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**Calcium Release from Presynaptic Internal Stores is Required for
Ethanol to Increase Spontaneous Gamma-aminobutyric Acid Release
onto Cerebellum Purkinje Neurons**

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Nonstandard Abbreviations:

aCSF- artificial cerebrospinal fluid

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ANOVA- analysis of variance

2-APB- 2-aminoethyl diphenylborinate

AP5- 2-amino-5-phosphonovaleric acid

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

Ca²⁺_{ext} – calcium external

CB- cannabinoid

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

CdCl₂- cadmium chloride

DMSO- dimethyl sulfoxide

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

EtOH- ethanol

GABA- Gamma-aminobutyric Acid

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

IP₃R- inositol 1,4,5-trisphosphate receptor

K⁺_{ext} -potassium external

mIPSC- miniature inhibitory postsynaptic current

Ry- ryanodine

RyR- ryanodine receptor

SERCA- sarco(endo)plasmic reticulum calcium ATPase

Thaps- thapsigargin

TTX- tetrodotoxin

VDCC- voltage dependent calcium channel

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ABSTRACT

Recent data have demonstrated that ethanol increases gamma-aminobutyric acid (GABA) release in many brain regions, but little is known about the mechanism responsible for this action. Consistent with previous results, ethanol increased miniature inhibitory postsynaptic current (mIPSC) frequency at the interneuron-Purkinje cell synapse in the slice and in mechanically dissociated neurons. These data suggest that ethanol is increasing spontaneous GABA release at this synapse. It is generally accepted that ethanol increases levels of intracellular calcium, and changes in intracellular calcium can alter neurotransmitter release. Therefore, we examined the contribution of calcium-dependent pathways to the effect of ethanol on spontaneous GABA release at the interneuron-Purkinje cell synapse. Ethanol continued to increase mIPSC frequency in a nominally calcium free extracellular solution and in the presence of a voltage dependent calcium channel inhibitor, cadmium chloride. These data suggest that influx of extracellular calcium does not play a critical role in the mechanism of ethanol-enhanced spontaneous GABA release. However, a sarco/endoplasmic-reticulum calcium ATPase pump inhibitor (thapsigargin), an inositol 1,4,5-trisphosphate receptor antagonist (2-aminoethoxydiphenylborate), and a ryanodine receptor antagonist (ryanodine) significantly reduced the ability of ethanol to increase mIPSC frequency. In addition, ethanol was still able to increase mIPSC frequency in the presence of intracellular BAPTA and a cannabinoid receptor antagonist (AM-251); thus, retrograde messengers are not involved in ethanol-enhanced spontaneous GABA release. Overall, these data suggest that calcium release from presynaptic internal

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stores plays a vital role in the mechanism of ethanol-enhanced spontaneous GABA release at the interneuron-Purkinje cell synapse.

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INTRODUCTION

Many of the sedative behavioral effects of ethanol mimic those of the benzodiazepines and barbiturates (Frye and Breese, 1982; Liljequist and Engel, 1982), which enhance the effect of gamma-aminobutyric acid (GABA) by acting directly on the GABA_A receptor (Mohler and Okada, 1977; Briley and Langer, 1978). This functional comparison suggested that the behavioral consequences of ethanol involved a GABAergic mechanism.

As a result, subsequent research focused on ethanol acting directly on the GABA_A receptor; however, this research produced mixed results. While early studies found that ethanol enhanced GABA-induced Cl⁻ currents (Suzdak et al., 1986; Aguayo, 1990; Reynolds et al., 1992), a majority of later studies failed to find a direct effect of ethanol at physiologically relevant concentrations on GABA-induced Cl⁻ currents (Sigel et al., 1993; Mori et al., 2000; Criswell et al., 2003). Because of these inconsistent results, focus has been placed on ethanol interacting with specific GABA_A receptor subunits (for reviews see Boehm et al., 2006; Lovinger and Homanics, 2007). These studies led to the observation that at low concentrations ethanol acts directly on GABA_A receptors containing δ and $\beta 3$ subunits associated with $\alpha 4$ or $\alpha 6$ subunits, which are predominately located on extrasynaptic sites (for review see Wallner et al., 2006). However, this effect of ethanol has not always been reproducible (Borghese et al., 2006; Botta et al., 2007). Overall, while ethanol may interact with specific postsynaptic GABA_A receptors, the GABAergic profile of ethanol appears to be more convoluted than previously thought (Weiner and Valenzuela, 2006).

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Recent data have demonstrated that ethanol increases both evoked and spontaneous GABA release in many brain regions (for reviews see Criswell and Breese, 2005; Siggins et al., 2005; Weiner and Valenzuela, 2006). These observations suggest that GABA release contributes to the GABAergic profile of ethanol. Because this concept has only recently been investigated, little is known about the mechanism through which ethanol acts to enhance GABA release.

It is generally accepted that physiologically relevant ethanol concentrations increase intracellular calcium levels (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). 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 could occur through an increase in extracellular calcium influx or through an increase in calcium release from internal stores. Consequently, we examined whether these calcium-dependent pathways contribute to the mechanism by which ethanol enhances spontaneous GABA release at the interneuron-Purkinje cell synapse.

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METHODS

Preparation of slices. Sprague Dawley rats, 12-20 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, 5 sucrose (pH to 7.4 with NaOH). The cerebella were isolated and parasagittal slices, 400 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 (BX50WI, 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 LUMPlanFI 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

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following composition (in mM):150 KCl, 3.1 MgCl₂, 15 HEPES, 5 K-ATP, 5 EGTA, 15 phosphocreatine (pH to 7.4 with KOH). For the 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid (BAPTA) experiments, the internal solution consisted of the following composition (in mM):105 KCl, 3.1 MgCl₂, 15 HEPES, 5 K-ATP, 30 BAPTA tetrapotassium salt (Sigma), 15 phosphocreatine (pH to 7.4 with KOH). Data were displayed on an oscilloscope (V-212, Hitachi, Japan), digitized at 5 kHz, and stored on a personal computer. The recordings were performed 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). In all experiments, 1 μM tetrodotoxin (TTX, Sigma), 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, Sigma) and 50 μM D-2-amino-5-phosphonopentanoate (AP5, Sigma) were included in all solutions attached to the drug pencil. Addition of 50 μM bicuculline methochloride (Tocris, Ellisville, MO), a GABA_A antagonist, abolished the mIPSCs (n=4, data not shown), which confirmed that the mIPSCs were GABAergic. The capacitance was monitored continuously throughout the recordings, and a decrease of 25% or more was sufficient to exclude the recording from analysis. Only one data point per slice was collected.

Mechanically dissociated neuron preparation. Slices were 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 (~.2 mm amplitude at 10 Hz) for 3 minutes. When the resulting mechanical forces break neurons free from the matrix, most of the dendritic tree is sheared off as well as the distal parts of axons; however, the presynaptic terminals remain attached to the soma (Akaike and Moorhouse, 2003). The slice was then removed from the chamber and the

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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 MgCl₂ and 10 mM glucose (pH to 7.4 with NaOH).

Drug preparation and drug delivery system. Cadmium chloride (Sigma) was made up as a concentrated (1000x) solution in distilled water and stored at room temperature. Ryanodine (Calbiochem, San Diego, CA) was made up as a concentrated stock solution (10x) in aCSF and stored at -20°C. Thapsigargin (Tocris), AM-251 (Tocris), 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 (Tocris) was made up as concentrated stock solution (5000x) in dimethyl sulfoxide (DMSO) and stored at -20°C. The final concentration of DMSO used in the experiments was less than 0.1%, which did not alter the properties of the mIPSCs (n=4, data not shown). The drugs were diluted in aCSF and then inserted into sealed 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) and positioned 150-250 µm from the cell tested.

Ethanol protocol for mIPSC experiments. After the membrane of the cell was broken, the drug pencil was turned on to a control solution that included TTX, CNQX, and AP5 (see above). Once steady state was obtained (determined from the average of repetitive 20 second sweeps), a pre-control perfusion was recorded for 30 to 120

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seconds before the application of ethanol. After completion of the pre-control recording, the ethanol solution was turned on and maximal ethanol effects were seen within five minutes in the slice and 30 seconds in the mechanically dissociated neurons. Once steady state was obtained, the ethanol response was recorded for 30 to 120 seconds. When the ethanol application was turned off, the ethanol washed out within five minutes in the slice and within 30 seconds in the mechanically dissociated neuron preparation. Once steady state was obtained, the washout was recorded for 30 to 120 seconds. This same ethanol protocol was used throughout in the presence of different antagonists and solutions. 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 (see Figure 1), to determine if the antagonist was having an effect on lower ethanol concentrations.

Data analysis for mIPSC experiments. The data were expressed as the mean \pm S.E.M. The “% Change in mIPSC Frequency” on the y-axis represents the change in mIPSC frequency induced by ethanol compared to an averaged control. The averaged control consists of the “pre control” (i.e. before ethanol) value and the “washout” value (i.e. after ethanol). The percent (%) change in mIPSC frequency, decay time and amplitude were calculated as follows: $100 * ((\text{“ethanol response”} / ((\text{“pre-control”} + \text{“washout”}) / 2)) - 100)$. The data were analyzed with miniAnalysis software (version 5.6.4; Synaptosoft, Decatur, GA).

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Statistics. Student's t-test, paired 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.

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RESULTS

Ethanol selectively increases mIPSC frequency at the interneuron-Purkinje cell synapse in the slice and in the mechanically dissociated neuron preparation. In

agreement with a previous report from our laboratory (Ming et al., 2006), ethanol increased the mIPSC frequency at the interneuron-Purkinje cell synapse in the slice. Compared to control ($99.39 \pm 2.8\%$, $n=8$), the mIPSC frequency of cerebellar Purkinje neurons was significantly increased by 50 mM ($110.93 \pm 1.98\%$, $n=9$), 75 mM ($118.57 \pm 3.52\%$, $n=9$) and 100 mM ($122.87 \pm 3.8\%$, $n=12$) ethanol, but not by 25 mM ($102.16 \pm 3.06\%$, $n=12$) ethanol, as illustrated in Figure 1C. In contrast, none of the ethanol concentrations had an effect on mIPSC decay time and amplitude (data not shown, see Ming et al., 2006). A representative neuron demonstrating the effect of 100 mM ethanol on mIPSC frequency is shown in Figure 1A, while the lack of effect of ethanol on mIPSC decay time and amplitude from the same representative neuron is shown in Figure 1B.

Mechanically dissociated neurons were also used to study the involvement of calcium-dependent pathways in the mechanism of ethanol-enhanced spontaneous GABA release. While baseline mIPSC decay time, rise time, and amplitude were all different in the mechanically dissociated neuron preparation compared to the slice, the baseline mIPSC frequency was not significantly different (see Table 1). Similar to the data collected in the slice, 50 mM ($136.44 \pm 9.14\%$, $n=9$) and 100 mM ($121.96 \pm 11.39\%$, $n=10$) ethanol significantly increased the mIPSC frequency of mechanically dissociated cerebellar Purkinje neurons compared to control ($89.25 \pm 6.17\%$, $n=9$, Fig. 2B), while having no effect on mIPSC decay time and amplitude (data not shown). A representative neuron demonstrating the effect of 100 mM ethanol on mIPSC frequency

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in a mechanically dissociated neuron is shown in Figure 2A. It should be noted that the effect of 50 mM ethanol on the mIPSC frequency with the mechanically dissociated neuron preparation is significantly greater than the effect of 50 mM ethanol on the mIPSC frequency with the slice ($p < .05$, Student's t-test). With the ethanol-induced increase in spontaneous GABA release onto cerebellar Purkinje neurons documented in the slice and in mechanically dissociated neurons, we explored the contribution of calcium-dependent pathways to this ethanol effect.

Extracellular calcium influx is not required for ethanol to increase mIPSC

frequency in the mechanically dissociated neuron preparation.

A nominally calcium free extracellular ($0 \text{ mM Ca}^{2+}_{\text{ext}}$) solution was used to eliminate all sources of extracellular calcium that could contribute to the ethanol-induced increase in mIPSC frequency. To conduct this experiment in a slice, extended exposure to the $0 \text{ mM Ca}^{2+}_{\text{ext}}$ solution is necessary to ensure that the solution is reaching the neuron; however, extended exposure to the $0 \text{ mM Ca}^{2+}_{\text{ext}}$ solution can also reduce intracellular calcium levels, which is not desirable because the contribution of extracellular calcium needed to be investigated independently. Therefore, the mechanically dissociated neuron preparation was utilized because it allows for almost instantaneous access of the solution to the neuron and dramatically reduces the possibility of the $0 \text{ mM Ca}^{2+}_{\text{ext}}$ solution affecting levels of intracellular calcium. In the presence of the $0 \text{ mM Ca}^{2+}_{\text{ext}}$ solution, 50 mM ($168.7 \pm 11.67\%$; $n=9$) and 100 mM ($165.80 \pm 13.7\%$; $n=8$) ethanol were still able to significantly increase mIPSC frequency compared to control ($93.26 \pm 3.79\%$; $n=7$, Fig 3B). Interestingly, the effect of 50 and 100 mM ethanol on mIPSC

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frequency was significantly enhanced in the presence of the 0 mM $\text{Ca}^{2+}_{\text{ext}}$ solution compared to the effect of 50 mM and 100 mM ethanol on mIPSC frequency in control conditions (* $p < .05$, Student's t test). Compared to control ($1.38 \pm .40$ Hz; $n=11$), the 0 mM $\text{Ca}^{2+}_{\text{ext}}$ solution did not significantly decrease baseline mIPSC frequency ($.90 \pm .23$ Hz ; $n=7$), although there was a trend towards a decrease. To confirm that the solution around the neuron was free of calcium, it was determined if the 0 mM $\text{Ca}^{2+}_{\text{ext}}$ solution could block calcium-dependent GABA release. Compared to control ($.45 \pm .16$ Hz; $n=4$), addition of a high potassium (K^+ , 15 mM) HEPES buffered solution increased spontaneous IPSC GABA release (5.24 ± 1.3 Hz; $n=4$), while the 0 mM $\text{Ca}^{2+}_{\text{ext}}$ solution with the same high K^+ concentration returned GABA release to baseline values ($.33 \pm .03$ Hz; $n=4$).

Similar experiments were conducted with cadmium chloride (CdCl_2), a non-specific voltage-dependent calcium channel inhibitor, to confirm the lack of importance of extracellular calcium influx in the mechanism of ethanol-enhanced GABA release. As expected, 50 mM ($140.61 \pm 11.64\%$; $n=10$) and 100 mM ($150.3 \pm 10.31\%$; $n=9$) ethanol still significantly increased mIPSC frequency in the presence of 50 μM CdCl_2 compared to control ($95.15 \pm 8.53\%$; $n=11$, Fig 3B). However, in the presence of 50 μM CdCl_2 the effect of ethanol did not always wash out, as demonstrated with the representative neuron in Figure 3A. Because this lack of a washout could affect the calculated % change in mIPSC frequency (see methods), all of the CdCl_2 mechanically dissociated neuron data were reanalyzed without the washout included. This additional analysis resulted in the same conclusions made from the data with the washout included. Compared to control ($1.38 \pm .40$ Hz; $n=9$), 50 μM CdCl_2 did not significantly decrease

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baseline mIPSC frequency ($.92 \pm .22$ Hz ; n=11), although there was a trend towards a decrease. The same control with the high K^+ HEPES buffered solution was carried out with $50 \mu\text{M CdCl}_2$. Compared to control ($.99 \pm .63$ Hz; n=3), addition of a high K^+ HEPES buffered solution increased spontaneous IPSC GABA release (14.1 ± 2.78 Hz; n=3; * $p < .05$, paired Student's t test), while addition of $50 \mu\text{M CdCl}_2$ to the high K^+ HEPES buffered solution returned GABA release to baseline values ($1.52 \pm .74$ Hz; n=3). These data collectively suggest that influx of extracellular calcium is not required for ethanol to increase mIPSC frequency.

In the slice 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 internal stores because the inositol 1,4,5-trisphosphate receptors (IP_3Rs) and ryanodine receptors (RyRs) continually release calcium from internal stores (Galante and Marty, 2003). However, depleting internal calcium stores 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 a high potassium extracellular (K^+_{ext} , 15 mM) solution was utilized 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 exposure of the slice to the

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high K^+_{ext} solution had no effect on the ability of 100 mM ethanol to increase mIPSC frequency after the high K^+_{ext} solution had been washed out ($127.82 \pm 5.04\%$; $n=3$). After completing the high K^+_{ext} solution protocol in the presence of thapsigargin, ethanol was not able to increase mIPSC frequency ($105.16 \pm 4.69\%$; $n=11$; Fig 4A). A representative neuron demonstrating the lack of effect of ethanol on mIPSC frequency in the presence of thapsigargin is shown in Figure 4B. Compared to control ($2.18 \pm .43$ Hz; $n=9$), depleting internal stores did not have an effect on the baseline mIPSC frequency ($2.13 \pm .36$ Hz ; $n=11$).

Because depletion of internal calcium stores prevented ethanol from increasing spontaneous GABA release, we next determined whether inhibition of the IP_3 Rs and RyRs would prevent ethanol from increasing spontaneous GABA release. The IP_3 R antagonist, 2-APB (14 μ M), significantly blocked the ability of 100 mM ethanol to increase mIPSC frequency ($105.14 \pm 2.72\%$; $n=10$; Fig. 4A). Application of 2-APB (14 μ M) did not have a significant effect on baseline mIPSC frequency ($1.83 \pm .29$ Hz ; $n=10$) compared to control ($2.18 \pm .43$ Hz; $n=9$).

Subsequently, we determined whether inhibition of the RyRs would prevent ethanol from increasing mIPSC frequency. At a concentration of 100 μ M, 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 ($108.65 \pm 4.62\%$; $n=13$; Fig

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4A). Compared to control ($2.18 \pm .43$ Hz; $n=9$), 100 μ M ryanodine did not have an effect on baseline mIPSC frequency ($2.22 \pm .23$ Hz ; $n=13$).

In the slice retrograde messengers do not contribute to the ethanol-induced

increase in mIPSC frequency. Because bath application of thapsigargin, 2-APB and ryanodine affects both presynaptic and postsynaptic calcium levels, calcium-dependent retrograde messengers could be responsible for the presynaptic effect of ethanol in lieu of ethanol acting directly on the presynaptic site. Therefore, BAPTA (30 mM) was included in the internal solution to buffer changes in postsynaptic calcium levels that could contribute to the release of calcium-dependent retrograde messengers.

Compared to control ($102.36 \pm 2.76\%$, $n=7$), 50 mM ($119.98 \pm 5.57\%$, $n=8$) and 100 mM ($123.85 \pm 4.3\%$, $n=6$) ethanol were still able to significantly increase mIPSC frequency with 30 mM BAPTA in the internal solution (Fig 5B). A representative neuron showing the effect of 50 mM ethanol on mIPSC frequency with 30 mM BAPTA in the internal solution is shown in Figure 5A. In addition, inclusion of BAPTA in the internal solution significantly increased the baseline mIPSC slow decay time (control: $11.75 \pm .32$ ms vs. BAPTA: 14.12 ± 1.12 ms, Fig 5C) and significantly decreased the baseline mIPSC amplitude (control: $15.4 \pm .65$ pA vs. BAPTA: 12.19 ± 1.12 pA, Fig 5C) and frequency (control: $2.5 \pm .26$ Hz vs. BAPTA: $1.75 \pm .27$ Hz, Fig 5C). These data suggest that BAPTA was reaching the postsynaptic neuron but was not inhibiting ethanol-enhanced spontaneous GABA release.

Cannabinoids (CBs) are the most widely recognized retrograde messengers in the cerebellum, and activation of CB receptors inhibits spontaneous GABA release from

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the interneurons (Fig 6B and Yamasaki et al., 2006). Because it has been proposed that endogenous CB release from postsynaptic neurons does not always require the presence of calcium (Hashimotodani et al., 2007), the contribution of CBs might not have been eliminated in the intracellular BAPTA experiments. Therefore, a CB receptor antagonist, AM-251 (5 μ M), was used to test this possibility. In the presence of AM-251, 50 mM ($115.34 \pm 3.36\%$, $n=7$) and 100 mM ($129.4 \pm 7.13\%$, $n=7$) ethanol significantly increased mIPSC frequency compared to control ($100.9 \pm 3.58\%$, $n=7$, Fig 6A). To document that this concentration of AM-251 (5 μ M) was effective as a CB receptor antagonist, the ability of AM-251 to block the CB-induced decrease in mIPSC frequency was tested. A CB agonist, WIN-55212-2 (5 μ M), significantly reduced ($.94 \pm .2$ Hz, $n=6$) the mIPSC frequency compared to control ($1.68 \pm .24$ Hz, $n=6$) at the interneuron-Purkinje cell synapse (Yamasaki et al., 2006), and 5 μ M AM-251 was sufficient to reverse this effect of WIN- 55212-2 ($1.63 \pm .26$ Hz, $n=6$, Fig 6B).

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DISCUSSION

In addition to ethanol increasing the mIPSC frequency of cerebellar Purkinje neurons (Ming et al., 2006), ethanol also increases the mIPSC frequency of central nucleus of the amygdala neurons (Roberto et al., 2003), basolateral amygdala neurons (Zhu and Lovinger, 2006), hippocampal CA1 pyramidal neurons (Sanna et al., 2004; Li et al., 2006), brainstem motor neurons (Sebe et al., 2003), and cerebellar granule cell neurons (Carta et al., 2004). However, in cultured cortical neurons ethanol actually decreases mIPSC frequency (Moriguchi et al., 2007). Therefore, information regarding the mechanism of this ethanol-induced, brain-region specific increase in spontaneous GABA release could be useful in defining the GABAergic behavioral profile of ethanol.

While ethanol increases mIPSC frequency at the interneuron-Purkinje cell synapse (Fig 1 and 2), it has no effect on mIPSC decay time or amplitude. 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 increase in mIPSC frequency indicates that ethanol is increasing spontaneous GABA release, whereas the lack of ethanol effect on mIPSC decay time indicates that ethanol is not altering the functioning of the postsynaptic GABA_A receptors in our recording conditions. In addition, ethanol did not alter the mIPSC amplitude, which further supports the conclusion that the change in mIPSC frequency by ethanol can be interpreted as ethanol having a presynaptic effect. While we did not observe a postsynaptic action of ethanol in either the slice or the mechanically dissociated neuron preparation, the intracellular milieu of the postsynaptic neuron is altered during whole-cell voltage clamp recordings (for

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review see Sarantopoulos et al., 2004); therefore, postsynaptic effects of ethanol cannot be ruled out at this synapse. Because ethanol increases the mIPSC frequency of mechanically dissociated cerebellar Purkinje neurons, it is not possible that ethanol is acting through glia to increase mIPSC frequency. Overall, our work and the work of others suggest that ethanol increases spontaneous GABA release in multiple brain regions through a presynaptic mechanism.

Voltage-dependent calcium channels, receptor-operated channels (ROCs) and store-operated channels (SOCs) increase intracellular calcium levels by allowing extracellular calcium to flow into the neuron (Fig 7). A 0 mM $\text{Ca}^{2+}_{\text{ext}}$ solution was used to eliminate the functionality of these channels to determine their importance in the mechanism of ethanol-enhanced spontaneous GABA release. Ethanol continued to increase mIPSC frequency in the presence of the 0 mM $\text{Ca}^{2+}_{\text{ext}}$ solution (Fig 3B), which suggests that extracellular calcium influx does not play a role in this ethanol mechanism. Interestingly, the effect of ethanol on mIPSC frequency in the presence of the 0 mM $\text{Ca}^{2+}_{\text{ext}}$ solution was actually enhanced compared to the effect of ethanol on mIPSC frequency in the control conditions. In the presence of the 0 mM $\text{Ca}^{2+}_{\text{ext}}$ solution, only “extracellular-calcium insensitive” mIPSCs are present. We predict that ethanol is specifically increasing the frequency of the extracellular-calcium insensitive mIPSCs. Therefore, when the extracellular calcium sensitive mIPSCs are eliminated in the presence of the 0 mM $\text{Ca}^{2+}_{\text{ext}}$ solution, a larger ethanol effect is unmasked. In addition, inhibition of the VDCCs had no effect on the ability of ethanol to increase mIPSC frequency (Fig 3B). Therefore, overall we conclude that extracellular calcium

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influx does not contribute to the mechanism of ethanol-enhanced spontaneous GABA release at the interneuron-Purkinje cell synapse.

In Figures 2 and 3, the mechanically dissociated neuron preparation was utilized to explore the contribution of extracellular calcium influx to the mechanism of ethanol-enhanced spontaneous GABA release. This preparation was advantageous because it allowed instantaneous access of the 0 mM $\text{Ca}^{2+}_{\text{ext}}$ solution and CdCl_2 to the neuron, which dramatically reduces the possibility of also reducing levels of intracellular calcium. However, there are some things to consider when using the mechanically dissociated neuron preparation. It has been proposed that the mechanical dissociation procedure could alter presynaptic terminal excitability (Akaike and Moorhouse, 2003), and the baseline mIPSC decay time, rise time, and amplitude are different in a mechanically dissociated neuron preparation compared to the slice (see Table 1). However, baseline mIPSC frequency was not different between the two preparations, suggesting that the presynaptic components of both preparations are similar.

However, it is important to note that 50 mM ethanol increases mIPSC frequency to a higher degree in the mechanically dissociated neuron preparation. Previously, ethanol has been shown to increase GABA release in a mechanically dissociated neuron preparation from the basolateral amygdala (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) hypothesize that

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because it takes a longer amount of time to see the ethanol effect in the slice, tolerance to the ethanol effect will start to develop, resulting in an overall smaller effect of ethanol in the slice compared to a mechanically dissociated neuron preparation.

Because influx of extracellular calcium was not required for ethanol to increase spontaneous GABA release, our focus shifted next to calcium release from internal stores. After using the thapsigargin protocol to deplete internal stores of calcium, there was no significant change in baseline mIPSC frequency. It has been reported that there is an increase in baseline mIPSC frequency when thapsigargin is applied for 20 minutes or less (Bardo et al., 2002; Li et al., 2004). These latter data are consistent with calcium still being released from internal stores while thapsigargin is blocking calcium reuptake through the SERCA pump (Fig 7). However, after a period of time the effect of thapsigargin on mIPSC frequency subsides when even more calcium is depleted from internal stores (Li et al., 2004), which is consistent with our current data. When the internal stores had been depleted of calcium, ethanol was not able to increase spontaneous GABA release (Fig 4A). Therefore, these data are consistent with calcium release from internal stores playing a vital role in the mechanism of ethanol-enhanced spontaneous GABA release.

After determining that calcium release from internal stores plays an imperative role in ethanol-enhanced spontaneous GABA release, we wanted to investigate whether the IP₃R and RyRs were also involved in this ethanol mechanism. The IP₃R antagonist, 2-APB, significantly blocked the ethanol-induced increase in mIPSC frequency (Fig. 4A). Even though 2-APB is the most widely used membrane permeable IP₃R antagonist, it has selectivity issues with respect to intracellular calcium signaling

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that needed to be considered before we interpreted these results. When 2-APB concentrations higher than 90 μM are used, there is a non-specific calcium leak from internal stores and slight inhibition of the SERCA pump (Missiaen et al., 2001). This non-specific effect of 2-APB offers an explanation for the large increase in miniature excitatory postsynaptic current frequency (Simkus and Stricker, 2002) and mIPSC frequency (unpublished results) seen with 2-APB concentrations higher than 80 μM . To circumvent these non-specific effects of 2-APB, a low concentration of 2-APB (14 μM) was utilized that does not increase baseline mIPSC frequency. However, 2-APB has additional non-specific effects that could occur at any concentration, including inhibition of SOCs as well as transient receptor potential (TRP) channels (Lievremont et al., 2005). Entry of calcium through SOCs is activated when internal stores are depleted of calcium; therefore, inhibition of the IP_3Rs would reduce the normal functioning of the SOCs because inhibition of the IP_3Rs prevents depletion of internal calcium stores. In addition, TRP channels have been implicated in this store operated calcium entry mechanism (Zhu et al., 1996). 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, in the present experiments the possibility that 2-APB was acting through the SOCs and the TRP channels to block the effect of ethanol on spontaneous GABA release can be ruled out because removal of extracellular calcium did not prevent ethanol from increasing spontaneous GABA release (Fig 3B). Therefore, these data suggest that calcium release from the IP_3Rs is playing a role in ethanol-enhanced spontaneous GABA release. The fact that depletion

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of internal calcium stores was sufficient to block the effect of ethanol on GABA release further supports this view (Figure 4A).

Inhibition of RyRs also prevented ethanol from increasing spontaneous GABA release (Fig 4A). This result is not surprising considering that there is a high density of RyRs present at the presynaptic component of the interneuron-Purkinje cell synapse (Llano et al., 2000). Calcium release from the RyRs is affected by calcium release from the IP₃Rs, and vice versa, because the amount of calcium in close proximity to each receptor affects its ability release calcium (Berridge, 1998). Therefore, at this time we cannot distinguish between the relative importance of the IP₃Rs and RyRs; however, the results strongly suggest that internal calcium stores play a central role in ethanol-enhanced spontaneous GABA release.

Because thapsigargin, 2-APB and ryanodine were applied to the bath, these drugs could affect both presynaptic and postsynaptic calcium levels. A change in postsynaptic calcium could alter release of calcium-dependent retrograde messengers, which could account for the effect of ethanol on mIPSC frequency. Ethanol was still able to increase mIPSC frequency with BAPTA in the internal solution (Figure 5B), which suggests that calcium-dependent retrograde messengers are not responsible for the effect of ethanol on spontaneous GABA release and is consistent with previous results (Zhu and Lovinger, 2006). However, inclusion of BAPTA in the internal solution would not necessarily prevent CB release because it has been proposed that CB release does not always require the presence of postsynaptic calcium (Hashimotodani et al., 2007). As a result, a separate control was conducted to test for the contribution of CBs to the mechanism of ethanol-enhanced spontaneous GABA release. Because

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the CB receptor antagonist, AM-251, did not alter the ability of ethanol to increase spontaneous GABA release, we concluded that CBs are not playing a major role in the mechanism of ethanol-enhanced spontaneous GABA release (Fig 6A).

In conclusion, the present study suggests that ethanol is acting presynaptically to increase spontaneous GABA release and that this mechanism involves calcium release from internal stores. To the best of our knowledge, this is the first evidence of calcium release from internal stores being necessary for ethanol-enhanced spontaneous GABA release. However, the exact interaction occurring between ethanol and internal calcium stores has yet to be elucidated. Further knowledge of the mechanism of ethanol-enhanced GABA release will provide information that can be applied towards delineating the mechanisms contributing to the GABAergic behavioral profile of ethanol.

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FOOTNOTES

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LEGENDS FOR FIGURES

Figure 1. In the slice ethanol increases mIPSC frequency and has no effect on mIPSC amplitude and decay time. A, a trace from a representative neuron demonstrating that 100 mM ethanol increases mIPSC frequency. B, a trace from the same representative neuron showing that ethanol has no effect on mIPSC decay time or amplitude. C, there is an increase in mIPSC frequency at 50, 75, and 100 mM ethanol (* $p < .05$, one-way ANOVA, Dunnett post hoc test).

Figure 2. In mechanically dissociated neurons, ethanol increases mIPSC frequency. A, a trace from a representative neuron demonstrating that 100 mM ethanol (EtOH) increases mIPSC frequency. B, there is an increase in mIPSC frequency at 50 and 100 mM ethanol (* $p < .05$, one-way ANOVA, Dunnett post hoc test).

Figure 3. In mechanically dissociated neurons, ethanol still increases mIPSC frequency in a nominally calcium free solution and in the presence of a VDCC inhibitor. A. Left, a trace from a representative neuron demonstrating that 50 mM ethanol (EtOH) increases mIPSC frequency in the presence of 50 μ M cadmium chloride (CdCl_2). Right, a cumulative frequency histogram from the same representative neuron demonstrating that, despite the lack of a washout, ethanol still increases mIPSC frequency compared to the averaged control ((pre-control+ washout)/2). B, compared to their controls, 50 and 100 mM ethanol still increase mIPSC frequency in a nominally calcium free solution (0 mM $\text{Ca}^{2+}_{\text{ext}}$) solution and in the presence of 50 μ M CdCl_2 (* $p < .05$, one-way ANOVA, Dunnett post hoc test).

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Figure 4. In the slice inhibition of calcium release from internal stores prevents ethanol from increasing mIPSC frequency. A, the ability of 100 mM ethanol (EtOH) to increase mIPSC frequency is prevented by 1 μ M thapsigargin (thaps), 100 μ M ryanodine (Ry), and 14 μ M 2-APB (* p <.05, one-way ANOVA, Dunnett post hoc test). B, a trace from a representative neuron showing that 100 mM ethanol has no effect on mIPSC frequency in the presence of 1 μ M thapsigargin.

Figure 5. In the slice ethanol still increases mIPSC frequency with BAPTA in the internal solution. A, a trace from a representative neuron demonstrating that 50 mM ethanol (EtOH) still increases mIPSC frequency when 30 mM BAPTA is included in the internal solution (BAPTA_{int}). B, ethanol (50 and 100 mM) still increases mIPSC frequency in the presence of 30 mM BAPTA_{int} (* p <.05, one-way ANOVA, Dunnett post hoc test). C, in the presence of 30 mM BAPTA_{int}, there is an increase in baseline mIPSC decay time and a decrease in baseline mIPSC amplitude and frequency (* p <.05, Student's t test).

Figure 6. In the slice ethanol still increases mIPSC frequency in the presence of a cannabinoid receptor antagonist. A, ethanol (50 and 100 mM) still increases mIPSC frequency in the presence of 5 μ M AM-251 (* p <.05, one-way ANOVA, Dunnett post hoc test). B, AM-251 (5 μ M) reverses the decrease in mIPSC frequency induced by WIN 55212-2 (5 μ M, * p <.05, paired Student's t test).

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Figure 7. A summary of the antagonists used to inhibit pathways that result in increases in presynaptic intracellular calcium levels. The nominally calcium free (0 mM $\text{Ca}^{2+}_{\text{ext}}$) solution tested for the involvement of all channels that increase intracellular calcium levels through influx of extracellular calcium—namely the voltage dependent calcium channels (VDCCs), the store operated channels (SOCs) and the receptor operated channels (ROCs). VDCCs were also inhibited with cadmium chloride (CdCl_2). To test for the involvement of calcium release from internal stores, thapsigargin was used in a protocol that depleted internal stores of calcium through inhibition of the sarco(endo)plasmic reticulum calcium ATPase (SERCA) pump. To specifically test for the involvement of the receptors that release calcium from internal stores, 2-APB was used to inhibit calcium release from the inositol 1,4,5-trisphosphate receptors (IP_3Rs), while ryanodine was used to inhibit calcium release from the ryanodine receptors (RyRs).

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Table 1

A comparison of mIPSC properties in the mechanically dissociated neuron and in the slice at the interneuron-Purkinje cell synapse.

	Frequency (Hz)	Fast Decay (ms)	Slow Decay (ms)	Rise Time (ms)	Amplitude (pA)
Slice	2.18 ± .43	11.88 ± .59	11.89 ± .59	2.62 ± .13	15.87 ± 1.63
MDNs	1.38 ± .40	5.56 ± .52*	16.91 ± 1.99*	1.01 ± .06*	29.41 ± 3.87*

MDNs, mechanically dissociated neurons; *p<.05, Student's t test (comparison between slice (n=8) and MDN (n=9) data).

Figure 1

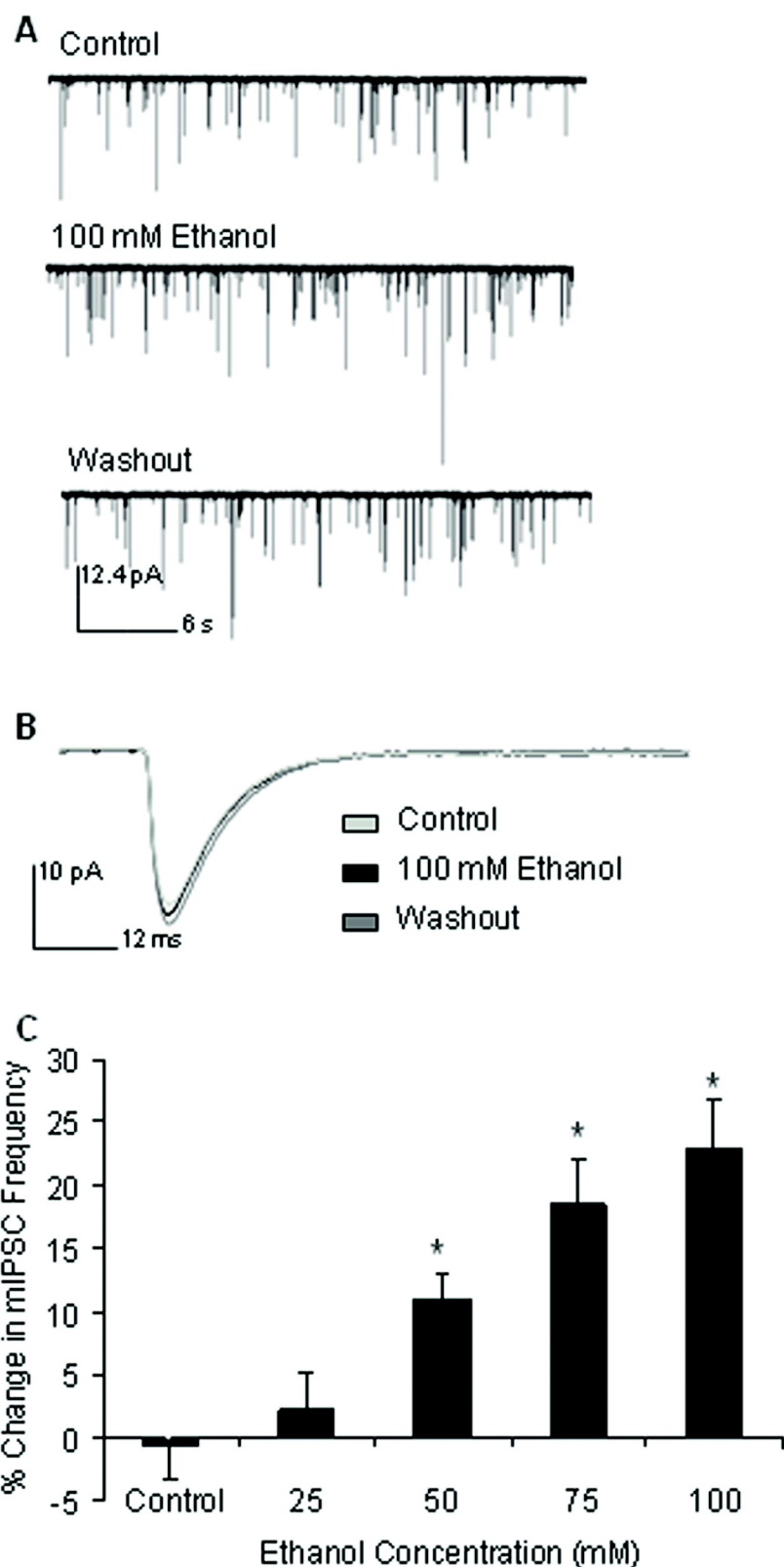


Figure 2

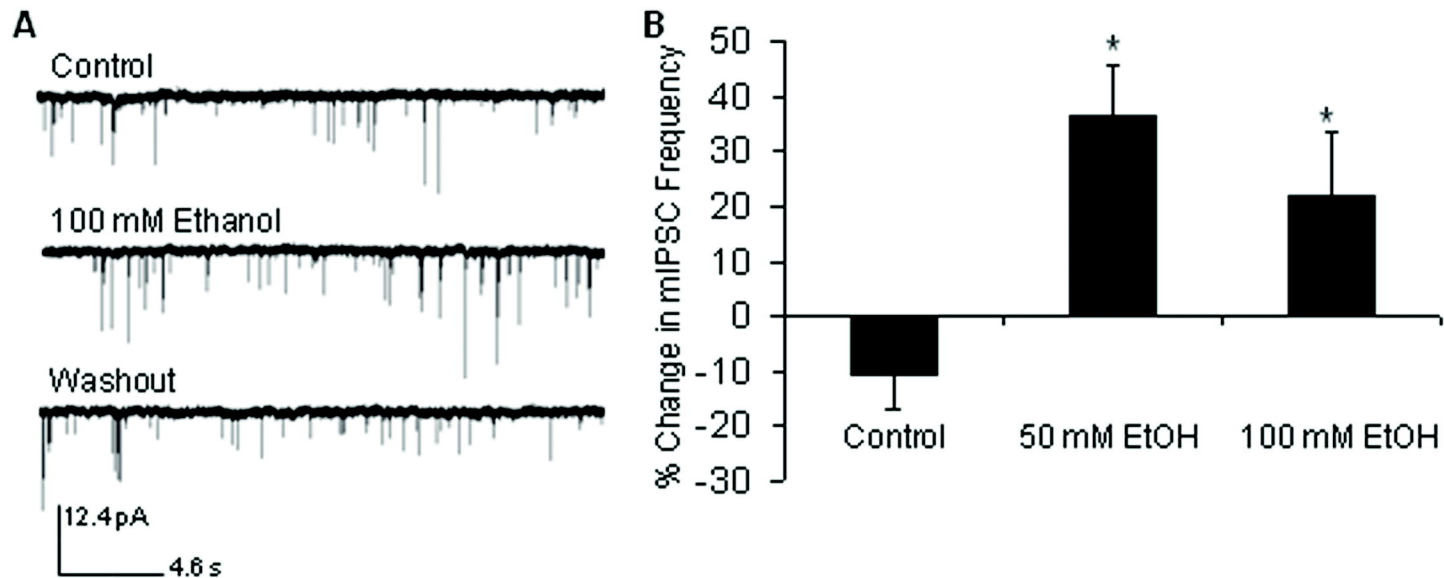


Figure 3

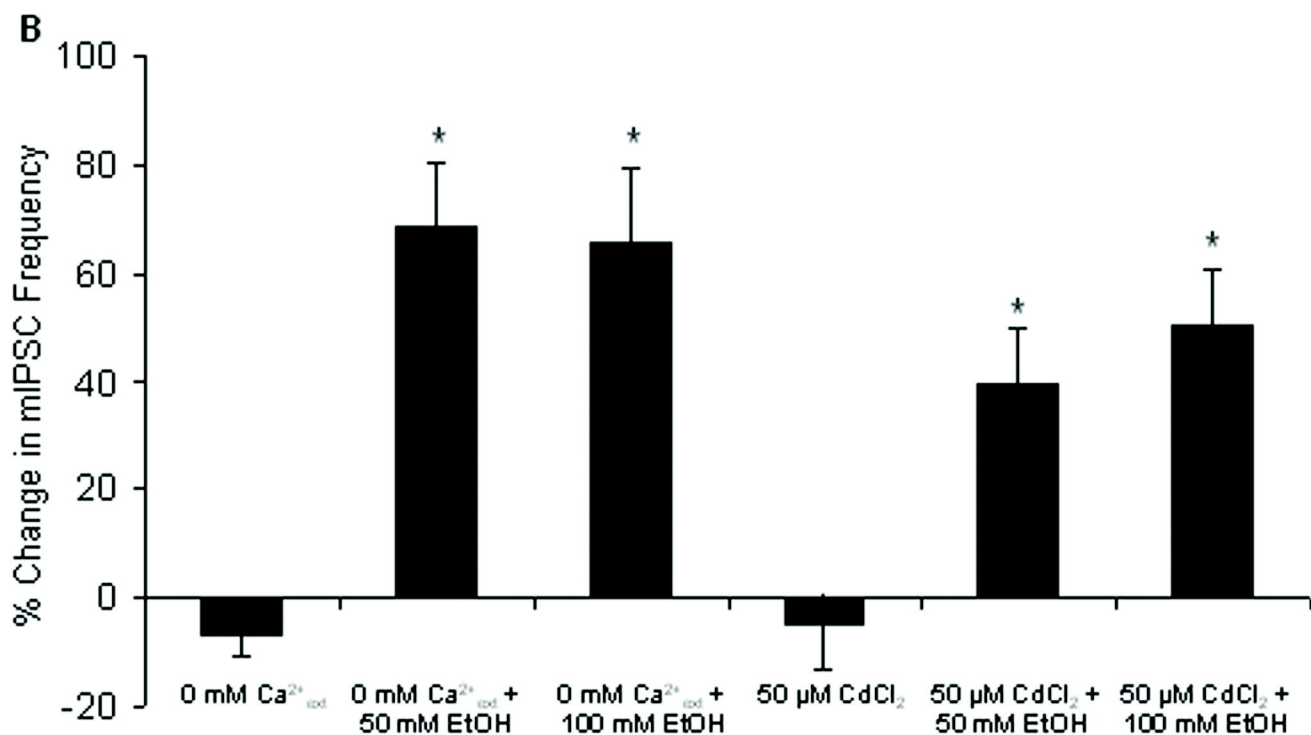
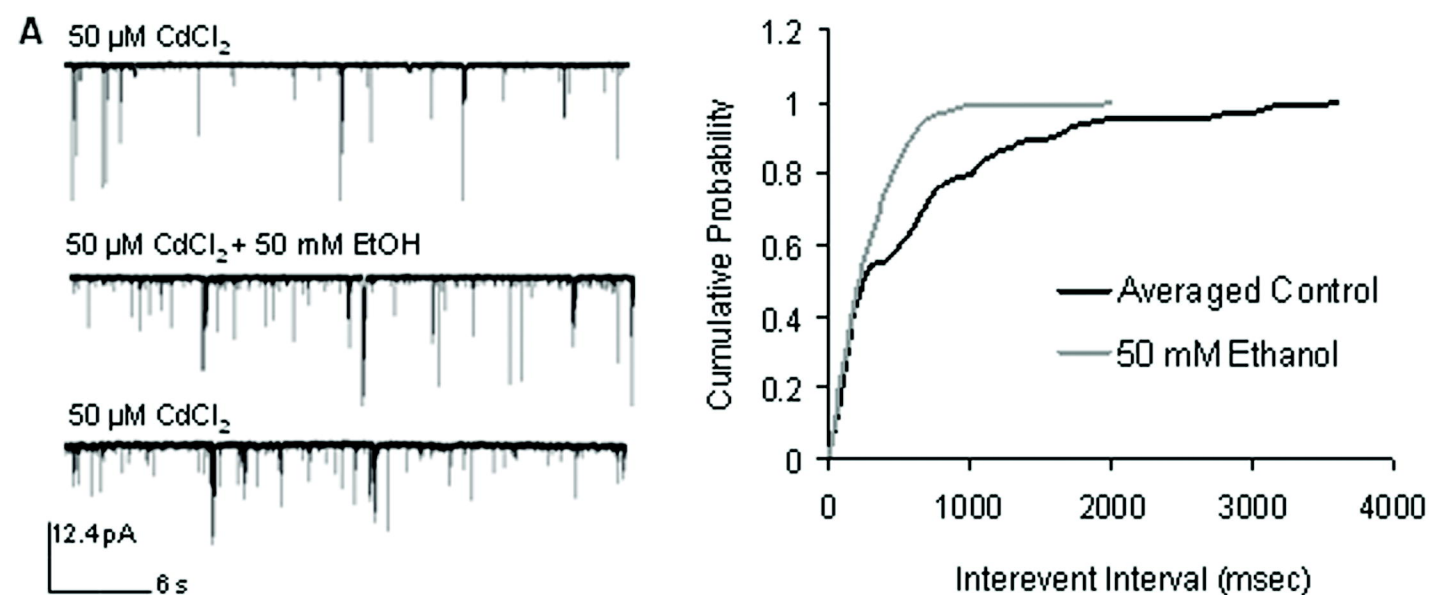


Figure 4

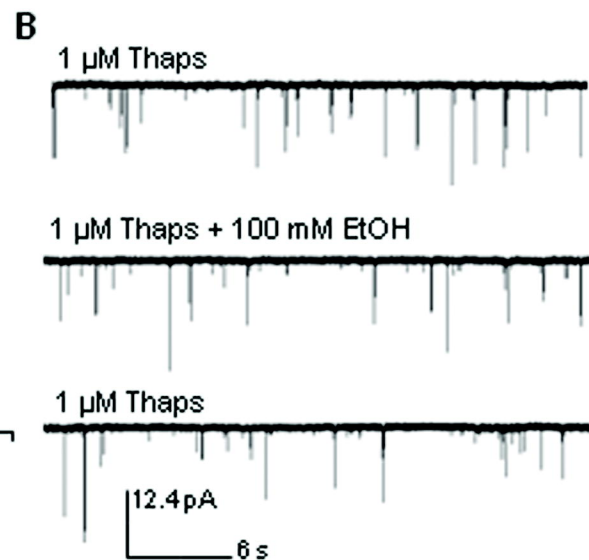
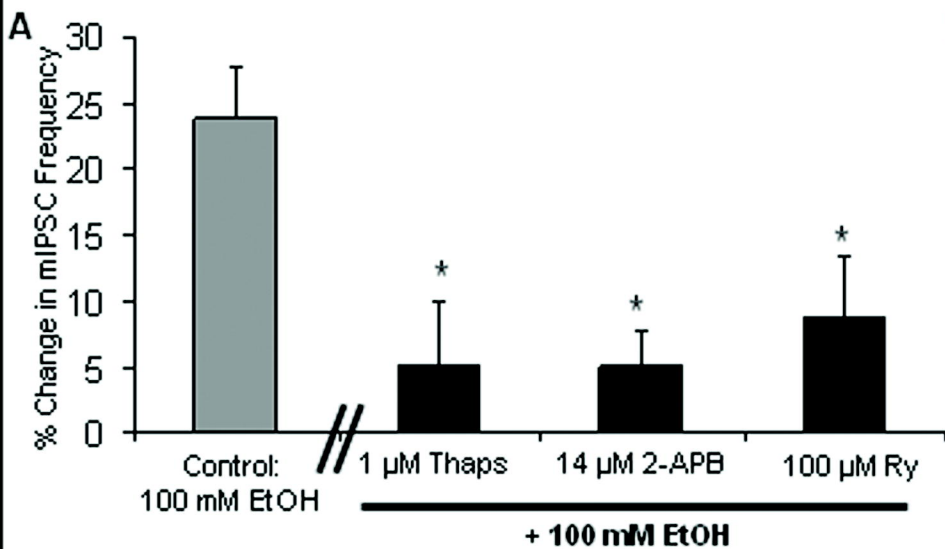


Figure 5

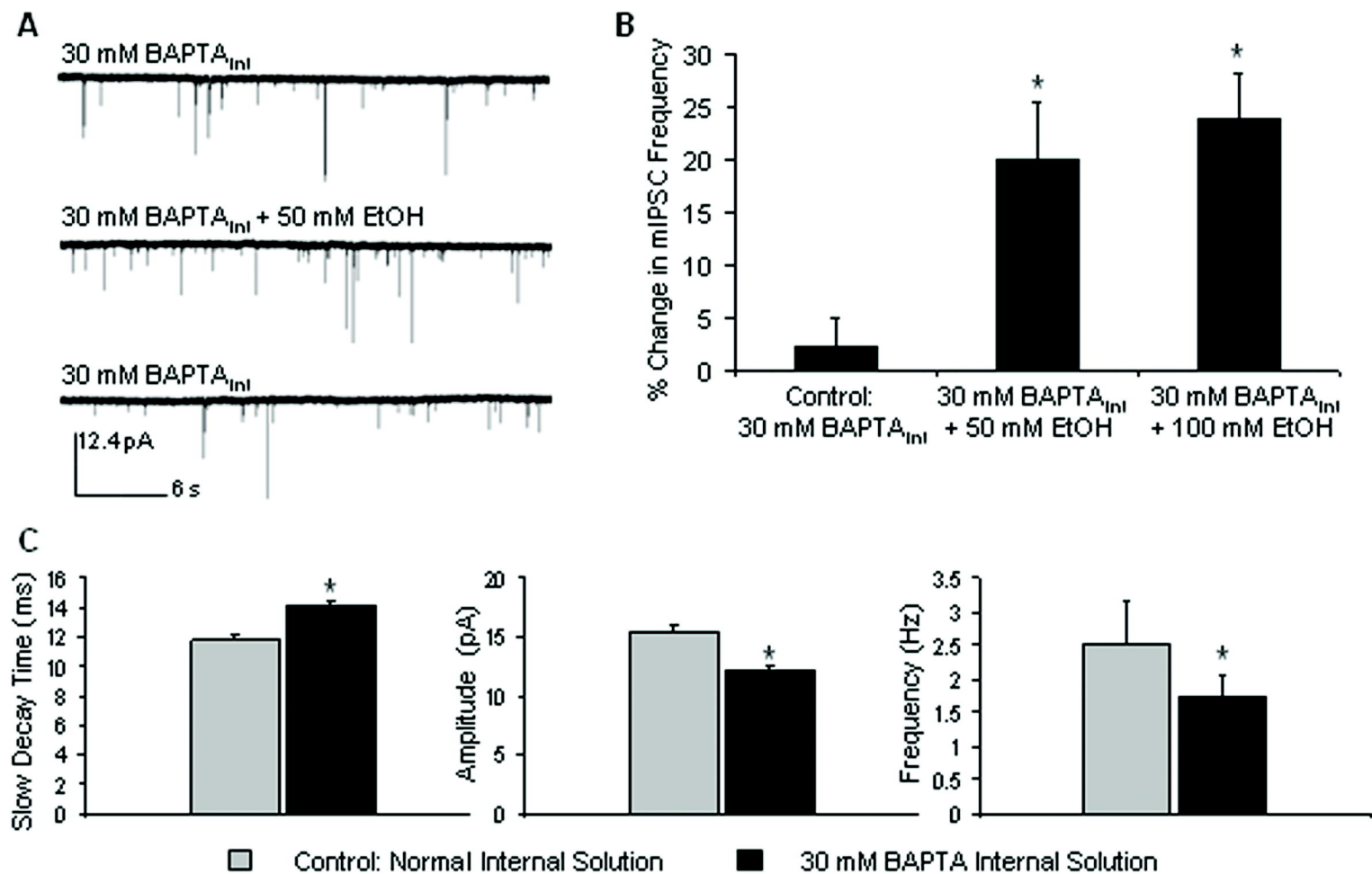


Figure 6

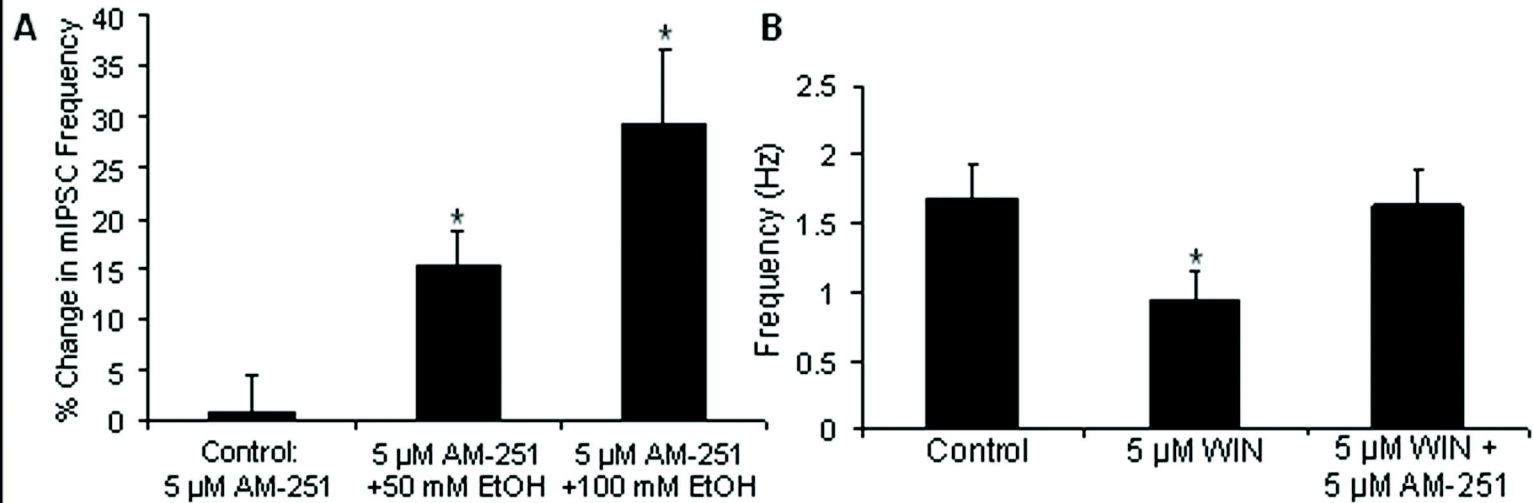


Figure 7

