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BRAIN REGIONAL DIFFERENCES IN THE EFFECT OF ETHANOL ON GABA RELEASE FROM PRESYNAPTIC TERMINALS

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Running title page

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Regionally Specific GABA Release by Ethanol

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Abbreviations: GABA, Gamma-aminobutyric acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol tetraacetic acid; 2-APV, 2-amino-5-phosphonovaleric acid; CNQX , 6-cyano-7-nitroquinoxaline-2,3-dione; TTX, Tetrodotoxin; ACSF, artificial cerebrospinal fluid; PPR, paired-pulse ratio; mIPSC, miniature inhibitory postsynaptic current; sIPSC, spontaneous inhibitory postsynaptic current; SNR, substantia nigra reticulata

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Abstract

While ethanol has behavioral actions consistent with increased GABAergic function, attempts to demonstrate a direct enhancement of GABA-gated currents by ethanol have produced mixed results. Recent work has suggested that a part of the GABAergic profile of ethanol may result from enhanced GABA release from presynaptic terminals. The present study examines the effect of ethanol on GABA release in several brain regions to assess the regional nature of ethanol-induced GABA release. Whole-cell voltage clamp recording of spontaneous inhibitory post-synaptic currents (sIPSCs) from mechanically dissociated neurons, and miniature inhibitory post-synaptic currents (mIPSCs) and paired-pulse ratio (PPR) from a slice preparation were used to quantify GABA release. Ethanol produced a concentration-dependent increase in the frequency of sIPSCs recorded from mechanically dissociated cerebellar Purkinje neurons and mIPSCs from substantia nigra neurons without having an effect on sIPSCs recorded from lateral septal or cerebrocortical neurons. This regional difference in the effect of ethanol on GABA release was confirmed with PPR recording from brain slices. These data indicate that ethanol can act on presynaptic terminals to increase GABA release in some brain regions while having little or no effect on GABA release in others. This regional difference is consistent with earlier *in vivo* studies in which ethanol affected neural activity and sensitivity to GABA in some, but not all, brain sites.

Introduction:

Effects of ethanol on mood, coordination and sedation are similar to those of the benzodiazepines and barbiturates (Frye et al., 1979); furthermore, benzodiazepines substitute for ethanol in drug discrimination tests (Grant et al., 2000). Because benzodiazepines and barbiturates are known to act by enhancing the inhibitory action of Gamma-aminobutyric acid (GABA; Martz et al., 1983), it has been assumed that at least some of the behavioral actions of ethanol are mediated by an effect of ethanol on GABA function (Criswell and Breese, 2005; Siggins et al., 2005; Weiner and Valenzuela, 2006). In support of this view, early studies found that ethanol enhanced the effects of GABA in synaptoneurosomes and cultured neurons (Suzdak et al., 1986; Aguayo, 1990; Reynolds et al., 1992), and enhanced the effect of GABA on neuronal discharge rate *in vivo* (Bloom and Siggins, 1987; Givens and Breese, 1990). Based on these results, a general conclusion was that ethanol acted postsynaptically on $GABA_A$ receptor function. However, a number of studies failed to find a direct effect of ethanol on GABA-gated currents from neurons (Sigel et al., 1993; Peoples and Weight, 1999; Mori et al., 2000; Criswell et al., 2003; Borghese et al., 2006). More recently, effects of low concentrations of ethanol on tonic currents mediated by $GABA_A$ receptors containing the $\alpha4,\beta x,\delta$ or $\alpha6,\beta x,\delta$ subunit combination have been reported (Wallner et al., 2003; Chandra et al., 2006; Wallner et al., 2006). However, even this finding has raised controversy (Borghese et al., 2006; Botta et al., 2007a; Botta et al., 2007b). Irrespective of any postsynaptic action ethanol may possess, earlier *in* vivo extracellular recordings indicated that ethanol reliably enhances GABA function in some brain regions while being ineffective in others (McCown et al., 1985; Bloom and Siggins, 1987; Givens and Breese, 1990; Ebert et al., 1994).

An emerging consensus indicates that ethanol can act presynaptically to enhance GABA function by increasing GABA release (Crowder et al., 2002; Roberto et al., 2003; Ziskind-Conhaim et al., 2003; Criswell and Breese, 2005; Siggins et al., 2005; Ming et al., 2006; Zhu and Lovinger, 2006). However, few studies have directly compared effects of ethanol on GABA release across brain regions. Differential sensitivity to the presynaptic action of ethanol to release GABA could contribute to a regional effect of ethanol on GABA function, which could influence specific behaviors (McCown et al., 1985). The present study uses whole-cell voltage clamp recording of GABA mediated inhibitory postsynaptic currents from mechanically dissociated neurons or neurons in a slice preparation and paired pulse ratio (PPR) recording from a slice preparation to examine the effect of ethanol on presynaptic GABA release in selected brain regions.

Methods

Preparation of brain slices. Sprague Dawley rats, 13-20 days old, were anesthetized with an i.p. injection of 75% urethane and decapitated. The brains were rapidly removed and placed in ice-cold bicarbonate-buffered artificial cerebrospinal fluid (ACSF) of the following composition: 124 mM NaCl, 3.25 mM KCl, 1.25 mM KH_2PO_4, 2 mM $CaCl_2$, 20 mM $NaHCO_3$, 2 mM $MgSO_4$, and 10 mM glucose. Parasagital slices (400 μm) through cerebellum, or coronal sections through the lateral septum, substantia nigra and medial frontal cortex were cut with a vibrating tissue slicer (Vibratome, series 1000). The slices were equilibrated in a beaker containing ACSF gassed with 95% O_2/5% CO_2 for at least 1 hour at room temperature before initiating electrophysiological recording.

Preparation and properties of mechanically dissociated neurons. Slices, as described above, were transferred to a recording chamber containing a 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffered ACSF (145 mM NaCl, 5 mM KCl, 10 mM HEPES, 2 mM $CaCl_2$ and 10 mM glucose, pH=7.4; 340 mosmoles/L). A 0.3 mm probe was touched to the surface of the submerged slice over the cells to be dissociated and vibrated using a piezoelectric transducer (~0.5 mm amplitude at 10 to 50 Hz for 2 min). The slice was then removed from the chamber and the mechanically dissociated neurons were allowed to settle to the bottom of the recording chamber.

The mechanical dissociation of neurons strips the dendritic tree and most of the axon from neurons. The micrographs in Figure 1 illustrate representative neurons dissociated from the cerebellum, lateral septum and medial frontal cortex. While the axon and dendritic tree are removed during the dissociation, many of the boutons from presynaptic neurons remain attached to the soma (Akaike and Moorhouse, 2003). As a result of the presynaptic terminal being present, spontaneous inhibitory postsynaptic currents (sIPSCs) can be recorded in the absence of inputs from other neurons.

Electrophysiological recording. Electrophysiological studies were performed under voltage-clamp (-60 mV for cortical, substantia nigra reticulata, and lateral septal neurons and -70 mV for cerebellar Purkinje neurons) in the whole-cell configuration using an Axopatch-1D amplifier. Recording pipettes were fabricated from N 51A capillary glass (Drummond Scientific; Broomall, PA). The internal solution included 150 mM KCl, 3.1 mM $MgCl_2$, 15 mM HEPES, 2 mM K-ATP, 5 mM EGTA, 15 mM phosphocreatine, and 50 U/ml creatine phosphokinase, adjusted to pH 7.4 with KOH with an osmolality of 310 mosmoles/L. Seals were formed on the neurons with

electrodes having a tip resistance of 2-4 MΩ. Data were displayed on an oscilloscope, digitized at either 2 or 5 kHz, and stored on a personal computer. Recordings were performed at room temperature in a bath where the neurons were superfused at 0.5-1 ml/min.

Recording of sIPSCs from mechanically dissociated cells. Neurons mechanically dissociated from the cerebral cortex, cerebellum and lateral septum were bathed in the HEPES buffered ACSF at a rate of 0.5 ml/min. Control solution or drugs were applied by an array of fused silica tubes (200 μm tip diameter moved by a micromanipulator), or by a perfusion pencil (250 μm tip diameter, Automate Scientific, Inc, Sarasota, FL) placed approximately 200 μm upstream from the cell.

Recording of mIPSCs and paired-pulse ratio from brain slices. Brain slices from cerebellum, lateral septum or medial frontal cortex were prepared as described above. Brain slices were then placed in a recording chamber and perfused with bicarbonate-buffered ACSF at a rate of 1 ml/min. Neurons were visualized using an infrared camera under Namarski or oblique illumination. For mIPSC recordings, TTX (500 nM) and CNQX (10 μM) were applied to the recording site by a drug application pencil (250 μm diameter) to block sodium currents and excitatory glutamate-gated currents (mEPSCs), respectively. Because spontaneous NMDA-gated currents are not observed under the present recording conditions, AP-5 was not included. Whole-cell voltage-clamp recording was established and mIPSCs were recorded for 1 min intervals in the presence and absence of ethanol (25-100 mM).

PPR was recorded in the absence of TTX. Platinum-Iridium stimulating electrodes were lowered into the vicinity (150 μm) of a cell to be recorded and paired-pulse stimulation (0.2 ms duration and 50 ms inter-stimulus interval) was delivered at 0.1 Hz. Following placement of the stimulating electrodes, whole-cell patch was established as described for the isolated cells and after a 5 min adaptation period to the 2-APV (50 μM) and CNQX (10 μM), PPR recording was performed. Following a minimum of 5 PPR records obtained at 10 sec intervals, ethanol (25, 50 or 100 mM in bicarbonate buffered ACSF) was delivered by the drug pencil and 5 min later a second series of PPR values was recorded. The ethanol was washed out for 5 min and a final set of PPR values was obtained after washout. Data from only one neuron was collected from each slice.

Analysis. Rate, amplitude and decay-time of sIPSCs were analyzed using Mini Analysis (version 5.6.4; Synaptosoft, Decatur, GA). Numerical data are given as mean ± SEM, and *n* represents the number of cells tested. Percent change during ethanol administration was calculated as 100 times the value for ethanol divided by the mean of the pretest and washout

values. Differences between means were compared with ANOVA followed by Dunnet ,Tukey HSD or LSD post hoc comparisons as appropriate. Linear trends were analyzed by the Pierson correlation. Calculated *p* values of less than 0.05 were accepted as evidence of a significant difference.

RESULTS

Blockade of sIPSCs and mIPSCs by bicucullilne. Both the sIPCSs recorded from mechanically dissociated neurons in the absence of TTX and the mIPSCs recorded from the slice preparation in the presence of TTX result from the spontaneous release of neurotransmitter from the presynaptic terminal (Akaike and Moorhouse, 2003**;** Zhu and Lovinger, 2006). When tested in a subset of neurons, the spontaneous currents recorded from SNR neurons in the slice preparation or from mechanically dissociated cerebellar Purkinje, lateral septal or cerebrocortical neurons were blocked by bicuculline indicating that they were mediated by $GABA_A$ receptors (Figure 1B). Thus, the frequency of mIPSCs from neurons in the SNR slice or sIPSCs from the mechanically dissociated neurons gives a good index of spontaneous release of GABA from presynaptic terminals.

Effects of ethanol on GABAergic (sIPSCs) from mechanically dissociated Purkinje neurons. Figure 2A illustrates that 50 mM ethanol increased the rate of sIPSCs recorded from a mechanically dissociated cerebellar Purkinje neuron with a return to baseline upon washout. Figure 2B demonstrates that this facilitation of sIPSCs by ethanol is concentration related, results similar to those obtained previously in cerebellar slices (Ming et al., 2006; Kelm et al., 2007)**.** In contrast to the change in frequency of sIPSCs by ethanol, Table 1 shows that amplitude and decay times were unchanged by ethanol. These latter results indicate that ethanol does not have measurable postsynaptic effects on GABA-gated currents under the present conditions.

Effects of ethanol on paired-pulse ratio (PPR) from cerebellar Purkinje neurons. To determine whether the effect of ethanol on spontaneous GABA release extended to evoked release, the ability of ethanol to increase GABA release onto cerebellar Purkinje neurons using PPR recording in slices was examined. Figure 2C shows a representative example of a paired-pulse recording from a cerebellar Purkinje neuron. Under control conditions, the response to the second pulse was greater than that to the first (paired-pulse facilitation). This result is consistent with previous work (Liu, 2007) that showed paired-pulse facilitation from cerebellar Purkinje neurons at this age. During exposure of the slice to 50 mM ethanol, (Figure 2C), the response to the second pulse was smaller than that seen with the first pulse (paired-pulse depression). Removal of the ethanol restored the original PPR (Figure 2C). Figure 2D indicates that the mean PPR was significantly depressed by 50 mM ethanol when averaged across several cerebellar Purkinje neurons. This decrease in the ratio of the second response to the first during ethanol

administration indicates an increase in stimulated GABA release (Siggins et al., 2005). Thus, the PPR data are consistent with the ethanol-induced increase in sIPSC frequency from the mechanically dissociated Purkinje neurons.

Effect of ethanol on sIPSCs and PPR from lateral septal neurons. Because earlier *in vivo* studies failed to find an effect of ethanol on enhancement of GABA responsiveness from lateral septal neurons (Givens and Breese, 1990 a,b), we next examined the effect of ethanol on sIPSC frequency from mechanically dissociated lateral septal neurons. Figure 3A provides a representative record from a lateral septal neuron illustrating the lack of effect of 50 mM ethanol on sIPSC frequency. A concentration-response relationship for the effect of ethanol on frequency of lateral septal sIPSCs is shown in Figure 3B. There was no significant effect of any concentration of ethanol tested on lateral septal sIPSC frequency, including a concentration of 100 mM ($P > 0.05$). Likewise, as shown in Table 1**,** there was no effect of ethanol on amplitude or decay time of the sIPSCs from lateral septal neurons.

Because $GABA_B$ receptors on presynaptic terminals can interfere with the ability of drugs to increase GABA release (Ariwodola and Weiner, 2004; Zhu and Lovinger, 2006) we examined the effect of 100 mM ethanol on sIPSC frequency in the presence of a $GABA_B$ antagonist. Ethanol did not cause a significant increase in sIPSC frequency (21.3 ± 19.3%, $n = 8$; $P > 0.05$) in the presence of a $GABA_B$ antagonist (CGP-53432; 10 μM). These results indicate that stimulation of $GABA_B$ receptors was not responsible for the lack of an effect of ethanol on GABA release in the lateral septum.

To provide further evidence for this lack of effect of ethanol on GABA release, we conducted paired-pulse recording from lateral septal neurons. As shown in a representative trace from lateral septal neurons, Figure 3C demonstrates that under control conditions the response to the second pulse is considerably greater than that to the first (paired-pulse facilitation). Unlike the PPR data from cerebellar Purkinje neurons, ethanol did not alter the pattern of PPR of this lateral septal neuron. Figure 3D demonstrates the lack of effect of the 50 mM ethanol on PPR when averaged across several lateral septal neurons. These two approaches clearly indicate a lack of effect of behaviorally relevant concentrations of ethanol on stimulated GABA release from this brain region.

Effects of ethanol on sIPSCs from mechanically dissociated cerebrocortical neurons: Mechanically dissociated neurons from the medial frontal cortex exhibit sIPSCs resulting from

release of GABA by attached presynaptic terminals. However, when ethanol was applied to these neurons, the rate of sIPSCs was unchanged (Figure 4). Figure 4A shows representative traces of sIPSC activity from a cerebrocortical neuron before, during and after ethanol exposure. Figure 4B shows the concentration response relationship for the effect of ethanol on sIPSC rate in these cortical neurons. These results demonstrate that ethanol did not increase GABA release from presynaptic terminals attached to these neurons. Additionally, as shown in Table 1**,** ethanol had no effect on sIPSC amplitude or decay time from cerebrocortical neurons.

Effect of ethanol on mIPSCs recorded from neurons in a substantia nigra reticula slice. To demonstrate that effects of ethanol on GABAergic mIPSCs could be detected in a slice preparation as well as to examine another brain region, mIPSCs were recorded from substantia nigra reticulata (SNR) neurons in the slice. Figure 5A shows traces of mIPSC activity from a SNR neuron before, during and after ethanol exposure. Figure 5B shows a concentration-related increase in mIPSC frequency by ethanol from SNR neurons. Under these recording conditions, ethanol did not alter either mIPSC amplitude or decay time from SNR neurons (Table 1).

Because the effect of 50 mM ethanol on mIPSC frequency was not statistically reliable, we examined that same effect in the presence of a $GABA_B$ antagonist. In contrast to data from lateral septal neurons, the effect of 50 mM ethanol on mIPSC frequency in the presence of the $GABA_B$ agonist (CPG-53432 10 μM) was significantly different from controls (41.4 ± 23.3% for the ethanol group vs -3.7 ± 3.8%; n = 13 for untreated controls). These data suggest that negative feedback from $GABA_B$ action at the presynaptic terminal may have decreased the effect of ethanol on GABA release in the SNR slice preparation.

DISCUSSION

In agreement with previous work evaluating mIPSCs in the cerebellar slice preparation (Ming et al., 2006; Kelm et al., 2007), ethanol enhanced the frequency of sIPSCs but did not affect sIPSC amplitude or decay time. Although the mechanically dissociated neurons in the present study were tested in the absence of TTX, a similar increase in frequency of mechanically dissociated Purkinje cell mIPSCs was reported by Kelm et al. (2007) in the presence or absence of TTX. Thus, voltage-sensitive sodium channels on the pre-terminal axon or on the presynaptic terminal are not necessary for the effect of ethanol on mIPSCs or sIPSCs. The decrease in PPR during ethanol administration to cerebellar Purkinje neurons is consistent with the elevated frequency of sIPSCs and supports the concept that ethanol increases both spontaneous and stimulated GABA release. Likewise, ethanol enhanced the frequency of mIPSCs from neurons in the SNR slice without affecting mIPSC amplitude or decay time. These observations at these two brain sites are consistent with ethanol increasing release of GABA from presynaptic terminals onto the cerebellar Purkinje and SNR neurons with no measurable effect of ethanol on the postsynaptic receptor. This lack of an effect of ethanol on postsynaptic GABA receptor function is in agreement with earlier conclusions where ethanol did not alter the effect of direct application of GABA to neurons from these brain sites (Criswell et al., 2003). However, because this preparation was from young animals and was optimized for detection of presynaptic events, it may not be representative of *in vivo* neural function. Therefore, due to alterations in the internal environment of the postsynaptic neuron due to whole-cell patch recording, including dialysis of intracellular molecules and high chloride concentrations, these later results should be viewed with caution (Weiner and Valenzuela, 2006). Just as found in the cerebellum and the SNR, previous work has demonstrated increased GABA release in several other regions of brain including the brainstem (Sebe et al., 2003), basolateral amygdala (Zhu and Lovinger, 2006), hippocampus (2006) and central amygdala (Roberto et al., 2003). Thus, these data collectively provide convincing data that ethanol can facilitate presynaptic release of GABA at several sites in brain.

In contrast to the ability of ethanol to increase GABA release from presynaptic terminals in the cerebellum, SNR and other brain sites, ethanol did not affect sIPSCs from lateral septal or cerebrocortical neurons. Additionally, ethanol did not change the PPR in the lateral septum. These observations provide evidence that ethanol does not enhance spontaneous or stimulated GABA release from these sites at the ethanol concentrations tested. Further, ethanol did not affect sIPSC amplitude or decay time at these latter brain sites indicative that ethanol did not affect postsynaptic function. This latter finding in the lateral septum is also in agreement with

previous data that ethanol does not affect post-synaptic receptors on neurons at this brain site (Criswell et al., 2003). However, as noted earlier, this preparation was optimized for detection of presynaptic events and only a sample of neurons easily patched is represented. Thus, there may be specific neurons or conditions under which either presynaptic or postsynaptic effects of ethanol can be observed at these brain sites. Similar to the lack of effect of ethanol on presynaptic terminals in the lateral septum and cerebral cortex affecting GABA release, Proctor et al. (2006) failed to find an effect of ethanol on PPR from CA-1 hippocampal neurons in mice.

Collectively, these varying results provide evidence that ethanol is having a regionally specific effect on GABA release in brain. While the mechanism underlying the effect of ethanol on presynaptic release of GABA from cerebellar Purkinje neurons has not been resolved, recent work has implicated the release of calcium from internal stores (Kelm et al., 2007). This observation provides a potential lead to determine if this mechanism associated with calcium release from internal stores applies to other brain sites where ethanol increases GABA release. Likewise, it will be of interest to determine if this mechanism is absent in neurons where ethanol does not influence GABA release.

Earlier work in the cerebellar slice preparation and in mechanically dissociated neurons from the amygdala found a similar enhancement of GABA release by ethanol in the presence of TTX (Ming et al., 2006; Zhu and Lovinger, 2006; Kelm et al., 2007). This indicates that an effect of ethanol on TTX sensitive Na^+ channels on the presynaptic terminal or preterminal axon is not required for an effect on GABA release. This conclusion was supported in the present investigation by the clear increase in mIPSC frequency by ethanol observed in the SNR slice in the presence of TTX (Figure 5).

It is conceivable that ethanol could regulate GABA release by acting on the postsynaptic neuron to release a retrograde messenger such as NO (Shin and Linden, 2005) or an endogenous cannabinoid (Galante and Diana, 2004). However, the lack of NMDA receptors on cerebellar Purkinje neurons (Shin and Linden, 2005) argues against NMDA receptor mediated NO release contributing to the effect of ethanol on GABA release at that site. Similarly, because the dendritic tree of the Purkinje neuron is removed during the mechanical dissociation, thereby eliminating the usual space-clamp problems, the voltage-clamp recording should minimize depolarization-induced cannabinoid release from mechanically dissociated neurons in cerebellum or other brain sites (Galante and Diana, 2004). In support of this view, Kelm et al., (2007) demonstrated a similar increase in mIPSC frequency from cerebellar Purkinje neurons by ethanol in the presence of a CB1 receptor antagonist.

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Presynaptic $GABA_B$ receptors can provide feedback inhibition of GABA release and the activation of these receptors by endogenous GABA in some brain regions could decrease the ability of ethanol to elicit GABA release (Ariwodola and Weiner, 2004; Zhu and Lovinger, 2006). Therefore, differential $GABA_B$ receptor inhibition of GABA release represents a potential mechanism for the lack of effect of ethanol in some brain regions; moreover, we observed this effect in the SNR (Figure 5). However, in the lateral septum the observed lack of an effect of ethanol on GABA release in the presence of a $GABA_B$ antagonist argues against this explanation. Additionally, $GABA_B$-mediated feedback inhibition of increased GABA release would only be expected if the ethanol initially caused at least some increase in GABA release. Thus, a mechanism distinct from $GABA_B$ receptor inhibition must be sought to elucidate the mechanism of the regional specificity associated with the action of ethanol to release GABA from presynaptic neurons.

The noted regional specificity of ethanol to release GABA has considerable relevance to actions of ethanol on the CNS and to drugs that influence GABA function. The ability of ethanol to release GABA could account for a part of the behavioral GABAmimetic profile of ethanol resembling that of benzodiazepines and barbiturates (Frye and Breese, 1982), both of which are known to act primarily by a GABAergic mechanism (Martz et al., 1983). Additionally, behavioral actions of ethanol are additive or superadditive with these GABAergic compounds, and they substitute for ethanol in discrimination studies (Frye and Breese, 1982; Frye et al., 1983; Martz et al., 1983; Grant et al., 2000). While met with controversy (Wallner et al., 2003; Borghese et al., 2006), the ability of ethanol to enhance the action of GABA on receptors containing α6β3δ and α4β3δ subunits could be enhanced by the increased release of GABA by ethanol. Thus, in this case, “spill over” from increased GABA release by ethanol could have a direct effect on these extrasynaptic $GABA_A$ receptors to increase inhibitory tone (Wei et al., 2003; Wei et al., 2004). This scenario would be dependent upon a brain site where ethanol released GABA and where these ethanol sensitive $GABA_A$ receptors are localized. Previous work has shown that substantia niga and cerebellar Purkinje neurons are highly sensitive to enhancement of currents gated by exogenously applied GABA by neuroactive steroids (Criswell et al., (2003). Thus, there are receptors present that could respond to the increased GABA release by ethanol.

The regional differences in the ability of ethanol to release GABA could offer an explanation of earlier studies showing regionally specific effects of ethanol on GABA function using *in vivo* extracellular recording (Bloom and Siggins, 1987; Givens and Breese, 1990; Criswell et al., 1993). Further, this regional specificity for GABA release by ethanol may account

for the selective effect of ethanol on specific behaviors. That is, only behaviors mediated by a brain region sensitive to this action of ethanol would by modified by ethanol. A caution for this interpretation is that concentrations of ethanol below 50 mM did not induce a reliable increase in mIPSC frequency *in vitro*—a finding that might be expected for this action of ethanol to contribute to all aspects of its GABAmimetic profile (Frye et al., 1979). Future studies should be able to define the accuracy of the view that release of GABA from presynaptic terminals in specific brain regions is a critical contributor to the GABAmimetic profile of ethanol.

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LEGENDS FOR FIGURES

Fig. 1. **Morphology and GABA-gated currents of mechanically dissociated neurons.**

A photomicrograph of a mechanically dissociated cerebellar Purkinje neuron surrounded by granule neurons is shown on the left (**A**). Mechanically dissociated neurons from the lateral septum and cerebral cortex are depicted in the middle and right, respectively (**A**). The calibration bars are 50 μm.

Representative traces illustrating inhibition of sIPSCs by the GABA antagonist bicuculline (50 μM) are shown for each neuronal type (**B**). A pre-test control (**PRE**) trace, a trace during application of bicuculline (**BIC**) and a trace following a 1 min washout (**WASH**) are shown for a cerebellar Purkinje neuron (**Purkinje**), a lateral septal neuron (**Lateral Septum**), a cerebrocortical neuron (**Cortex**) and a substantia Nigra reticulata neuron (**SNR**). Note the differing time and current calibration bars for each brain region. Overall, bicuculline decreased the frequency of sIPSCs by 97 ± 1.0% for Purkinje neurons (n = 11), by 98 ± 1.8% for lateral septal neurons (n = 6), by 97 ± 2.7% for cerebrocortical neurons (n = 6) and by 100 ± 0% for substantia nigra reticulata neurons (n = 4). Substantia Nigra neurons were recorded in a slice preparation while all other neurons were mechanically dissociated.

Fig. 2. Ethanol increases frequency of sIPSCs and decreases PPR from cerebellar Purkinje neurons.

When ethanol was applied to mechanically dissociated cerebellar Purkinje neurons, GABA release was increased as indicated by an increased rate of sIPSCs (**A**). Representative traces of sIPSCs are shown from a Purkinje neuron prior to ethanol administration (**PRE**), during ethanol administration (**EtOH**) and after washout (**WASH**). Means for the percent change in rate averaged across 10 to 18 neurons for varying concentrations of ethanol are shown in **B.** Control values represent repeated delivery of ACSF to the cell using the drug delivery pencil while experimental values represent the effect of 25, 50 and 100 mM ethanol delivered in ACSF.

* Significantly different from control ($P < 0.05$, Dunnet test)

The effect of ethanol on PPR is shown in **C.** When pulse pairs were applied at a 50 ms inter-stimulus interval under control conditions (**PRE**), the second pulse produced a greater response than the first (paired-pulse facilitation). During application of 50 mM ethanol (**EtOH**), the second stimulus produced a smaller effect than the first (paired-pulse depression). The decrease in paired-pulse-ratio is consistent with an increase in stimulus induced GABA release.

On washout, the paired-pulse ratio returned to control values (**WASH**). Mean amplitude for the initial pulse was 65 ± 9.9; n = 9. The mean (n = 9) PPRs under control (mean of pretest and wash; **Avg Cont**) and ethanol (**50 mM EtOH**) conditions are illustrated in **D.** * $P < 0.05$ compared to control.

Fig. 3. Ethanol does not have an effect on the frequency of the sIPSCs and the PPR from lateral septal neurons.

When ethanol was applied to mechanically dissociated lateral septal neurons, GABA release was not affected as indicated by a lack of effect on rate of sIPSCs (**A**). Representative traces are shown from a lateral septal neuron prior to ethanol administration (**PRE**), during application of 50 mM ethanol (**EtOH**) and following washout (**WASH**). When data were averaged across 6 to 10 neurons (**B**) there was no effect of 25, 50 or 100 mM ethanol on sIPSC frequency ($P > 0.1$).

Representative paired-pulse traces from a lateral septal neuron (**C**) are shown prior to ethanol administration (**PRE**), during application of 50 mM ethanol (**EtOH**) and following washout (**WASH**). Note that a similar paired-pulse facilitation was observed in each instance. Mean amplitude for the initial pulse was 84 ± 20 pA; n = 6. Mean PPR for lateral septal neurons in the presence **(50 mM EtOH)** and absence (mean of pretest and wash; **Avg Cont**) of ethanol is shown in **D**. There was no effect of the 50 mM ethanol on the PPR from the lateral septal neurons ($P > 0.1$; n = 6).

Fig. 4. Ethanol does not have an effect on the frequency of the sIPSCs from medial frontal cortex neurons.

Representative traces of sIPSCs (**A**) were recorded from a cerebrocortical neuron prior to ethanol administration (**PRE**), during application of 50 mM ethanol (**EtOH**) and following washout (**WASH**). When data were averaged across 10 to 12 neurons (**B**), there was no effect of 25, 50 or 100 mM ethanol on sIPSC frequency ($P > 0.1$).

Fig. 5. Ethanol increases the frequency of mIPSCs from substantia nigra reticulata neurons.

Representative traces of mIPSCs (**A**) recorded from a substantia nigra reticulata neuron prior to ethanol administration (**PRE**), during application of 50 mM ethanol (**EtOH**) and following washout (**WASH**). While there was a significant linear trend with an n of 9-13 per group (**B**, r = 0.48, n =

47), indicating a concentration-related effect of ethanol, only the 100 mM ethanol concentration produced a significant increase in mIPSC frequency ($P < 0.05$ Tukey HSD test). In the presence of a $GABA_B$ antagonist, the 50 mM ethanol concentration also produced a statistically reliable increase in frequency of mIPSCs (see text).

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TABLES

Table 1, Effect of Ethanol on Amplitude and Decay Times across Brain Regions

SITE	CONT AMP	EtOH AMP	CONT DECAY	EtOH DECAY
Purkinje	31.4 ± 4.0 pA	29.6 ± 4.2 pA	6.1 ± 0.53 ms	5.8 ± 0.41 ms
Cortex	46.43 ± 6.7 pA	40.85 ± 4.7 pA	16.42 ± 2.7 ms	13.39 ± 2.1 ms
Lateral Septum	23.9 ± 9.7 pA	19.0 ± 5.17 pA	13.29 ± 1.22 ms	13.48 ± 1.15 ms
Substantia Nigra	87.5 ± 10.1 pA	98.8 ± 13.7 pA	13.54 ± 1.86 ms	13.26 ± 1.62 ms

Amplitude for control sIPSCs or mIPSCs (CONT AMP) and during 100 mM ethanol (EtOH AMP) did not differ significantly at any of the three sites $P > 0.05$). Similarly, the decay times under control conditions (CONT DECAY) and during ethanol administration (EtOH DECAY) did not differ significantly at any of the four sites ($P > 0.05$).

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Fig. 1

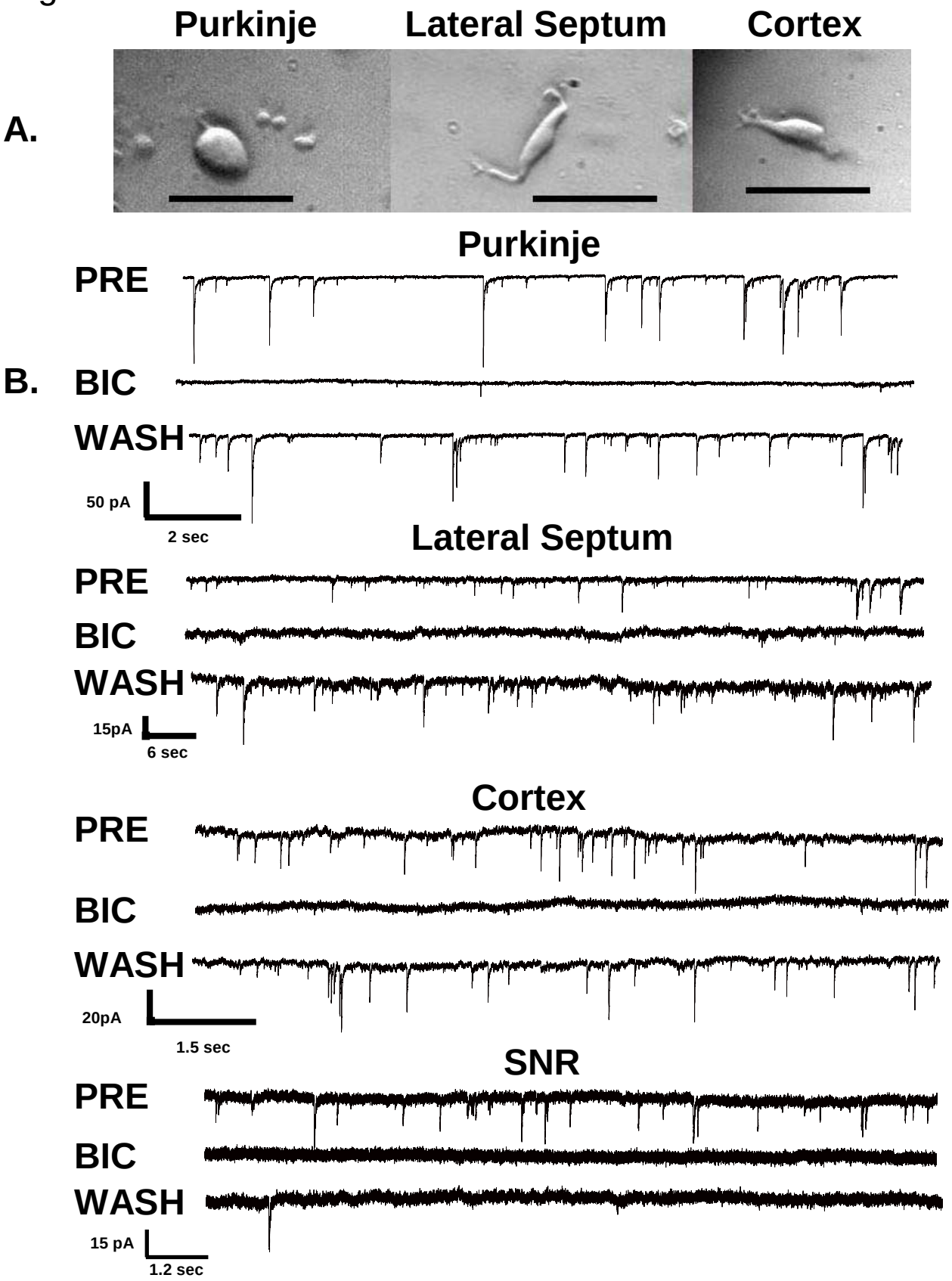

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Fig. 2

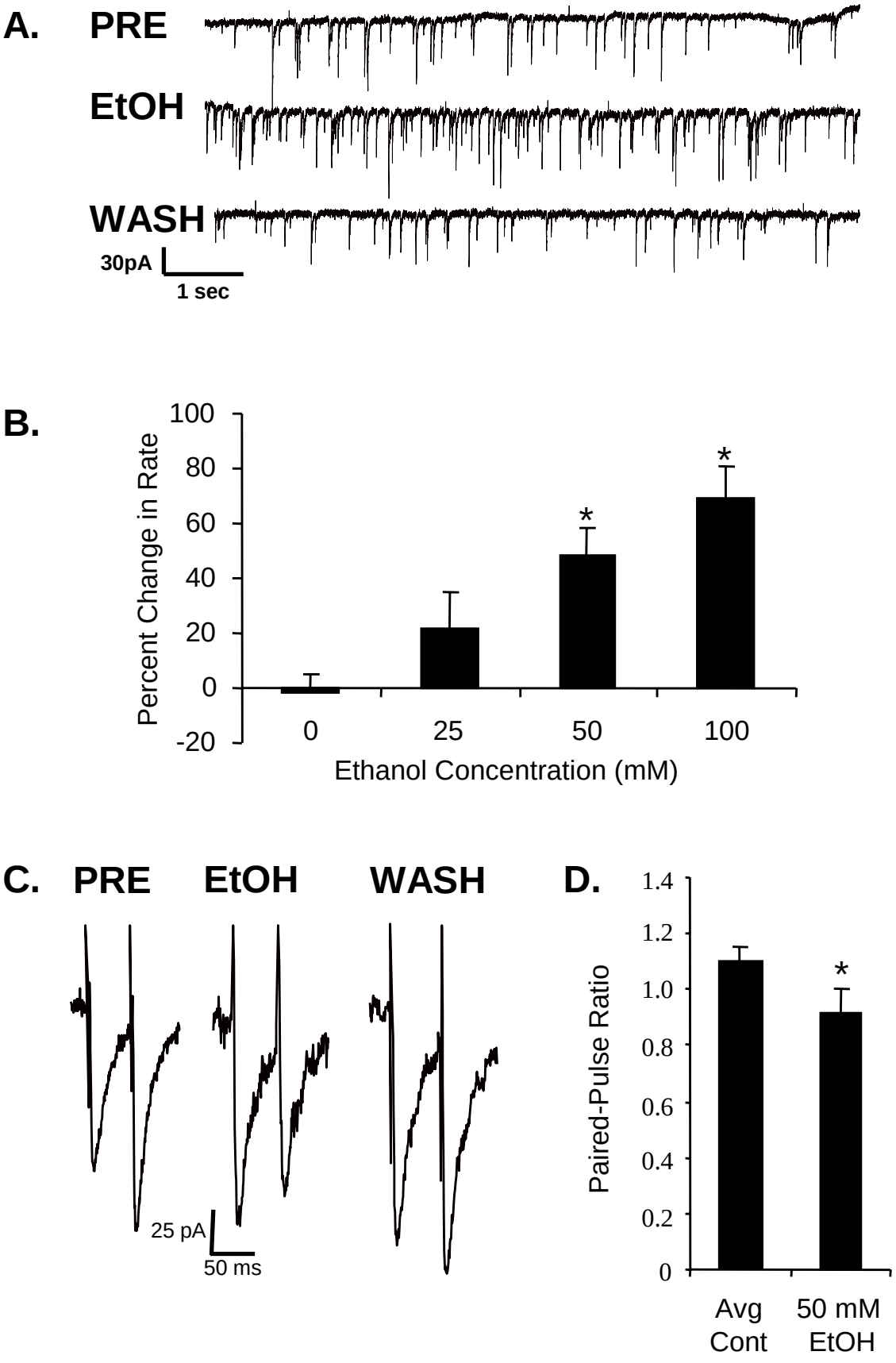

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Fig. 3

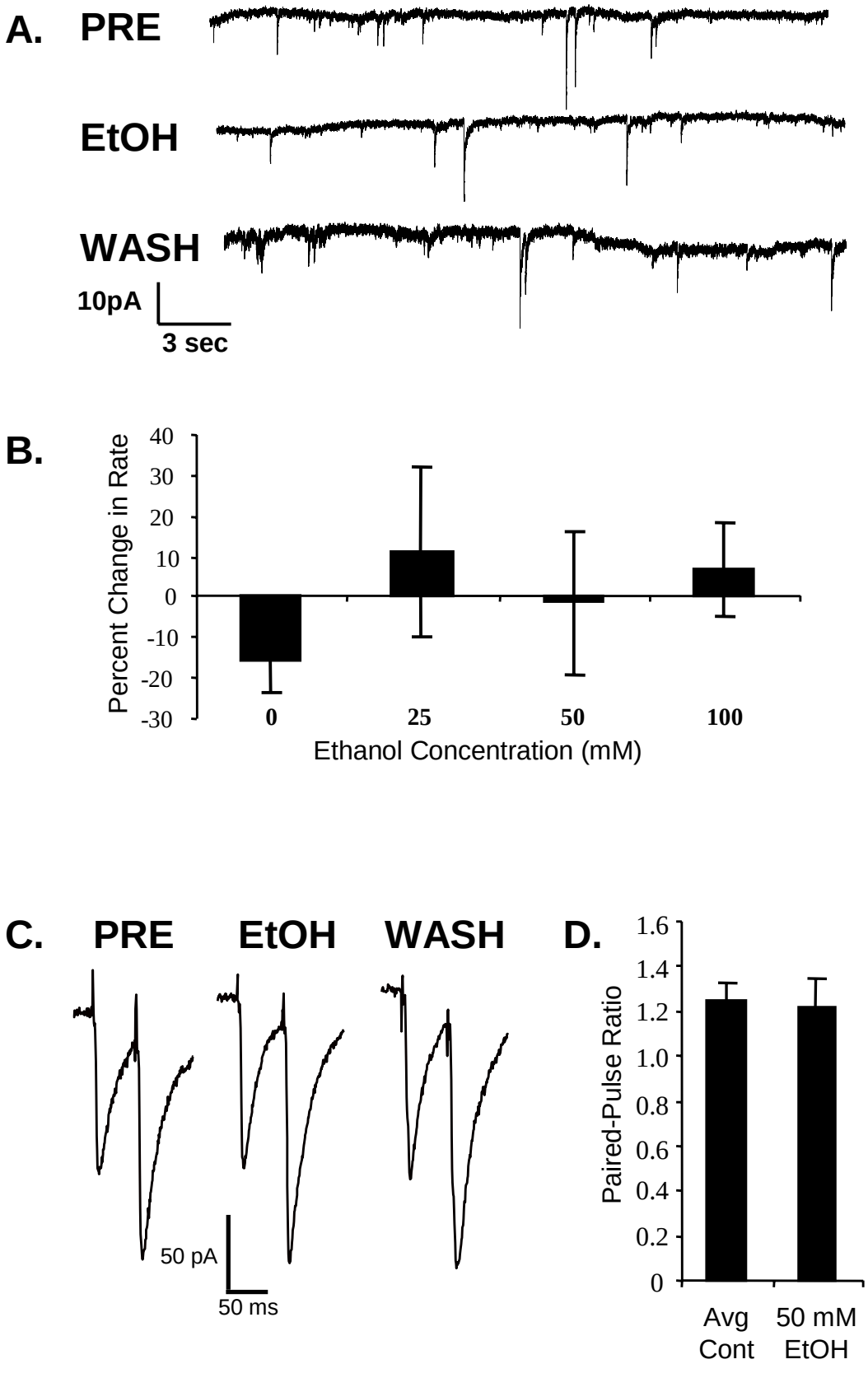

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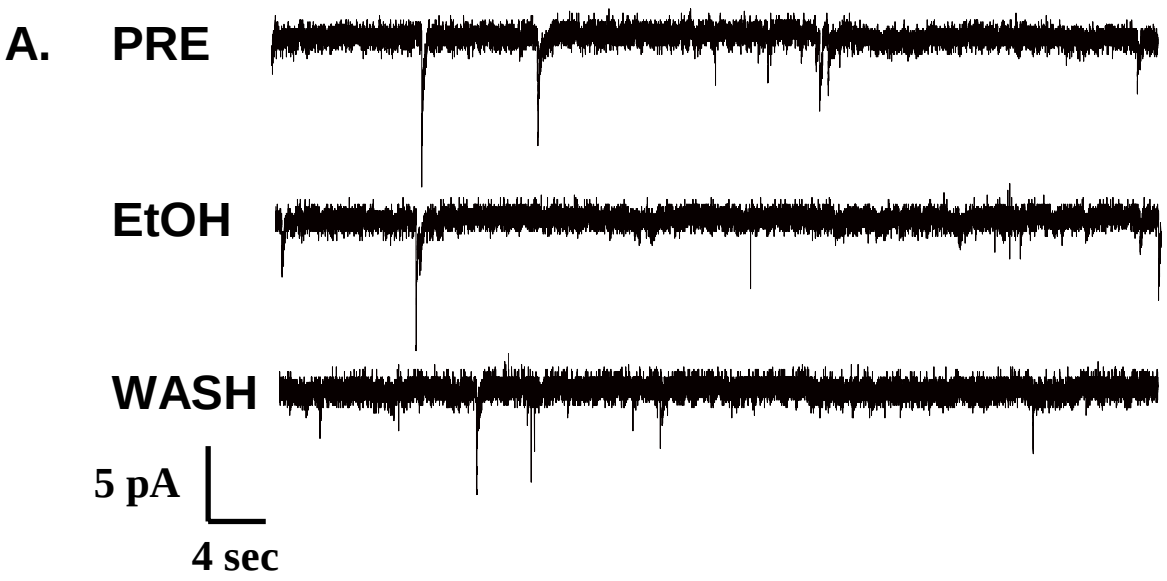
Fig. 4



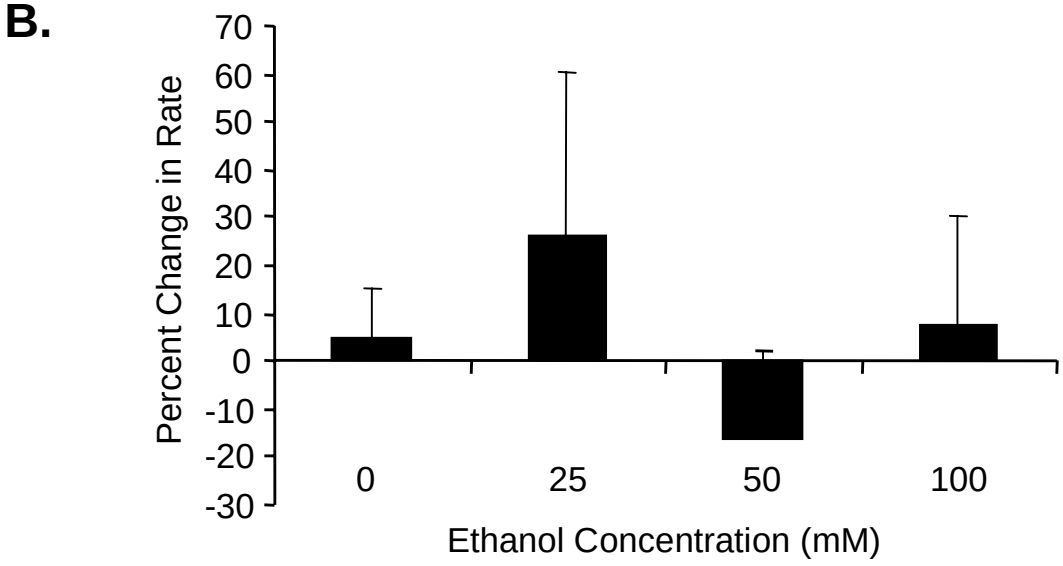


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Fig. 5