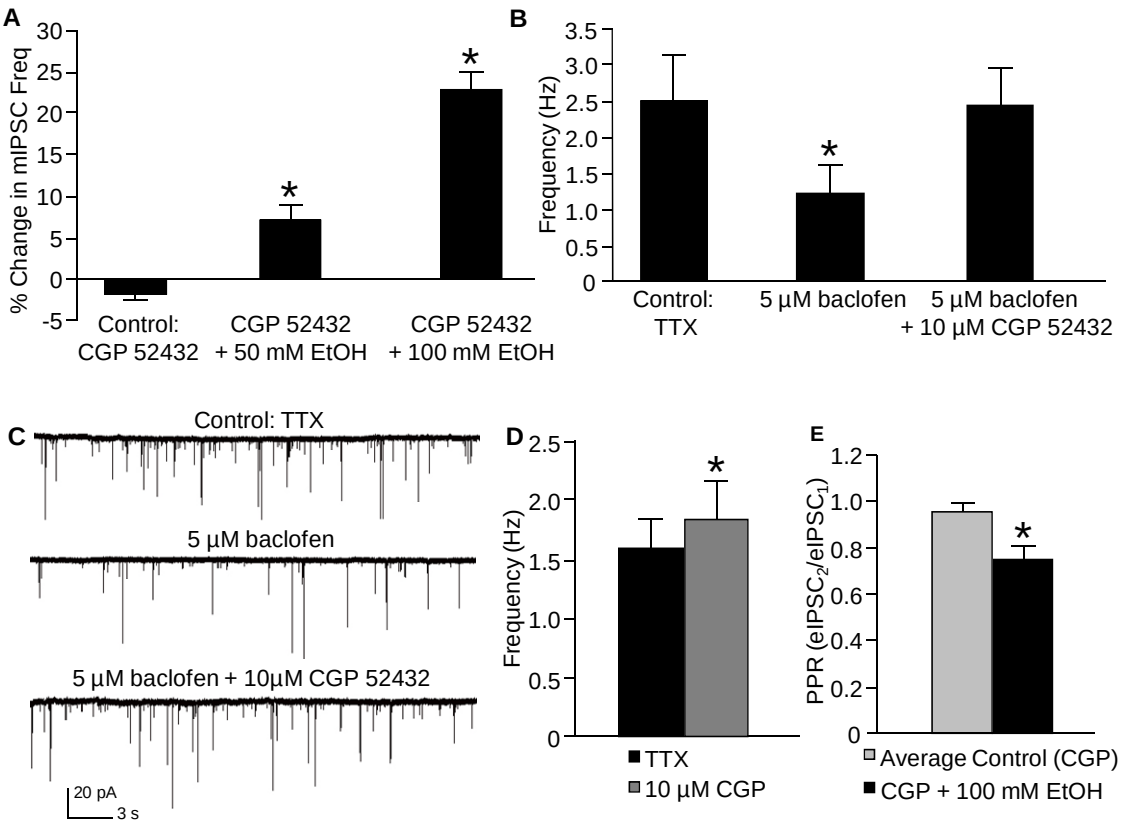
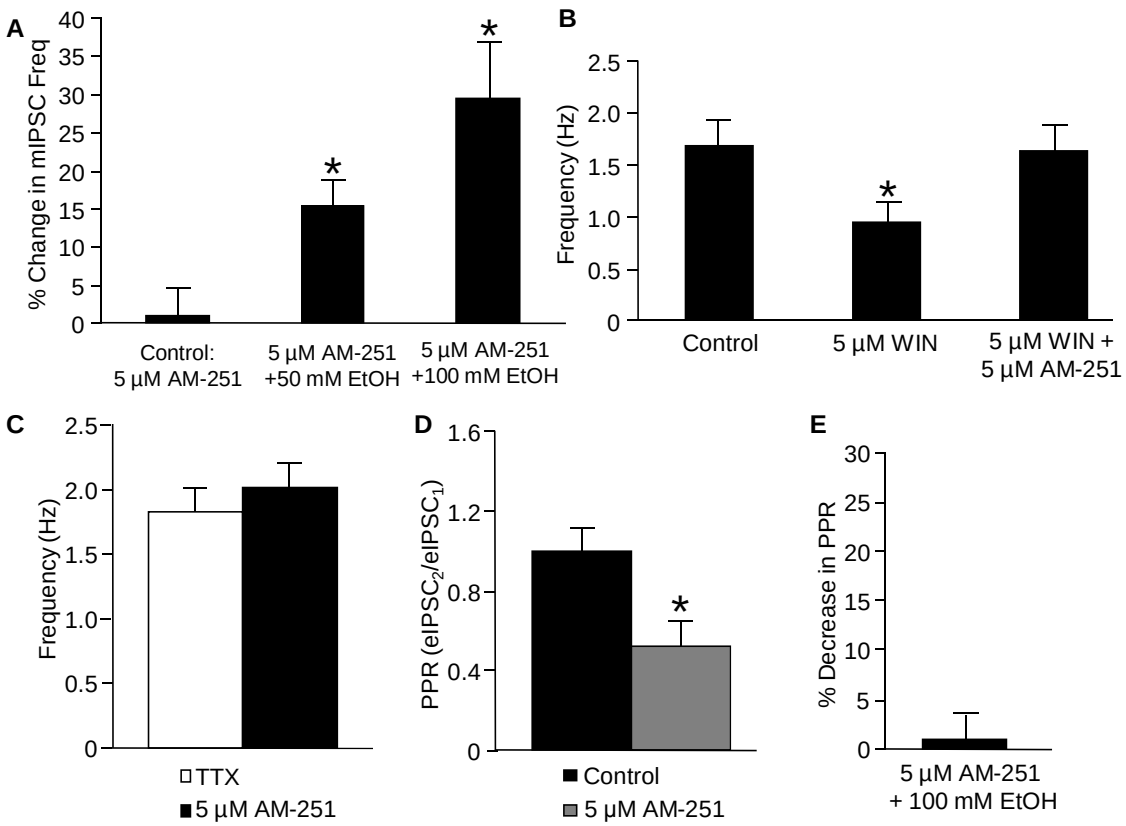


Figure 5.2. A $GABA_B$ receptor antagonist did not affect the ethanol-induced increase in mIPSC frequency or decrease in PPR. A, CGP 52432 (CGP, 10 µM) did not alter the ability of 50 and 100 mM ethanol to increase mIPSC frequency (*, p<.05, one-way ANOVA, Dunnett's post hoc test). B, the reduction in mIPSC frequency by baclofen (5 µM) was reversed by CGP (*, p<.05, paired Student's t test). C, a trace from a representative neuron showing the effect of baclofen on mIPSC frequency and the ability of CGP to reverse it. D, CGP increased baseline mIPSC frequency (*, p<.05, paired Student's t test). E, CGP did not affect the ability of 100 mM ethanol to decrease the PPR (*, p<.05, paired Student's t test).



<u>Figure 5.3.</u> A cannabinoid receptor antagonist had no effect on the ethanol-induced increase in mIPSC frequency but blocked the ethanol-induced decrease in PPR. A, AM-251 (5 µM) did not alter the ability of 50 and 100 mM ethanol (EtOH) to increase mIPSC frequency (*, $p<.05$, one-way ANOVA, Dunnett's post hoc test). B, the reduction in mIPSC frequency by WIN (5 µM) was reversed by AM-251 (*, $p<.05$, paired Student's t test). C, AM-251 had no effect on baseline mIPSC frequency. D, AM-251 significantly decreased the PPR (*, $p<.05$, paired Student's t test). E, AM-251 blocked the ability of 100 mM ethanol to decrease the PPR (*, $p<.05$, paired Student's t test).

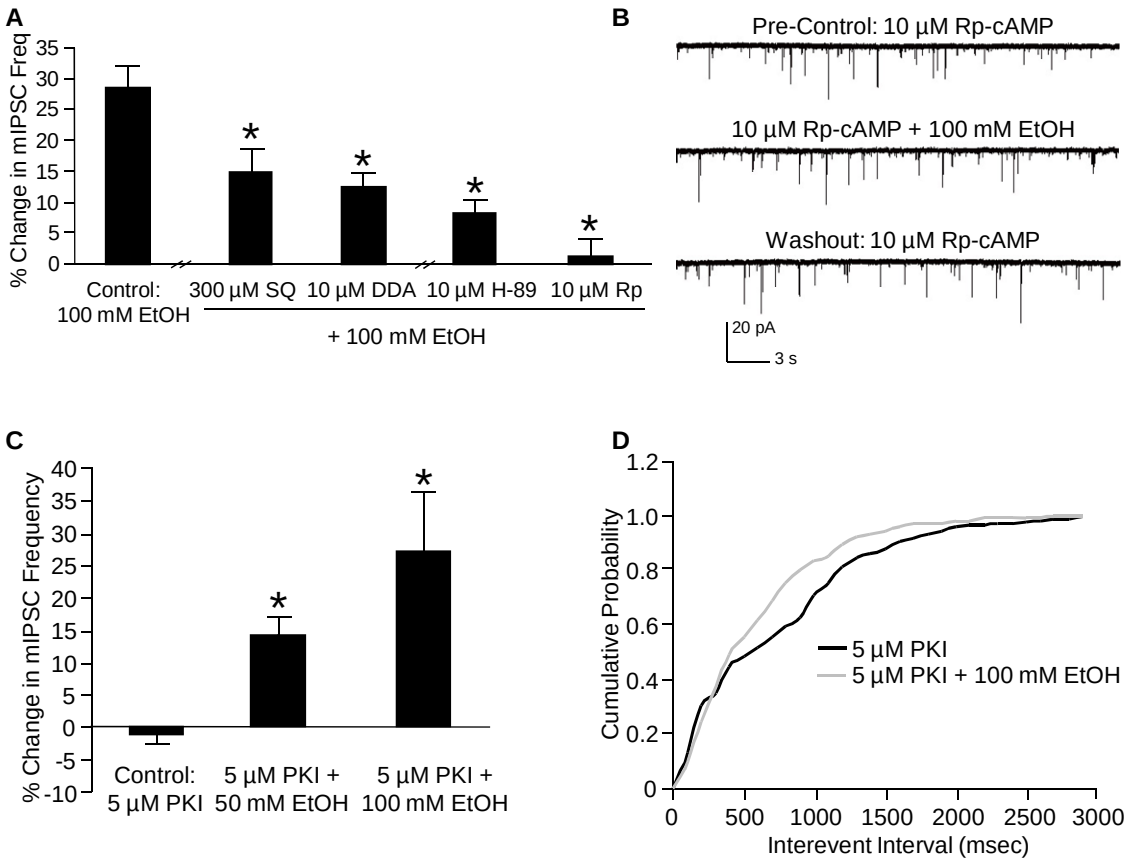


Figure 5.4. Adenylate cyclase and protein kinase A (PKA) antagonists prevented ethanol from increasing mIPSC frequency. A, SQ 22,536 (SQ, 300 µM), DDA (10 µM), H-89 (10 µM) and Rp-cAMP (Rp, 10 µM) prevented 100 mM ethanol (EtOH) from increasing mIPSC frequency (*, $p<.05$, one-way ANOVA, Dunnett's post hoc test). B, a trace from a representative neuron demonstrating the effect of 100 mM ethanol on mIPSC frequency in the presence of Rp-cAMP. C, ethanol (50 and 100 mM) increased mIPSC frequency when 5 µM PKI was in the pipette internal solution (*, $p<.05$, one-way ANOVA, Dunnett's post hoc test). D, a cumulative frequency histogram from a representative neuron demonstrating the effect of ethanol on the interevent interval curve with PKI in the pipette internal solution.

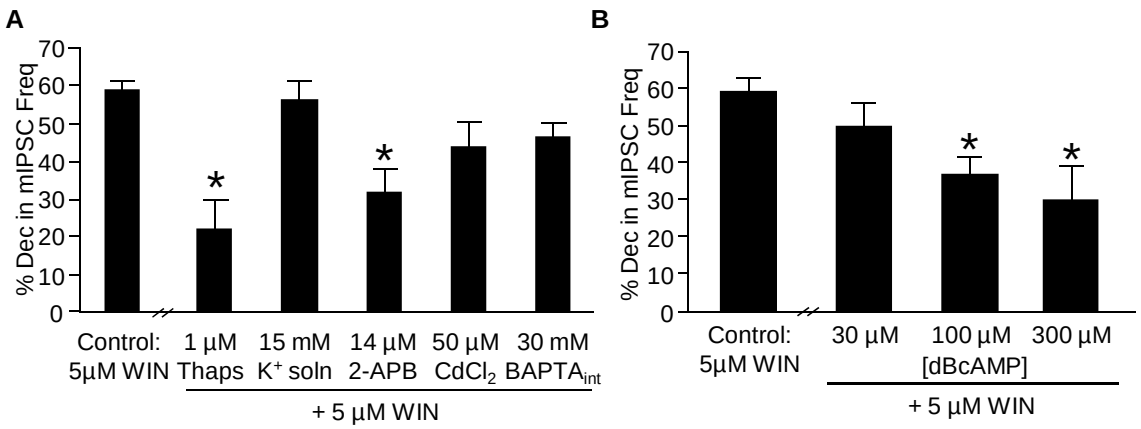


Figure 5.5. Inhibition of calcium release from internal stores and activation of PKA prevented WIN 55,212-2 from decreasing mIPSC frequency. A, thapsigargin (Thaps, 1 µM) and 2-APB (14 µM) inhibited WIN 55,212-2 (WIN, 5 µM) from decreasing mIPSC frequency, while the high potassium extracellular solution control (K^+ soln, 15 mM), cadmium chloride ($CdCl_2$, 50 µM), and BAPTA in the pipette internal solution ($BAPTA_{int}$, 30 mM) were without effect (*, $p<.05$, one-way ANOVA, Dunnett's post hoc test). B, the ability of WIN to decrease mIPSC frequency was significantly reduced in the presence of 100 µM and 300 µM dBcAMP, while there was not a significant effect at 30 µM (*, $p<.05$, one-way ANOVA, Dunnett's post hoc test).

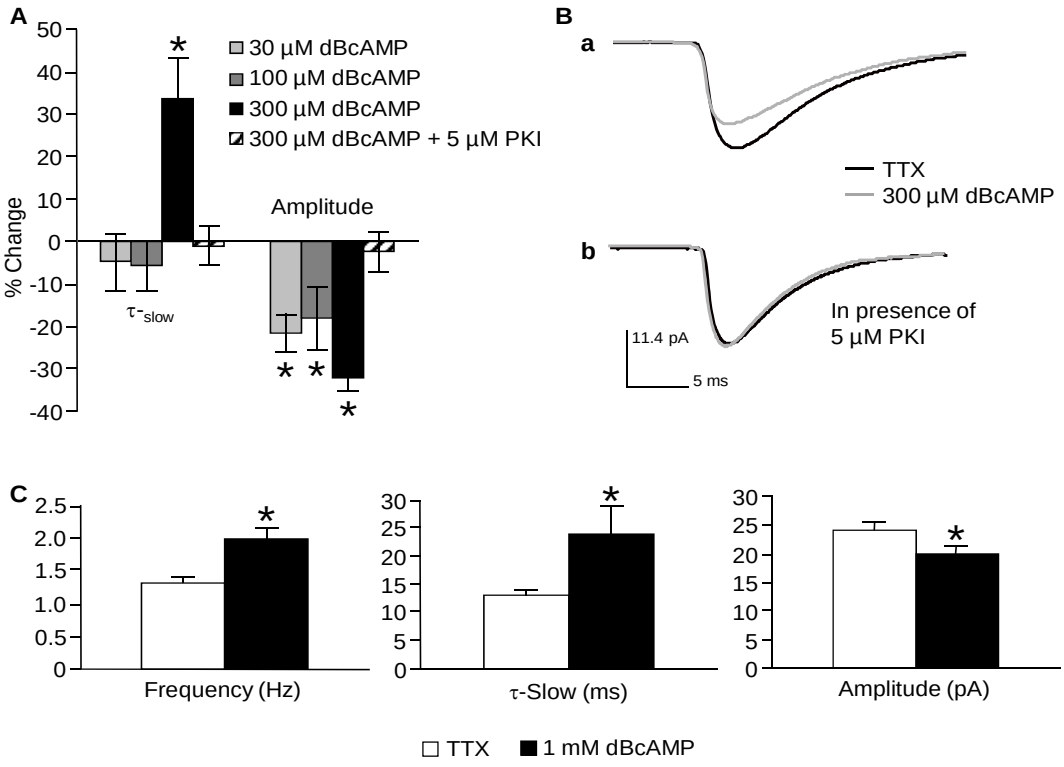


Figure 5.6. Activation of PKA affected baseline mIPSC properties. A, baseline mIPSC slow decay time (τ-slow) was increased in the presence of 300 µM dBcAMP, and 30, 100 and 300 µM dBcAMP decreased baseline mIPSC amplitude (*, p<.05, paired Student's t test). The effect of 300 µM dBcAMP on mIPSC τ-slow and amplitude was blocked when 5 µM PKI was included in the pipette internal solution. B, a trace from a representative neuron showing the effect of 300 µM dBcAMP on mIPSC τ-slow and amplitude (a) and the ability of 5 µM PKI in the pipette internal solution to block this effect (b). C, 1 mM dBcAMP increased baseline mIPSC frequency, increased baseline mIPSC τ-slow and decreased baseline mIPSC amplitude (*, p<.05, paired Student's t test).

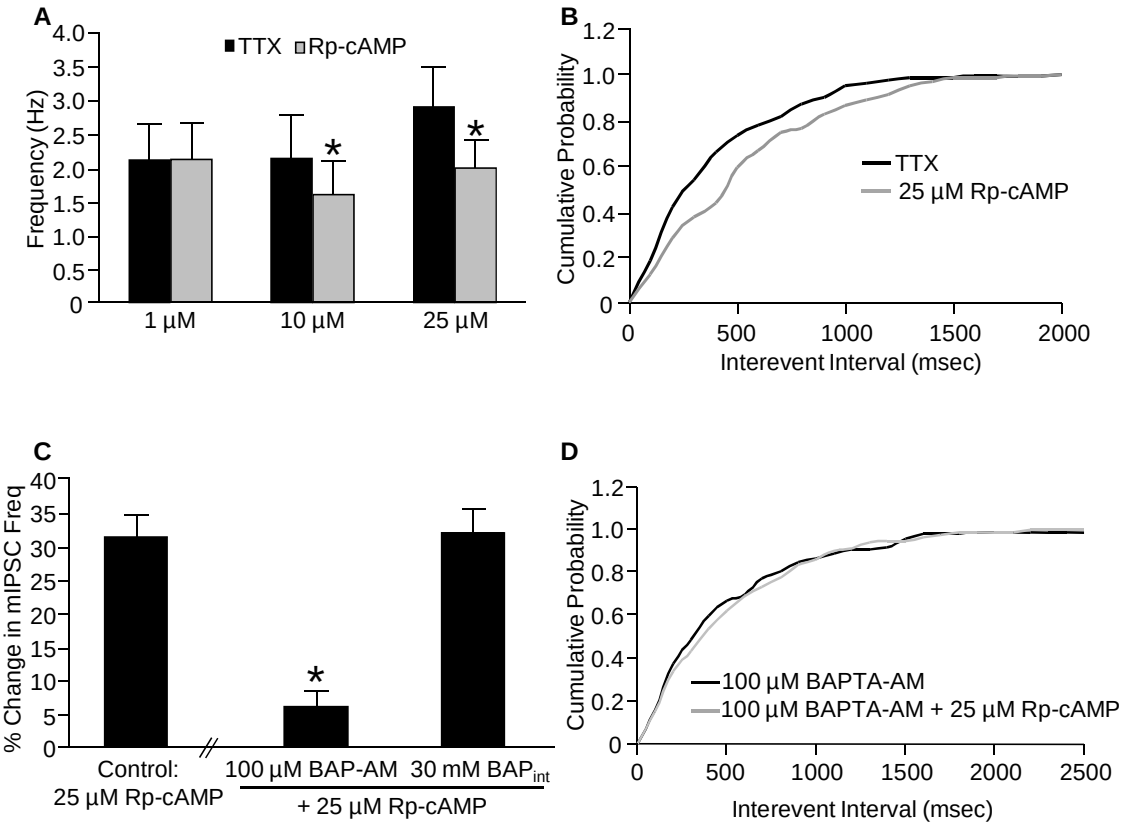


Figure 5.7. BAPTA-AM prevented Rp-cAMP from decreasing mIPSC frequency. A, Rp-cAMP (10 and 25 µM) significantly reduced baseline mIPSC frequency, while 1 µM Rp-cAMP was without effect (*, $p<.05$, paired Student's t test). B, a cumulative frequency histogram from a representative neuron demonstrating the effect of 25 µM Rp-cAMP on the interevent interval curve. C, pre-incubation of the slice with BAPTA-AM (BAP-AM, 100 µM) decreased the effect of 25 µM Rp-cAMP on mIPSC frequency, while BAPTA in the internal solution ($BAPTA_{int}$, 30 mM) did not have an effect (*, $p<.05$, one-way ANOVA, Dunnett's post hoc test). D, a cumulative frequency histogram from a representative neuron demonstrating the effect of 25 µM Rp-cAMP on the interevent interval curve in the presence of 100 µM BAPTA-AM.

Chapter VI: The Role of the PLC/PKC Pathway in Ethanol-enhanced GABA Release

INTRODUCTION

I have provided evidence that calcium release from internal stores and the adenylate cyclase/PKA pathway play an essential role in ethanol-enhanced spontaneous GABA release at the cerebellar interneuron-Purkinje cell synapse. Calcium is released from internal stores through activation of the IP_3Rs and RyRs. The RyR is activated by calcium alone, while the IP_3R also requires IP_3 (Berridge, 1998; Berridge et al., 2003). This IP_3 can come from activation of phospholipase C (PLC), which catalyzes the conversion of phosphoinositol 4,5-bisphosphate into diacylglycerol (DAG) and IP_3 (Kiselyov et al., 2003).

The released calcium from activation of the IP_3R and DAG contribute to the activation of typical PKC isoforms. When PKC is in the inactive state, a pseudosubstrate within the regulatory domain is bound to the catalytic domain (House and Kemp, 1987). When DAG binds to the PKC C1 domain and two cofactors, calcium and phosphatidylserine, bind to the C2 domain, there is a conformational change that frees the pseudosubstrate domain from the catalytic domain (Kishimoto et al., 1980; Boni and Rando, 1985; Ono et al., 1989; Bell and Burns, 1991). In addition to the dissociation of the regulatory domain from the catalytic domain, phosphorylation and translocation to the appropriate subcellular location are necessary for PKC activation (Dutil et al., 1998; Le Good et al.,

1998; Mochly-Rosen and Gordon, 1998). Once PKC is activated, it can phosphorylate a number of substrates, including the IP_3R (Patterson et al. 2004). Phosphorylation of the IP_3R increases the amount of calcium released from the internal stores (Mignery et al. 1990; Bugrim 1999; Bardo et al. 2006), forming a calcium-mediated feedback loop between activation of the IP_3R and PKC.

There is no evidence supporting that ethanol binds directly to the IP_3Rs and RyRs, with the more likely alternative being that ethanol indirectly modulates calcium release from internal stores. Therefore, activation of PLC and PKC could be playing a role in the ethanol-mediated increase in GABA release. The present study investigates the role of PLC and PKC in ethanol-enhanced spontaneous GABA release and determines the role of PKC in GABAergic neurotransmission.

RESULTS

Inhibition of PLC or PKC prevents ethanol from increasing mIPSC frequency. In the presence of the phosphoinositol (PI)-specific PLC antagonist, edelfosine (10 μM), the ability of ethanol to increase mIPSC frequency was significantly blocked (8.4 ± 4.1%, n = 9, Fig. 6.1A) compared to control (28.7 ± 3.4%, n = 12, Fig. 6.1A). A trace from a representative neuron showing that 10 μM edelfosine blocks ethanol from increasing mIPSC frequency is shown in Fig. 6.1B. In addition, a PKC general antagonist, chelerythrine (1 μM) or a PKCε-specific antagonist, εV1-2 (0.5 μM), prevented ethanol from increasing mIPSC frequency (chelerythrine: 7.2 ± 4.8%, n = 8; εV1-2: 9.1 ± 2.9%, n = 8, Fig. 6.1A). These results suggest that both PLC and PKC play a role in ethanol-enhanced spontaneous GABA release.

To determine if the PKC antagonists were acting at the presynaptic terminal and not the postsynaptic neuron, the membrane impermeable PKC antagonist, PKC(19-36), was included in the pipette internal solution, which limits exposure to the PKC antagonist to the postsynaptic neuron. With 20 μM PKC(19-36) in the pipette internal solution, both 50 (18.9 ± 4.4%, n = 6) and 100 mM (26.3 ± 3.4%, n = 6) ethanol significantly increased mIPSC frequency compared to control (0.7 ± 2.0%, n = 6, Fig. 6.1C). A trace from a representative neuron showing the effect of ethanol on mIPSC frequency with 20 μM PKC(19-36) in the pipette internal solution is shown in Fig. 6.1D. These results suggest that the PKC antagonist is acting presynaptically to block ethanol-enhanced spontaneous GABA release.

PKC antagonists affect baseline mIPSC properties. The general PKC antagonist, chelerythrine (1 µM and 10 µM) did not have an effect on baseline mIPSC frequency, fast decay time and amplitude (Fig. 6.2A, B, and D). There was an effect of 10 µM chelerythrine on mIPSC slow decay time (control: 12.8 ± 0.7 Hz, chelerythrine: 14.0 ± 0.7 Hz, n = 5, Fig. 6.2B). While a lower concentration of εV1-2 (0.5 µM) had no effect on mIPSC frequency, a higher concentration of εV1-2 (5 µM) increased mIPSC frequency (control: 2.5 ± 0.6 Hz, εV1-2: 3.1 ± 0.5 Hz, n = 5, Fig. 6.2A). Interestingly, 0.5 µM εV1-2 significantly increased mIPSC fast decay time (control: 9.2 ± 0.3 Hz, εV1-2: 10.6 ± 0.5 Hz, n = 14, Fig. 6.2B) and decreased mIPSC amplitude (control: 28.1 ± 2.0 Hz, εV1-2: 19.1 ± 1.4 Hz, n = 5, Fig. 6.2D), while there was no effect on these mIPSC parameters at 5 µM εV1-2. Overall, these results suggest that the general PKC antagonist and PKCε specific antagonist have differing effects on GABAergic neurotransmission.

PKC agonists affect baseline mIPSC properties. To further investigate the role of PKC in generating spontaneous GABA release, the effect of two PKC agonists, PMA and SC9, on GABAergic neurotransmission was determined. These two PKC agonists have different mechanisms of action: PMA activates PKC by binding to the C1 domain, while SC9 binds to the C2 domain (Ito et al., 1986; Ono et al., 1989). Both 3 µM PMA (control: 2.7 ± 0.5 Hz, PMA: 4.2 ± 0.7 Hz, n = 7) and 10 µM PMA (control: 2.7 ± 0.7 Hz, PMA: 4.4 ± 0.9 Hz, n = 7)

significantly increased mIPSC frequency (Fig. 6.3A). Likewise, both 25 µM SC9 (control: 1.8 ± 0.3 Hz, SC9: 2.1 ± 0.4 Hz, n = 8) and 100 µM SC9 (control: 2.1 ± 0.5 Hz, SC9: 2.6 ± 0.5 Hz, n = 9) significantly increased mIPSC frequency (Fig. 6.3A). There was no effect of any PKC agonist concentration on mIPSC slow decay time, but 25 µM SC9 (control: 9.4 ± 0.5 Hz, SC9: 10.1 ± 0.4 Hz, n = 8, Fig. 6.3B) significantly increased mIPSC fast decay time. There was also a reduction in mIPSC amplitude at 10 µM PMA (control: 25.1 ± 3.5 pA, PMA: 19.4 ± 2.3 pA, n = 7, Fig. 6.3D). Overall, these results suggest that PKC agonists affect GABAergic neurotransmission, including spontaneous GABA release.

Activation of PKA and PKC is necessary for ethanol to increase mIPSC frequency and cross-talk is not occurring between PKA and PKC. To determine if there is cross-talk occurring between PKA and PKC in the mechanism of ethanol-enhanced spontaneous GABA release, I determined if a PKC agonist could reverse the effect of a PKA antagonist on ethanol-enhanced spontaneous GABA release, and vice versa. As a control, the effect of a PKC agonist and a PKA agonist on ethanol-enhanced spontaneous GABA release was investigated. Interestingly, both the PKA agonist and PKC agonist significantly blocked ethanol from increasing spontaneous GABA release compared to control (100 µM dBcAMP: 12.9 ± 2.3%, n = 5; 300 µM dBcAMP: 5.9 ± 2.5%, n = 9; 25 µM SC9: 0.8 ± 3.8%, n = 7; Fig. 6.4A). A representative neuron demonstrating that 300 µM dBcAMP blocks ethanol from increasing mIPSC frequency is shown in Figure 6.4B. Because PKA and PKC agonists and

antagonists blocked the ethanol effect, I was not able to conduct the cross-talk experiments.

As an alternative, I determined if the PKC and PKA agonist-induced increase in mIPSC frequency could be blocked with a PKA and PKC antagonist, respectively. The effect of the PKC agonist, SC9 (25 µM), on mIPSC frequency (17.7 ± 5.5%, n = 8, Fig. 6.4C) was not reversed in the presence of the PKA antagonist, Rp-cAMP (25 µM, 17.6 ± 3.2%, n = 6). Similarly, the effect of the PKA agonist, dBcAMP (1mM), on mIPSC frequency (50.2 ± 12.1%, n = 8, Fig. 6.4C) was not reduced in the presence of the PKC antagonist, chelerythrine (1 µM, 44.9 ± 7.3%, n = 6). A cumulative probability curve from a representative neuron shows that 1 mM dBcAMP still shifts the curve to the left in the presence of 1 µM chelerythrine (Fig. 6.4D). These results suggest that the PKC agonist-induced increase in spontaneous GABA release is not dependent on activation of PKA, and the PKA agonist-induced increase in spontaneous GABA release is not dependent on activation of PKC.

DISCUSSION

The PI-PLC antagonist, edelfosine, blocked ethanol from increasing spontaneous GABA release, which suggests that PLC contributes to ethanol-enhanced spontaneous GABA release. Edelfosine has varying effects on intracellular calcium levels based on the cell type, proliferation state, and the edelfosine concentration used (Lohmeyer and Workman, 1993; Bergmann et al., 1994; Alonso et al., 1997; Jan et al., 1999). A between cell comparison found no effect of edelfosine on spontaneous GABA release compared to neurons exposed to control conditions. If there was a large increase in intracellular calcium after exposure to edelfosine, one would expect to see a large increase in spontaneous GABA release. Additional experiments were attempted with the general PLC antagonist, U73122, but there was an overall increase in GABA release (data not shown), suggesting that there were effects occurring through a PLC-independent pathway. Consistent with these data, PLC-independent effects have been reported for U73122 (Pulcinelli et al., 1998; Lockhart and McNicol, 1999). Based on this evidence, I conclude that PLCβ is necessary for ethanol-enhanced spontaneous GABA release. This conclusion is consistent with the IP_3R data and internal calcium store data presented in Chapter 4.

A general PKC antagonist and a PKCε specific antagonist inhibited ethanol-enhanced GABA release, which is similar to what is seen in the central nucleus of the amygdala (Bajo et al., 2008). This interaction between ethanol and PKCε at the molecular level is consistent with what is seen behaviorally in PKCε null mice that are exposed to alcohol. Specifically, these mice consume

less alcohol and are supersensitive to the behavioral effects of alcohol compared to wild-type controls (Hodge et al., 1999). While the current results suggest that PKCε is necessary for ethanol-enhanced spontaneous GABA release, it does not suggest that PKCε is the only PKC isoform involved in this ethanol mechanism. PKCε is an atypical PKC isoform, which differ from typical PKC isoforms by not requiring calcium for activation (for review see Steinberg, 2008). Typical PKC isoforms, which are the isoforms most likely to be activated by the PLCβ pathway, are also involved in generating the behavioral response to ethanol. In contrast to the PKCε null mice, PKCγ null mice are less sensitive to the intoxicating effects of alcohol and consume more alcohol than wild-type mice (Harris et al., 1995; Bowers and Wehner, 2001). PKCδ null mice are also less sensitive to the intoxicating effects of alcohol (Choi et al. 2008). Therefore, multiple PKC isoforms contribute to the behavioral effects of alcohol and the isoforms can have opposite effects on alcohol-related behaviors. Moreover, it is possible that multiple PKC isoforms play a role in ethanol-enhanced spontaneous GABA release.

The PKC general antagonist had no effect on baseline spontaneous GABA release, while the PKCε specific antagonist increased spontaneous GABA release. These results are consistent with a study conducted in the amygdala that used the same PKCε specific antagonist in addition to PKCε null mice (Bajo et al., 2008). These results suggest that PKCε regulates the amount of GABA released from the presynaptic terminal. Furthermore, because the PKC general antagonist had no effect on spontaneous GABA release, it is likely that another

PKC isoform increases the amount of GABA released to cancel out the inhibitory effect of PKCε. These results are consistent with the opposing effects of the PKC isoforms on alcohol-related behaviors (see above).

At the lower εV1-2 concentration, there was an increase and a decrease in mIPSC fast decay time and mIPSC amplitude, respectively. The change in both mIPSC fast decay time and amplitude mostly likely reflects a change in the functioning of the postsynaptic $GABA_A$ receptors. Consistent with this, there is evidence of PKCε regulating $GABA_A$ receptor sensitivity to ethanol through phosphorylation of the $GABA_A$ receptor γ_2 subunit (Qi et al., 2007). Interestingly, both of these effects on mIPSC properties are not present at a higher εV1-2 concentration. One explanation is that a nonspecific effect is occurring at the higher concentration, such as inhibition of another PKC isoform, that counteracts the PKCε effect on postsynaptic $GABA_A$ receptors. It should be noted that the ethanol studies with εV1-2 were conducted at the lower concentration. Overall, it appears that the PKC isoforms have different effects on GABAergic neurotransmission.

Two PKC activators, PMA and SC9, both increased spontaneous GABA release, with PMA increasing spontaneous GABA release to a much higher extent. PMA is a phorbol ester that binds to the C1 domain of PKC, while SC9 binds to the C2 domain (Ito et al., 1986; Ono et al., 1989). The C1 domain sequence is conserved in other proteins, which raises the possibility that PKC agonists acting at the C1 domain, such as PMA, are having nonspecific effects. For instance, the C1 domain is conserved in the Munc-13 protein, which has

effects on the neurotransmitter release machinery (Betz et al., 1998; Brose and Rosenmund, 2002; Kazanietz, 2002); moreover, phorbol esters modulate neurotransmitter release through both Munc-13 and PKC (Lou et al., 2008). Therefore, it is possible that PMA does not selectively activate PKC to affect spontaneous GABA release. Because SC9 acts at the C2 domain, which is another domain conserved in a number of proteins (including one that plays a role in neurotransmitter release), additional studies will be needed to determine if this effect on spontaneous GABA release is PKC specific (Rizo and Sudhof, 1998). For example, one could determine if the PKC agonist effect on spontaneous GABA release can be inhibited by a general PKC antagonist. Interestingly, the effect of the PKCε antagonist on mIPSC frequency, fast decay time and amplitude goes in the same direction as the effect of the general PKC agonist on mIPSC frequency, fast decay time and amplitude. Overall, the results from the PKC agonist and antagonist experiments suggest that PKC plays a role in the generation of spontaneous GABA release.

Next I wanted to determine if there is overlap in the pathways involved in ethanol-enhanced spontaneous GABA release. Cross-talk occurs between PKA and PKC at the GABAergic nucleus basalis of Meynert synapses (Kubota et al., 2003); furthermore, there are ethanol effects that involve cross-talk between these protein kinases. One mechanism involves ethanol increasing adenylate cyclase isoform 7 activity through a PKCδ-mediated mechanism, which leads to activation of PKA (Tabakoff et al., 2001). Another example involves ethanol inducing PKCε translocation to the cytosol through a PKA-dependent mechanism

(Yao et al., 2008). Specifically, this translocation of PKCε is thought to involve PKA activation of PLCβ. Therefore, it is tempting to speculate that the mechanism of ethanol-enhanced GABA release depends on cross-talk occurring between PKA and PKC.

Experiments were designed to determine if cross-talk is occurring between PKA and PKC in this ethanol mechanism. A required control for these experiments was to determine whether ethanol increases spontaneous GABA release in the presence of a PKC agonist or a PKA agonist. Both agonists blocked the ability of ethanol to increase spontaneous GABA release; therefore, the cross-talk experiments were not conducted because the agonists were supposed to "rescue" the block induced by the antagonist. However, these results imply that ethanol must induce a change in the activation state of the protein kinases to increase spontaneous GABA release and cannot simply interact with a downstream pathway that was activated by the protein kinase.

As an alternative to the experiment described above, I tested if a PKC antagonist could block the effect of a PKA agonist (and vice versa) on spontaneous GABA release. Neither antagonist was effective at blocking the effect of the opposite agonist, which suggests that cross-talk is not occurring between PKA and PKC to modulate GABA release at the interneuron-Purkinje cell synapse.

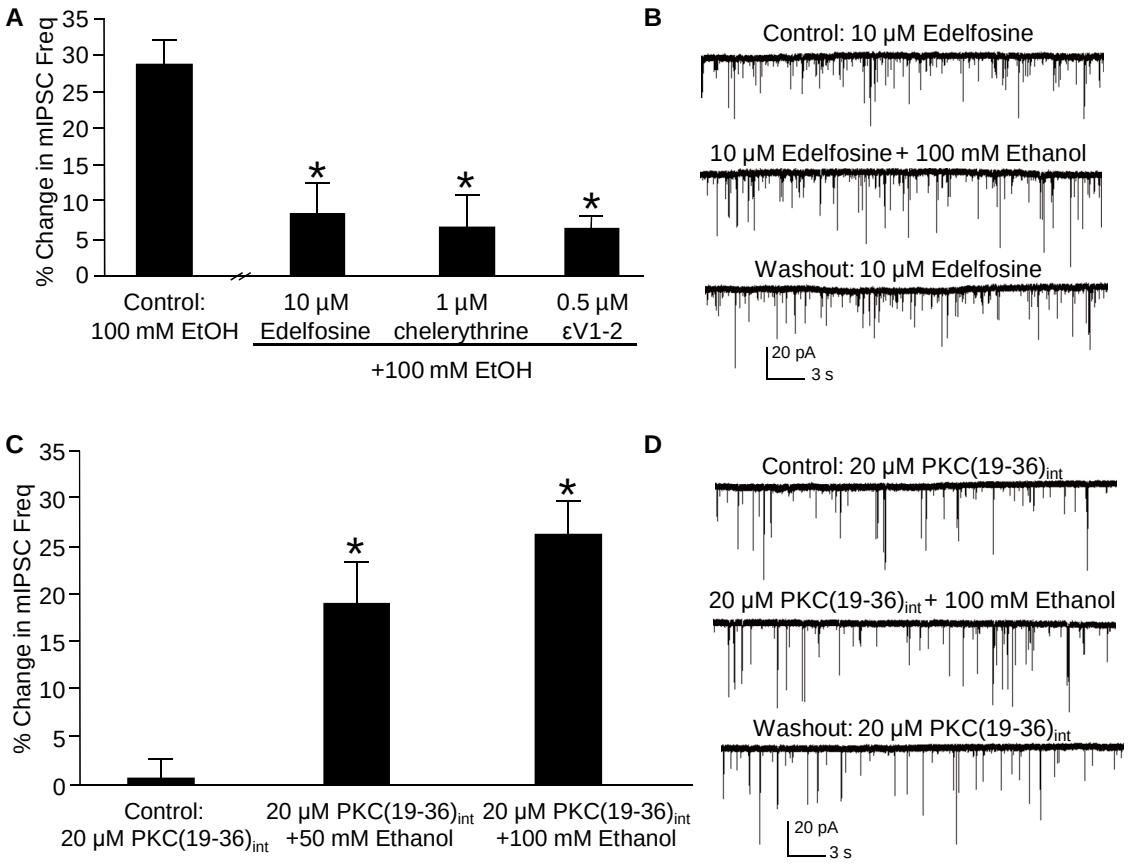


Figure 6.1. Phospholipase C and protein kinase C antagonists prevented ethanol from increasing mIPSC frequency. A, edelfosine (10 µM), chelerythrine (1 µM), and εV1-2 (0.5 µM) prevented 100 mM ethanol from increasing mIPSC frequency compared to control (*, $p<.05$, one-way ANOVA, Dunnett's post hoc test). B, a trace from a representative neuron demonstrating the effect of 100 mM ethanol on mIPSC frequency in the presence of edelfosine (10 µM). C, ethanol (50 and 100 mM) increased mIPSC frequency when 20 µM PKC(19-36) was in the pipette internal solution ($PKC(19\text{-}36)_{int}$; *, $p<.05$, one-way ANOVA, Dunnett's post hoc test). D, a trace from a representative neuron showing the effect of 100 mM ethanol on mIPSC frequency when PKC(19-36) was included in the pipette internal solution.

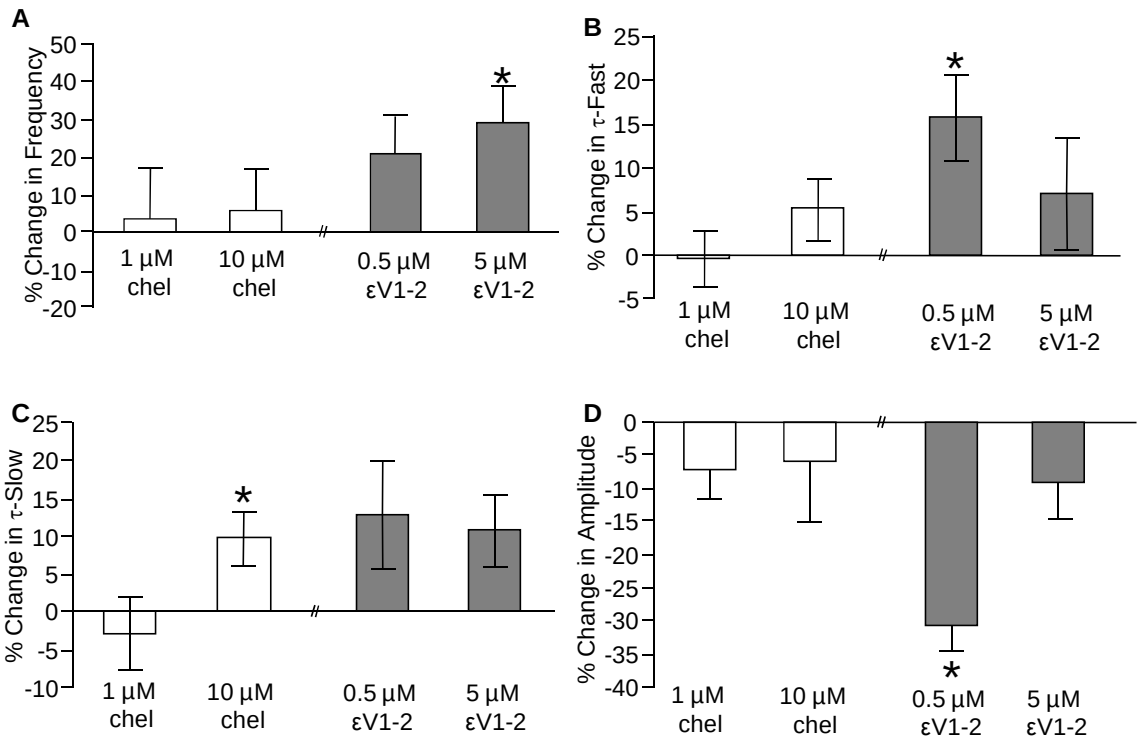


Figure 6.2. Inhibition of PKC affected baseline mIPSC properties. A, the higher concentration of εV1-2 (5 µM) increased mIPSC frequency (*, p<.05, paired Student's t test). B, there was a significant increase in mIPSC fast decay time (τ-fast) at 0.5 µM εV1-2 (*, p<.05, paired Student's t test). C, there was an increase in mIPSC slow decay time (τ-slow) at 10 µM chelerythrine (chel; *, p<.05, paired Student's t test). D, εV1-2 (0.5 µM) decreased mIPSC amplitude (*, p<.05, paired Student's t test).

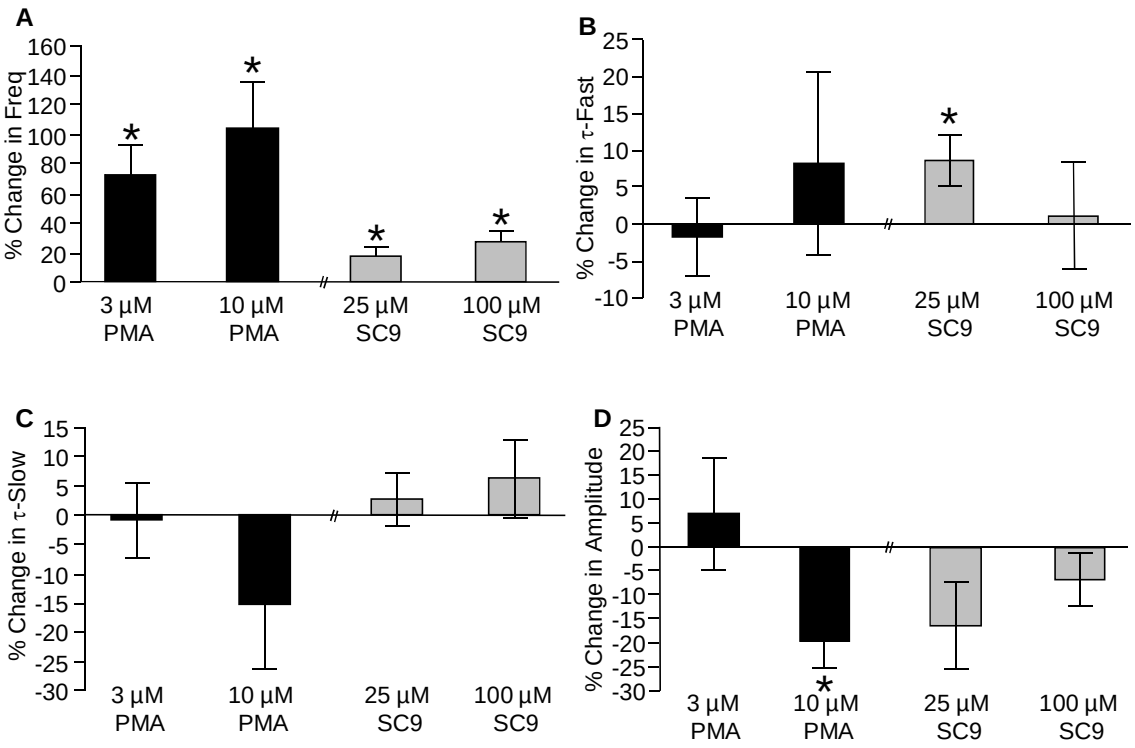


Figure 6.3. Activation of PKC affected baseline mIPSC properties. A, all concentrations of the PKC agonists that were tested increased mIPSC frequency (*, p<.05, paired Student's t test). B, there was a significant increase in mIPSC fast decay time (τ-fast) at 25 µM SC9 (*, p<.05, paired Student's t test). C, no effect was seen on mIPSC slow decay time (τ-slow) with either of the PKC agonists. D, PMA (10 µM) decreased mIPSC amplitude (*, p<.05, paired Student's t test).

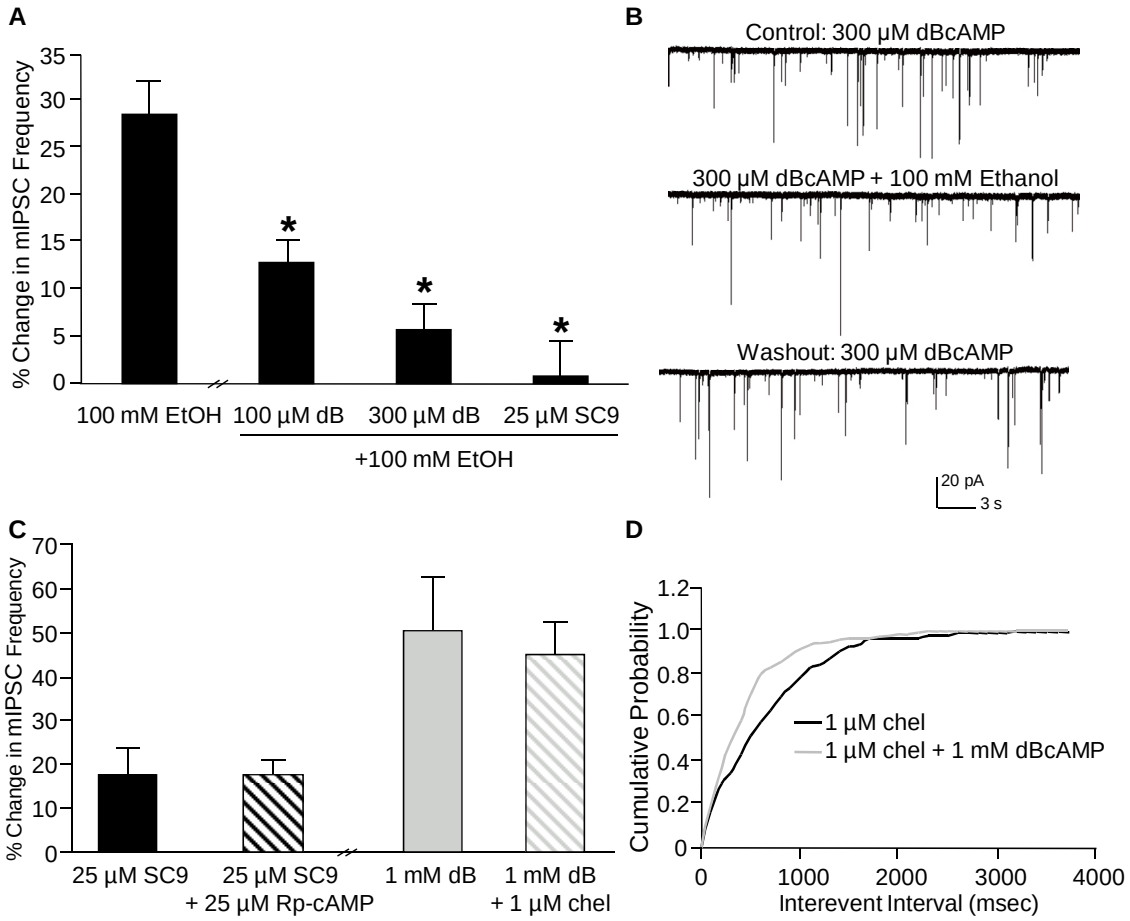


Figure 6.4. Activation of PKA and PKC was necessary for ethanol to increase mIPSC frequency and PKA and PKC were not dependent on each other to increase mIPSC frequency. A, dBcAMP (dB, 100 µM and 300 µM) and SC9 (25 µM) prevented 100 mM ethanol from increasing mIPSC frequency (*, $p<.05$, one-way ANOVA, Dunnett's post hoc test). B, a trace from a representative neuron showing the effect of 100 mM ethanol on mIPSC frequency in the presence of dBcAMP. C, Rp-cAMP (25 µM) did not prevent SC9 (25 µM) from increasing mIPSC frequency (*, $p<.05$, Student's t test). Likewise, chelerythrine (chel, 1 µM) did not prevent dBcAMP (1mM) from increasing mIPSC frequency (*, $p<.05$, Student's t test). D, a cumulative frequency histogram from a representative neuron demonstrating the effect of dBcAMP on the interevent interval curve in the presence of chelerythrine.

Chapter VII: General Discussion

The intoxicating effects of alcohol are partially mediated through modulation of the GABAergic system. A person who is less sensitive to the intoxicating effects of alcohol is prone to developing alcoholism (Schuckit, 2009), so understanding the molecular mechanisms contributing to alcohol intoxication will further our understanding of this disease. Within the past few years it was discovered that the GABAergic profile of ethanol consists of more than a direct ethanol interaction with the postsynaptic $GABA_A$ receptors and can involve activation of extrasynaptic $GABA_A$ receptors as well as an increase in GABA release from presynaptic terminals. The current studies provided information regarding the mechanism responsible for the ethanol-induced increase in GABA release.

With the use of whole-cell voltage clamp recordings, it was established that ethanol increases spontaneous and evoked GABA release at the interneuron-Purkinje cell synapse. Activation of cannabinoid 1 receptors or $GABA_B$ receptors, both of which are $G\alpha_i$-linked GPCRs, inhibits ethanol-enhanced spontaneous GABA release. There was no tonic activation of the cannabinoid receptors, and while there was tonic activation of the $GABA_B$ receptors, inhibiting the $GABA_B$ receptors did not affect ethanol-enhanced spontaneous GABA release. The ability of ethanol to increase spontaneous

GABA release is dependent on calcium release from internal stores, adenylate cyclase, PKA, PLC and PKC; moreover, these intracellular messengers act at the presynaptic terminal. Influx of extracellular calcium into the neuron is not involved in this ethanol mechanism. Because there are potential nonspecific effects that can occur with use of the IP_3R antagonist, electron microcopy was used to show that IP_3Rs are located in the presynaptic terminals at this synapse. It was also determined that both PKA and PKC contribute to the generation of spontaneous GABA release and cross-talk is not occurring between these two intracellular messengers.

Is there a connection between the pathways?

The current results suggest that the mechanism of ethanol-enhanced spontaneous GABA release at the interneuron-Purkinje cell synapse involves activation of the $G\alpha_q$-linked pathway, the $G\alpha_s$-linked pathway and calcium release from internal stores. The contribution of each is required for ethanol to increase spontaneous GABA release but no one pathway is necessarily sufficient. In Chapter 6 I attempted to perform PKA/PKC cross-talk experiments, but this was unsuccessful because both the agonists and antagonists inhibit ethanol-enhanced spontaneous GABA release. However, the experiments provided useful information: the mechanism of ethanol-enhanced spontaneous release involves an increase in activated PKA and PKC. Therefore, either ethanol is acting upstream to activate PKA and PKC or is activating PKA and PKC directly. If ethanol is acting upstream to activate PKA and PKC, two likely candidates are

adenylate cyclase and PLC, respectively, because data support the involvement of both in this ethanol mechanism. I found that a PKA antagonist did not block the PKC agonist from increasing spontaneous GABA release, and vice versa, which suggests that the two protein kinases are not dependent on each other to increase spontaneous GABA release. Therefore, while cross-talk could still occur in the mechanism of ethanol-enhanced spontaneous GABA release, it is not a necessity because both kinases are capable of independently increasing spontaneous GABA release.

How does calcium release from internal stores fit into this mechanism? I hypothesize that ethanol does not bind directly to the IP_3R and RyR to increase spontaneous GABA release. As mentioned previously, PKC has a binding site on the IP_3R and PKA has binding sites on the IP_3R and the RyR (Mignery et al., 1990; Patterson et al., 2004; Sobie et al., 2006). When either binds to its respective site, there is an increase in the amount of calcium released from the internal stores (Mignery et al., 1990; Bugrim, 1999; Bardo et al., 2006). The link between calcium and PKC is well-established, and I provided evidence that the PKA effect on spontaneous GABA release is dependent on calcium (Chapter 5). Therefore, I propose that ethanol activates PKA and PKC and these two kinases phosphorylate the IP_3Rs and RyRs to increase the amount of calcium released from the internal stores, which increases the amount of spontaneous GABA release. This proposed mechanism does not limit the actions of PKA and PKC to the internal calcium stores and it is likely that both have other means of regulating spontaneous GABA release. For instance, PKA and PKC have known

effects on the neurotransmitter release machinery (Trudeau et al., 1996; Chheda et al., 2001; Seino and Shibasaki, 2005; Lou et al., 2008).

All the current ethanol experiments were conducted with the general protocol of inhibiting an intracellular messenger with the appropriate antagonist and determining if ethanol could increase spontaneous GABA release under these conditions. For example, I demonstrated that BAPTA-AM and antagonists that inhibit calcium release from the internal stores block ethanol-enhanced spontaneous GABA release. As a result of these data, I inferred that ethanol was increasing intracellular calcium to increase spontaneous GABA release. These experiments could be taken a step further by determining if ethanol directly increases calcium release in the presynaptic terminal with two-photon calcium imaging. If I found that ethanol does increase calcium release from internal stores using this technique, it would be interesting to see if a PKA and PKC antagonist could block this ethanol effect. This experiment would directly test the hypothesis that PKA and PKC are mediating the ethanol-induced increase in calcium release from internal stores.

Yin and Yang: the ethanol effect on GABA and glutamate

During alcohol intoxication, ethanol increases GABA neurotransmission and decreases glutamate neurotransmission (see Chapter 1). The present work and the work of others have provided evidence that ethanol can increase GABA release from the presynaptic terminal. Moreover, ethanol can decrease glutamate release from the presynaptic terminal, although this effect is not as

well characterized. In spinal motorneurons, ethanol inhibits both NMDA receptor-mediated and non-NMDA receptor mediated spontaneous glutamate release, and within the same neuron increases spontaneous GABA release (Ziskind-Conhaim et al., 2003). Ethanol reduces glutamate neurotransmission at the crayfish neuromuscular junction by decreasing the release of glutamate-filled vesicles (Strawn and Cooper, 2002). Ethanol inhibits NMDA receptor-mediated spontaneous and evoked glutamate release in the hippocampus but has no effect on non-NMDA receptor mediated spontaneous glutamate release (Hendricson et al., 2003; Hendricson et al., 2004). There was no effect of ethanol on glutamate release onto the central nucleus of the amygdala neurons unless the animals had undergone chronic ethanol treatment (Roberto et al., 2004). There was no effect of ethanol on glutamate release onto cerebellar Purkinje cells (Carta et al., 2006; Belmeguenai et al., 2008). Ethanol also had no effect on action potential-independent glutamate release in the VTA, but there was an increase in action potential-dependent glutamate release (Xiao et al., 2009). Therefore, current evidence supports that ethanol decreases glutamate release, but like the effect of ethanol on GABA release, it happens in a brain region-specific fashion.

What mechanism is responsible for the different ethanol effect on GABA and glutamate release?

That ethanol affects GABA and glutamate release in opposite directions poses a conundrum because internal calcium stores, the $G\alpha_q$-linked pathway and

the $G\alpha_{i/s}$-linked pathway modulate baseline GABA and glutamate release in the same manner (Malenka et al., 1986; Hopkins and Johnston, 1988; Weisskopf et al., 1994; Bouron, 1999; Hori et al., 1999; Emptage et al., 2001; Simkus and Stricker, 2002; Lee et al., 2008). Therefore, ethanol cannot be activating the same intracellular messengers to increase and decrease GABA and glutamate release, respectively.

It is possible that ethanol is acting through different intracellular messengers. This seems unlikely since I found that a number of intracellular messengers are involved in ethanol-enhanced GABA release. However, one possibility is extracellular calcium because influx of extracellular calcium does not play a role in ethanol-enhanced spontaneous GABA release (Chapter 4). Therefore, it is possible that influx of extracellular calcium into the neuron is necessary to generate spontaneous glutamate release and ethanol is preventing this influx to cause a decrease in glutamate release. This is something that can be tested in the future in brain regions that show an ethanol-induced decrease in glutamate release.

The other possibility is that ethanol is acting through the same intracellular messengers but is having an opposite effect on them. Ethanol could be acting upstream of a particular messenger or activating the intracellular messenger directly. There is evidence of an “ethanol-responsive domain” within adenylate cyclase (Yoshimura et al., 2006), but so far this is the only support for such a domain existing on an intracellular messenger. If ethanol binds directly to the intracellular messengers to regulate GABA and glutamate release, ethanol would

need to differentiate between the intracellular messengers in the GABAergic and glutamatergic terminals as well as have an opposite effect on these messengers. To differentiate between terminals, ethanol would need some type of “sensor” to recognize if a certain terminal is GABAergic or glutamatergic, but there is no support for an “ethanol sensor” existing. Another possibility is that the intracellular messenger isoforms are divided between the GABA and glutamate terminals. While isoform expression can vary by brain region, there is no current evidence suggesting that the isoforms are divided between terminals.

Even if the isoforms were divided between the terminals, ethanol would need to have an opposite effect on the isoforms to regulate spontaneous GABA release. The most likely candidate is PKC because PKCε and PKCγ have opposing effects on alcohol-induced behaviors (Harris et al., 1995; Hodge et al., 1999; Bowers and Wehner, 2001), and I provided data that suggest there are PKC-isoform specific effects on spontaneous GABA release (Chapter 6). Ethanol does have different effects on the adenylate cyclase isoforms, but the isoforms are either insensitive or activated by ethanol- there is no opposite effect on activation (Yoshimura and Tabakoff, 1995). Therefore, because there is no current evidence suggesting that ethanol can differentiate between the two terminal types and because there is minimal evidence suggesting that intracellular messenger isoforms could account for this effect, it is unlikely that ethanol is activating the intracellular messengers directly to mediate the increase and decrease in GABA and glutamate release, respectively. There is more

evidence supporting that ethanol acts upstream of the intracellular messengers, which is discussed in more detail below.

The role of GPCRs in the ethanol effect on neurotransmitter release

One hypothesis to explain the opposing role of ethanol on GABA and glutamate release is that activation of different presynaptic GPCRs mediates the ethanol effect at each terminal. Activation of the $G\alpha_i$ and $G\alpha_s$–linked GPCRs has opposing effects on the adenylate cyclase/PKA pathway, and activation of these GPCRs is necessary for ethanol to affect GABA and glutamate release in some brain regions. For instance, activation of the CRF1 receptor, which is a $G\alpha_s$-linked GPCR, is necessary for ethanol to increase GABA release in the amygdala. Interestingly, endocannabinoid release from the postsynaptic neuron, which activates the presynaptic $G\alpha_i$-linked cannabinoid 1 receptors, is necessary for ethanol to decrease glutamate release in the hippocampus (Basavarajappa et al., 2008). In the nucleus accumbens, activation of presynaptic $GABA_B$ receptors is necessary for ethanol to inhibit NMDA receptor-mediated glutamate neurotransmission (Steffensen et al., 2000). These results provide examples of activation of a presynaptic $G\alpha_s$-coupled GPCR being necessary for ethanol to increase GABA release and activation of a presynaptic $G\alpha_i$-coupled GPCR being necessary for ethanol to decrease glutamate release. Additionally, antagonists for the δ-opioid receptor and the $GABA_B$ receptor, both of which are $G\alpha_i$-linked GPCRs, augment the ability of ethanol to increase GABA release in the amygdala and hippocampus (Ariwodola and Weiner, 2004; Zhu and Lovinger,

2006; Kang-Park et al., 2007). Therefore, activation of $G\alpha_i$-coupled GPCRs can attenuate the ethanol-induced increase in GABA release.

Interestingly, Xiao and Ye (2008) found that ethanol *decreases* GABA release in the VTA, but in the presence of a μ-opioid receptor agonist, which activates a $G\alpha_i$-coupled GPCR, ethanol *increases* GABA release. The authors suggest that the μ-opioid receptor agonist "silences" a population of GABAergic neurons that decrease GABA release in the presence of ethanol, which allows for a different population of GABAergic neurons- one that increases GABA release in the presence of ethanol- to dominate. This study is in contrast to other studies in the VTA that found that ethanol increases GABA release in the absence of a μ-opioid receptor agonist (Melis et al., 2002; Thiele et al., 2008). Xiao and colleagues also found that ethanol *increases* glutamate release in the VTA, and this mechanism is dependent on activation of the dopamine 1 receptors, which are $G\alpha_s$-linked GPCRs (Xiao et al., 2009). Overall, these results suggest that activation of $G\alpha_i$-linked GPCRs does not always selectively decrease glutamate release and activation of $G\alpha_s$-linked GPCRs does not always selectively increase GABA release, at least in the VTA. The data of Xiao and colleagues are the only evidence of ethanol decreasing and increasing GABA and glutamate release, respectively, in the brain. While it is entirely possible that this effect is seen in other brain regions, more evidence will be needed to support that this effect is extended to other regions of the brain. Regardless, these data provide further support for the important role that GPCRs play in the effect of ethanol on neurotransmitter release.

So how are these GPCRs activated by ethanol? Ethanol does not nonspecifically increase activation of every GPCR throughout the brain. For example, both μ-opioid receptor null mice and a μ-opioid receptor antagonist increase baseline GABA release but have no effect on ethanol-enhanced GABA release in the central nucleus of the amygdala (Kang-Park et al., 2009). Additionally, there is tonic activation of $GABA_B$ receptors in the cerebellum and VTA, but inhibiting these $GABA_B$ receptors has no effect on ethanol-enhanced spontaneous GABA release (Chapter 5; Thiele et al., 2008). Because ethanol does not always activate a given presynaptic GPCR, even when activation of the GPCR affects GABA release, I find it unlikely that ethanol is binding to an "ethanol binding site" on the GPCRs. This concept was suggested previously for the $GABA_B$ receptors because of differences in the activation of presynaptic and postsynaptic $GABA_B$ receptors by ethanol (Ariwodola and Weiner, 2004).

Another possibility is that ethanol increases the concentration of the endogenous agonist that activates the GPCR. This would explain why there is tonic activation of a GPCR at a presynaptic terminal, which provides evidence that the particular GPCR is there and can affect neurotransmitter release, while there is no contribution of this same GPCR to the effect of ethanol on neurotransmitter release: ethanol does not change the amount of endogenous agonist that reaches the presynaptic GPCR. Therefore, despite the fact that a presynaptic GPCR is present, it is not necessarily involved in this ethanol mechanism. However, this hypothesis fails to explain how ethanol increases spontaneous GABA release in the mechanically dissociated neuron preparation

(Zhu and Lovinger, 2006; Fig. 3.2). Potential explanations include ethanol increasing release of a retrograde messenger or activating non-GABAergic presynaptic terminals that are still attached to the Purkinje cell in the mechanically dissociated neuron preparation. Both of these explanations seem unlikely and therefore weaken the hypothesis that ethanol is increasing the concentration of an endogenous agonist to activate the GPCRs. More work will be necessary to determine the interaction between ethanol and the GPCRs that would be consistent with what is known about the ethanol effect on neurotransmitter release.

The role of calcium in the ethanol effect on neurotransmitter release

In addition to the GPCRs, calcium signaling could also play an important role in determining if ethanol is going to affect neurotransmitter release at a particular synapse. In normal external calcium concentrations, a cannabinoid agonist decreases spontaneous GABA release at the interneuron-Purkinje cell synapse and has no effect on spontaneous glutamate release at the parallel fiber-Purkinje cell synapse (Yamasaki et al., 2006). However, if the calcium concentration in the extracellular solution is increased, the cannabinoid agonist decreases glutamate release at the parallel fiber-Purkinje cell synapse. The authors conclude that cannabinoids selectively interact with “calcium-enhanced” neurotransmitter release, and at normal external calcium concentrations there is calcium-enhanced spontaneous GABA release but not calcium-enhanced

spontaneous glutamate release onto Purkinje cells (Carter et al., 2002; Yamasaki et al., 2006).

Yamasaki and colleagues found that this effect is not limited to cannabinoid receptors- activation of $GABA_B$ receptors and group 3 mGluRs have no effect on spontaneous glutamate release onto Purkinje cells in normal external calcium concentrations, but when the calcium concentration in the extracellular solution is increased, activation of $GABA_B$ receptors and group 3 mGluRs decreases glutamate release. All of these receptors are $G\alpha_i$-linked GPCRs, which provides an additional link between this pathway and calcium. Consistent with these data, I provided evidence for PKA being involved in the cannabinoid-mediated decrease in spontaneous GABA release, and the PKA antagonist-induced decrease in spontaneous GABA release occurred through a calcium-dependent mechanism (Chapter 5).

A likely source for these calcium-enhanced events is calcium release from internal stores because inhibition of calcium release from internal stores suppresses the cannabinoid effect on spontaneous GABA release at the interneuron-Purkinje cell synapse (Yamasaki et al., 2006; Chapter 5). I provided evidence that the ability of ethanol to increase spontaneous GABA release at the interneuron-Purkinje cell synapse is dependent on calcium release from internal stores (Chapter 4). Calcium release from internal stores does not affect glutamate release at the parallel fiber-Purkinje cell synapse (Carter et al., 2002), and at the parallel fiber-Purkinje cell synapse ethanol has no effect on glutamate release (Belmeguenai et al., 2008). Therefore, similar to the cannabinoids,

ethanol could selectively alter “calcium-enhanced” neurotransmitter release that is due to calcium release from the internal stores.

This concept of “calcium-enhanced” neurotransmitter release works to explain how ethanol modulates neurotransmitter release onto Purkinje cells, and more studies will be needed before this theory can be applied to the whole brain. An interesting future experiment would be to see if the presence of a high calcium extracellular solution could reverse the lack of ethanol effect on mIPSC frequency in the cortex, thalamus and lateral septum (Criswell et al., 2008; Jia et al., 2008). Additionally, one could investigate if the presence of a high calcium extracellular solution could reverse the reported lack of ethanol effect on glutamate release in the amygdala and cerebellum (Roberto et al., 2004; Carta et al., 2006; Belmeguenai et al., 2008). If the experiments are successful, one could determine if blocking calcium release from the internal stores in the presence of the high calcium extracellular solution could revert ethanol back to having no effect on release.

The big picture: the interneuron-Purkinje cell synapse and beyond

While I have focused on the effect of ethanol at the presynaptic terminal, one should keep in mind that this change in neurotransmitter release affects the activation of postsynaptic ionotropic and metabotropic receptors. A change in the amount of neurotransmitter binding to the ionotropic receptors can depolarize or hyperpolarize the postsynaptic neuron, which affects the excitability of the cell. A change in the amount of neurotransmitter binding to the metabotropic

receptors can have a number of effects on the neuron, which are generally longer lasting than the effects induced by the ionotropic receptors. When ethanol increases the amount of GABA released from the basket and stellate cells, there is an increase in activation of the Purkinje cell $GABA_A$ receptors. The increase in chloride flux would hyperpolarize the Purkinje cell, and this is consistent with the ethanol-induced decrease in Purkinje cell firing (Siggins and French, 1979; Sorensen et al., 1980; George and Chu, 1984). Decreased Purkinje cell firing leads to motor incoordination, which also occurs during alcohol intoxication (Servais et al., 2005; Levin et al., 2006).

Because the goal of the current work was to determine the intracellular messengers involved in ethanol-enhanced GABA release, analysis of action potential-independent release was ideal since there is a direct readout for changes at the presynaptic terminal. However, neurotransmission involves action potential-dependent release, so conducting evoked neurotransmitter release studies will be necessary when trying to figure out the effect of ethanol throughout the brain. Additionally, experiments should be done to explore the contribution of presynaptic GPCRs to the ethanol effect on neurotransmitter release in different brain regions. To start, studies should be undertaken to determine what GPCR(s) is/are activated at the interneuron-Purkinje cell synapse to allow ethanol to increase GABA release. It will likely involve activation of a $G\alpha_s$- or $G\alpha_q$-linked GPCR because activation of either receptor is consistent with an increase in neurotransmitter release.

Most importantly, it should be determined if chronic alcohol treatment affects the ethanol-induced increase in GABA release. For example, if rats were made alcohol-dependent before the electrophysiology experiments, would ethanol increase GABA release to the same extent? If so, is it occurring through the same mechanism? One could also determine if there is an effect on baseline GABA release after chronic ethanol treatment. Comparing these results to the results from alcohol naïve rats will give us an idea of the neuroadaptations that are occurring during chronic alcohol use, which would contribute to our understanding of the development of alcoholism.

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