The Role of Protein Kinase A in the Ethanol-induced Increase in Spontaneous GABA Release onto Cerebellum Purkinje Neurons

M. Katherine Kelm¹
Hugh E. Criswell²
George R. Breese³

Departments of Pharmacology¹,³ and Psychiatry²,³, Bowles Center for Alcohol Studies¹,²,³
School of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina

Running Head: PKA and Ethanol-enhanced GABA Release

Contact Information:
Mary Katherine Kelm
The University of North Carolina at Chapel Hill
Bowles Center for Alcohol Studies
CB # 7178, Thurston-Bowles Building
Chapel Hill, NC 27599-7178
Telephone: 919-843-9478
Fax: 919-966-5679
Email: katie_kelm@med.unc.edu
ABSTRACT

Ethanol increases miniature inhibitory postsynaptic current frequency and decreases the paired-pulse ratio, which suggests that ethanol increases both spontaneous and evoked release, respectively. We have shown previously that ethanol increases GABA release at the rat interneuron-Purkinje cell synapse and that this ethanol effect involves calcium release from internal stores; however, further exploration of the mechanism responsible for ethanol-enhanced GABA release was needed. We found that a cannabinoid receptor 1 (CB1) agonist, WIN-55,212, and a GABA-B receptor agonist, baclofen, decreased baseline spontaneous GABA release and prevented ethanol from increasing spontaneous GABA release. The CB1 receptor and GABA-B receptor are Galpha-i-linked G protein coupled receptors with common downstream messengers that include adenylate cyclase and protein kinase A (PKA). Adenylate cyclase and PKA antagonists blocked ethanol from increasing spontaneous GABA release, while a PKA antagonist limited to the postsynaptic neuron did not block ethanol from increasing spontaneous GABA release. These results suggest that presynaptic PKA plays an essential role in ethanol-enhanced spontaneous GABA release. Similar to ethanol, we found that the mechanism of the cannabinoid-mediated decrease in spontaneous GABA release involves internal calcium stores and PKA. A PKA antagonist decreased baseline spontaneous GABA release. This effect was reduced after incubating the slice with a calcium chelator, BAPTA-AM, but was unaffected when BAPTA was limited to the postsynaptic neuron. This suggests that the PKA antagonist is acting through a presynaptic, calcium-dependent mechanism to decrease spontaneous GABA release. Overall, these results suggest that PKA activation is necessary for ethanol to increase spontaneous GABA release.

Keywords: cannabinoids, adenylate cyclase mIPSCs, electrophysiology, interneuron-Purkinje cell synapse
INTRODUCTION

There is substantial evidence that the acute behavioral effects of alcohol (ex: lethargy, anxiolysis and incoordination) involve modulation of the GABAergic system (Aguayo et al. 2002; Criswell and Breese 2005; Grobin et al. 1998; Mihic 1999; Siggins et al. 2005; Weiner and Valenzuela 2006). Previous research investigating the effect of ethanol on GABAergic function focused on ethanol acting directly on GABA\textsubscript{A} receptors with little emphasis on possible indirect mechanisms. One recently discovered indirect mechanism includes ethanol altering the amount of GABA released from presynaptic terminals in a number of brain regions \textit{in vitro} (Criswell and Breese 2005; Siggins et al. 2005; Weiner and Valenzuela 2006). Interestingly, ethanol does not increase GABA release in every brain region (Criswell et al. 2008; Jia et al. 2008; Moriguchi et al. 2007), which is in agreement with earlier \textit{in vivo} studies that demonstrated ethanol increases GABA function in select brain regions (Bloom and Siggins 1987; Criswell et al. 1993; Givens and Breese 1990). The ability of ethanol to increase GABA release in a brain region-specific manner is consistent with alcohol-induced behaviors that are linked to certain brain regions (McCown et al. 1986). Despite this role for GABA release in the GABAergic profile of ethanol, much remains unknown about the mechanism responsible for the ethanol-induced increase in GABA release.

Progress was made towards elucidating this mechanism with the discovery that calcium release from internal stores plays an essential role in ethanol-enhanced spontaneous GABA release at the rat cerebellar interneuron-Purkinje cell synapse; moreover, this ethanol action is not dependent on the influx of extracellular calcium or on calcium-dependent retrograde messengers (Kelm et al. 2007). However, the manner in which ethanol interacts with internal calcium stores is uncertain. While internal stores release calcium through activation of the inositol 1,4,5-trisphosphate receptors (IP\textsubscript{3}Rs) and ryanodine receptors (RyRs), there is no current evidence suggesting that ethanol interacts directly with these receptors. The amount
of calcium released from the IP$_3$Rs and RyRs is regulated by a number of factors, including calcium itself, nucleotides and protein kinases (Bardo et al. 2006; Patterson et al. 2004). 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 G protein coupled receptors (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 delta 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; Kang-Park et al. 2007; Zhu and Lovinger 2006). Consistent with these results, activation of the corticotrophin-releasing factor 1 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$_3$R (Mignery et al. 1990; Patterson et al. 2004) and RyR (Sobie et al. 2006), and phosphorylation of these receptors leads to increased calcium release (Bardo et al. 2006; Bugrim 1999). In addition, there is evidence for PKA acting at the neurotransmitter release
machinery to regulate synaptic transmission (Chheda et al. 2001; Seino and Shibasaki 2005; Trudeau et al. 1996). 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 from the presynaptic terminals of rat cerebellar interneurons.
METHODS

Preparation of slices. Sprague-Dawley rats, 13-20 days old, were anesthetized with an intraperitoneal (i.p.) 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 CaCl₂, 1 MgCl₂, 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 NaH₂PO₄, 24 NaHCO₃, 6 MgSO₄, 0.5 CaCl₂, 10 glucose, and gassed with 95% O₂/5% CO₂. 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 KH₂PO₄, 10 glucose, 2 MgSO₄, 20 NaHCO₃, 2 CaCl₂, and gassed with 95% O₂/5% CO₂. 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°C) 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 internal solution consisted of the following composition (in mM): 150 KCl, 3.1 MgCl₂, 15 HEPES, 5 K-ATP, 5 EGTA, 15 phosphocreatine. The internal solution pH was adjusted to 7.4 with KOH and the osmolarity was around 310 mOsm. The internal solution
composition for the BAPTA experiments was described previously (Kelm et al. 2007). For the paired-pulse studies, 5 mM N-(2,6-Dimethylphenylcarbamoylmethyl)triethylammonium bromide (QX-314, Sigma) 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.** Tetrodotoxin (TTX, Sigma), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, Sigma), H-89 (Sigma), D-2-amino-5-phosphonopentanoate (AP5, Sigma), CGP 52432 (Tocris, Ellisville, MO), (R)-baclofen (Tocris), cadmium chloride (CdCl₂, Sigma), dibutyryl-cAMP sodium salt (dBcAMP, Tocris) and Rp-Adenosine 3′,5′-cyclic monophosphorothioate triethylammonium salt hydrate (Rp-cAMP, Sigma) were made up as concentrated stock solutions (1000x) in distilled water and stored at -20°C (except for TTX, which was stored at 4°C). 9-(Tetrahydro-2-furanyl)-9H-purin-6-amine (SQ 22,536, Sigma) was made up as a concentrated stock solution (500x) in aCSF and stored at -20°C. L-(−)-Noradrenaline (+)-bitartrate salt monohydrate (norepinephrine, Sigma) was made the day of use as a concentrated stock solution (1000x) in distilled water. Protein kinase inhibitor-(6-22)-amide (PKI, Tocris) was made up as a concentrated stock solution (1000x) in distilled water and was added to the pipette internal solution on the day of use. Thapsigargin (Tocris), 2′,3′-Dideoxyadenosine (DDA, Tocris), 1,2-Bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis(acetoxymethyl ester) (BAPTA-AM, Sigma), and 2-aminoethoxydiphenylborate (2-APB,
Tocris) were made up as concentrated stock solutions (1000x) in dimethyl sulfoxide (DMSO) and stored at -20°C. WIN 55,212-2 mesylate (WIN, Tocris) was made up as a concentrated stock solution (5000x) in dimethyl sulfoxide (DMSO) and stored at -20°C. The final concentration of DMSO used in the experiments was less that 0.1%, which does not alter the miniature inhibitory postsynaptic current (mIPSC) properties (Kelm et al. 2007). When BAPTA-AM, SQ 22,536, DDA and H-89 were used in an experiment, there was at least a 30 minute drug pre-incubation period with the slice before starting the experiment. The drug stock solutions were diluted in aCSF and inserted into sealed syringes. CNQX (10 μM) and AP5 (50 μM) were added to all solutions inserted into the syringes. The sealed syringes were 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 about 150 μm from the experimental Purkinje cell. The membrane of the cell was broken into, and the control solution was delivered through the multi-barrel perfusion pencil. After allowing the cell to stabilize, a paired-pulse (PP) stimulation (0.2 ms duration and 50 ms inter-stimulus interval) was delivered at 0.1 Hz, which generated a PP record of two closely spaced evoked inhibitory postsynaptic currents (eIPSCs). A maximum stimulation was applied to determine the maximal current response, and half the maximal current response 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 an averaged PP trace 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 (eIPSC₂/eIPSC₁). The PPR value for each cell in a given group (pre-control, ethanol, washout) were averaged together and represented as the mean ± S.E.M. The “averaged control” values were calculated from the pre-control and washout ((pre-control + washout)/2).

**Protocol and analysis for mIPSC experiments.** After the membrane of the cell was broken into, the control solution, which included 1 µM TTX in addition to the CNQX and AP5, was delivered through the multi-barrel perfusion pencil. Once steady state was obtained (determined from the average of at least two repetitive recordings), a pre-control perfusion was recorded for 60 seconds. For the ethanol experiments, in addition to the pre-control recording, an ethanol recording and washout recording were collected. 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 results section went through the same protocol (pre-control, ethanol, washout) but were never subjected 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. All data were expressed as the mean ± S.E.M. If the control or pre-control baseline mIPSC frequency was lower than 0.5 Hz (except for experiments with the
control or pre-control value including exposure to WIN or baclofen), the experiment was excluded from analysis. The data were analyzed with miniAnalysis software. A bi-exponential fit for mIPSC decay times was determined using the miniAnalysis software, and fast ($\tau_{\text{fast}}$) and slow decay ($\tau_{\text{slow}}$) were analyzed separately.

**Statistics.** Paired Student’s t-test, Student’s t-test, one-way analysis of variance (ANOVA) and Dunnett post hoc test were performed as indicated. A two-tailed $p$ value less than .05 was accepted as statistically significant.
RESULTS

Ethanol increases mIPSC frequency and decreases the PPR.

Similar to previous results (Criswell et al. 2008; Kelm et al. 2007; Ming et al. 2006), 50 mM (14.2 ± 2.2%, n = 7) and 100 mM (28.7 ± 3.4%, n = 12) ethanol significantly increased mIPSC frequency compared to control (-0.53 ± 1.8%, n = 8) at the interneuron-Purkinje cell synapse as illustrated in Fig. 1A. In agreement with an earlier report (Ming et al. 2006), neither of the ethanol concentrations had an effect on mIPSC decay time or amplitude (data not shown). A cumulative probability curve from a representative neuron demonstrates that 100 mM ethanol shifted the distribution of the interevent interval curve to the left, which is interpreted as ethanol increasing mIPSC frequency (Fig. 1B). These results suggest that ethanol increases spontaneous GABA release at this synapse.

In addition to increasing mIPSC frequency, ethanol decreased the PPR at this same synapse. 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 Fig. 1C. A decrease in the PPR by ethanol is interpreted as an increase in evoked GABA release (Siggins et al. 2005). 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 Fig. 1D, 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 (i.e. PPR) compared to the pre-control and washout. These results suggest that ethanol increases evoked GABA release at the interneuron-Purkinje cell synapse.

Activation of Gαi-coupled GPCRs prevents ethanol from increasing mIPSC frequency.
Because of previous research demonstrating a link between G\(\alpha_{i/s}\)-coupled GPCRs and ethanol-enhanced GABA release (Ariwodola and Weiner 2004; Kang-Park et al. 2007; Nie et al. 2004; Roberto and Siggins 2006; Zhu and Lovinger 2006), we determined whether this observation was also true at the interneuron-Purkinje cell synapse. Both GABA\(_{B}\) receptors and cannabinoid receptors 1 (CB1) are located in the molecular layer of the cerebellum, where the presynaptic interneurons are located, and activation of both receptors inhibits baseline mIPSC frequency at this synapse (Harvey and Stephens 2004; Takahashi and Linden 2000; Yamasaki et al. 2006). Similar to these results, application of a GABA\(_{B}\) receptor agonist, baclofen (5 µM), and 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: 0.64 ± 0.2 Hz, n = 4; control: 2.9 ± 0.8 Hz, WIN: 1.1 ± 0.3 Hz, n = 13; Fig. 2A) 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 Fig. 2B. 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. 2C) compared to control. Shown in Fig. 2D 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 the G\(\alpha_i\)-linked pathway blocks ethanol-enhanced spontaneous GABA release (Fig. 2C), we wanted to determine if tonic activation of these G\(\alpha_i\)-linked GPCRs was masking the extent of the ethanol effect on both spontaneous and evoked GABA release. Our laboratory has shown that blockade of CB1 receptors does not affect ethanol-enhanced spontaneous GABA release (Kelm et al. 2007); however, we have not investigated GABA\(_B\) receptors. Compared to control (-1.8 ± 0.8%, n = 6, Fig. 3A), 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 the GABA\(_B\) receptor antagonist CGP 52432 (10 µM) to a similar extent to that seen in the absence of CGP 52432.

To see if an effective concentration of CGP 52432 was used, we determined if CGP 52432 antagonized the effect of baclofen on mIPSC frequency. 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. 3B). A trace from a representative neuron showing the effect of baclofen on mIPSC frequency and the ability of CGP 52432 to block it is shown in Fig. 3C. 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. 3D), suggesting that there is tonic activation of GABA\(_B\) receptors that can affect spontaneous GABA release. In the presence of CGP 52432, 100 mM ethanol significantly decreased the PPR (20.7 ± 3.1%, n = 10, Fig. 3E) 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.

Inhibition of adenylate cyclase and PKA blocks ethanol from increasing mIPSC frequency.
Next we determined if inhibiting adenylate cyclase and PKA 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 Fig. 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. It has been shown previously that SQ 22,536 can inhibit the norepinephrine-induced increase in mIPSC frequency at the interneuron-Purkinje cell synapse (Harvey and Stephens 2004), which activates Gαs-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.

To determine the role of PKA in ethanol-enhanced GABA release, we used two PKA antagonists, H-89 and Rp-cAMP, which 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 ethanol from increasing mIPSC frequency (8.2 ± 2.1%, n = 7 and 1.4 ± 2.5%, n = 10, respectively, Fig. 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 Fig. 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 non-specific effects that can start to occur at even 10 µM (Lochner and Moolman 2006). Overall, these results suggest that adenylate cyclase and PKA play 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, we included the membrane impermeable PKA antagonist, PKI, 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. 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. 4D). Because of the lack of PKI effect, there was concern that PKI was not reaching the postsynaptic neuron; however, PKI was able to block the effect of a PKA agonist in a separate experiment (see Fig. 5C). These results suggest that PKA is acting presynaptically to block 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. 2A), and this effect involves calcium release from RyRs (Yamasaki et al. 2006). Therefore, we 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, we used a sarcoendoplasmic reticulum calcium ATPase (SERCA) pump inhibitor, thapsigargin. The SERCA pump refills the internal stores with calcium, so inhibition of the pump leads to depletion of calcium from the internal stores. During exposure to thapsigargin, we applied a high potassium extracellular solution to deplete internal calcium stores at a faster rate by depolarizing the presynaptic terminals, which increases the rate of calcium release from the IP₃Rs and RyRs (Kelm et al. 2007; Simkus and Stricker 2002).
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. 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 IP₃R antagonist, 2-APB (14 µM), significantly reduced the ability of WIN to decrease mIPSC frequency (31.5 ± 6.3%, n = 8, Fig. 5A). As mentioned above, a similar effect has been shown with a RyR antagonist (Yamasaki et al. 2006). The voltage dependent calcium channel inhibitor, CdCl₂ (50 µM), did not significantly prevent WIN from decreasing mIPSC frequency (43.3 ± 6.9%, n = 8, Fig. 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). Our laboratory has shown that these concentrations of CdCl₂ and BAPTA are effective in this model system (Kelm et al. 2007). 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 we determined if PKA plays a role in the cannabinoid-induced decrease in mIPSC frequency. 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. 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 = -.53, p<.05), which suggests that dBcAMP inhibits WIN from decreasing spontaneous GABA release in a dose-dependent manner.

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 \( \tau_{\text{slow}} \) (Fig. 5C). With 300 \( \mu \text{M} \) dBcAMP, there was a significant decrease in baseline mIPSC amplitude (32 ± 2.9\%, n = 6. Fig. 5C) and an increase in baseline mIPSC \( \tau_{\text{slow}} \) (33.7 ± 9.4\%, n = 6). A trace from representative neuron showing that 300 \( \mu \text{M} \) dBcAMP increases mIPSC \( \tau_{\text{slow}} \) and decreases mIPSC amplitude is shown in Fig. 5Da. There was no effect on baseline mIPSC frequency or on baseline mIPSC \( \tau_{\text{fast}} \) at any dBcAMP concentration tested (data not shown). The change in mIPSC \( \tau_{\text{slow}} \) and amplitude appears to be due to a postsynaptic PKA mechanism because inclusion of 5 \( \mu \text{M} \) PKI in the internal solution blocked 300 \( \mu \text{M} \) dBcAMP from increasing mIPSC \( \tau_{\text{slow}} \) (-.97 ± 4.4\%, n = 7) and decreasing mIPSC amplitude (2.3 ± 4.9\%, n = 7, Fig. 5C). A trace from a representative neuron showing this PKI effect is in Fig. 5Db. These results suggest that the PKA agonist is having a PKA-dependent, postsynaptic effect that is manifested through a change in mIPSC \( \tau_{\text{slow}} \) and amplitude.

**Buffering presynaptic calcium prevents a PKA antagonist from decreasing baseline mIPSC frequency.**

Because of the established role of PKA in neurotransmitter release (Seino and Shibasaki 2005), we determined if a PKA antagonist decreases baseline mIPSC frequency. Both 10 \( \mu \text{M} \) and 25 \( \mu \text{M} \) Rp-cAMP significantly decreased mIPSC frequency (by 23.3 ± 6.8\%, n = 7 and 31.2 ± 3.6\%, n = 11, respectively), while 1 \( \mu \text{M} \) Rp-cAMP was without effect (0.61 ± 4.8\%, n = 4, Fig. 6A). At these concentrations of Rp-cAMP, no changes in mIPSC decay time or amplitude were observed (data not shown). In Fig. 6B a cumulative probability curve from a representative neuron demonstrates that 25 \( \mu \text{M} \) Rp-cAMP shifts the distribution of the interevent interval curve to the right, which is interpreted as Rp-cAMP decreasing mIPSC frequency. At 100 \( \mu \text{M} \) 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, we 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. 6C) 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. 6D). 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. 6C). 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

Consistent with previous results (Criswell et al. 2008; Kelm et al. 2007; Ming et al. 2006), ethanol dose-dependently increased both evoked and spontaneous GABA release at the interneuron-Purkinje cell synapse. Our laboratory has shown previously that internal calcium stores play an important role in ethanol-enhanced GABA release (Kelm et al. 2007); however, it has not been determined how ethanol interacts with these internal calcium stores. The present experiments provide new insight into the mechanism responsible for ethanol-enhanced GABA release.

Involvement of the Gαi-linked GPCR pathway in ethanol-enhanced GABA release

The Gαi-coupled GPCR agonists, WIN 55,212-2 and baclofen, blocked ethanol from increasing spontaneous GABA release at the interneuron-Purkinje cell synapse. Consistent with these results, it has been recently presented 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 spontaneous IPSCs in the hippocampus (Ariwodola and Weiner 2004). However, in the ventral tegmental area baclofen does not inhibit ethanol from increasing spontaneous IPSCs, despite the fact that baclofen affects baseline GABA release (Theile et al. 2008).

Because activation of Gαi-linked GPCRs blocked ethanol from increasing spontaneous GABA release onto cerebellar Purkinje cells, we were curious to see if tonic activation of Gαi-coupled GPCRs was preventing ethanol from fully eliciting GABA release at the interneuron-Purkinje cell synapse. Previous work from our laboratory showed that blockade of CB1 receptors does not affect the ability of ethanol to increase GABA release (Kelm et al. 2007). In the current study, we found that, despite tonic activation of the GABA_B receptors, a GABA_B receptor antagonist does 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 ventral tegmental area (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.

**Adenylate cyclase and PKA play an important role in the mechanism of ethanol-enhanced GABA release**

Adenylate cyclase and PKA, which are downstream messengers of the G\(\alpha_i/s\) G proteins, were found to play an essential role in 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, we are confident that this PKA effect is presynaptic. While this work provides a link between ethanol-enhanced GABA release and the adenylate cyclase-PKA pathway, more research is needed to determine how ethanol is interacting with this pathway.

There is considerable evidence connecting the adenylate cyclase/PKA pathway to certain effects of ethanol (Newton and Messing 2006; Pandey 1998). 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* exposure to ethanol induces a long-lasting potentiation of GABAergic synapses in the ventral tegmental area (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.
(Fee et al. 2006; Lai et al. 2007; Misra and Pandey 2006; Thiele et al. 2000; Wand et al. 2001). Therefore, there is evidence of the adenylate cyclase/PKA pathway playing an important role in multiple ethanol actions extending from molecular to behavioral.

**CBs and ethanol act in opposite directions through similar downstream messengers to alter spontaneous GABA release**

To learn more about the mechanism behind the ethanol-induced increase in GABA release, we studied the mechanism responsible for the WIN-induced decrease in GABA release. Previously, calcium release from RyRs was shown to play 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$_3$Rs 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$_2$ had no significant effect on baseline mIPSC frequency (Takahashi and Linden 2000). Additionally, 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 presynaptic.

We used a PKA agonist, dBcAMP, to determine the role of PKA in the cannabinoid-mediated suppression of spontaneous GABA release. Interestingly, during the application of 300 µM dBcAMP, baseline mIPSC $\tau$$_{\text{slow}}$ and amplitude were increased and decreased, respectively. Both decreases 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 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 by the membrane impermeable PKA antagonist in the pipette internal solution. 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. 6A, 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.

A PKA antagonist decreases spontaneous GABA release through a calcium-dependent mechanism

The PKA antagonist, Rp-cAMP (10 \(\mu\)M and 25 \(\mu\)M), inhibited baseline spontaneous GABA release, which is consistent with the established role for PKA in neurotransmitter release (Seino and Shibasaki 2005). Additionally, similar effects of a PKA antagonist on baseline spontaneous GABA release are seen in the hippocampus and hypothalamus (Jeong et al. 2003; Lee et al. 2008). This PKA antagonist effect was not seen in the tuberomammillary nucleus, but this could be due to the low concentration of the PKA antagonist used in this study (Yum et al. 2008). At 1 \(\mu\)M 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 it 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 plays a role in the PKA-antagonist mediated decrease in spontaneous GABA release. Two possible calcium-dependent mechanisms related to PKA involve PKA phosphorylating the IP₃R and RyR to increase calcium release from internal stores (Bardo et al. 2006; Bugrim 1999; Mignery et al. 1990; Patterson et al. 2004; Sobie et al. 2006) and/or PKA phosphorylating a protein in the neurotransmitter release machinery that is involved in calcium dependent exocytosis (Chheda et al. 2001; Trudeau et al. 1996).

We 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 extracellular solution control protocol had the same effect. One possible explanation is that the presynaptic depolarization induced by the high potassium extracellular solution altered the phosphorylation state of different 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.

Is cross-talk between PKA and PKC involved in ethanol-enhanced GABA release?

Recent data have shown that protein kinase C (PKC)ε is necessary for ethanol to increase GABA release in the central nucleus of the amygdala (Bajo et al. 2008). We have shown previously that internal calcium stores play an important role in ethanol-enhanced GABA release, and in the current study we found that the Gq-coupled pathway also plays a role in this ethanol mechanism. Taken together, these data suggest that calcium release from
internal stores, PKCε, adenylate cyclase and PKA may all play a role in ethanol-enhanced GABA release. It is possible that ethanol is activating these components separately and/or there could be cross-talk occurring between them. Cross-talk occurs between PKA and PKC at the GABAergic nucleus basalis of Meynert synapses (Kubota et al. 2003). Moreover, there are ethanol effects that involve cross-talk between these protein kinases. One mechanism involves ethanol increasing adenylate cyclase isoform 7 (AC7) activity through PKCδ-mediated phosphorylation of AC7, which leads to activation of PKA (Tabakoff et al. 2001). Another example of an ethanol cross-talk mechanism involves ethanol inducing PKCε translocation to the cytosol through a PKA-dependent mechanism (Yao et al. 2008). This translocation of PKCε is thought to involve PKA activation of phospholipase Cβ. Therefore, it is tempting to speculate that the mechanism of ethanol-enhanced GABA release depends upon cross-talk occurring between PKA and PKC, but more work will be needed before any firm conclusions are reached.

Role of brain region-specificity in the GABAergic actions of ethanol

While we have presented data regarding the mechanism of ethanol-enhanced spontaneous GABA release at the interneuron-Purkinje cell synapse, it is unknown if this mechanism can be applied to every brain region where ethanol increases GABA release. When collectively looking at all of the experiments that have studied ethanol-enhanced GABA release, effects can vary by brain region. Specifically, ethanol increases GABA release in many (Carta et al. 2004; Criswell et al. 2008; Li et al. 2006; Roberto et al. 2003; Sanna et al. 2004; Sebe et al. 2003; Theile et al. 2008; Zhu and Lovinger 2006; Ziskind-Conhaim et al. 2003), but not all (Criswell et al. 2008; Jia et al. 2008; Moriguchi et al. 2007), brain regions. Additionally, activation and inhibition of GABA_B receptors alters ethanol-enhanced GABA release in a brain region-specific manner (Fig 2; Fig 3; Ariwodola and Weiner 2004; Theile et
Therefore, these findings suggest that, depending on the brain region, multiple mechanisms can affect ethanol-enhanced GABA release. As a result, the mechanism of ethanol-enhanced GABA release should be explored in each brain region where ethanol increases GABA release. While much remains uncertain regarding the mechanism of ethanol-enhanced GABA release, thinking in terms of the effect of ethanol in each brain region could help isolate the potentially differing mechanisms involved in this ethanol action and provide information regarding the behavioral GABAergic profile of ethanol.
ACKNOWLEDGEMENTS

None.

GRANTS

This work was supported by NRSA Predoctoral Fellowship AA 17025 and grants R01 AA 11605, RO1 AA 14284 and RO1 AA 14949 from the National Institute on Alcohol Abuse and Alcoholism.

DISCLOSURES

None.
REFERENCES


**Criswell HE and Breese GR.** A conceptualization of integrated actions of ethanol contributing to its GABAmimetic profile: a commentary. *Neuropsychopharmacology* 30: 1407-1425, 2005.

**Criswell HE, Ming Z, Kelm MK, and Breese GR.** Brain Regional Differences in the Effect of Ethanol on Gaba Release from Presynaptic Terminals. *J Pharmacol Exp Ther*, 2008.

**Criswell HE, Simson PE, Duncan GE, McCown TJ, Herbert JS, Morrow AL, and Breese GR.** Molecular basis for regionally specific action of ethanol on gamma-aminobutyric acidA receptors: generalization to other ligand-gated ion channels. *J Pharmacol Exp Ther* 267: 522-537, 1993.


Grobin AC, Matthews DB, Devaud LL, and Morrow AL. The role of GABA(A) receptors in the acute and chronic effects of ethanol. *Psychopharmacology (Berl)* 139: 2-19, 1998.


FIGURE LEGENDS

Figure 1. Ethanol increases miniature inhibitory postsynaptic current (mIPSC) frequency and decreases the paired-pulse ratio (PPR). A, compared to control, 50 and 100 mM ethanol decreased mIPSC frequency (\(^*\), \(p<.05\), one-way ANOVA, Dunnett’s post hoc test). B, a cumulative frequency histogram from a representative neuron demonstrating that the ethanol (100 mM) application shifted the distribution of the mIPSC interevent interval curve to the left, indicating that ethanol increases mIPSC frequency. C, the PPR was decreased at 50, 75 and 100 mM ethanol (\(^*\), \(p<.05\), paired Student’s \(t\) test). D, traces from a representative neuron demonstrating that ethanol decreased the ratio of the amplitude of the second evoked inhibitory postsynaptic current (eIPSC) to the amplitude of the first eIPSC compared to pre-control and washout.

Figure 2. WIN 55,212-2 (WIN) and baclofen decrease baseline mIPSC frequency and prevent ethanol from increasing mIPSC frequency. A, WIN (5 \(\mu\)M) and baclofen (5 \(\mu\)M) reduced baseline mIPSC frequency (\(^*\), \(p<.05\), paired Student’s \(t\) test). B, a cumulative frequency histogram from a representative neuron demonstrating that WIN caused a rightward shift of the interevent interval curve, which suggests that WIN decreases mIPSC frequency. C, WIN and baclofen prevented 100 mM ethanol from increasing mIPSC frequency compared to control (\(^*\), \(p<.05\), one-way ANOVA, Dunnett’s post hoc test). D, a cumulative frequency histogram from a representative neuron demonstrating that ethanol did not shift the interevent interval curve in the presence of WIN, indicating that WIN blocks the effect of ethanol on mIPSC frequency.

Figure 3. A GABA\(_B\) antagonist does not affect the ethanol-induced increase in mIPSC frequency or decrease in PPR. A, CGP 52432 (CGP, 10 \(\mu\)M) did not alter the ability of 50 and 100 mM ethanol to increase mIPSC frequency compared to control (\(^*\), \(p<.05\), 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 that baclofen inhibits mIPSC frequency and CGP reverses this effect. 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).

Figure 4. Adenylate cyclase and protein kinase A (PKA) antagonists prevent 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 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 that Rp-cAMP prevents ethanol from increasing mIPSC frequency. 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 that ethanol still shifted the curve to the left when PKI was included in the pipette internal solution.

Figure 5. Inhibition of calcium release from internal stores and activation of PKA prevent WIN 55,212-2 (WIN) from decreasing mIPSC frequency. A, thapsigargin (Thaps, 1 µM) and 2-APB (14 µM) inhibited WIN (5 µM) from decreasing mIPSC frequency, while the high potassium extracellular solution control (K⁺ soln, 15 mM), cadmium chloride (CdCl₂, 50 µM), and BAPTA in the pipette internal solution (BAPTAint, 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). C, baseline mIPSC τslow 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 \( \tau_{\text{slow}} \) and amplitude was blocked when 5 µM PKI was included in the pipette internal solution. D, a trace from a representative neuron showing the effect of 300 µM dBcAMP on mIPSC \( \tau_{\text{slow}} \) and amplitude (a.) and the ability of 5 µM PKI in the pipette internal solution to block this effect (b.).

**Figure 6.** BAPTA-AM prevents Rp-cAMP from decreasing mIPSC frequency. A, Rp-cAMP (10 and 25 µM) was able to significantly reduce 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 that 25 µM Rp-cAMP shifted the distribution of the interevent interval curve to the right, which indicates that Rp-cAMP decreases mIPSC frequency. C, pre-incubation of the slice with BAPTA-AM (BAP-AM, 100 µM) was able to decrease the effect of 25 µM Rp-cAMP on mIPSC frequency compared to control, while BAPTA in the internal solution (BAPTA\(_{\text{int}}\), 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 that 100 µM BAPTA-AM prevented 25 µM Rp-cAMP from shifting the curve.
Figure 1

A. Bar graph showing % Inc in mIPSC Frequency for different concentrations of EtOH compared to control. The bar for 100 mM EtOH is significantly higher than the control and 50 mM EtOH.

B. Cumulative probability plot of interevent interval (msec) showing data for control and 100 mM EtOH. The cumulative probability for 100 mM EtOH is lower than control.

C. Bar graph showing PPR (eIPSC²/eIPSC₁) for different concentrations of EtOH. The bars for 75 mM and 100 mM EtOH are significantly lower than control.

D. Graph showing electrophysiological traces before and after exposure to 100 mM EtOH. The trace after exposure shows a decrease in amplitude and a change in shape, indicating a change in neuronal function.
Figure 2

A. Bar graph showing frequency (Hz) of mIPSC in control and drug-treated conditions. Control: 100 mM EtOH, 5 µM WIN, 5 µM baclofen. * indicates significance.

B. Cumulative probability plots showing interevent interval (msec) for TTX and 5 µM WIN.

C. Bar graph showing percent increase in mIPSC frequency for control conditions: 100 mM EtOH, 5 µM WIN, 5 µM baclofen + 100 mM Ethanol. * indicates significance.

D. Cumulative probability plots showing interevent interval (msec) for 5 µM WIN and 5 µM WIN + 100 mM EtOH.
Figure 3

A. % Inc in mIPSC Frequency

B. Frequency (Hz)

C. Control: TTX

D. Frequency (Hz)

E. PPR (eIPSC$_2$/eIPSC$_1$)

- Control: CGP 52432
- CGP 52432 + 50 mM EtOH
- CGP 52432 + 100 mM EtOH

- Control: TTX
- 5 µM baclofen
- 5 µM baclofen + 10 µM CGP 52432

- TTX
- 10 µM CGP

- Average Control (CGP)
- CGP + 100 mM EtOH
Figure 4

A. Bar graph showing the percentage increase in mIPSC frequency. The control is 100 mM EtOH, and treatments include 300 µM SQ, 10 µM DDA, 10 µM H-89, 10 µM Rp, and the combination of 10 µM Rp-cAMP + 100 mM EtOH.

B. Graph showing the effect of pre-control (10 µM Rp-cAMP), 10 µM Rp-cAMP + 100 mM EtOH, and washout (10 µM Rp-cAMP) on mIPSCs. The traces are recorded over 3 seconds with 20 pA sensitivity.

C. Bar graph showing the percentage increase in mIPSC frequency. The control is 5 µM PKI, and treatments include 5 µM PKI + 50 mM EtOH and 5 µM PKI + 100 mM EtOH.

D. Cumulative probability graph comparing 5 µM PKI and 5 µM PKI + 100 mM EtOH for interevent interval (msec).
Figure 5

A. % Dec in mIPSC Frequency

<table>
<thead>
<tr>
<th>Condition</th>
<th>% Dec</th>
<th>Error Bars</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control: 5 µM WIN</td>
<td>60</td>
<td>±10</td>
</tr>
<tr>
<td>1 µM Thaps</td>
<td>50</td>
<td>±5</td>
</tr>
<tr>
<td>15 mM K⁺ soln</td>
<td>40</td>
<td>±4</td>
</tr>
<tr>
<td>14 µM 2-APB</td>
<td>30</td>
<td>±3</td>
</tr>
<tr>
<td>50 µM CdCl₂</td>
<td>20</td>
<td>±2</td>
</tr>
<tr>
<td>30 mM BAPTA_{int}</td>
<td>10</td>
<td>±1</td>
</tr>
</tbody>
</table>

B. % Dec in mIPSC Frequency

<table>
<thead>
<tr>
<th>Condition</th>
<th>% Dec</th>
<th>Error Bars</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control: 5 µM WIN</td>
<td>60</td>
<td>±10</td>
</tr>
<tr>
<td>30 µM [dBcAMP] + 5 µM WIN</td>
<td>50</td>
<td>±5</td>
</tr>
<tr>
<td>100 µM [dBcAMP] + 5 µM WIN</td>
<td>40</td>
<td>±4</td>
</tr>
<tr>
<td>300 µM [dBcAMP] + 5 µM WIN</td>
<td>30</td>
<td>±3</td>
</tr>
</tbody>
</table>

C. % change

<table>
<thead>
<tr>
<th>Condition</th>
<th>% change</th>
<th>Error Bars</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control: 5 µM WIN + 5 µM WIN</td>
<td>0</td>
<td>±0</td>
</tr>
<tr>
<td>1 µM Thaps</td>
<td>5</td>
<td>±1</td>
</tr>
<tr>
<td>15 mM K⁺ soln</td>
<td>10</td>
<td>±2</td>
</tr>
<tr>
<td>14 µM 2-APB</td>
<td>15</td>
<td>±3</td>
</tr>
<tr>
<td>50 µM CdCl₂</td>
<td>20</td>
<td>±4</td>
</tr>
<tr>
<td>30 mM BAPTA_{int}</td>
<td>25</td>
<td>±5</td>
</tr>
</tbody>
</table>

D. Amplitude

- a. TTX
- b. 300 µM dBcAMP

In presence of 5 µM PKI

<table>
<thead>
<tr>
<th>Current</th>
<th>Time (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 pA</td>
<td>5 ms</td>
</tr>
</tbody>
</table>