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A Conceptualization of Integrated Actions of Ethanol Contributing to its GABAmimetic Profile: A Commentary

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Early behavioral investigations supported the contention that systemic ethanol displays a GABAmimetic profile. Microinjection of GABA agonists into brain and *in vivo* electrophysiological studies implicated a regionally specific action of ethanol on GABA function. While selectivity of ethanol to enhance the effect of GABA was initially attributed an effect on type-1-benzodiazepine (BZD)-$GABA_A$ receptors, a lack of ethanol's effect on GABA responsiveness from isolated neurons with this receptor subtype discounted this contention. Nonetheless, subsequent work identified $GABA_A$ receptor subtypes, with limited distribution in brain, sensitive to enhancement of GABA at relevant ethanol concentrations. In view of these data, it is hypothesized that the GABAmimetic profile for ethanol is due to activation of mechanisms associated with GABA function, distinct from a direct action on the majority of postsynaptic $GABA_A$ receptors. The primary action proposed to account for ethanol's regional specificity on GABA transmission is its ability to release GABA from some, but not all, presynaptic GABAergic terminals. As systemic administration of ethanol increases neuroactive steroids, which can enhance GABA responsiveness, this elevated level of neurosteroids is proposed to magnify the effect of GABA released by ethanol. Additional factors contributing to the degree to which ethanol interacts with GABA function include an involvement of $GABA_B$ and other receptors that influence ethanol-induced GABA release, an effect of phosphorylation on GABA responsiveness, and a regional reduction of glutamatergic tone. Thus, an integration of these consequences induced by ethanol is proposed to provide a logical basis for its *in vivo* GABAmimetic profile.

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INTRODUCTION

Ingestion of ethanol results in a dose-dependent reduction of central nervous system (CNS) activity. For several decades, it was presumed that this CNS depression was due to a major effect of ethanol on GABA mechanisms. Owing to its regional specificity *in vivo* to enhance the effect of GABA, it was initially hypothesized that ethanol had a direct effect on type-1-$GABA_A$ receptors, which represent the majority of the $GABA_A$ receptors in the CNS. Subsequently, while it became apparent that this view was not viable, recent studies implicated a direct effect of ethanol on selected benzodiazepine (BZD)-insensitive receptor subtypes. However, pursuit of new initiatives has provided alternatives to a direct effect of ethanol on the majority of $GABA_A$ receptors. The present commentary will first provide an historical perspective of attempts to define ethanol's ability to influence this neural system in brain, followed by an overview of findings from recent initiatives to clarify the means by which ethanol displays its GABAmimetic profile.

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INTERACTION OF ETHANOL WITH THE GABA SYSTEM *IN VIVO*

GABA (γ-amino-butyric acid) is the primary inhibitory neurotransmitter in brain (Mody *et al*, 1994; Sieghart, 1995). Evidence accumulated over several decades suggested that ethanol influences GABA function (Allan and Harris, 1987; Frye and Breese, 1982; Liljequist and Engel, 1982; Martz *et al*, 1983; Mereu and Gessa, 1985; Nestoros, 1980; Simson *et al*, 1991; see Crews *et al*, 1996). Most convincing was the early recognition that behavioral consequences of moderate doses of ethanol had similarities to those of BZDs and barbiturates (Breese *et al*, 1983; Frye *et al*, 1979, White *et al*, 1997), drugs known to rely on $GABA_A$ receptor function (see Harris, 1990; Ticku, 1989). Further, BZDs and barbiturates enhanced ethanol-induced motor impairment

(Martz *et al*, 1983) and substituted for ethanol in drug discrimination studies (Grant *et al*, 2000; Shannon *et al*, 2004). On the other hand, a BZD-inverse agonist minimized ethanol-induced sedation (McCown and Breese, 1989; Suzdak *et al*, 1986a; Ticku and Kulkarni, 1988) and $GABA_A$ receptor antagonists decreased the antipunishment action of ethanol (Koob *et al*, 1986, 1988; Liljequist and Engel, 1984). Samson *et al* (1987) reported that a BZD-inverse agonist reduced ethanol self-administration—a common procedure used to evaluate motivation to drink ethanol (Samson, 1986; Samson *et al*, 1988). Likewise, a BZD-inverse agonist and a ligand blocking $GABA_A$ receptor function decreased responding for ethanol (Petry, 1995; Rassnick *et al*, 1993).

Another area that has been associated with an action of ethanol on GABA function is withdrawal from chronic ethanol. Withdrawal results in an increased sensitivity to induction of seizures (Cooper *et al*, 1979; Frye *et al*, 1983b; McCown *et al*, 1985, Allan and Harris, 1987). BZDs and other drugs with GABAmimetic action reduced such withdrawal-related hyper-excitability (McCown *et al*, 1985; Ticku and Burch, 1980). Conversely, $GABA_A$ receptor antagonists (Allan and Harris, 1987; Brouillet *et al*, 1991) and BZD-receptor inverse agonists (Mehta and Ticku, 1989; Becker and Anton, 1989) exacerbated seizure susceptibility associated with ethanol withdrawal. Withdrawal from chronic ethanol also results in an anxiety-like response (File *et al*, 1989; Criswell and Breese, 1989)—a response blocked by BZDs and GABAmimetic drugs (Cooper *et al*, 1979; Criswell and Breese, 1989). Additionally, flumazenil, a BZD-receptor antagonist, blocked the anxiogenic consequence of ethanol withdrawal (File *et al*, 1989; Criswell and Breese, 1990, 1993).

Collectively, these functional and behavioral studies of GABAmimetics and GABA antagonists on the acute and chronic actions of ethanol offered strong support for the hypothesis that at least a part of the action of ethanol was mediated by effects on neural functions associated with GABA transmission.

REGIONAL SPECIFICITY OF ETHANOL ON FUNCTIONS ALTERED BY GABAMIMETIC DRUGS

The next series of investigations sought to define brain site(s) responsible for enhancement of ethanol-induced sedation by GABAmimetic drugs. A potential brain site involved in the sedative action of ethanol came from research with thyrotropin releasing factor (TRH), a peptide known to reduce sleep time induced by ethanol (Breese *et al*, 1974; Cott *et al*, 1976). Since microinjection of TRH into the medial septum antagonized measures of sedation induced by ethanol (Breese *et al*, 1984; McCown *et al*, 1986), this site was chosen to examine the effect of drugs affecting GABA function on loss of aerial righting and sleep-time induced by systemic administration of ethanol. Microinjection of muscimol into the medial septum markedly enhanced these measures of ethanol-induced sedation (McCown *et al*, 1986; Givens and Breese, 1990b). Conversely, microinjection of the GABA antagonist, bicuculline, into the medial septum antagonized the effect of ethanol on sedation (Breese *et al*, 1984; Givens and Breese, 1990b). In contrast to results obtained in the medial septum, muscimol microinjected into the lateral septum was without an effect on sleep time (Givens and Breese, 1990b).

Subsequent work was undertaken to define specific brain sites responsible for the action of GABAmimetic drugs to minimize the increased susceptibility to audiogenic seizures following withdrawal from chronic ethanol exposure (Cooper *et al*, 1979; Frye *et al*, 1983b). As lesioning of inferior colliculus, a prominent relay nucleus in the auditory pathway, prevented audiogenic seizures in rodents (Wada *et al*, 1970; Henry *et al*, 1972), the possible involvement of this brain site in the ability of systemically administered GABA receptor agonists to reduce audiogenic seizure activity during withdrawal from chronic ethanol was investigated (Frye *et al*, 1983a). Muscimol microinjected into the inferior colliculus antagonized seizure activity during withdrawal from chronic ethanol, consistent with a reduction in GABA function within this brain site during withdrawal (Frye *et al*, 1983b, 1986). Such reduction in seizure susceptibility was not observed when GABAmimetics were introduced into other brain regions (Frye *et al*, 1983b).

Brain sites responsible for GABA transmission supporting self-administration and discrimination of ethanol have also been studied. McBride *et al* (1999) reviewed the literature concerning intracranial self-administration of ethanol in comparison to the consequence of intracranial administration of drugs that influence $GABA_A$ receptor function. The effect of drugs influencing GABA function implicated the extended amygdala in ethanol self-administration (Hyytia and Koob, 1995; Roberts *et al*, 1996). Utilizing intracranial self-administration of ethanol, Rodd *et al* (2005) described regional heterogeneity within the ventral tegmental area. To define sites that support the discriminative stimulus for ethanol, Hodge and Cox (1998) found that muscimol substituted for ethanol when microinjected into the amygdala, but not when microinjected in the prelimbic cortex.

Thus, microinjection of drugs affecting GABA function provided evidence that the sedation induced by acute ethanol and the increased seizure susceptibility following withdrawal from chronic ethanol depend upon its action on GABA function within specific regions of brain (see Frye *et al*, 1983b, 1986; McCown *et al*, 1986). Likewise, examination of drugs affecting $GABA_A$ receptor function implicated differing brain regions for self-administration and ethanol discrimination of ethanol (Hyytia and Koob, 1995; McBride *et al*, 1999; Roberts *et al*, 1996; Rodd *et al*, 2004, 2005). In further support of a regional specificity of ethanol on GABA transmission, work demonstrated that chronic ethanol treatment altered expression of $GABA_A$ receptor subunit mRNAs in some brain regions, but not others (Devaud *et al*, 1995; Grobin *et al*, 2000a, b; Montpied *et al*, 1991; Morrow *et al*, 1992; Mhatre and Ticku, 1992; Papadeas *et al*, 2001).

IN VIVO ELECTROPHYSIOLOGICAL MEASURES OF ETHANOL ACTION ON GABA FUNCTION

Another approach taken to evaluate the interaction of ethanol with GABA function employed *in vivo* extracellular

electrophysiological recording of spontaneous activity of neurons in anesthetized animals across a variety of brain regions. For example, Bloom and Siggins (1987) described regionally distinct effects of ethanol on cell firing ranging from increased firing to depression when recording from inferior olive, hippocampal, cerebellar, and locus coeruleus neurons. In accord with this observation, Givens and Breese (1990a) showed that systemic administration of ethanol depressed neural activity in the medial septum, but not the lateral septum, whereas others described ethanol increases in firing rate of neurons in the ventral tegmental area (Brodie *et al*, 1990; Clark and Little, 2004). Mereu and Gessa (1985) found that low doses of systemically administered ethanol inhibited firing of neurons in the substantia nigra reticulata—an effect enhanced by muscimol and eliminated by bicuculline.

Other studies examined the effect of ethanol on the GABA-induced changes in firing rate of neurons. Nestoros (1980) found that intravenous administration of low doses of ethanol, as well as its electro-osmotic application, enhanced GABA-induced inhibition from cerebro-cortical neurons. Subsequently, Givens and Breese (1990a) observed that systemically administered (1.5 g/kg) and iontophoretic-applied ethanol enhanced the inhibitory action of GABA on single unit activity of neurons in the medial septum, but not from lateral septal neurons. This effect of ethanol on cellular responsiveness to GABA in these distinct brain regions was consistent with earlier data demonstrating that muscimol microinjection into the medial septum facilitated ethanol-induced sedation, whereas microinjection into the lateral septum did not (Breese *et al*, 1984; Givens and Breese, 1990b; McCown *et al*, 1986). Criswell *et al* (1993, 1995) examined the effect of ethanol on GABA responsiveness in several additional regions of brain and demonstrated that ethanol enhanced the effect of GABA on spontaneous activity from neurons in some, but not all, brain regions. Figure 1 provides an example of rate-meter records demonstrating that ethanol can have distinct effects on GABA-induced firing rate when ethanol is applied locally to differing neurons within the ventral pallidum (Criswell *et al*, 1995). The demonstration of regional specificity of ethanol on neural rate (Bloom and Siggins, 1987; Givens and Breese, 1990a) and of its influences on $GABA_A$ receptor-related function (Criswell *et al*, 1993, 1995; Givens and Breese, 1990a, b) was a major advance in understanding the complex pharmacology of ethanol.

In the *in vivo* studies, the specific effect of ethanol on GABA-induced changes in neuronal firing rate occurred following either systemic (Bloom and Siggins, 1987; Givens and Breese, 1990a, b; Mereu and Gessa, 1985; Nestoros, 1980) or central application of 'relevant' ethanol concentrations to neurons (Criswell *et al*, 1993, 1995; Givens and Breese, 1990a; Lin *et al*, 1991; Nestoros, 1980). Since locally applied ethanol enhanced GABA-induced inhibition, ethanol was able to produce this effect without acting at a distant site (Criswell *et al*, 1993, 1995; Givens and Breese, 1990b; Lin *et al*, 1991). Siggins *et al* (1987a) reported that systemic ethanol (2 g/kg) had differing effects on firing of inferior olive neurons in rats anesthetized with urethane compared to the response obtained to ethanol in rats anesthetized with other anesthetics. Therefore, a potentially complicating factor in many *in vivo* studies of ethanol was that animals were anesthetized with urethane or other anesthetics. However, the effects of ethanol on medial septal area neurons in freely moving rats were found to be similar to those in rats anesthetized with urethane (Givens and Breese, 1990a). Nonetheless, the possibility that urethane or other anesthetics influence single-unit activity changes induced *in vivo* by GABA application after ethanol has yet to be eliminated.

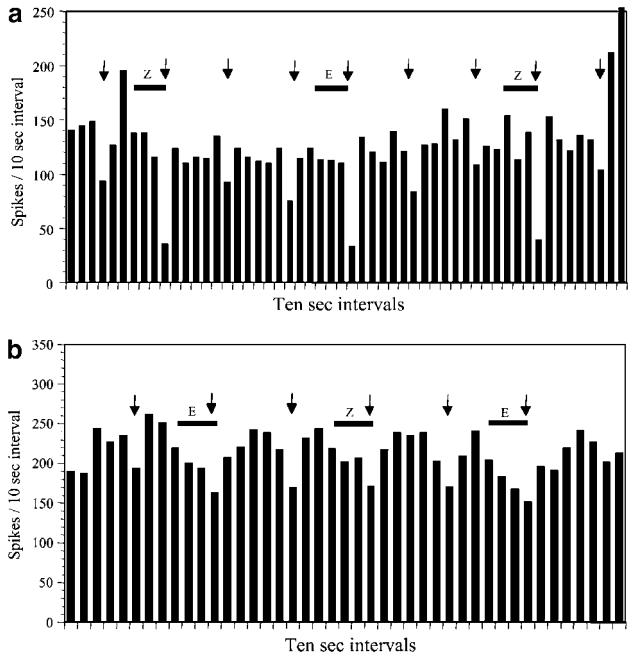

Figure 1 Selective enhancement of GABA-mediated inhibition of single unit activity by ethanol in the ventral pallidum. Vertical bars represent action potentials during a 10 s period. GABA (EC_{30} current) was applied iontophoretically with a constant current for a 10 s period every 60 s as indicated by the arrows. Alcohol (E) or zolpidem (Z) were applied every 40 s shown by the horizontal bars. The alcohol and zolpidem were applied 30 s prior to GABA application. Note that the neuron in the left trace (a) responded to these drugs, whereas the neuron in the right trace (b) did not respond to either ethanol or zolpidem. This finding indicates that some, but not all, neurons at this site respond to ethanol. Modified from Criswell *et al* (1995).

HYPOTHESIS FOR ETHANOL ACTION ON A TYPE-1-BZD RECEPTOR

The $GABA_A$ receptor is assembled as a heteropentamere (Macdonald and Olsen, 1994; Hevers and Lüddens, 1998) from a collection of differing receptor subunits—six α, three β, three γ, three ρ, and δ, ξ, π, and θ subunits (Benke *et al*, 1991b; Hevers and Lüddens, 1998, 2002; Khan *et al*, 1994; Korpi *et al*, 2002; Lüddens and Korpi, 1995; McKernan and Whiting, 1996; Rudolph *et al*, 2001; Sieghart, 1995; Wisden *et al*, 1992). The distribution of these differing assortments of receptor subunits was found to be heterogeneous, allowing for diversity of $GABA_A$ receptor complexes (Fritschy and Mohler, 1995; Mckernan and Whiting, 1996; Rudolph *et al*, 2001; Wisden *et al*, 1992). Two independent lines of research suggested that the regional

differences in the effect of ethanol on GABA function might be due to a selective effect of ethanol on a specific $GABA_A$ receptor subtype.

In an initial study, Wafford *et al* (1991) found that when various sets of $GABA_A$ subunits were assembled in oocytes, only that combination containing the $\alpha_1\beta_1\gamma_{2L}$ subunits was affected by ethanol—a finding confirmed in L(tk$^-$) cells (Harris *et al*, 1995c). Additionally, Harris *et al* (1997) found that ethanol enhanced muscimol-induced chloride flux in L(tk$^-$) cells transfected with $\alpha_1\beta_{2/3}\gamma_{2L}$ $GABA_A$ receptor subunits. From immunoprecipitation (Benke *et al*, 1991a) and immunohistochemical studies of brain (Fritschy *et al*, 1992), the α_1 receptor was found to combine with $\beta_{2/3}$ and γ_2 $GABA_A$ receptor subunits (see review by Mohler *et al*, 1995). This $GABA_A$ receptor combination ($\alpha_1\beta_X\gamma_2$) reportedly is the most abundant BZD/$GABA_A$ receptor in brain (Duncan *et al*, 1995; Lüddens and Korpi, 1995; Mckernan and Whiting, 1996). Binding of zolpidem, as a BZD-receptor agonist selective for $GABA_A$ receptors that contained $\alpha_1\beta_X\gamma_2$ receptor subunits (Wafford *et al*, 1993; Criswell *et al*, 1997), localized with the $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptor subunit combination (Criswell *et al*, 1995; Duncan *et al*, 1995). Importantly, zolpidem binding was found in the medial septum, a site where muscimol increased ethanol-induced sedation, but not in the lateral septum, a site where muscimol did not have this action (Givens and Breese, 1990b; McCown *et al*, 1986).

Based upon finding zolpidem binding in a brain area where ethanol enhanced GABA inhibition of neural rate *in vivo* (Givens and Breese, 1990b), the second line of research utilized extracellular recording to test whether subunit composition of $GABA_A$ receptors sensitive to the action of zolpidem (Horne *et al*, 1992; Wafford *et al*, 1993) would be sensitive to ethanol enhancement of GABA inhibition. This investigation demonstrated that ethanol enhanced the effect of GABA in brain regions where zolpidem binding was high, but not in regions where zolpidem binding was low (Criswell *et al*, 1993, 1995). When effects of ethanol and zolpidem were tested on the same neuron, neurons sensitive to zolpidem were also sensitive to ethanol enhancement of the effect of GABA across several brain regions (Criswell *et al*, 1995; see Figure 1). Thus, it appeared that the effect of zolpidem on GABA responsiveness was capable of predicting the action of ethanol to enhance inhibitory responses to GABA on neural firing in selected sites in brain (Criswell *et al*, 1993, 1995).

It is well known that the effect of ethanol on GABA-induced depression of cerebellar Purkinje neurons can be variable (see Bloom and Siggins, 1987; Freund *et al*, 1993; Lee *et al*, 1995; Lin *et al*, 1994; Yang *et al*, 1998, 2000), even though these neurons have only the type-1-BZD receptor (Fritschy and Mohler, 1995; Itier *et al*, 1996; Lüddens and Korpi, 1995). Therefore, the concordance between effects of zolpidem and ethanol to potentiate GABA-induced depressions of neuronal activity did not appear to hold for cerebellar Purkinje neurons (Lin *et al*, 1991, 1994; Yang *et al*, 1998, 2000). Subsequently, it was found that when the action of ethanol was concomitantly modulated by beta-adrenergic (Freund and Palmer, 1997; Lin *et al*, 1991, 1994; Yang *et al*, 1998) or $GABA_B$ (Yang *et al*, 2000) receptor agonists, ethanol markedly enhanced the GABA-induced inhibition of firing rate from Purkinje neurons (see phosphorylation section). Thus, with this clarification, ethanol action on Purkinje neurons was consistent with the hypothesis that the ethanol enhancement of the effect of GABA *in vivo* related to ethanol having a direct effect on $GABA_A$ receptors sensitive to zolpidem. Since other $GABA_A$ receptor subunits had differing distributions from that of the α_1 subunit forming the type-1-BZD receptor (Fritschy *et al*, 1992; Wisden *et al*, 1992), it was proposed that this differing distribution of $GABA_A$ receptor subtypes accounted for the regional specificity of ethanol to enhance GABA responsiveness (Criswell *et al*, 1993, 1995).

ARGUMENT FOR AN ACTION OF ETHANOL ON BZD-INSENSITIVE $GABA_A$ RECEPTOR SUBTYPES

Based upon a variety of investigations, an argument could be made that the furosemide-sensitive receptor, a BZD-insensitive receptor (Kleingoor *et al*, 1991), might also be sensitive to ethanol enhancement of GABA function (Harris *et al*, 1995b, Ticku and Burch, 1980). This view is based upon the inverse agonist, RO15-4513, which binds to the furosemide-sensitive receptor (Mehta and Ticku, 1988; Mhatre *et al*, 1988), antagonizing the deficit in righting reflex induced by ethanol (Bonetti *et al*, 1989; Suzdak *et al*, 1986a; see review by Ticku and Kulkarni, 1988). Additionally, *in vivo*, the BZD-inverse agonists, RO15-4513 and FG7142, blocked the ethanol-induced depression of Purkinje neurons in the cerebellum (Palmer *et al*, 1988; see review by Palmer and Hoffer, 1990). The RO15-4513 and other BZD inverse agonists are now known to bind to receptors with α_6- and α_4-$GABA_A$ receptor subunits (Derry *et al*, 2004; Knoflach *et al*, 1996; Mhatre and Ticku, 1992; Yang *et al*, 1995), providing evidence that the reversal of ethanol action was related to their effect on these BZD-insensitive $GABA_A$ receptor subtypes.

After chronic ethanol, an alteration in the expression of α_4-receptor subunit occurs in some, but not all, brain regions (Grobin *et al*, 2000a, b; Kumar *et al*, 2002; Montpied *et al*, 1991; Papadeas *et al*, 2001) and the α_6-receptor subunit is increased in the cerebellum (Mhatre and Ticku, 1992; Morrow *et al*, 1992). In respect to the increase in the expression of the α_4- and α_6-receptor subunits following chronic ethanol exposure, there is often a concomitant downregulation of the α_1-receptor subunit (Grobin *et al*, 2000a, b; Mhatre and Ticku, 1992; Morrow *et al*, 1992; Papadeas *et al*, 2001). While the underlying basis of the shift in these alpha-subunits for $GABA_A$ receptors after chronic ethanol is unknown, this change in $GABA_A$ receptor subunits is consistent with a close relationship between ethanol action *in vivo* and GABA function.

IN VITRO EXAMINATION OF ETHANOL ACTION ON $GABA_A$ RECEPTOR FUNCTION

A number of observations utilizing *in vitro* approaches were consistent with ethanol enhancing GABA function. For example, it was observed that ethanol enhanced GABA-induced chloride flux in synapto-neurosomes (Suzdak *et al*, 1986b). Other studies of cultured neurons seemed consistent with ethanol having an action on $GABA_A$ receptors at

concentrations below 100 mM (Aguayo, 1990; Reynolds and Prasad, 1991; Reynolds *et al*, 1992; see review by Mihic, 1999). Durand *et al* (1981) found that ethanol depressed the evoked population spike from CA1 neurons in slices consistent with an effect on GABA function. Enhancement of GABA-gated currents by ethanol from acutely dissociated cerebellar Purkinje and retinal bipolar cells and ganglion cells was also observed (Nishio and Narahashi, 1990; Sapp and Yeh, 1998; Yeh and Kolb, 1997). Signore and Yeh (2000) found ethanol potentiation of GABA ($>10\%$) in a small number (nine of 44) of pyramidal neurons in the pyriform cortex. Roberto *et al* (2003) recently reported that 44 mM ethanol enhanced the effect of exogenously applied GABA to amygdaloid neurons, indicative that ethanol had a direct postsynaptic action on $GABA_A$ receptors at this site. Finally, the specific $\alpha_4\beta_X\delta$ and $\alpha_6\beta_X\delta$ $GABA_A$ receptor subunits (BZD-insensitive receptor subtypes), when expressed in oocytes, exhibited a direct action of low ethanol concentration enhancement of GABA responsiveness (Sundstrom-Poromaa *et al*, 2002; Wallner *et al*, 2003). More will be said later about this latter observation.

In contrast to studies thus far outlined, other *in vitro* investigations suggested that ethanol was not influencing $GABA_A$ receptors directly (Frye and Breese, 1982; Palmer and Hoffer, 1990; Siggins *et al*, 1987b; White *et al*, 1990). In accord with this view, several studies failed to demonstrate an enhancement of GABA responsiveness from isolated neurons, cultured neurons, and cells transfected with specific $GABA_A$ receptor subunits. Indicative of a lack of effect of ethanol on $GABA_A$ receptor function, Siggins *et al* (1987b), utilizing an intracellular approach to measure GABA-gated currents from CA1- and CA3 pyramidal cells in hippocampal slices, were unable to find a direct effect of ethanol on GABA responsiveness. Likewise, Frye *et al* (1994) were unable to demonstrate an effect of pharmacologically active concentrations of ethanol on GABA-gated currents from neurons isolated from medial septal nucleus, a brain site where earlier *in vivo* recording showed enhancement of the inhibitory action of GABA by ethanol (Givens and Breese, 1990b; Criswell *et al*, 1993, 1995). Perfusion of 100 mM ethanol did not augment the effect of exogenous GABA applied to cultured cortical neurons (Marszalec *et al*, 1998)—an observation confirmed by Ming *et al* (2001). In a study comparing ethanol to longer chain alcohols, Peoples and Weight (1999) found that longer chain alcohols enhanced GABA-gated currents at pharmacologically relevant concentrations, whereas the extrapolated EC_{50} for ethanol was 2.1 M—several times the lethal concentration. In a comprehensive study of neurons from differing regions of the CNS, Mori *et al* (2000) concluded that longer chain alcohols had reliable effects on GABA-gated currents at physiologically relevant concentrations, while ethanol did not. Additionally, a wide range of ethanol concentrations failed to enhance GABA-gated currents from neurons acutely dissociated from a number of brain regions previously found sensitive to zolpidem (Criswell *et al*, 2003). This point is illustrated in Figure 2. The topic of ethanol action on $GABA_A$ receptors has been reviewed by Aguayo *et al* (2002).

Most important to the concept that zolpidem predicted the action of ethanol on GABA function (Criswell *et al*,

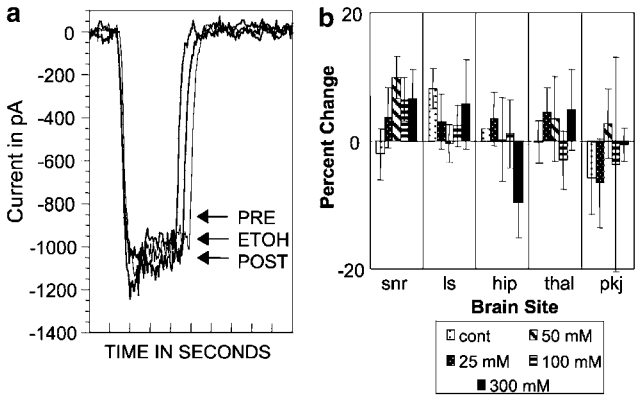

Figure 2 Demonstration that ethanol does not enhance GABA responsiveness from isolated Purkinje neurons and neurons from other brain regions. For this investigation, neurons were enzymatically dissociated from 12 to 18 day Sprague–Dawley rats. An approximate EC_{20} concentration of GABA was used in all cases. Illustration (a) on the left shows a lack of effect of 100 mM ethanol on GABA-gated current from a cerebellar Purkinje neuron. The right illustration (b) expressed as percent of control shows concentration–response curves for the effect of ethanol on neurons from substantia nigra reticulata (snr), lateral septum (ls) hippocampus (hip) thalamus (thal) and cerebellar Purkinje neurons (pkj). There was no significant effect of ethanol on GABA-gated currents at any concentration tested in these latter brain regions. Modified from Criswell *et al* (2003).

1995), ethanol at reasonable concentrations did not alter the effect of GABA responsiveness when the $\alpha_1\beta_2\gamma_{2L}$ combination was expressed in various cell lines (see Criswell *et al*, 2003; Kleingoor *et al*, 1991; Mihic *et al*, 1994; Mori *et al*, 2000; Sapp and Yeh, 1998; Sigel *et al*, 1993) or in oocytes (Harris *et al*, 1997)—a contrast to the positive effect found when the $\alpha_1\beta_1\gamma_{2L}$ subunit combination was expressed in oocytes (Wafford *et al*, 1991) or L(tk$^-$) cells (Harris *et al*, 1995c). Consequently, the view concerning the specificity for ethanol to affect the type-1-BZD receptor directly to enhance GABA function was placed in doubt. Given this conclusion that a direct effect of ethanol on the zolpidem receptor is not responsible for the GABAmimetic profile of ethanol (Criswell *et al*, 2003), the mechanism likely underlying ethanol enhancement of the ability of GABA to inhibit neuronal activity *in vivo* at brain sites with type-1-BZD receptors required resolution.

In contrast to work on dissociated neurons, several studies demonstrated that evoked release of GABA in a slice preparation was sensitive to ethanol (Carlen *et al*, 1982; Bloom and Siggins, 1987; Proctor *et al*, 1992; Roberto *et al*, 2003; Wan *et al*, 1996; Weiner *et al*, 1994a, b, 1997a, b). Carlen *et al* (1982) reported enhanced inhibitory postsynaptic potentials (IPSPs) from CA1 hippocampal cells by ethanol, an observation later confirmed by a number of laboratories (Proctor *et al*, 2004; Wan *et al*, 1996; Weiner *et al*, 1994a, b, 1997a). However, Siggins *et al* (1987b) reported that ethanol most often reduced hippocampal CA1 and CA3 neuronal IPSPs induced by stimulation of the hilar mossy fiber pathway. Proctor *et al* (1992) reported that ethanol did not enhance IPSPs from the hippocampus, but enhanced this measure from neurons in the cortex. Subsequently, Weiner *et al* (1997a) reported differing

sensitivity of subpopulations of rat CA1 pyramidal cells to evoked $GABA_A$ receptor mediated IPSCs by ethanol (see Wu *et al*, 2004). Wan *et al* (1996) found that IPSCs from CA1 pyramidal cells were only sensitive to ethanol enhancement in the presence of a $GABA_B$ antagonist, providing a mechanism for the variability reported (see subsequent section on $GABA_B$ receptor influences). In another brain region, investigators (Nie *et al*, 2004; Roberto *et al*, 2003) reported that ethanol enhanced IPSCs from amygdaloid neurons.

Even though the majority of responses to GABA from isolated neurons were not affected by ethanol (Bloom and Siggins, 1987; Criswell *et al*, 2003; Frye *et al*, 1994; Mori *et al*, 2000; Peoples and Weight, 1999), under the appropriate conditions, work indicated that the effect of ethanol on *in vivo* single unit recording of neural firing provided consistent results within a brain region (Bloom and Siggins, 1987; Criswell *et al*, 1995; Givens and Breese, 1990a; Nestoros, 1980; Palmer and Hoffer, 1990). Based upon knowing that ethanol has a local effect on GABA function when measuring neural rate *in vivo*, it was reasoned that ethanol would affect spontaneous single unit activity in a slice preparation. In this respect, Figure 3 demonstrates that ethanol reduced neural activity of a cerebellar Purkinje neuron in a slice and enhanced the action of GABA on this measure. Based upon these data demonstrating a local action of ethanol in the slice, previous data that ethanol is without effect on enhancement of GABA from isolated Purkinje neurons, and the positive effects of ethanol on evoked IPSCs in several brain regions, *the neural action of ethanol on GABA transmission in vivo and in slices was assumed to depend upon mechanisms not available to individual neurons enzymatically isolated from brain or to cells transfected with the majority of $GABA_A$ receptor subunits.*

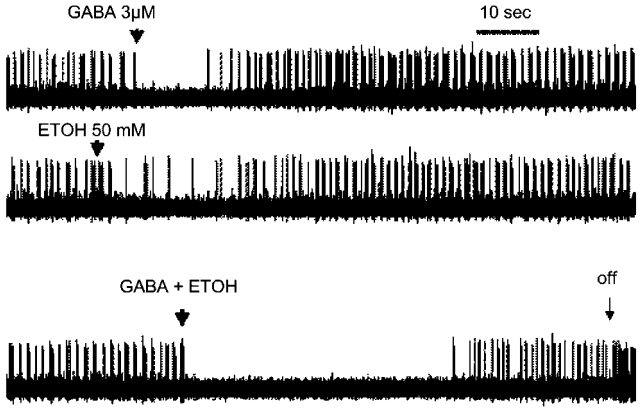

Figure 3 Effect of ethanol on neural rate from cerebellar Purkinje neurons. This figure illustrates the effect of GABA (top), ethanol (middle; 50 mM) and the combination of GABA and ethanol (bottom) on extracellularly recorded action potentials from a cerebellar Purkinje neuron in a slice preparation. Drugs were administered at the time point shown by the arrow and remain present for the remainder of the record. Note that both GABA and ethanol cause a temporary decrease in rate that returns over time. The combination of GABA and ethanol causes a much longer period of inhibition. The reason for the return of neural activity over time is not presently known but may involve local circuits that invoke homeostatic control (unpublished data).

HYPOTHESIS FOR THE GABAMIMETIC PROFILE OF ETHANOL

For the most part, the apparent discrepancies concerning ethanol action on GABA function could be related to the *in vitro* preparations used to evaluate the action of ethanol, the region of brain investigated, factors responsible for maintaining IPSPs, or the presence of a specific receptor subtype within a given brain region being sensitive to ethanol. Regardless, with current evidence not being persuasive that ethanol has a direct effect on zolpidem-sensitive receptors to account for its GABAmimetic profile, new initiatives were needed to understand the overwhelming behavioral and *in vivo* electrophysiological studies linking ethanol to a GABA-like action. Of course, one proposed component that could contribute to its GABAmimetic profile would be a direct action of ethanol on selected BZD-insensitive $GABA_A$ receptors (Sundstrom-Poromaa *et al*, 2002; Wallner *et al*, 2003). However, as will be discussed, these BZD-insensitive $GABA_A$ receptors are believed to have limited localization in brain (Peng *et al*, 2002). While these selected BZD-insensitive receptors likely support the action of ethanol on GABA function in specific regions of brain, they cannot contribute to actions of ethanol related to $GABA_A$ receptors containing an α_1-receptor subunit. Therefore, *for systemic ethanol to display its GABAmimetic profile, it is hypothesized that ethanol affects mechanisms associated with GABA function distinct from a direct effect on the majority of postsynaptic $GABA_A$ receptors (*Criswell and Breese, 2005). These neural mechanisms must be capable of supporting ethanol-depression of neural rate (Givens and Breese, 1990b) and the action of ethanol on evoked IPSCs (Roberto *et al*, 2003; Weiner *et al*, 1997a) from some, but not all, neurons within and across brain regions (Breese *et al*, 1984; McCown *et al*, 1986; Givens and Breese, 1990a, b; Weiner *et al*, 1997a). Potential neural mechanisms proposed to account for characteristics contributing to the GABAmimetic profile of ethanol are illustrated in Figure 4 and discussed in the legend.

In earlier work (Bloom and Siggins, 1987; Siggins *et al*, 1987a, b), a possible presynaptic action of ethanol was suggested. Therefore, based upon this view, another action of ethanol distinct from a direct action on $GABA_A$ receptors that could contribute to its GABAmimetic profile would be an ability to release GABA from presynaptic terminals (see Figure 4). Consistent with this view, Marszalec *et al* (1998) found that perfusion of 100 mM ethanol augmented $GABA_A$ receptor mediated IPSCs from rat cortical neurons, even though the effect of GABA applied to the neuron was not altered. Subsequently, Roberto *et al* (2003, 2004a) provided evidence that ethanol enhanced the effect of stimulus-induced GABA-mediated IPSPs and mIPSCs from neurons in slices from the central nucleus of the amygdala and caused release of GABA into microdialysates—clear evidence that ethanol could facilitate GABA release. This action of ethanol to release GABA can apparently be modified at presynaptic sites (see designation in Figure 4), by $GABA_B$ (Ariwodola and Weiner, 2004; Wan *et al*, 1996) and corticotrophin releasing factor (CRF; Nie *et al*, 2004) receptors. Additionally, other neurotransmitter receptors affected by ethanol can influence neural circuits that influence GABA transmission (Carta *et al*, 2003, 2004;

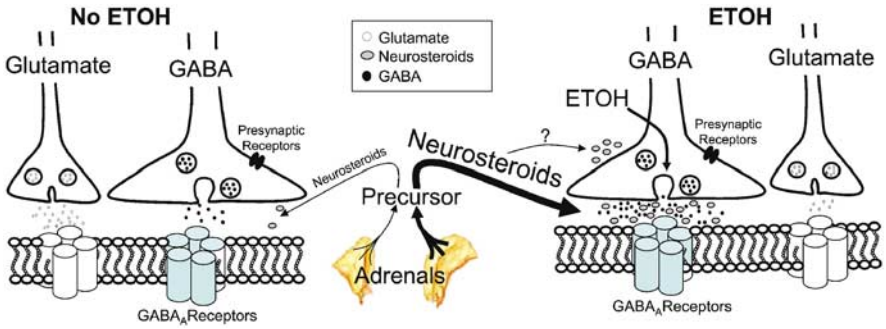

Figure 4 Proposed mechanisms for the *in vivo* GABAmimetic action of ethanol. As noted in the text, a direct action of ethanol on $\alpha_4\beta_3\delta$ or $\alpha_6\beta_3\delta$ $GABA_A$ receptors (Hanchar *et al*, 2004; Sundstrom-Poromaa *et al*, 2002; Wallner *et al*, 2003) could be a means by which ethanol is capable of enhancing GABA transmission in selected brain regions. Additionally, systemic administration of ethanol is proposed to result in an increase in GABA release from presynaptic terminals, as illustrated (right side under ETOH). This release of GABA by ethanol is thought to occur in some, but not all, regions of brain (see text). Data are noted in the text that presynaptic influences (note 'presynaptic receptor' designation in the figure) on $GABA_B$ (see Ariwodola and Weiner, 2004) and other transmitter receptors (Nie *et al*, 2004) can influence GABA released by ethanol. Additionally, following ethanol activation of the hypothalamic-pituitary-adrenal (HPA) axis (denoted by the darkened arrows), ethanol increases neurosteroid precursors from the adrenal, which in turn results in increased neurosteroids in brain (Barbaccia *et al*, 1999; Khisti *et al*, 2003; O'Dell *et al*, 2004). Since neuroactive steroids enhance GABA responsiveness (Lambert *et al*, 2001, 2003; Paul and Purdy, 1992), it is proposed that the ethanol-induced enhancement of neurosteroid presence in brain synergizes the effect of GABA released by ethanol (Criswell and Breese, 2005). Finally, other possible means by which ethanol is capable of enhancing the effect of GABA is by reducing excitatory drive or by influencing phosphorylation of $GABA_A$ receptors (see these topics in Additional Considerations). The relationship of each of these proposed mechanisms to the regional specificity of ethanol is discussed.

Crowder *et al*, 2002; Lomniczi *et al*, 2000; Yang *et al*, 1996).

Since acute ethanol increases neurosteroid levels in brain (see Morrow *et al*, 2004) and neurosteroids enhance the effect of GABA (Criswell *et al*, 2003; Harrison and Simmonds, 1984; Majewska *et al*, 1986), the increase in neurosteroids induced by acute ethanol exposure would be expected to synergize the response to GABA released by ethanol (see Figure 4). Furthermore, a reduction in functions associated with glutamate transmission would also contribute to facilitating the GABAmimetic profile of ethanol by decreasing the excitation that can counteract GABA inhibition (Figure 4, legend). This possibility is supported by earlier reports that ethanol inhibits evoked EPSPs (Bloom and Siggins, 1987; Brancucci *et al*, 2004; Maldve *et al*, 2004; Siggins *et al*, 1987a, b; Ziskind-Conhaim *et al*, 2003). Another consideration could be influences of phosphorylation on GABA transmission (Freund and Palmer, 1997; Weiner *et al*, 1994a, 1997b).

Collectively, these actions of ethanol associated with influences on GABA function are proposed to contribute to its GABAmimetic profile after systemic administration. Critical to understanding the overall action of ethanol is defining the basis of its regionally specific action to influence GABA function and elucidating how this specificity in various brain regions affects distinct functions of the CNS. The current status of mechanisms in Figure 4 and legend, which are proposed to contribute to the GABAmimetic action of ethanol *in vivo,* are discussed below in relation to its regional specificity on GABA transmission.

PROPOSED ROLE FOR BZD-INSENSITIVE $GABA_A$ RECEPTORS IN ETHANOL ACTION

As noted earlier, low ethanol concentrations (3–30 mM) enhance the effects of GABA from transfected $\alpha_4\beta_X\delta$ and $\alpha_6\beta_X\delta$ $GABA_A$ receptor subunits expressed *in vitro* in oocytes (Sundstrom-Poromaa *et al*, 2002; Wallner *et al*, 2003). Consistent with ethanol having an effect on neurons with BZD-insensitive receptors, low concentrations of ethanol also selectively augmented the tonic GABA inhibition of granule cells that contain the α_4 and δ $GABA_A$ receptor subunits, but did not have an effect on CA1 pyramidal cells containing the $\alpha_5\beta_3\gamma_{2/3}$ subunits (Wei *et al*, 2004). Likewise, hippocampal neurons with increased levels of the α_4-receptor subunit following progesterone withdrawal were sensitive to low doses of ethanol (Smith *et al*, 2004; Sundstrom-Poromaa *et al*, 2002). Upregulation of the α_4-receptor subunit has been associated with increased sensitivity to the anxiolytic action of ethanol (Sundstrom-Poromaa *et al*, 2002).

It is recognized that GABA has an extremely high affinity for some BZD-insensitive receptors containing the δ subunit, but a relatively low efficacy (Wallner *et al*, 2003). In some cases, the BDZ-insensitive receptors carry a major portion of the inhibitory current (Mody, 2001; Nusser and Mody, 2002; Hamann *et al*, 2002). Further, it is proposed that $GABA_A$ receptors present at extrasynaptic sites on neurons (Mody, 2001; Nusser *et al*, 1998; Nusser and Mody, 2002) are endogenous $\alpha_4\beta_X\delta$ and $\alpha_6\beta_X\delta$ subtypes, which would be probable targets of ethanol action (Hanchar *et al*, 2004). Thus, in brain areas with high concentrations of BDZ-insensitive receptors containing the δ subunit, ethanol could have a major effect on GABA function. Given the sensitivity of these BZD-insensitive receptor subtypes to ethanol, work like that previously undertaken with 200 mM ethanol to identify critical sites for ethanol action (Mihic *et al*, 1997) should be performed at low ethanol concentrations (ie <60 mM) on the BZD-insensitive $GABA_A$ receptor subtypes to define the molecular basis of the difference between these receptors and those subtypes only sensitive to a high level of ethanol (ie >100 mM).

The effect of ethanol in knockout mice with various subunits making up BZD-insensitive receptor subtypes provided important insight into their contribution to the GABAmimetic profile of ethanol. The effects of ethanol in various $GABA_A$-receptor knockout mouse models have been reviewed (Boehm *et al*, 2004). The $GABA_A$ receptor δ knockout mice exhibit normal anxiolytic action to ethanol (Mihalek *et al*, 2001), but deficits in seizure sensitivity and a reduction in the anticonvulsant action of ethanol (Mihalek *et al*, 2001; Spigelman *et al*, 2002). The discriminative stimulus by ethanol, as well as that for other $GABA_A$ receptor ligands, was not altered in δ-knockout mice (Shannon *et al*, 2004). Finally, an absence of the δ- and the α_6-$GABA_A$ receptor subunits did not alter ethanol-induced sleep time (Mihalek *et al*, 2001) or righting reflex (Homanics *et al*, 1997, 1998). Clearly, not all behavioral responses associated with ethanol action are affected by specific removal of subunits forming the BZD-insensitive receptors (Homanics *et al*, 1997, 1998; Mihalek *et al*, 2001; Shannon *et al*, 2004; Spigelman *et al*, 2002). The potential of these $GABA_A$ receptor subtypes to provide an understanding of ethanol action was discussed by Harris and Mihic (2004).

The restricted anatomical localization of the α_6, α_4, and δ-$GABA_A$ receptor subunits (Gutierrez *et al*, 1996; Laurie *et al*, 1992; Peng *et al*, 2002), and these combinations contributing less than 5% of the total $GABA_A$ receptor pool (Hanchar *et al*, 2004), likely explains the limited effects removal of the subunits associated with the BZD-insensitive receptors have on function. Nonetheless, definition of specific brain regions influenced by an action of ethanol on these BZD-insensitive receptors, which could support selected functions associated with the GABAmimetic profile of ethanol, remains an open issue. However, the complexity of this interpretation is exemplified by the recent observation that GABA responses from the $GABA_A$ receptor on granule cells in the cerebellum, which are thought to contain $\alpha_6\beta_X\delta$ subunits (Hamann *et al*, 2002), were not affected by ethanol (Carta *et al*, 2004). However, Hanchar *et al* (2005) recently published data that a variant of the $GABA_A$ receptor [α_6(R100)$\beta_3\delta$] on cerebellar granule cells was more sensitive to ethanol than the native receptor. The basis of this apparent conflict concerning the effect of ethanol on this receptor subtype on the cerebellar granule neurons requires further investigation (see discussion by Carta *et al*, 2004).

In contrast to the lack of an effect in α_6 and δ knockout mice on ethanol-induced sedation (Homanics *et al*, 1997, 1998; Mihalek *et al*, 2001), deletion of the α_1-$GABA_A$ receptor subunit minimizes the sedative effect of ethanol (Kralic *et al*, 2003) indicative of a role of this receptor subtype in the sedative properties of ethanol. Owing to the lack of sensitivity of BZD-insensitive receptors to zolpidem (Derry *et al*, 2004; Knoflach *et al*, 1996; Yang *et al*, 1995), these $GABA_A$ receptors containing the $\alpha_4/\alpha_6/\beta_X\delta$ subunits would not be viable candidates for ethanol action in brain regions with type-1-BZD (zolpidem) receptors—sites where ethanol was shown earlier *in vivo* to support enhancement of GABA (Criswell *et al*, 1993, 1995). Therefore, additional initiatives undertaken were directed at clarifying the means by which ethanol might display its influence on GABA transmission at sites lacking BZD-insensitive receptors by a mechanism distinct from a direct effect on $GABA_A$ receptor function.

EVIDENCE FOR ETHANOL-INDUCED RELEASE OF GABA—A MAJOR CONTRIBUTOR

A logical contribution, consistent with the GABAmimetic profile of ethanol, would be that ethanol releases GABA from presynaptic terminals. Such an action would certainly result in a GABA-related response. The first potential evidence that ethanol might affect mechanisms related to GABA release was the demonstration that ethanol altered levels of GABA in the cortex and cerebellum following systemic administration (Gordon, 1967). In contrast to this finding, Frye and Breese (1982) did not find a change in GABA levels after acute ethanol administration in three brain regions, including the cerebellum. However, measurement of tissue level after ethanol would require sufficient release of GABA to reduce content. Therefore, alternative approaches were required to test this hypothesis.

A direct approach utilized to evaluate the potential action of ethanol on presynaptic release of GABA was microdialysis. However, various investigations using microdialysis were unable to demonstrate an elevation of GABA by acute ethanol from nucleus accumbens, ventral pallidum, or the ventral tegmental area (Cowen *et al*, 1998; Dahchour *et al*, 1994; Heidbreder and De Witte, 1993; Piepponen *et al*, 2002). On the other hand, Roberto *et al* (2004a) demonstrated an ethanol dose-related release of GABA from amygdala utilizing this approach. One possibility for this apparent discrepancy among the various dialysis studies would be ethanol having a selective effect on GABA release in some, but not all, brain regions—a possibility to be delineated in future experiments.

In spite of the ambiguity of these latter approaches to define an ethanol induction of GABA release, several recent investigations utilizing alternative procedures provided credence for a direct effect of ethanol on presynaptic terminals to release GABA. In this respect, Melis *et al* (2002) found that ethanol increased the frequency of miniature inhibitory postsynaptic potentials (mIPSPs) from ventral tegmental neurons, a finding in accord with GABA release, but in apparent conflict with data collected with microdialysis (Cowen *et al*, 1998). Likewise, Ziskind-Conhaim *et al* (2003) demonstrated that 70 mM ethanol increased the number of mIPSPs related to GABA release from neurons in slices of rat spinal cord. A similar presynaptic action of ethanol to increase the frequency of GABA-related mIPSCs was observed from all neurons in slices containing the central nucleus of the amygdala (Roberto *et al*, 2003), a finding consistent with the ethanol-induced increase of GABA in microdialysates from this site (Roberto *et al*, 2004a). Ethanol also increased the frequency of mIPSCs from CA1 pyramidal neurons in slices of hippocampus (Sanna *et al*, 2004), and from brain stem motor neurons (Sebe *et al*, 2003). In addition to the work on mIPSCs performed in slices (see Roberto *et al*, 2003, 2004a; Nie *et al*, 2004; Carta *et al*, 2004), the use of mechanically dissociated neurons that have terminals attached (see Akaike and Moorhouse, 2003) has allowed confirmation of the conclusion that ethanol indeed has the ability to release GABA

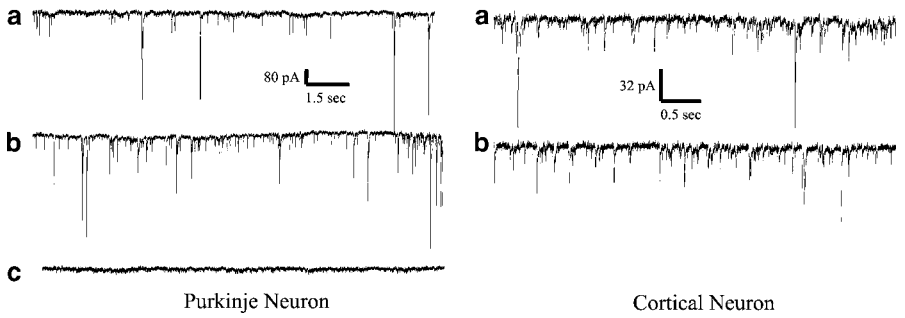

Figure 5 Ethanol increases mIPSCs from isolated cerebellar Purkinje neurons (left), but not from cortical neurons (right): blockade of ethanol effect by bicuculline (left). On the left (cerebellar Purkinje), the top trace (a) shows a representative 15 s sample of mIPSCs from a mechanically dissociated cerebellar Purkinje neuron from rat brain. The middle trace (b) shows the increase in frequency of mIPSCs from 3.1 Hz in the absence of ethanol (a) to 10.0 Hz in the presence of 50 mM ethanol (b). Trace C demonstrates a block of mIPSPs induced by ethanol with bicuculline. The ethanol did not change the amplitude or decay time of mIPSCs from the cerebellar Purkinje neurons. On the right (Cortex), the top trace (a) shows a 5 s sample of mIPSCs from a mechanically dissociated cortical neuron. The bottom trace (b) illustrates the lack of increase in frequency of mIPSCs from 13.1 Hz in the absence of ethanol (a) to 11.6 Hz in the presence of 50 mM ethanol (b). There was also no change in amplitude or of decay time. All recordings were made in the presence of 500 nM tetrodotoxin to eliminate the influence of preterminal Na^+ currents. Criswell *et al* (2004) has shown that the increased frequency of mIPSCs from cerebellar Purkinje neurons is concentration related (from Criswell *et al*, 2004 and unpublished data).

directly from presynaptic terminals. Utilizing this approach, Criswell *et al* (2004) provided evidence that ethanol increased the frequency of mIPSCs from cerebellar Purkinje neurons. An example is presented in Figure 5. Since these mIPSCs were blocked by bicuculline, postsynaptic $GABA_A$ receptors were activated by the ethanol-induced release of GABA (Figure 5). Zhu and Lovinger (2004) reported similar ethanol enhancement of the frequency of mIPSCs from neurons mechanically dissociated from the basolateral amygdala.

Thus, these recent findings *in vitro* showing that ethanol increases the frequency of mIPSCs from neurons in slices and from mechanically dissociated neurons provide needed evidence that ethanol is capable of releasing GABA from presynaptic terminals. *However, our laboratory found that ethanol did not enhance miniature currents from isolated cortical neurons* (Figure 5, Cortex)—a *finding suggesting that ethanol does not release GABA from terminals in all brain locations.* In accord with this view, Dubois *et al* (2004) found that ethanol decreased the frequency of mIPSCs of cultured septal neurons without changing decay kinetics. These latter observations would be consistent with *in vivo* electrophysiological recording showing that ethanol has regionally specific actions on GABA function (Bloom and Siggins, 1987; Givens and Breese, 1990a, b; Criswell *et al*, 1993, 1995); however, the extent that ethanol has a regional action on GABA release has yet to be defined.

The demonstrated presynaptic action of ethanol to increase the frequency of GABA-induced mIPSCs (Criswell *et al*, 2004; Roberto *et al*, 2003, 2004a; Siggins *et al*, 2005; Zhu and Lovinger, 2004) supports the view that ethanol can affect a mechanism related to GABA function distinct from a postsynaptic action on $GABA_A$ receptors (see Figure 4). These findings can also explain the positive interaction of ethanol with modulators of $GABA_A$ receptor function, such as barbiturates and BZDs (Akaike *et al*, 1990; Puia *et al*, 1991), by their enhancing the effect of GABA released by ethanol. Since Sebe *et al* (2003) and Li *et al* (2003) described a developmental consequence of ethanol action to influence GABA transmission, testing the role of presynaptic release of GABA by ethanol in this developmental process should be investigated. For the present, the mechanism by which ethanol induces release of GABA from some, but not all, terminals is uncertain, but is a finding that should receive attention. Likewise, the mechanism of the specificity for this action on GABA release not being universal for all neurotransmitter release must also be considered. The next question that arose was whether any other mechanisms or actions of ethanol could contribute to the GABAmimetic profile of ethanol by affecting the degree to which GABA is released.

OTHER NEURAL MECHANISMS INFLUENCING GABA RELEASE: RELATION TO ETHANOL ACTION

$GABA_B$ Receptor Involvement

Several studies demonstrated that $GABA_B$ receptor antagonists enhance the ability of ethanol to facilitate GABA transmission in the hippocampus (Ariwodola and Weiner, 2004; Wan *et al*, 1996; Wu *et al*, 2004) and nucleus accumbens (Nie *et al*, 2000). Melis *et al* (2002) linked the long-lasting potentiation of GABAergic synapses on dopaminergic neurons in the ventral tegmental area by systemic ethanol to an effect on presynaptic $GABA_B$ receptors. Ariwodola and Weiner (2004) recently suggested that the effect of ethanol to facilitate GABA transmission is limited because of GABA feedback on presynaptic $GABA_B$ receptors. Wu *et al* (2004) reported that the presence of $GABA_B$ receptors accounted for the difference in sensitivity to ethanol influences on GABA transmission in differing subfields of the hippocampus (see Weiner *et al*, 1997a). Additionally, $GABA_B$ receptors did not influence GABA release from neurons in the amygdala (Roberto *et al*, 2003). Thus, the involvement of $GABA_B$ receptors on GABA release in various brain regions may not be universal, suggestive that the presence or absence of presynaptic $GABA_B$ receptors may be an important determinant for the regional specificity of ethanol to affect GABA transmission (Ariwodola and Weiner, 2004).

Other Presynaptic Receptor Involvement

In addition to GABA itself influencing GABA release via $GABA_B$ receptors, other neurotransmitter systems acting at presynaptic sites can influence GABA release. Nie *et al* (2004) showed that CRF is involved in controlling the presynaptic action of ethanol in amygdala, implying that CRF influences GABA release. Yang *et al* (1996) demonstrated *in vivo* that nicotine inhibition in medial septum involved activation of GABA release by presynaptic nicotinic cholinergic receptors—a means by which ethanol and nicotine may interact. Defining the potential role of ethanol action on nicotinic receptor influences on GABA transmission could be a means to understand the high incidence of nicotine addiction in alcoholics. Missing from consideration is testing the effect of ethanol on the myriad of other neurotransmitters and mechanisms that appear to influence presynaptic release of GABA (Cossart *et al*, 2001; Gitler *et al*, 2004; Ho *et al*, 2003; Kerchner *et al*, 2001; Meir *et al*, 1999; Miller, 1998; Nakamura *et al*, 2003; Saitow *et al*, 2001; Satake *et al*, 2000).

Circuit Dependent Influences

In addition to work demonstrating direct presynaptic influences of ethanol to affect GABA transmission, other studies describe circuit-dependent effects of ethanol on GABA release. For example, Lomniczi *et al* (2000) reported that incubation of hypothalamic tissue with ethanol resulted in GABA release—a change proposed to relate to ethanol-induced release of endorphin. Crowder *et al* (2002) reported that ethanol concentrations as low as 20 mM inhibit kainate-induced inhibition of $GABA_A$ IPSCs from CA1 hippocampal neurons (see Weiner *et al*, 1999). Subsequently, Carta *et al* (2003) reported that ethanol inhibition of kainate receptor excitation of GABA-containing interneurons reduced GABA release on CA1 neurons. In an investigation of cerebellar granule cells, Carta *et al* (2004) described an ethanol effect on a GABAergic inhibition of cerebellar granule cells via a release of GABA due to activation of Golgi cells—a circuit-dependent mechanism blocked by TTX.

Collectively, defining the possible influence of ethanol on presynaptic mechanisms that influence GABA release, as well as its actions on other neurotransmitter systems that indirectly influence release, is important to understand ethanol action on GABA transmission. Defining the relationship of these actions to the regional specificity in various brain regions could be a fertile area of future research.

NEUROSTEROID INVOLVEMENT IN THE GABAMIMETIC PROFILE OF ETHANOL: ENHANCEMENT OF THE EFFECT OF GABA RELEASED BY ETHANOL

Progesterone released from the adrenal gland (Holzbauer *et al*, 1985) serves as a precursor for neurosteroids in brain (see Barbaccia *et al*, 2001; Mellon *et al*, 2001). Acute administration of ethanol activates the hypothalamic-pituitary-adrenal (HPA) axis and increases plasma precursors for neurosteroids, resulting in elevated neurosteroids in brain (Barbaccia *et al*, 1999; Morrow *et al*, 2001)—a change blocked by adrenalectomy (Khisti *et al*, 2002, 2003) and by combining adrenalectomy and gonadectomy (O'Dell *et al*, 2004). As seen in male and female rats (Morrow *et al*, 1999), ethanol has a like effect to increase allopregnanolone levels in both male and female adolescent humans (Torres and Ortega, 2003, 2004). As it is well known that neurosteroids enhance the action of GABA (Harrison and Simmonds, 1984; Majewska *et al*, 1986), the increase in brain neurosteroids induced by acute ethanol could presumably contribute to its GABAmimetic profile.

Even though Gabriel *et al* (2004) found that allopregnanolone did not influence ethanol-induced place preference, other work emphasized a relationship of neurosteroid action to ethanol pharmacology by demonstrating that discrimination for ethanol generalized to neurosteroids (Ator *et al*, 1993; Bowen *et al*, 1999; Grant *et al*, 1996). Additionally, exogenous neurosteroid altered the reinforcement responding for ethanol (Janak *et al*, 1998) and reinstated previously extinguished responding for ethanol (Nie and Janak, 2003). Further, a neurosteroid enhanced ethanol consumption in two-bottle preference test (Sinnott *et al*, 2002). These findings, which demonstrate an interaction of ethanol and neurosteroids, are consistent with these compounds sharing influences on $GABA_A$ receptors.

A variety of other functional investigations, in which neurosteroid production was inhibited, also supported the view that central neurosteroids interact with actions of ethanol. VanDoren *et al* (2000) were able to reduce the anticonvulsant action of ethanol and completely block the effect of moderate concentrations of ethanol on medial septal neurons by minimizing the production of neurosteroids with the 5α-reductase inhibitor finasteride. Likewise, Tokunaga *et al* (2003) demonstrated a reduction of ethanol action on neural activity in the hippocampus by inhibiting neuroactive steroid production. However, finasteride did not affect duration of loss of righting by ethanol (Khisti *et al*, 2003; VanDoren *et al*, 2000), possibly because the enzyme blocked by this synthesis inhibitor is localized to specific brain regions not related to this functional measure.

Since adrenalectomy reduces neurosteroids as noted previously (Khisti *et al*, 2002, 2003; O'Dell *et al*, 2004), this approach provided an alternative to evaluate the role of neurosteroids in the action of ethanol. An early study by Bowers *et al* (1991) noted that adrenalectomy increased bicuculline-induced seizure sensitivity in long-sleep and short-sleep mice. A subsequent study demonstrated that adrenalectomy reduced the sedative action of ethanol (Khisti *et al*, 2003). This adrenalectomy-induced deficiency of ethanol action was reversed by administration of the neurosteroid, allopregnanolone (3-alpha-hydroxy-5-alpha-pregnan-20-one; Khisti *et al*, 2003). Even though adrenalectomy did not completely eliminate the sedation induced by ethanol (Khisti *et al*, 2003), the reduction of ethanol action by adrenalectomy implied that a significant part of the sedation related to ethanol is dependent upon its activation of the HPA axis to increase neurosteroids. Based upon the findings that reducing neurosteroid content in brain minimized sedation and other actions of ethanol, Morrow *et al* (1999, 2001, 2004) suggested that the increase in neurosteroids following acute ethanol administration contributes to its actions.

A missing link for understanding neurosteroid contribution to the GABAmimetic profile of ethanol was the specific means of their involvement—a circumstance that has not been given the attention it deserves. While reports suggested a direct interaction between ethanol and neurosteroids on $GABA_A$ receptor function (Akk and Steinbach, 2003; Criswell *et al*, 1999), recent data from our laboratory questions this observation (Criswell *et al*, 2003). Further, it should be recognized that the concentration of neurosteroid following ethanol (<100 nM) is likely insufficient to have a direct effect on $GABA_A$ receptors to gate current in the absence of GABA (Callachan *et al*, 1987; Cottrell *et al*, 1987; Puia *et al*, 1990). This circumstance minimizes the possibility that a direct activation of $GABA_A$ receptor function by neurosteroids is responsible for the neurosteroid contribution to the GABAmimetic profile of ethanol. However, given the recent report consistent with a direct effect of neurosteroids on $GABA_A$ receptor gating (Shu *et al*, 2004), some caution must be taken with this interpretation.

A clue to the mechanism by which the ethanol-induced increase in neurosteroids could influence GABA function was a study by Kang *et al* (1998), who noted that a neurosteroid enhanced evoked IPSCs from hippocampal neurons. Likewise, Vicini *et al* (2002) found that a neurosteroid enhanced decay time of mIPSCs. A finding directly relevant to the mechanism by which ethanol enhances GABA transmission was the study by Sanna *et al* (2004), who found that the effect of ethanol on decay time of mIPSCs in the hippocampus was dependent upon brain steroidogenesis. Utilizing mechanically dissociated cerebellar Purkinje neurons, Figure 6 shows that the neuroactive steroid, alphaxalone, enhances the decay time of mIPSCs induced by 50 mM ethanol without affecting amplitude. Collectively, such data are consistent with the proposal that the ethanol-induced increase in neurosteroids, by its activation of the HPA axis, enhances (synergizes) the responsiveness of the GABA released by ethanol from presynaptic terminals. *These combined actions are proposed to be the primary contributors to the GABAmimetic profile of ethanol* (Figure 4). Further, the specificity of neurosteroids on differing $GABA_A$ receptor subtypes (see Belelli *et al*, 2002; Bianchi and Macdonald, 2003; Lambert *et al*, 2001, 2003; Spigelman *et al*, 2003; Stell *et al*, 2003; Wallner *et al*, 2003) could conceivably contribute to the regional specificity of ethanol on GABA function.

In addition to enhancing the effect of GABA, Haage and Johansson (1999) and Haage *et al* (2002) stated that neurosteroids increase the frequency of mIPSCs from preoptic nerves, suggestive of a presynaptic action of these compounds to release GABA. This neurosteroid enhancement of mIPSCs from medial preoptic neurons has been confirmed by Uchida *et al* (2002). Sulfated neuroactive steroids can inhibit GABA release from hippocampal pyramidal cells by acting on a sigma receptor (Mtchedlishvili and Kapur, 2003). While our laboratory has confirmed the ability of a sulfated neurosteroid to inhibit GABA release (unpublished data from Purkinje neurons), our laboratory and others (Cooper *et al*, 1999; Puia *et al*, 2003) have not observed a change in frequency of mIPSCs at low concentrations of unsulfated neuroactive steroids from terminals influencing hippocampal or cerebellar Purkinje neurons. If neurosteroids are found to release GABA from some, but not all, brain regions, this potential action on GABA function would be an additional way the ethanol-induced increase in central neurosteroids could contribute to its regional specificity on GABA function—a possibility that must be tested.

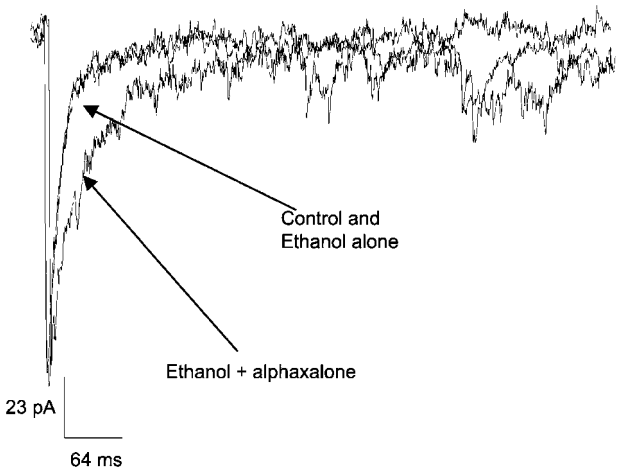

Figure 6 Alphaxalone, a neuroactive steroid, increases decay time of mIPSCs associated with ethanol-induced GABA release. The traces show the scaled mean of 12–14 mIPSCs recorded from a cerebellar Purkinje neuron after ethanol application (50 mM; ethanol alone) in the absence (control) and presence of 100 nM alphaxalone (ethanol + alphaxalone). The control and ethanol traces overlap indicating a lack of effect of ethanol on decay time. However, addition of the 100 nM alphaxalone in the presence of ethanol prolonged the decay time. The curves were best fit by a double exponential curve with $\tau_1 = 3.31$ ms and $\tau_2 = 49.5$ ms for the control condition (ethanol alone) and $\tau_1 = 2.86$ ms and $\tau_2 = 125.8$ ms in the presence of alphaxalone. Alphaxalone had similar effects on increasing the decay time of the mIPSCs in the absence of ethanol. This finding illustrates a direct action of alphaxalone on the $GABA_A$ receptor complex to increase the effectiveness of GABA in the presence of ethanol (unpublished data).

ADDITIONAL CONSIDERATIONS

In addition to the factors already discussed, there are other aspects of ethanol pharmacology that may contribute to its GABAmimetic profile that require further investigation. Selected factors are discussed below.

Phosphorylation and Ethanol Action on GABA Function

One area that needs clarification is the role of phosphorylation on GABA transmission and ethanol's effect on this process. Some $GABA_A$ receptors have consensus sites for phosphorylation (Browning *et al*, 1990; Kellenberger *et al*, 1992). Phosphorylation of these sites results in an alteration in GABA-related currents (Kellenberger *et al*, 1992; Leidenheimer *et al*, 1992). In a seminal publication, Weiner *et al* (1994b) reported that ethanol enhancement of IPSCs from hippocampal CA1 neurons was modulated by PKC, an observation later confirmed (Weiner *et al*, 1997b). In contrast to this consistent finding, mice with the PKCε and PKCγ isoforms provided differing effects on ethanol action. Removal of PKCε gene enhanced the behavioral

response to ethanol (Hodge *et al*, 1999; Proctor *et al*, 2003), suggestive that phosphorylation by this enzyme facilitates its GABAmimetic profile. On the other hand, mutant mice lacking the γ isoform of PKC exhibit a decrease in behavioral responses to ethanol and the action of ethanol on $GABA_A$ receptor function is abolished (Harris *et al*, 1995a), suggestive that removal of this PKC isoform results in a reduction of ethanol action on GABA function. Consistent with these *in vivo* results, Proctor *et al* (2003) reported that ethanol (80 mM) enhancement of IPSCs from CA1 hippocampal neurons was absent in the PKCγ null mice, but potentiated in PKCε null mice. Needing clarification is whether differing isoforms of PKC have alternative means of affecting the action of ethanol on GABA function (eg altering either pre- or postsynaptic functions). Whether the differing isoforms of PKC have a differing regional specificity to alter the action of ethanol on GABA function has not been investigated. Additionally, the role of PKA in modifying ethanol action requires further examination—a need given the suggestion that this enzyme may be responsible for beta-adrenergic stimulation that allows ethanol action to enhance GABA function from cerebellar Purkinje neurons (Freund *et al*, 1993; Freund and Palmer, 1997). Additionally, altered phosphorylation can modulate inhibitory synaptic currents associated with neurosteroid action (Vicini *et al*, 2002). Definition of the basis of these differing findings concerning phosphorylation modifying the action of ethanol on GABA transmission is likely to further clarify whether this process contributes to ethanol's regional specificity.

Ethanol on Excitatory Drive

In whole animals, the GABAmimetic action of ethanol *in vivo* could also be related to reduce excitatory function. In this respect, Ziskind-Conhaim *et al* (2003) found a decrease in the number of glutamate mediated miniature excitatory postsynaptic currents (mEPSCs) from spinal cord neurons, under the same conditions as those in which GABA-related minipotentials were increased. Likewise, Maldve *et al* (2004) found ethanol inhibition of vesicular release at excitatory synapses and Brancucci *et al* (2004) found that ethanol reduced the efficacy of excitatory glutamatergic transmission on dopaminergic neurons in the substantia nigra pars compacta. In the ventral tegmental area, Stobbs *et al* (2004) found that ethanol had inhibitory effects on excitatory glutamatergic neurotransmission. Additionally, ethanol is reported to diminish glutamate release in nucleus accumbens (Piepponen *et al*, 2002; Yan *et al*, 1998), to reduce the probability of glutamate release at the crayfish neuromuscular junction (Strawn and Cooper, 2002), and to minimize potassium stimulated glutamate release in the guinea pig hippocampus (Butters *et al*, 2001). On the other hand, Roberto *et al* (2004b) found that acute ethanol (5–66 mM) did not affect paired pulse facilitation of EPSPs and EPSCs in naïve rat central amygdala and that infusion of ethanol by reverse microdialysis did not affect glutamate in dialysates from this site. Collectively, these limited findings suggest that acute ethanol is capable of reducing glutamate release in some, but not all, brain regions. Nonetheless, acute ethanol can also reduce the postsynaptic effects of NMDA and non-NMDA receptors mediating excitation (see Crews *et al*, 1996)—another change that would reduce excitatory drive and allow greater effectiveness of GABA released by ethanol. As with GABA transmission, a number of presynaptic mechanisms can influence glutamate release (see Brown *et al*, 2004; Jones and Wonnacott, 2004). Since ethanol increases neurosteroids, another aspect that should receive future attention is the possibility that pregnenolone sulfate may enhance spontaneous glutamate release (see Meyer *et al*, 2002), while reducing release of GABA (Mtchedlishvili and Kapur, 2003). Such an action would likely relate to adaptations induced by chronic ethanol exposure.

SUMMARY

From this commentary, the view that an integration of various actions of ethanol that influence GABA function contributes to its GABAmimetic profile appears reasonable (Criswell and Breese, 2005). However, it is emphasized that documentation of this integration is in its infancy. In view of the reported specificity of ethanol action within and at differing sites in brain (Bloom and Siggins, 1987; Givens and Breese, 1990a, b; Criswell *et al*, 1993, 1995; Weiner *et al*, 1997a), additional investigation of the potential regional specificity for each of the mechanisms proposed to collectively influence the *in vivo* GABAmimetic profile of ethanol should be defined. Given reports that ethanol can affect ion channels that influence membrane potential (see Blednov *et al*, 2001; Davies *et al*, 2003; Kobayashi *et al*, 1999; Lewohl *et al*, 1999; Carlen *et al*, 1982; Gruol *et al*, 1997), this area also warrants further investigation, particularly given that small changes in membrane potential can have potent effects on neural activity (North, 1989). Likewise, further attention should be given to whether GABA uptake is affected by ethanol, as an effect on this function could be a means by which ethanol could influence the effectiveness of GABA released by ethanol. The focus proposed should allow identification of specific brain regions responsible for various functions affected by ethanol, extending our understanding of ethanol's varied physiological and functional consequences—particularly those that relate to its GABAmimetic profile. Ultimately, delineation of the contribution of each of the proposed mechanisms influencing the acute pharmacology of ethanol might provide future clues for addressing alcohol abuse.

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