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Actions of Acute and Chronic Ethanol on Presynaptic Terminals

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Abstract

This article presents the proceedings of a symposium entitled “The Tipsy Terminal: Presynaptic Effects of Ethanol” (held at the annual meeting of the Research Society on Alcoholism, in Santa Barbara, CA, June 27, 2005). The objective of this symposium was to focus on a cellular site of ethanol action underrepresented in the alcohol literature, but quickly becoming a “hot” topic. The chairs of the session were Marisa Roberto and George Robert Siggins. Our speakers were chosen on the basis of the diverse electrophysiological and other methods used to discern the effects of acute and chronic ethanol on presynaptic terminals and on the basis of significant insights that their data provide for understanding ethanol actions on neurons in general, as mechanisms underlying problematic behavioral effects of alcohol. The 5 presenters drew from their recent studies examining the effects of acute and chronic ethanol using a range of sophisticated methods from electrophysiological analysis of paired-pulse facilitation and spontaneous and miniature synaptic currents (Drs. Weiner, Valenzuela, Zhu, and Morrisett), to direct recording of ion channel activity and peptide release from acutely isolated synaptic terminals (Dr. Treistman), to direct microscopic observation of vesicular release (Dr. Morrisett). They showed that ethanol administration could both increase and decrease the probability of release of different transmitters from synaptic terminals. The effects of ethanol on synaptic terminals could often be correlated with important behavioral or developmental actions of alcohol. These and other novel findings suggest that future analyses of synaptic effects of ethanol should attempt to ascertain, in multiple brain regions, the role of presynaptic terminals, relevant presynaptic receptors and signal transduction linkages, exocytotic mechanisms, and their involvement in alcohol’s behavioral actions. Such studies could lead to new treatment strategies for alcohol intoxication, alcohol abuse, and alcoholism.

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Keywords

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OVER THE PAST 30 years or so abundant electrophysiological studies using a variety of CNS models have supported the idea that the synapse is the most sensitive site for ethanol action (Deitrich et al., 1989; Shefner, 1990; Siggins et al., 1987a, 1987b, 1999; Siggins and Bloom, 1981; Weight, 1992). Many of these studies were highlighted in an important symposium (the "Drunken Synapse") and the resulting book (Liu and Hunt, 1999) on the synaptic effects of ethanol. However, most synaptic research in the past involved ethanol actions on postsynaptic transmitter receptors, and particularly those for γ-aminobutyric acid (GABA) and glutamate, but few studies addressed the possible presynaptic loci of ethanol action, particularly at the level of the synaptic terminal. A recent NIAAA 2002 workshop on presynaptic ethanol effects, organized by D. Twombly, helped address this lack, but studies on presynaptic ethanol effect were relatively preliminary at that time, and to our knowledge no publication came out of that meeting. Therefore, the present symposium was organized to address the emerging new evidence that presynaptic terminals play a role in acute and chronic ethanol effects.

As exemplified by the studies presented below, a variety of more or less direct methods have been developed to estimate drug effects on presynaptic terminals. These include the following: (1) direct recording of ion channel activity and peptide release from acutely isolated synaptic terminals (see Treistman and Pietrzykowski below); (2) electrophysiological analyses of paired-pulse facilitation (PPF) of evoked synaptic currents and of spontaneous and miniature synaptic currents (see publications by Roberto et al., 2003, 2004; also Weiner below, Valenzuela below, Morrisett below, Moore, et al. in another 2005 RSA symposium); (3) direct microscopic observation of vesicular release of dyes (see Morrisett below); (4) isolated neurons and "neuron-bouton" preparations (see Zhu and Lovinger below and Criswell and Breese, in another 2005 RSA symposium); and (5) adjunctive methods such as bath assays, microdialysis, and electrochemical detection (see Roberto et al., 2004; Parsons et al. at another 2005 RSA symposium; Luthman et al., 1993; Weiss et al., 1993). More details on these important methods can be found in the recent review by Siggins et al. (2005).

A burgeoning number of alcohol-related studies using these methods have appeared in the last 2 to 3 years. For this symposium, we attempted to choose presentations representative of each of the above methods; however, time constraints forced us to omit several key studies from different labs. For example, the work of Roberto et al. (2003, 2004), using analyses of PPF and miniature inhibitory postsynaptic currents (mIPSC), showed that low acute ethanol concentrations (11–44 mM) increased vesicular GABA release from terminals in the central amygdala, and chronic ethanol led to an even greater GABA release. Furthermore, several more recent studies have led to important insights into the loci and mechanisms of this ethanol action. For example, new evidence from Nie et al. (2004) and Moore et al. (reported at another 2005 RSA symposium) suggests that presynaptic receptors for neuro-peptides mediate (CRF) or regulate (opioids) ethanol's augmentation of GABA release in the central

amygdala. In addition, Sebe et al. (2003) showed that 100 mM ethanol increased mIPSC frequencies but decreased mIPSC amplitudes in brain stem motoneurons (MNs). Thus, in some neurons, ethanol may enhance GABA release presynaptically while reducing GABA responses postsynaptically. Related findings by Ziskind-Conhaim et al. (2003) showed that, in neonatal spinal MNs, 70 mM ethanol increased GABAergic and glycinergic mIPSC frequencies (with no effect on mIPSC amplitudes) but decreased glutamatergic miniature excitatory postsynaptic currents (mEPSC) frequencies (but not amplitude). Thus, in the same postsynaptic neurons ethanol may enhance presynaptic-GABA release but may decrease glutamate release.

These and other important insights into the presynaptic effects of ethanol, as highlighted by the studies that follow, suggest that a major central synaptic effect of acute and chronic ethanol is exerted at presynaptic terminals. We anticipate that a continuing survey of neurons from other brain and spinal regions will lead to a more general formulation of these important presynaptic actions of ethanol.

ALCOHOL TOLERANCE IN BK CHANNELS OF NEURONAL TERMINALS

Steven N. Treistman and Andrzej Z. Pietrzykowski

The hypothalamic–neurohypophysial system (HNS) has proven to be a remarkably good system in which to understand the acute and chronic actions of ethanol. Magnocellular neurons (MCN), located in the supraoptic nucleus, extend their axonal projections (the supraoptic–hypophysial tract) to the neurohypophysis (NH, also known as the posterior pituitary or neural lobe). The NH consists of numerous MCN terminals, from which 2 peptide hormones, arginine-vasopressin (AVP, also known as antidiuretic hormone) and oxytocin (OT), are released. The release of these hormones is modulated by ethanol (Knott et al., 2000). Plasma levels of AVP and OT in animals, including humans, are depressed after acute exposure to ethanol, increasing diuresis. These phenomena appear to reflect ethanol's inhibition of AVP release, which can be observed in vitro (Knott et al., 2000). However, animals previously exposed to an ethanol-containing diet develop tolerance to acute ethanol-induced reduction of AVP and OT release.

The release of neurohormones from the terminals is a highly complex process in which calcium-activated potassium (BK) and voltage-gated calcium channels (VGCC) play a pivotal role. We are using electrophysiological and molecular biology methods to understand the molecular underpinnings of the development of tolerance to alcohol. We observed, using whole-cell patch-clamp techniques, that prior exposure to alcohol decreased sensitivity to rechallenge with the drug for both VGCC and BK. In addition, current density in the terminal membrane increased for the VGCC current and decreased for the BK current, consistent with tolerance development. Other channels examined did not exhibit altered current density, demonstrating the selectivity of this aspect of tolerance to VGCC and BK channels. These results were obtained in terminals from rats that had been on an ethanol-containing diet for 2 to 3 weeks.

To better understand the spatial and temporal characteristics of the development of tolerance, we used a cultured HNS explant model. The HNS explant, obtained from rat

juveniles, is sculpted to contain only the elements of the supraoptic–hypophysial tract and allows for the following: (1) control over temporal parameters of drug exposure and withdrawal, (2) exclusion of extrinsic factors (metabolic and neural inputs from outside the HNS), and (3) the possibility for selective application of a drug to either the cell bodies or the terminals. Using this model, we first focused on ethanol effects on the BK channel. BK forms a functional dyad with the VGCC providing a negative feedback for Ca entry and limiting peptide hormone release. The central role of BK in ethanol intoxication has recently been highlighted by a genetic study in Caenorhabditis elegans (Davies et al., 2003), which showed that behaviors of BK gain-of-function mutants resemble those of ethanol-intoxicated animals. We examined whether 24 hours chronic ethanol treatment of the explant mimicked changes in BK channel properties similar to those observed in terminals isolated from animals on an ethanol diet (Knott et al., 2002). Acute ethanol increased the open probability of BK channels from freshly isolated terminals and did not change channel unitary current amplitudes, in agreement with previous findings. Interestingly, 24 hours of 20 mM ethanol treatment of the explant produced significantly reduced BK channel potentiation by subsequent acute ethanol challenge. Thus, the chronic tolerance to ethanol observed in HNS explants resemble that observed in the whole animal model, establishing that this form of tolerance was intrinsic to the HNS, and did not require input from supra-HNS elements in the nervous system or animal (Pietrzykowski et al., 2004).

In addition to the decreased potentiation by acute drug challenge, BK current densities obtained in terminals from explants exposed to ethanol for 24 hours were significantly lower than from terminals from naive explants. The decrease in current density observed could have resulted either from altered individual channel properties or from a down-regulation of functional channels (or both). We observed that the single-channel properties remained unchanged. We next measured expression of BK channel protein in terminals, using a specific antibody directed against the main (pore-forming) α subunit of the channel. Although the total BK expression was not changed by chronic ethanol, it was shifted from the membrane to the interior of the terminal. In addition, we found that chronic ethanol disturbed the expression pattern of BK channels in the terminal membrane, causing their declustering and lateral diffusion, significantly diminishing the amount of BK channel in each cluster (Pietrzykowski et al., 2004). Thus, it is a change in population and distribution properties of the channel, rather than alterations in individual channel properties, that appear to be responsible for the reduction in current density.

Next, we determined the time course of the development of tolerance by evaluating its 2 components. Two distinct time courses were observed as follows: (1) a rapid reduction in ethanol potentiation of BK evident within 12 minutes of drug exposure and sustained for the 24 hours period and (2) a slower developing decrease in BK current density showing partial reduction in current density after 6 hours of ethanol exposure and reaching a near-minimal value after 24 hours (Pietrzykowski et al., 2004).

Thus, the use of the HNS explant allowed us to precisely characterize the development of tolerance to ethanol and distinguish its 2 components. It is possible that ethanol exposure can exert its prolonged effects on BK channel density not only by modulating BK channels already present in the plasma membrane but also through the regulation of BK protein

production. Alternative splicing of premRNAs in the nucleus is a powerful regulatory mechanism involved in the functional diversification of proteins. We used the HNS explant to determine whether the splicing of BK channel is affected by ethanol. Using classical PCR, we first established that, in fresh HNS tissue, alternatively spliced exons are inserted into BK mRNA at 3 splice sites (# 3, # 4, and # 6). They code inserts within the BK channel protein as follows: site # 3—a 4 amino acid (aa)-long insert; site # 4—3, 58, or 61 aa-long inserts (the latter 2 known as STREX-1 and -2, respectively); site # 6—a 27 aa-long insert (p27) (Ha et al., 2000; Tseng- Crank et al., 1994; Xie and McCobb, 1998). Interestingly, after 24 hours of ethanol treatment, we observed a completely different splicing pattern of BK mRNA, with only the following isoforms present: (1) STREX-1 and (2) Insertless (the completely spliced-out isoform). Next, we used real-time PCR to increase the temporal resolution of the measurement of ethanol regulation of BK alternative splicing. We observed that the expression of both STREX-1 and p27 exons is quickly regulated, diminishing as soon as 15 minutes after ethanol application. The p27 exon is most sensitive to the ethanol treatment and is completely excluded from BK α mRNA after 24 hours, while the STREX exon stabilizes at 25% of its original expression level after the same time of ethanol exposure.

The regulation of BK channel alternative splicing by ethanol begs the question of whether a terminal can be a translation site. Is BK protein synthesized from a newly modified mRNA in the cell body or in the terminal? To answer this question, several criteria must be fulfilled as follows: (1) presence of mRNA, (2) ability to produce proteins de novo, (3) ability to export proteins to their destination, and (4) functional activity of newly produced proteins. There is extensive literature showing that vertebrate axons can satisfy all the above criteria. Specific mRNAs are sequestered in axons, and incorporation of labeled amino acids into proteins takes place in isolated axons (Bassell et al., 1998; Muslimov et al., 2002). Various components of the translation machinery are present in axons and terminals (including ribosomal proteins, rRNA, and translation factors), and newly synthesized functional proteins are incorporated into axonal plasma membranes (Brittis et al., 2002). In NH axons, ribosomes as well as several mRNAs have been detected (Trembleau et al., 1994, 1996). We decided to determine whether BK mRNA is present in the terminals. Using single-cell RT-PCR we, indeed, observed the presence of a specific BK channel α subunit mRNA in a single NH terminal.

The changes observed in BK channel protein distribution as well as the presence of BK channel mRNA in the NH terminal bring up the interesting question of terminal “capabilities” for adaptation to the drug and the development of tolerance. We already know that the BK channel ethanol sensitivity in the HNS is compartmentalized, with ethanol-sensitive BK channels present in the terminals, and ethanol-insensitive BK channels located in the soma (Dopico et al., 1999). We have also determined that the individual terminal is capable of developing acute tolerance, over a time course of minutes. Is the terminal fully capable of developing and maintaining all aspects of tolerance, or rather is a connection with the cell body essential? Does the presence of BK mRNA in the terminal play a role in the development of tolerance? Does the site of transcription in the cell body need to “see” the drug for tolerance to develop? All of these important questions can be answered by the selective application of ethanol to the cell body or terminal compartment of a magnocellular

neuron. We are currently using a compartmentalized culture device, which allows a selective, compartment-specific, long-term exposure of the HNS explant to alcohol for that purpose.

In summary, our results indicate that the development of tolerance to ethanol in neurohypophysial terminals, characterized by altered ethanol sensitivity and channel density, can be selectively demonstrated for several ion channels. These 2 components of tolerance have distinct time courses (at least for the BK channel). Chronic ethanol also regulates BK channel alternative splicing, and RNA message can be found inside the NH terminal. The selective application of ethanol to cell body versus axonal terminal gives us an exciting opportunity to explore the capabilities of presynaptic terminals in the development of tolerance to alcohol.

PRESYNAPTIC MECHANISMS UNDERLYING ETHANOL ACTIONS AT GABAERGIC SYNAPSES IN RAT AND MONKEY HIPPOCAMPUS

Jeff Weiner

There is now considerable evidence that ethanol intoxication, dependence, and withdrawal are mediated, in part, by alterations in GABAergic synaptic inhibition (Criswell and Breese, 2005; Siggins et al., 2005). Thus, numerous studies have demonstrated that pharmacologically relevant concentrations of ethanol can significantly potentiate GABAergic synaptic inhibition in many brain regions (e.g. Carta et al., 2004; Roberto et al., 2003; Weiner et al., 1997). In addition, long-term ethanol exposure has been shown to result in alterations in GABAergic synaptic transmission that may reflect compensatory adaptation to the acute effects of this drug (Cagetti et al., 2003; Roberto et al., 2004). Interestingly, several recent studies on hippocampal GABAergic synapses have provided some of the first evidence, albeit correlative, of possible links between the effects of ethanol on these hippocampal inhibitory synapses and the acute and long-term behavioral effects of this drug. For example, studies on hippocampal slices from several selectively inbred rodent lines (Poelchen et al., 2000) as well as transgenic mice with alterations in the expression of specific protein kinase C isoforms (Proctor et al., 2004) have revealed a strong correlation between the acute ethanol sensitivity of hippocampal GABAergic synapses and behavioral sensitivity to ethanol's hypnotic effects. In addition, a temporal relationship has been demonstrated between the behavioral changes associated with ethanol withdrawal (e.g., increased anxiety) and adaptive decreases in hippocampal GABAergic synaptic function (Cagetti et al., 2003). Taken together, these recent studies suggest an important role for GABAergic synapses as mediators of both acute and chronic effects of ethanol.

Given the flurry of recent studies highlighting the central role of GABAergic synapses in mediating ethanol effects, much effort has focused on elucidating the specific mechanisms through which ethanol alters GABAergic synaptic inhibition. The vast majority of early studies suggested that acute and long-term ethanol exposure resulted primarily in alterations in the postsynaptic GABAA receptors that underlie fast inhibitory postsynaptic potentials (i.e. acute, potentiation of GABAA receptor function; chronic, GABAA receptor subunit rearrangement, functional down-regulation of receptor activity). However, a number of

recent studies have begun to challenge this view and have demonstrated that, in addition to these postsynaptic effects, ethanol exposure may also result in profound alterations in presynaptic function at GABAergic synapses (see Siggins et al., 2005). To this end, a number of our recent electrophysiological studies in rat and monkey hippocampal slices have also identified presynaptic mechanisms that contribute to the short-term and long-term effects of ethanol on GABAergic synapses in this brain.

For example, we have previously demonstrated that ethanol, at concentrations well below 100 mM, significantly potentiates the area of proximal GABAA IPSCs recorded from rat hippocampal CA1 pyramidal neurons (Weiner et al., 1997) as well as rat and monkey dentate granule cells (Ariwodola et al., 2003). Recently, we have demonstrated that a significant component of these acute ethanol effects appear to be mediated via a presynaptic facilitation of GABA release. Analysis of tetrodotoxin (TTX)-resistant spontaneous IPSCs (mIPSCs) revealed a significant facilitatory effect of 80 mM ethanol on mIPSC frequency (presynaptic parameter) in recordings from rat CA1 pyramidal neurons and monkey dentate granule cells. In contrast, no significant change in either the amplitude or the area of mIPSCs (postsynaptic parameters) was observed in either neuronal population. These results are generally in agreement with another recent study in rat hippocampal CA1 neurons (Sanna et al., 2004) as well as other studies in the rat central nucleus of the amygdala (Roberto et al., 2003) and cerebellum (Carta et al., 2004), although a postsynaptic contribution was noted in some of those studies (Roberto et al., 2003; Sanna et al., 2004).

Other ongoing studies in our laboratory have shown that the acute ethanol facilitation of GABA release may actually serve to limit this drug's overall potentiating effect at these synapses through the activation of presynaptic GABAB autoreceptors. It was reported several years ago that blockade of GABAB receptors greatly enhances ethanol potentiation of evoked GABAA IPSCs in rat hippocampus (Wan et al., 1996) and nucleus accumbens (Siggins et al., 1999). We recently sought to identify the GABAB receptor–dependent mechanism responsible for limiting ethanol potentiation of hippocampal GABAergic synapses. Our studies revealed that, although proximal GABAA IPSCs were enhanced by ethanol in the absence of GABAB receptor blockade, pretreatment with a GABAB receptor antagonist significantly potentiated this effect. We next carried out a series of experiments to determine whether this facilitatory effect of the GABAB receptor antagonist was because of the blockade of presynaptic or postsynaptic GABAB receptors (Bowery and Enna, 2000). Most importantly, we found that, under normal recording conditions, in the absence of a GABAB receptor antagonist, 80m Methanol significantly increased the frequency, amplitude, and area of spontaneous GABAA IPSCs (sIPSCs). Although ethanol appeared to enhance both presynaptic and postsynaptic parameters of sIPSCs, recorded in the absence of TTX, the enhancement of sIPSC frequency was the most robust effect of ethanol and, notably, blockade of GABAB receptors only facilitated the effect of ethanol on sIPSC frequency (Ariwodola and Weiner, 2004). Taken together, these studies suggest that acute ethanol exposure increases ambient GABA levels sufficiently to engage presynaptic GABAB receptors, which, in turn, serve to reduce ethanol's overall facilitatory effect at these synapses by decreasing subsequent action potential–dependent GABA release.

We have also recently completed the first study on hippocampal synaptic neuroadaptation in a nonhuman primate model of long-term ethanol consumption. In this model, developed by the laboratory of Dr. Kathleen Grant, cynomolgus macaques are trained on operant panels to self-administer a 4% ethanol solution (Vivian et al., 2001). Once trained, monkeys are then given access to the panels for 22 hours each day, and many subjects in this paradigm routinely consume ethanol in doses exceeding 2 g/kg/d. In our first study, we characterized GABAergic synapses onto dentate granule cells in slices prepared from ethanol-drinking monkeys and age- and sex-matched controls immediately following 18 months of daily ethanol drinking. The initial electrophysiological findings revealed a significant increase in the ratio of pairs of GABAA IPSCs evoked at interevent intervals of 25, 50, and 250 milliseconds. These increases in paired-pulse ratios are consistent with a decrease in GABA release probability and are in agreement with the observed decrease in mIPSC frequency observed in rats following a noncontingent chronic intermittent ethanol regimen (Cagetti et al., 2003). In addition, despite the fact that the ethanol group had daily access to this drug for a year and a half, the acute potentiating effect of ethanol observed in slices prepared from these animals was identical to that of ethanol-naive controls. A similar lack of tolerance has also been reported at GABAergic synapses in the hippocampus (Kang et al., 1998) and the central nucleus of the amygdala (Roberto et al., 2004) in rodent studies of chronic ethanol exposure. Interestingly, although tolerance is known to develop to many of ethanol's behavioral effects, some actions, such as ethanol-mediated anxiolysis, do not decline following long-term ethanol treatment (McCool et al., 2003). It will be important in future studies to determine whether the persistence of certain behavioral effects associated with ethanol exposure, such as anxiolysis, is related to the fact that the ethanol sensitivity of GABAergic synapses do not decline even following prolonged ethanol treatment regimens.

Collectively, these ongoing studies add to a rapidly growing body of literature suggesting that presynaptic mechanisms contribute to the acute and long-term effects of ethanol on GABAergic synaptic inhibition. Under our recording conditions, the predominantly acute effect of ethanol on GABAergic synapses in rat and monkey hippocampus is a facilitation of GABA release. Moreover, a decrease in GABA release probability appears to be one of the most pronounced functional changes in these synapses following long-term ethanol self-administration. Additional studies will be needed to address the specific mechanisms underlying these presynaptic actions of ethanol. Elucidation of these mechanisms may reveal novel genes associated with increased risk of alcoholism and may point toward novel presynaptic targets for the development of pharmacotherapies for the treatment of alcoholism.

PRESYNAPTIC ACTIONS OF ETHANOL ON DEVELOPING HIPPOCAMPAL CIRCUITS

C. Fernando Valenzuela, Rafael Galindo, and Manuel Mameli

The hippocampus is important for learning and memory processes, and studies have demonstrated that it is particularly sensitive to the neuroteratogenic effects of ethanol (Berman and Hannigan, 2000). In this brain region, there is a developmentally regulated pattern of network-driven electrical activity known as the giant depolarizing potentials

(GDPs) (Ben-Ari, 2002; Ben-Ari et al., 1989). In immature neurons, GABAA receptors are excitatory because of a shift in the Cl^- equilibrium potential toward more depolarized potentials. Under this condition, there is an outward Cl^- flow through GABAA receptors. The intracellular concentration of Cl^- is higher in developing neurons because these cells express low levels of the K^+/Cl^- cotransporter, KCC2. Giant depolarizing potentials are generated by a complex mechanism in which the excitatory actions of GABA play a central role; however, glutamate receptors also contribute to the generation of these events. Pyramidal neurons are depolarized by GABA released from interneuronal terminals, which acts on intrinsically bursting pyramidal neurons to drive GDPs (Sipila et al., 2005). Glutamate released from pyramidal neurons participates in the generation of GDPs by increasing the excitability of interneurons. γ-aminobutyric acid released at interneuron-to-interneuron synapses also contributes to increasing firing of neuronal networks. Thus, GDPs are primarily generated by a positive feedback local circuit between interneurons and pyramidal neurons within the CA3 region. Giant depolarizing potentials are associated with Ca^{2+} transients mediated by voltage-gated Ca^{2+} channels. These transients contribute to activity-dependent modulation of neuronal growth and synaptogenesis. Indeed, it is well established that GABA acts as a trophic factor that regulates proliferation, migration, differentiation, and synaptic maturation (Owens and Kriegstein, 2002). For instance, GABA treatment has been shown to increase neurite extension in hippocampal neurons (Barbin et al., 1993). Therefore, alterations in this pattern of synaptic activity that is driven by the excitatory actions of GABA could have profound effects on the normal maturation of hippocampal circuits.

We characterized the effects of ethanol on immature network neuronal activity in the developing rat hippocampus. Using acute hippocampal slices from neonatal rats and patch-clamp electrophysiological techniques, it was discovered that ethanol dramatically disrupts GDPs in the CA3 region (Galindo et al., 2005). Ethanol, at concentrations as low as 10 mM, increased GDP frequency. This effect is likely mediated by an increase in action potential–dependent GABA release, given that we found that ethanol significantly increased the frequency of GABAA receptor–mediated spontaneous postsynaptic currents in pyramidal cells and interneurons. Ethanol did not affect the intrinsic excitability of either interneurons or pyramidal neurons. However, it increased the frequency of GABAA receptor–mediated miniature postsynaptic currents at inter-neurons of the CA3 stratum radiatum, suggesting that ethanol selectively increases the probability of GABA release at interneuron-to-interneuron synapses. These findings indicate that ethanol acts as a potent stimulant of local circuits in the immature CA3 region and that presynaptic mechanisms are mainly responsible for this effect.

In CA3 pyramidal neurons, we have also characterized the effect of ethanol on glutamate release from axonal terminals that originate at more distant sites (e.g., the dentate gyrus or the entorhinal cortex) (Mameli et al., 2005). We found that ethanol (50 mM) increased the paired-pulse ratio of both AMPA and *N*-methyl-D-aspartate (NMDA) receptor–dependent EPSCs evoked by stimulation of glut-amatergic afferents (i.e., mossy fibers and/or perforant path). Importantly, this effect was observed in neurons from neonatal but not juvenile rats. Specifically, this effect disappeared between postnatal days 10 and 15. Under basal

conditions, mEPSC frequency was very low and we did not detect an effect on the frequency of these events in the presence of ethanol. However, when the frequency of these events was increased by depolarizing presynaptic terminals with KCl, ethanol (50 mM) significantly decreased mEPSC frequency. This effect could not be detected under conditions in which mEPSC frequency was increased by the addition of sucrose, which acts via an osmotic effect that is Ca^{2+} independent (Stevens and Sullivan, 1998). Taken together, these findings suggested that ethanol decreases the probability of glutamate release by inhibiting a presynaptic voltage-gated Ca^{2+} ion channel, and to test this hypothesis, we assessed the effect of ethanol on NMDA receptor–dependent EPSCs evoked by stimulation of afferent glutamatergic fibers (Mameli et al., 2005). Control experiments established that ethanol does not inhibit NMDA receptor–dependent EPSCs in CA3 pyramidal neurons from neonatal rats. Application of the N-type Ca^{2+} channel blocker, ω-conotoxin-GVIA, decreased the amplitude of NMDA EPSCs by −50% and application of the P/Q-type Ca^{2+} channel blocker, ω-agatoxin-IVA, blocked the reminder of the EPSCs. Importantly, *ω*-conotoxin- GVIA had a minimal effect on the NMDA EPSCs of juvenile rats, while *ω*-agatoxin-IVA had a more substantial effect. Therefore, N-type Ca^{2+} channels have a more prominent role in supporting glutamate release in neonatal than in juvenile rats and this may explain the age dependency of the effect of ethanol on paired-pulse plasticity. Moreover, blockade of N-type Ca^{2+} channels occluded the effect of ethanol on glutamate release in neonatal neurons. Thus, these findings strongly suggest that ethanol decreases glutamate release at immature CA3 pyramidal neurons via inhibition of presynaptic N-type Ca^{2+} channels. These results are consistent with several reports indicating that these Ca^{2+} channels are sensitive targets of ethanol (Walter and Messing, 1999).

Fetal alcohol spectrum disorder is associated with deficits in learning and memory, and the hippocampus has been shown to be important for these processes. Studies have demonstrated that the CA3 region and other regions of the hippocampus are very sensitive to the neuro-teratogenic effects of ethanol (Berman and Hannigan, 2000; Livy et al., 2003). We postulate that presynaptic actions of ethanol on immature hippocampal circuits are likely to contribute to abnormal synapse refinement and maturation. These may underlie, at least in part, the hippocampal alterations that are produced by ethanol exposure during the brain growth spurt.

ETHANOL POTENTIATES GABA RELEASE IN AN ISOLATED NEURON-BOUTON PREPARATION FROM BASOLATERAL AMYGDALA

David M. Lovinger and Ping Jun Zhu

Evidence that ethanol potentiates GABAergic synaptic transmission via presynaptic actions has been accumulating in recent years. The majority of evidence supporting this mechanism comes from studies performed in brain slice preparations where neural circtuiry is intact, and neighboring cells can influence transmission at the synapse of interest (Ariwodola and Weiner, 2004, Nie et al., 2004, Siggins et al., 2005, Ziskind-Conhaim et al., 2003). We wished to determine whether ethanol potentiates GABAergic synaptic transmission in the basolateral amygdala (BLA), a brain region known to have roles in anxiety and fear conditioning (Davis et al., 1994). We also wished to examine ethanol effects at intact

synapses in the simplest possible preparation, to determine whether ethanol has direct effects at the synapses, or whether effects might be mediated indirectly via actions on other cellular elements in the slice preparation. To this end, we took advantage of a preparation in which neurons retaining attached synaptic boutons are isolated from brain slices using mechanical "vibrodissection."

Brain slices containing the BLA were made from P14–P18 rats. For brain slice experiments, whole-cell patchclamp recordings in BLA neurons were made in slices fully submerged in standard cerebrospinal fluid (aCSF) using a CsCl-based internal solution (as in Zhu and Lovinger, 2005). Recordings were performed at room temperature (21–23°C) on the stage of an upright microscope, and neurons were visualized using differential interference contrast optics. When examining inhibitory synaptic currents, excitatory synaptic transmission mediated by ionotropic glutamate receptors was blocked with a combination of AMPA and NMDA receptor antagonists. Ethanol was applied via bath superfusion driven by gravity.

Neurons were vibrodisssected from the BLA using a patch pipette that had been pulled and then polished to form a ball at the tip. The polished pipette was placed into the BLA portion of a brain slice under visualization with a dissecting microscope, and the pipette was moved laterally over a distance of 100 to 200 μm at a frequency of 6 to 10 Hz for −2 minutes using a micromanipulator driven by a piezoelectric bimorph (Zhu and Lovinger, 2005). The slice was then removed, leaving neurons that were completely isolated from other cells and bits of tissue. Whole-cell patch-clamp recordings were performed in the isolated neurons at room temperature on the stage of an inverted microscope under modulation contrast optics. Recordings were performed at room temperature using a standard HEPES-buffered saline extracellular solution, and a CsCl-based internal solution (as in Zhu and Lovinger, 2005). Antagonists of AMPA and NMDA ionotropic glutamate receptors were used to isolate GABAergic synaptic currents. Drugs, including receptor agonists and ethanol, were applied to neurons from a series of fused square glass pipettes situated −100 μM of the cell under study. Solution exchange was effected in −200 ms using a stepper motor-driven micromanipulator to reposition different barrels of the pipette in front of the cell. All recordings were made at a holding potential of −60 mV. For all experiments, data were recorded and digitized using Axon Instruments (Molecular Devices, Union City, CA) hardware and software. Measures of sIPSC frequency and amplitude were made using Mini Analysis software (Synaptosoft, Decatur, GA). Mechanical "vibrodissection" (Akaike and Moorhouse 2003) in the BLA yields isolated neurons that exhibit both GABAergic and glutamatergic transmission (Zhu and Lovinger, 2005). The majority of these neurons are BLA principal neurons that show firing with accommodation and voltage "sags" during hyperpolarization, which are characteristic of this cell type (Washburn and Moises, 1992). Spontaneous GABAergic synaptic transmission can be recorded as sIPSCs in these neurons using CsCl-filled whole-cell patch pipettes. The sIPSCs were completely blocked in the presence of 20 μM bicuculline, indicating that these synaptic responses are mediated by release of GABA and subsequent activation of GABAA receptors. Application of 20 or 100 mM ethanol increases sIPSC frequency and amplitude in this preparation, while 10 mM ethanol is without effect. The magnitude of potentiation of sIPSC frequency subsides with time during ethanol applications lasting 5 minutes, providing evidence of acute tolerance to

ethanol action in this preparation. Application of the GABAB receptor antagonist SCH50911 slows the onset of alcohol action and reduces the acute tolerance, consistent with involvement of this receptor in the acute tolerance to alcohol effects on GABAergic transmission in hippocampus (Ariwodola and Weiner, 2004). Ethanol potentiation of sIPSC frequency and amplitude was also observed in a BLA slice preparation during recordings from principal neurons, although acute tolerance was less pronounced in the slice. Ethanol potentiation of sIPSC frequency was retained in isolated cells in the presence of the sodium channel blocker tetrodotoxin (TTX, 1 μM), but ethanol did not increase synaptic current amplitude under this condition. A change in the frequency of spontaneous synaptic responses with no change in amplitude is generally indicative of a presynaptic locus of a change in transmission. This finding also indicates that ethanol does not potentiate transmission via an increase in presynaptic action potential firing. One advantage of using the isolated neuron preparation is that solutions can be exchanged around the cell with rapid solution exchange on the millisecond time scale. We applied GABA (0.3 μM) to neurons in the presence and absence of 20 or 100 mM ethanol. Ethanol application did not alter the amplitude of GABA-activated ion current in the isolated neuron preparation. This observation indicates that ethanol does not have a detectable influence on postsynaptic GABAA receptors, at least those activated by low agonist concentrations.

Our findings using mechanically isolated BLA neurons indicate that ethanol potentiates GABAergic synaptic transmission mainly via increased GABA release in this preparation. We cannot exclude the possibility that postsynaptic effects take place in other preparations and/ or brain regions, but we have not obtained any evidence that such actions contribute to the potentiation observed in our studies. Ethanol potentiation of GABAergic trans- mission in BLA could contribute to the anxiolytic effects of the drug (cf. McCool et al., 2003). The amygdala has also been implicated in control of ethanol intake (Moller et al., 1997; Roberts et al., 1996). Alterations in GABAergic transmission in this brain region may thus contribute to abuse liability of alcohol.

TICKLING THE TIPSY TERMINAL: ACUTE AND CHRONIC EXPOSURE AND ACTION POTENTIAL–DEPENDENT EFFECTS ON RELEASE

Tao A. Zhang, Adam H. Hendricson, and Richard Morrisett

This symposium was a very timely one, which addressed the varied literature on the presynaptic actions of ethanol at central synapses. Until quite recently, most neurobiologists would have agreed that intoxicating or even hypnotic levels of ethanol have little demonstrable effect on neurotransmitter release. This concerns release at central synapses operated by the 2 major fast neurotransmitters, glutamate and GABA. Neuronal circuits that are particularly responsible for the behavior effects of ethanol intoxication and depressant actions are primarily constructed of these 2 neurotransmitters within (but not limited to) cortical and limbic structures. My presentation focused on glutamatergic transmission and involved both NMDA and non-NMDA receptor–mediated synaptic events. Curiously, more than 12 years ago we compared ethanol modulation of pharmacologically isolated glutamatergic and GABAergic synaptic currents and observed no effects suggestive of any

presynaptic actions on either EPSCs or IPSCs (Morrisett and Swartzwelder, 1993). What has changed, and what are the major determining factors in this shift?

The short answer to this question is that we have not looked hard enough and that we overlooked the complexity of action potential–dependent and –independent effects on the release process. In my presentation, I describe 2 examples of glutamatergic transmission in which ethanol actions on action potential–dependent synaptic events are due to at least in part, modulation of release.

In the first example, NMDA quantal synaptic events, pharmacologically isolated with the non-NMDA antagonist, 6,7-dinitroquinoxaline-2,3-dione (DNQX), and GABA receptor blocker, picrotoxin, were recorded from acutely prepared slices of the nucleus accumbens (NAc) and evoked by local synaptic stimulation as described previously (Maldve et al., 2002; Zhang et al., 2005a). To measure NMDA mEPSCs under conditions whereby ethanol inhibition was representative of that occurring physiologically, we evoked NMDA mEPSCs in the presence of 0.6 mM Mg^{2+} (Calton et al., 1998; Morrisett et al., 1991). Also, we evoked responses in the presence of Sr^{2+}, which replaced Ca^{2+}; the mEPSCs that we analyzed occurred as asynchronous release events in the 200 to 1,000 milliseconds interval following stimulation similar to those recorded previously in the hippocampus (Hendricson et al., 2004). Conventional analysis of frequency and amplitude of mEPSCs enables one to deduce how ethanol might alter the presynaptic and postsynaptic function of the synapse; that is, alterations in release probability are reflected in changes in the frequency of mEPSCs and not amplitude.

When ethanol was perfused onto medium spiny neurons in NAc slices, we observed a concentration-dependent decrease in NMDA mEPSC frequency and amplitude. While this inhibitory effect was not statistically significant at 25 mM, it was highly significant at 50 and 75 mM for both synaptic measures; mEPSC frequency was maximally inhibited by about 35% (while amplitude was inhibited by about 25%). Furthermore, we also demonstrated that the presynaptic inhibitory actions of ethanol could be detected via paired-pulse facilitation of conventional Ca^{2+}-dependent NMDA EPSCs. As we have reported previously, activation of D1-dopamine receptors strongly reversed the effects of ethanol on NMDA mEPSCs. While we observed the postsynaptic changes in mEPSC amplitude as hypothesized, we were surprised to observe reversal of the presynaptic effects of ethanol by D1-like receptor activation as well. To verify this finding further, we performed strict analyses of all populations of events and compared absolute event amplitude distributions prior to and following ethanol treatment and D1 agonist addition. Such analyses revealed that the changes in mEPSC frequency that were detected from standard cumulative event distributions were most likely not due to detection errors. Therefore, not only did this analysis of quantal NMDA-receptor– mediated synaptic events reveal an action of ethanol to modulate the release of glutamate onto medium spiny neurons, but these data indicate that the presynaptic actions are modulated by D1-like receptor activation. Indeed, there is precedent for this interaction, and a report on these findings is available (Zhang et al., 2005).

In the second example, chronic ethanol exposure in CA1 pyramidal neurons of hippocampal organotypic explants was studied. Following several days of continual exposure to ethanol,

these explants display robust and long-lasting electrographic seizure discharges upon ethanol washout (Thomas et al., 1998). Subsequent application of NMDA induces neurotoxicity to a much greater extent than in normal ethanol-naive explants (Thomas and Morrisett, 2000). As above, we analyzed Sr^{2+}-supported pharmacologically isolated NMDA mEPSCs, and consistent with our previous acute ethanol studies (Hendricson et al., 2004), we also observed strong indications of ethanol actions on presynaptic function. *N*-Methyl-D-aspartate mEPSC frequency was enhanced by about 3-fold following ethanol washout from chronic exposure in comparison with that observed following acute exposure (about 70% increase in mEPSC frequency vs 25%, respectively). In comparison, mEPSC amplitude increased to a similar extent over a much larger range (about a 160% increase in mEPSC amplitude vs 42% following ethanol washout). In support of these mEPSC frequency data, an additional analysis of Ca^{2+}-dependent mixed AMPA EPSCs revealed that ethanol modulated paired-pulse facilitation as well, thereby supporting the conclusion that chronic ethanol exposure induces neuroadaptive changes at glutamatergic synapses involving both presynaptic and postsynaptic aspects.

Analysis of spontaneous AMPA-mediated mEPSCs, recorded in the presence of calcium and tetrodotoxin to block action potentials, revealed no changes in the frequency (or amplitude) of events following withdrawal from chronic exposure. These findings are consistent with our previous recordings of AMPA mEPSCs, whereby no direct effects of ethanol were noted on release or AMPA receptor function (Hendricson et al., 2004; Maldve et al., 2004). One common denominator here appears to be the presence of terminal excitability. Release events evoked in association with action potentials appear to be particularly ethanol sensitive, whereas basal AP-independent release events are not. Indeed, a good example of this is seen in the comparison of ethanol-insensitive basal AMPA mEPSC frequency, as we reported previously (Hendricson et al., 2003), with the ethanol-sensitive enhanced AMPA mEPSC frequency, which we also reported (Maldve et al., 2004). These recordings were performed under virtually identical conditions at the same synapse by different researchers in the laboratory and the only difference was that ethanol-sensitive AMPA mEPSC frequency was observed in the presence of depolarizing conditions using slightly elevated K^+.

Obviously, there are aspects to this range of ethanol sensitivities between basal and evoked events that we do not yet fully understand. For instance, in plasticity studies we have reliably seen that ethanol does not significantly modulate evoked synaptic transmission recorded using extracellular field potentials (Hendricson et al., 2002; Zhang et al., 2005b). These events are elicited at very similar stimulation intensities as other studies noted above on Ca^{2+}- supported conventional AMPA EPSCs or Sr^{2+}-supported NMDA mEPSCs; therefore, the level of terminal excitability between these different recording conditions is similar and would not appear to be the defining factor dictating ethanol modulation of release. At this point, the data set indicating presynaptic modulation of glutamate release is consistent to the extent that it cannot be discounted, and therefore our understanding of the conditions under which ethanol modulates synaptic transmission via presynaptic actions is still unclear and unresolved. The major question to be resolved concerns identifying the

factors whereby ethanol modulates release mediated by terminal excitation while leaving basal release unaffected.

SUMMARY

These combined results suggest that acute and chronic ethanol can either increase or decrease the probability of vesicular release of neurotransmitters from synaptic terminals in many central neurons. As yet it is unclear whether ethanol exerts these effects directly or indirectly via release of neuropeptides or other transmitters acting on presynaptic receptors. However, it is apparent from these combined findings that presynaptic receptors for peptides (CRF, opioids), amino acids (e.g. GABA-B), or monoamines (dopamine at D-1) can regulate the sensitivity of the terminals to ethanol. As pointed out above, these receptors could be involved in the genetic variability in the human response to ethanol. These findings also provide support for the idea that future analyses should attempt to ascertain the role of relevant signal transduction linkages such as G-protein-linked pathways and kinases for these presynaptic receptors. Indeed, the fact that the same concentration of ethanol can increase GABA release while decreasing glutamate release probability, as monitored in the same postsynaptic neuron, suggests that such different transduction or exocytotic mechanisms may be the targets for the ethanol effects on these disparate terminals. The large family of GPCRs, G-proteins, and kinases, which can act downstream in different and even opposing directions, could provide such a diverse substrate for alcohol action. Studies of ethanol effects on these elements should be vigorously pursued in the future.

These considerations also suggest that more research is needed on the exocytotic mechanisms, particularly with regard to alcohol's behavioral actions. Thus, more studies of the effects of ethanol on the vesicular release machinery (including vesicle docking and fusion), such as the synapsins, elements of the SNARE proteins, and relevant voltage-dependent Ca^{2+} channels, will be needed in the future. The use of more cell and molecular biological methods will likely be required for such analyses. Finally, attention to neurons from appropriate brain regions involved in discrete behavioral effects of ethanol is needed, at least initially for a correlative view of the possible different presynaptic mechanisms underlying these behavioral effects. Judging from the considerable evidence now accumulating for presynaptic effects of behaviorally relevant ethanol concentrations, such extensive studies could lead to new treatment strategies for a variety of alcohol-related disorders.

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