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The role of $GABA_A$ receptors in the acute and chronic effects of ethanol: a decade of progress

Sandeep Kumar[1], **Patrizia Porcu**[1], **David F. Werner**[1], **Douglas B. Matthews**[3], **Jaime L. Diaz-Granados**[3], **Rebecca S. Helfand**[3], and **A. Leslie Morrow**[1,2]

Sandeep Kumar: ; Patrizia Porcu: ; David F. Werner: ; Douglas B. Matthews: ; Jaime L. Diaz-Granados: ; Rebecca S. Helfand: ; A. Leslie Morrow: morrow@med.unc.edu

[1] Department of Psychiatry, Bowles Center for Alcohol Studies, University of North Carolina School of Medicine, 3027 Thurston-Bowles Building, CB #7178, Chapel Hill, NC 27599-7178, USA

[2] Department of Pharmacology, Bowles Center for Alcohol Studies, University of North Carolina School of Medicine, 3027 Thurston-Bowles Building, CB #7178, Chapel Hill, NC 27599-7178, USA

[3] Department of Psychology and Neuroscience, Baylor University, Waco, TX, USA

Abstract

The past decade has brought many advances in our understanding of $GABA_A$ receptor-mediated ethanol action in the central nervous system. We now know that specific $GABA_A$ receptor subtypes are sensitive to ethanol at doses attained during social drinking while other subtypes respond to ethanol at doses attained by severe intoxication. Furthermore, ethanol increases GABAergic neurotransmission through indirect effects, including the elevation of endogenous GABAergic neuroactive steroids, presynaptic release of GABA, and dephosphorylation of $GABA_A$ receptors promoting increases in GABA sensitivity. Ethanol's effects on intracellular signaling also influence GABAergic transmission in multiple ways that vary across brain regions and cell types. The effects of chronic ethanol administration are influenced by adaptations in $GABA_A$ receptor function, expression, trafficking, and subcellular localization that contribute to ethanol tolerance, dependence, and withdrawal hyperexcitability. Adolescents exhibit altered sensitivity to ethanol actions, the tendency for higher drinking and longer lasting GABAergic adaptations to chronic ethanol administration. The elucidation of the mechanisms that underlie adaptations to ethanol exposure are leading to a better understanding of the regulation of inhibitory transmission and new targets for therapies to support recovery from ethanol withdrawal and alcoholism.

Keywords

Alcohol; $GABA_A$ receptor; Neuroactive steroids; Protein kinase C

Introduction

The past decade has brought many advances in our understanding of ethanol's acute and chronic actions mediated by gamma-aminobutyric acid type A ($GABA_A$) receptors in the central nervous system (CNS). The field has uncovered the GABAergic mechanisms that underlie many of the behavioral effects of ethanol, including its anxiolytic, anticonvulsant, sedative-hypnotic, cognitive-impairing, and motor incoordinating actions. These mechanisms include direct and indirect effects on $GABA_A$ receptors as well as effects on GABA release and the

Correspondence to: A. Leslie Morrow, morrow@med.unc.edu.

synthesis and availability of endogenous neuroactive steroids. The effects of chronic ethanol exposure have also been more clearly elucidated in rodents, monkeys, and humans, and there is a better understanding of the intracellular mechanisms that mediate adaptations of $GABA_A$ receptors to ethanol. The purpose of this article is to highlight the advances of the past decade and to underscore new insights into the mechanisms of ethanol's actions and adaptations, particularly for their therapeutic relevance in the development of medications to support recovery from ethanol dependence and treatment of alcoholism.

$GABA_A$ receptors are a family of chloride ion channels that predominately mediate rapid inhibitory neurotransmission throughout the CNS. The neurotransmitter GABA binds to these receptors, changing their conformation state and thereby opening the pore to allow chloride ions (Cl^-) to pass down an electrochemical gradient. The flux of chloride ions hyperpolarizes the membrane leading to neuronal inhibition. However, it should be noted that, in developing animals, neurons have a higher intracellular chloride concentration at an early stage leading to an efflux of chloride and excitatory actions of GABA in immature neurons (Ben-Ari 2002).

$GABA_A$ receptors are heteromeric protein complexes consisting of several homologous membrane-spanning glycoprotein subunits. Molecular cloning has revealed multiple $GABA_A$ receptor subunits that can be divided by homology into subunit classes with several members: α(1–6), β(1–3), γ(1–3), δ, ε, θ, π, and ρ(1–3) (Olsen and Sieghart 2008; Sieghart and Sperk 2002). The large number of $GABA_A$ receptor subunits generates the potential for various subunit compositions that may account for variable sensitivity to modulatory drugs such as benzodiazepines, barbiturates, neuroactive steroids, and possibly ethanol and general anesthetics. The $GABA_A$ receptor α1 subunit is the most abundant α subunit in adult brain, highly expressed throughout most brain regions and is a component of ~50% of all $GABA_A$ receptors (Kralic et al. 2002a). The analysis of subunit composition by co-immunoprecipitation studies has demonstrated that 98% of α1 subunit-containing receptors are assembled with a γ or δ subunit (Sieghart and Sperk 2002). In contrast, a significant fraction of α4 subunit-containing receptors are comprised of α4 and β subunits only (Bencsits et al. 1999). $GABA_A$ receptors also associate with various other proteins that anchor the receptor, regulate phosphorylation state and trafficking, and modulate receptor function under various conditions. These protein interactions are not yet well understood but clearly influence $GABA_A$ receptor assembly, expression on the membrane surface, endocytosis, and channel function that ultimately controls the inhibitory tone of the CNS.

$GABA_A$ receptors can exist as either synaptic or extrasynaptic receptors that may facilitate rapid changes in inhibition. Most synaptic $GABA_A$ receptors are composed of two α, two β, and one γ subunit, where the γ subunit is located between an α and β subunit (Sieghart et al. 1999; Tretter et al. 1997) and contribute to synaptically mediated (phasic) inhibition. In contrast, tonic inhibition is due to highly sensitive $GABA_A$ receptors, activated by ambient extracellular GABA or from 'spillover' of GABA from synaptic signaling, thought to be in the range of 100 nM to 1 μM (Santhakumar et al. 2006; Tossman et al. 1986). The subunit composition of $GABA_A$ receptors at synaptic and extrasynaptic sites are thought to be different. Studies have shown that the α1 subunit is usually expressed in synaptic $GABA_A$ receptors while the α4 subunit predominantly occurs in extrasynaptic receptors (Mody 2001; Olsen and Sieghart 2009). $GABA_A$ receptor δ subunits are so far found to be exclusively present in extrasynaptic sites (Farrant and Nusser 2005). The α4 subunit has relatively low expression in the CNS (Wisden et al. 1992) and α4βδ $GABA_A$ receptors are exclusively extrasynaptic (Wei et al. 2003), whereas α4βγ2 may be localized at the synapse, as well as extrasynaptically (Hsu et al. 2003; Liang et al. 2006). Given this distinction, the study of synaptic vs. extrasynaptic $GABA_A$ receptors and the effect of ethanol on these receptors is essential to elucidate the mechanism involved in development of ethanol actions including tolerance and dependence. Additionally, the role of various subunits in $GABA_A$ receptor-mediated actions has been

studied by recombinant expression studies *in vitro* and by genetically modified mouse models and pharmacologic modulators *in vivo*. In this review, we address the role of various subunits in ethanol actions by referring to various subunit-containing receptors. For example, the α1 subunit-containing receptors will be referred to as α1-$GABA_A$ receptors.

Acute effects of ethanol on $GABA_A$ receptors

Behavioral evidence of GABAergic involvement in ethanol effects

Alcohol consumption produces many well-known behavioral effects including anxiolysis, impaired motor coordination, impaired cognitive function, sedation, hypnosis, anticonvulsant, and pro-aggressive action. Strikingly, many of these behavioral effects of ethanol overlap with the effects of $GABA_A$ receptor agonists and can be altered by $GABA_A$ receptor modifiers. $GABA_A$ receptor agonists (benzodiazepines, muscimol) increase ethanol responses whereas inverse agonists and antagonists (Ro15-4513, picrotoxin, bicuculline) decrease ethanol responses (see Grobin et al. 1998 for review).

Genetically modified mouse models such as transgenics and global knockouts have increased our understanding of $GABA_A$ receptor involvement in behavioral ethanol actions (see Boehm et al. 2006, 2004b; Crabbe et al. 2006 for more detailed reviews). For instance, the locomotor-stimulant effects of ethanol appear to be modulated by α1-$GABA_A$ receptors. The α1-$GABA_A$ receptor knockout mice have increased ethanol-induced activity (Blednov et al. 2003b; June et al. 2007; Kralic et al. 2003). Ethanol's sedative-hypnotic effects may also involve interactions with α1-$GABA_A$ receptors since male α1-$GABA_A$ receptor knockout mice have reduced loss of righting reflex duration (Blednov et al. 2003a). However, this effect was not observed in other studies (Kralic et al. 2003), possibly due to variation in genetic background of the strains used to create separate knockout mouse lines. Further, α2-$GABA_A$ receptors are implicated in the sedative-hypnotic effects of ethanol, as shown by reduced ethanol-induced loss of righting reflex in α2- $GABA_A$ receptor knockout animals (Boehm et al. 2004b). Studies using benzodiazepine-insensitive histidine101 to arginine (H101R) knockin mice have also indicated a role for α2-$GABA_A$ receptors in ethanol's sedative-hypnotic effects. The combined effect of subthreshold doses of diazepam and ethanol resulted in a loss of righting reflex in α1-, α3-, and α5-, but not α2- (H101R) $GABA_A$ receptor knockin mice (Tauber et al. 2003). Additionally, β2-$GABA_A$ receptors may also play a role in ethanol's sedative-hypnotic effects, as male β2-$GABA_A$ receptor knockout mice displayed reduced loss of righting reflex duration compared to controls (Blednov et al. 2003a). Surprisingly, given the similarity of benzodiazepine and ethanol effects on anxiety, the α1-, α2-, α4-, α5-, γ2L-, and δ-$GABA_A$ receptor knockout mice did not display changes in ethanol-induced anxiolysis (Boehm et al. 2004b; Chandra et al. 2008; Homanics et al. 1999a; Kralic et al. 2003; Mihalek et al. 2001). Further studies are clearly needed to elucidate whether specific GABAergic receptor subtypes mediate the anxiolytic effects of ethanol.

Several studies suggest that $GABA_A$ receptors may be involved in ethanol preference. α1-$GABA_A$ receptor knockouts consumed less ethanol in a two-bottle choice drinking study, but also showed reduced saccharin preference compared to wildtype mice (Blednov et al. 2003b; June et al. 2007). In studies of ethanol reinforcement, α1-$GABA_A$ receptor knockouts exhibited reduced operant responding for ethanol, although this was also accompanied by a reduced responding for sucrose (June et al. 2007). In contrast, α5- and δ-$GABA_A$ receptor knockouts showed reduced ethanol preference with no changes in saccharin preference (Boehm et al. 2004b; Mihalek et al. 2001; Stephens et al. 2005). Additionally, a recent study utilizing viral vectors (Rewal et al. 2009) demonstrates that reduction of α4-$GABA_A$ receptor subunits in the nucleus accumbens shell by RNA interference decreases ethanol consumption and preference. Further studies in other brain areas as well as studies targeting other subunits will be of great interest. Remarkably, many other ethanol-induced behaviors remain unaltered in mice with

genetically modified $GABA_A$ receptors. For instance, the δ-$GABA_A$ receptors do not appear necessary for the discriminative stimulus effects of ethanol or substitution by barbiturates, benzodiazepines, and neuroactive steroids in knockout mice (Shannon et al. 2004). One caveat to these studies is that observed effects or lack of effects may be due to compensatory changes that are sometimes observed in knockout models and should be taken into consideration. Nonetheless, these studies implicate $GABA_A$ receptor subtypes in some behavioral actions of ethanol.

Pharmacologic dissection of $GABA_A$ receptors in ethanol action has also identified a potential role for $GABA_A$ receptor subtypes in ethanol reinforcement. For instance, α1-$GABA_A$ receptors in the ventral pallidum are involved in the reinforcing properties of ethanol (Harvey et al. 2002). Systemic administration or infusion into the ventral pallidum of an α1 selective mixed benzodiazepine agonist–antagonist, 3-propoxy-β-carboline hydrochloride, reduces ethanol-maintained responding in ethanol-preferring rats. α1-$GABA_A$ receptors have also been implicated in ethanol-related heightened aggressive behavior (de Almeida et al. 2004). Administration of the benzodiazepine antagonist flumazenil or the preferential α1 antagonist, β-carboline-3-carboxylate-t-butyl ester, decreases ethanol-related aggression in mice trained to self-administer ethanol. The α5-$GABA_A$ receptors appear to play a role in the rewarding, motor-impairing, and sedative effects of ethanol in rats, since the selective inverse-agonist RY023 impacts these actions (Cook et al. 2005). Furthermore, interactions between ethanol and benzodiazepines increase spatial memory deficits in animals (Takiguchi et al. 2006) and humans (Simpson and Rush 2002) implicating γ2-$GABA_A$ receptors in spatial memory impairment. The development of more subtype specific/selective compounds will aid in further elucidating the role of specific $GABA_A$ receptor subtypes in ethanol action. Newer, more selective compounds such as L-838,417 (which has selectivity for α2-/α3-/α5-$GABA_A$ receptors) will be particularly useful in identifying the role of specific receptor subtypes (see Atack 2003 for review).

The discriminative stimulus paradigm has been used as an *in vivo* assay of receptor-mediated activity and can be a useful tool to define the neurotransmitter systems that underlie the behavioral effects of a given dose and class of drugs (Holtzman 1990). Positive modulators of $GABA_A$ receptors, like benzodiazepines, barbiturates, and certain neuroactive steroids, all produce ethanol-like discriminative stimulus effects in water vs. ethanol discrimination in pigeons, mice, rats, gerbils, and cynomolgus monkeys (Grant 1999; Grant et al. 1996, 2008a; Shelton and Grant 2002). Recent studies have aimed to understand the contribution of different $GABA_A$ receptor subtypes to the discriminative stimulus properties of ethanol. Studies in non-human primates show that ethanol and zolpidem share similar discriminative stimulus effects suggesting that ethanol's sedative-hypnotic actions may involve α1-$GABA_A$ receptors (Helms et al. 2008). However, zolpidem antagonism by Ro15-4513 may involve additional zolpidem-sensitive α2-/α3-/α5-$GABA_A$ receptor subtypes (Helms et al. 2008). In contrast, Platt and collaborators (Platt et al. 2005) reported a prominent role for the α5- but not the α1-$GABA_A$ receptors in the discriminative stimulus effects of ethanol and the ethanol-like effects of benzodiazepines in squirrel monkeys. However, the ethanol-like discriminative stimulus effects are complex. For instance, the discriminative stimulus effects of low doses of ethanol (1.0 g/kg) are predominantly mediated by $GABA_A$ receptor positive modulation, while higher doses (2.0 g/kg) require other systems such as *N*-methyl-D-aspartate (NMDA) receptors (Grant 1999). Indeed, ethanol's discriminative stimulus effects are mediated by interactions of $GABA_A$, NMDA, and metabotropic glutamate receptor subtype 5 (mGluR5), located in specific limbic brain regions such as nucleus accumbens core and amygdala (Besheer et al. 2003; Besheer and Hodge 2005; Hodge and Cox 1998).

Direct effects on $GABA_A$ receptor subtypes

Low dose effects of ethanol (≤30 mM) on tonic inhibition—While much work has shown that ethanol enhances $GABA_A$ receptor potentiation, the exact mechanism of ethanol actions on these receptors remains unclear. Recent work has addressed the contribution of specific $GABA_A$ receptor subtypes in distinct cellular localizations at ethanol concentrations typically found in social settings (3–30 mM). Several groups have shown that ethanol enhances the inhibition of α4/6βδ-$GABA_A$ receptors (found extra-synaptically), whereas receptors containing a γ2 subunit (found synaptically) are significantly less sensitive to the ethanol enhancement of GABAergic responses (Sundstrom-Poromaa et al. 2002; Wallner et al. 2003; Wei et al. 2004). Typically, δ-$GABA_A$ receptors are found extrasynaptically and are commonly associated with α4/α6 subunits (Jones et al. 1997; Sur et al. 1999; Wei et al. 2003). Additionally, these receptors have a greater affinity for GABA (Brown et al. 2002; Wallner et al. 2003) and knockout models display substantially reduced/absent tonic inhibition (Brickley et al. 2001; Chandra et al. 2006; Stell et al. 2003). Importantly, δ-$GABA_A$ receptor knockout mice also have reduced/ablated enhancement of tonic current by ethanol (Glykys et al. 2007). Therefore, it is likely that ethanol actions on α4/6βδ-$GABA_A$ receptors involve the modulation of tonic inhibition *in vivo*.

In parallel, the ‘ethanol antagonist’ Ro15-4513 has been shown to bind with high affinity to δ-$GABA_A$ receptors that are activated by low concentrations of ethanol (Hanchar et al. 2006). Ethanol-induced potentiation of α4βδ-$GABA_A$ receptors by low ethanol concentrations is blocked by Ro15-4513 (Wallner et al. 2006). Changes in tonic inhibition may alter ethanol-related behavioral responses. Hanchar et al. (2005) noted that a naturally occurring mutation found in outbred Sprague-Dawley rats (arginine100 to glutamine, R100Q; also found in Sardinian non-preferring rats and ethanol non-tolerant rats) substantially increases the sensitivity of α6β3δ-$GABA_A$ receptors to ethanol. Importantly, rats with the naturally occurring mutation exhibit greater enhancement of tonic inhibition in cerebellar granule cells as well as greater motor impairment due to ethanol. Moreover, it now appears that tonic current is not solely dependent on the presence of α4δ- or α6δ-$GABA_A$ receptors. α1δ-$GABA_A$ receptors also mediate ethanol-induced increases in tonic inhibition in hippocampal interneurons in α4-$GABA_A$ receptor knockout mice (Glykys et al. 2007). Further studies of ethanol actions on these receptors are needed.

High dose effects of ethanol (>30 mM) on tonic inhibition—While the previously described studies have indicated a major role of ethanol potentiation of GABA inhibition at α4/6βδ extrasynaptic receptors, these observations are controversial. In a recent study by Borghese et al. (2006a), rat and human α4β3δ-$GABA_A$ subunits expressed in oocytes, α4β3δ subunits stably transfected in fibroblasts, and slice recordings conducted in dentate granule cells all failed to exhibit enhanced $GABA_A$ receptor potentiation by low doses of ethanol. Further, effects of lower doses of ethanol (30 mM or less) are not observed in cerebellar granule cells (Casagrande et al. 2007; Yamashita et al. 2006) or ventrobasal thalamic neurons (Jia et al. 2008a) and dentate gyrus granule cells that express α4β2δ receptors (Liang et al. 2008). However, the latter study did observe some detectable effects at 10 mM ethanol. One possibility may be the orientation of receptor subunits within the $GABA_A$ receptor pentamer. However, Kaur et al. (2009) have shown that 30 mM ethanol did not display potentiation in concatenated α1β3δ receptors. In agreement with the lack of a low dose effect, autoradiographic experiments also suggest that Ro15-4513 binding is not altered by low concentrations of ethanol (Korpi et al. 2007). Additionally, the naturally occurring R100Q mutation in α6-$GABA_A$ receptors does not increase ethanol sensitivity at extrasynaptic receptors in outbred rats or in ethanol non-tolerant rats as described above (Botta et al. 2007a; Valenzuela et al. 2005). In agreement with the lack of a low dose effect, α4- and δ-$GABA_A$ receptor knockout mice do not show any major changes in acute ethanol-related behaviors (Chandra et al. 2008; Mihalek et al. 2001).

Nonetheless, electrophysiologic studies utilizing α4-$GABA_A$ receptor subunit knockout mice did not show ethanol-enhanced tonic inhibition compared to controls at 50 mM (Jia et al. 2008a; Liang et al. 2008). Many of these studies assessing low dose ethanol effects on extrasynaptic receptors and tonic inhibition are summarized in Table 1.

The discrepancies in ethanol actions observed across various laboratories may indicate very specific requirements for low dose effects of ethanol on extrasynaptic $GABA_A$ receptors. It is also possible that presynaptic ethanol actions may play a role. Recent studies suggest that increased firing onto cerebellar granule cells increases extrasynaptically available GABA (Botta et al. 2007b; Carta et al. 2004; Valenzuela et al. 2005) that may contribute to changes in tonic inhibition. Other mechanisms may include increases in endogenous modulators that are particularly potent at extrasynaptic $GABA_A$ receptors. Ethanol increases GABAergic neuroactive steroids in slice preparations (see 'Ethanol-induced elevation of neuroactive steroids'). Ethanol also increases taurine (De Witte et al. 1994), which has been shown to enhance tonic inhibition at low concentrations (Jia et al. 2008b). The possibility exists that other unknown chemicals are also released by ethanol. Alternatively, it is possible that the intracellular milieu of different preparations, including second messengers, kinases and phosphatases, may contribute to ethanol actions. Indeed, recent work by Choi et al. (2008) using knockout mice and stably transfected cells has shown that PKCδ activity may be required for ethanol enhancement of extrasynaptic $GABA_A$ receptor responses and this requirement may explain the lack of consistent effects in prior studies. Irrespective of the low ethanol dose discrepancy, it is clear that ethanol does enhance tonic inhibition, and that low dose actions of ethanol may have multiple mechanisms. Further studies are needed to clarify these discrepancies.

High dose effects of ethanol (>30 mM) on phasic inhibition—$GABA_A$ receptor subtypes associated with phasic inhibition are modulated by higher doses of ethanol. Using chimeric receptors, Mihic et al. (1997) identified a 45-amino-acid region spanning the second and third transmembrane domains required for the direct enhancement of glycine receptors by ethanol and volatile anesthetics. Moreover, the mutation of two residues (serine270 and alanine291) produces receptors insensitive to ethanol-induced potentiation. Interestingly, the volume of this putative ethanol 'binding pocket' dictates receptor sensitivity to ethanol (Wick et al. 1998; Ye et al. 1998). $GABA_A$ receptors possessing the serine270 mutation have altered receptor gating and are hypersensitive to GABA (Ueno et al. 2000). Moreover, knockin mice with the serine270 to histidine (S270H) mutation show marked abnormal behaviors, premature mortality, and prolonged miniature inhibitory post-synaptic currents (mIPSCs) (Homanics et al. 2005). Nonetheless, the mutation of leucine277 to alanine (L277A) in combination with S270H restored GABA sensitivity to near normal affinity while retaining ethanol and volatile anesthetic insensitivity *in vitro* (Borghese et al. 2006b). Importantly, gene knockin mice with both mutations show decreased sensitivity to ethanol potentiation (80 mM) and recover more quickly from ethanol's motor ataxic effects, but do not differ on many other ethanol-related behaviors (Werner et al. 2006). These studies implicate a molecular site of action on $GABA_A$ receptors found synaptically that contributes to high dose ethanol action.

A membrane-spanning region of the β3-$GABA_A$ receptor subunit is also implicated in $GABA_A$ receptor responses to higher amounts of ethanol. Work by Wallner et al. (2006) has indicated that while Ro15-4513 blocked ethanol-induced potentiation at low doses, higher concentrations of ethanol still enhanced $GABA_A$ receptor function in the presence of Ro15-4513. Mutations in equivalent positions in β1-$GABA_A$ receptors also modulate ethanol responses like intravenous anesthetic $GABA_A$ receptor modulators etomidate and propofol in β3-$GABA_A$ receptors (Jurd et al. 2003; Mihic et al. 1997). This evidence supports the notion that ethanol and Ro15-4513 may bind to the same site on $GABA_A$ receptors at low ethanol

concentrations whereas a second ethanol-binding site involving α and β subunits may be responsible for ethanol responses at higher concentrations.

Overall, a considerable amount of evidence continues to accumulate that suggests $GABA_A$ receptors may directly modulate some ethanol effects. More so, it is becoming apparent that different $GABA_A$ receptor subtypes not only respond to different ethanol concentrations but their cellular localization may contribute to various ethanol-related responses.

Indirect ethanol effects on $GABA_A$ receptor subtypes

Ethanol-mediated post-translational modification of $GABA_A$ receptors—Protein phosphorylation regulates $GABA_A$ receptor function under numerous physiologic conditions (Brandon et al. 2000; Kellenberger et al. 1992; Krishek et al. 1994; Kumar et al. 2005; Leidenheimer et al. 1992; Poisbeau et al. 1999), including alcoholism and epilepsy (Kumar et al. 2006; Niimura et al. 2005; Rakhade et al. 2008). Several protein kinases, including PKC and PKA, have consensus sites on $GABA_A$ receptor subunits and can phosphorylate these subunits (Brandon et al. 2000; McDonald and Moss 1997). Phosphorylation of $GABA_A$ receptor subunits can modify GABA binding to its receptor (Oh et al. 1999), channel conductance, and possibly internalization (Moss and Smart 2001). Additionally, studies using genetically modified mice clearly demonstrate that the pharmacological and behavioral effects of ethanol are partially mediated by PKC activity (Choi et al. 2008; Harris et al. 1995; Hodge et al. 1999; Proctor et al. 2003).

PKC phosphorylation: A large volume of recent work has focused on the role of PKC as a regulator of $GABA_A$ receptor function following ethanol exposure. Various PKC isoforms associate with $GABA_A$ receptors and therefore may alter receptor subunit phosphorylation and function. PKCβ associates with $GABA_A$ receptor β subunit and alters $GABA_A$ receptor phosphorylation and function (Connolly et al. 1999). PKCγ co-immunoprecipitates with α1- and α4-$GABA_A$ receptors in the cerebral cortex (Kumar et al. 2002) and PKCε co-localizes with various $GABA_A$ receptor subunits across the brain (Olive and Hodge 2000). Similarly, PKCδ alters the function of extrasynaptic $GABA_A$ receptors (Choi et al. 2008). Despite the existence of several isoforms of PKC, genetic deletion of PKCγ, PKCε, and PKCδ has clearly demonstrated that these isoforms are major players in ethanol-mediated modulation of $GABA_A$ receptor function both *in vitro* and *in vivo*. For example, PKCγ knockout mice show reduced sensitivity to the anxiolytic effects of both intoxicating and sedative doses of ethanol (Bowers et al. 2001), while PKCδ knockout mice are insensitive to the ataxic effects of ethanol (Choi et al. 2008) and PKCε knockout mice show increased ethanol sensitivity (Hodge et al. 1999). Furthermore, mice lacking the gene for PKCγ show a significant reduction in ethanol potentiation of GABA responses compared to responses in wildtype mice (Harris et al. 1995; Proctor et al. 2003). In contrast, ethanol and flunitrazepam potentiation of muscimol-stimulated chloride (Cl^-) uptake is greater in microsacs from PKCε knockout mice compared to wildtype controls (Hodge et al. 1999). Therefore, it appears that the ε and γ isoforms of PKC have opposing influences on ethanol enhancement of $GABA_A$ receptor function. PKCδ plays a critical role in ethanol effects on extrasynaptic $GABA_A$ receptors. PKCδ knockout mice display a reduction in ethanol-induced ataxia as well as ethanol-mediated tonic current in thalamic and dentate granule cell neurons (Choi et al. 2008). Taken together, it is likely that various PKC isoforms mediate phosphorylation of $GABA_A$ receptors and/or associated proteins and modulate $GABA_A$ receptor function following ethanol exposure.

Acute systemic ethanol administration directly alters PKC activity and expression in the brain (Kumar et al. 2006). However, ethanol administration to rats (2 g/kg) differentially alters PKCβ, γ, and ε expression and translocation to the P2 fraction of rat cerebral cortex (Kumar et al. 2006). PKCβ and PKCε expression in subcellular fractions exhibited responses to ethanol

that likely represent increased synthesis and translocation, since increased levels of these kinases in the P2 fraction would require translocation of the enzyme. On the other hand, PKCγ expression and translocation to the P2 fraction of cortex are reduced by ethanol. These effects were associated with alterations in phosphorylation of both $GABA_A$ and NMDA receptors and/or associated proteins. Hence, ethanol differentially alters PKC expression in an isoform-specific manner and these effects are likely to contribute to GABAergic (and glutamatergic) actions of ethanol.

Phosphorylation of various $GABA_A$ receptor subunits can alter receptor function without altering receptor expression. We have shown that acute ethanol administration (2 g/kg) decreased serine phosphorylation of $GABA_A$ receptor β subunits without altering the expression of these subunits in the P2 fraction of rat cerebral cortex (Kumar et al. 2006). This suggests that ethanol may alter the phosphorylation state of receptors to alter $GABA_A$ receptor function and these effects may contribute to ethanol actions. This is consistent with the observation of increased $GABA_A$ receptor function following ethanol exposure. Likewise, reduced phosphorylation of the $GABA_A$ receptor γ2 subunit enhances the action of ethanol (Qi et al. 2007). PKCε phosphorylates the $GABA_A$ receptor γ2 subunit at serine327 *in vitro* and γ2 serine327 phosphorylation is reduced in mice lacking PKCε. In addition, exposure to ethanol (4 g/kg) for 1 h increases γ2 serine327 phosphorylation in the cerebellum of wildtype mice, but not PKCε knockout mice and this leads to reduced $GABA_A$ receptor function in cerebellar microsacs (Qi et al. 2007). Finally, phosphorylation at serine327 on $GABA_A$ receptor γ2 subunits is required for PKCε-mediated modulation of $GABA_A$ receptor function (Qi et al. 2007). Further studies investigating post-translational modification of these receptors at PKC phosphorylation sites by specific PKC isoforms will lead to a better understanding of PKC-mediated effects of ethanol on $GABA_A$ receptors.

PKA phosphorylation: Accumulating evidence indicates that the cyclic adenosine monophosphate (cAMP)-dependent kinase PKA is involved in the neurobiological responses to ethanol. PKA is a tetramer composed of a regulatory homodimer and two catalytic subunits. PKA activation occurs when cAMP binds to the regulatory subunits of the PKA complex, liberating the catalytically active subunits, which diffuse throughout the cell and phosphorylate nearby proteins. Ethanol stimulates cAMP signaling through activation of adenosine A2a receptors. Since A2a receptors are coupled to stimulatory G proteins, this increases levels of intracellular cAMP followed by stimulation of PKA (see Diamond and Gordon 1994 for review). Recently, we have found that acute ethanol exposure increases the expression of PKA regulatory RIIα and RIIβ subunits in the membrane fraction of rat cerebral cortex and also alters $GABA_A$ receptor expression (Kumar et al. 2008). Therefore, it appears that ethanol alters PKA expression in a subunit specific manner and may affect $GABA_A$ receptor expression.

Recent pharmacological and genetic studies have provided additional evidence for the importance of the cAMP/PKA system in regulating the neurobiological responses to ethanol. PKA inhibition (using micro-infusions of the PKA inhibitor Rp diastereomer of adenosine 3′, 5′-cyclic mono-phosphorothioate, Rp-cAMPS) in the central amygdala prevents the development of anxiety in rats during ethanol withdrawal (Pandey et al. 2003). Other studies have shown that the PKA signaling pathway plays an important role in the modulation of several ethanol-induced behavioral actions such as loss of righting reflex (Maas et al. 2005; Thiele et al. 2000). For example, PKA regulatory RIIβ subunit mutant mice, which exhibit lower total cAMP-stimulated PKA activity, are less sensitive to the sedative-hypnotic effects of ethanol as assessed by the loss of righting reflex test (Lai et al. 2007). Furthermore, *in vivo* studies show that mutant *Drosophila* lacking production of the RII subunit of PKA are resistant to the intoxicating effects of ethanol (Park et al. 2000). In addition, PKA has been shown to alter $GABA_A$ receptor function. For example, PKA activation causes a decrease in GABA-activated currents in cultured cerebellar granule cells, hippocampal pyramidal cells,

and spinal cord neurons (Moss et al. 1992; Poisbeau et al. 1999; Porter et al. 1990; Robello et al. 1993). Taken together, it is clear that PKA also plays a role in ethanol-mediated behavior and may influence $GABA_A$ receptor function.

Phosphorylation by fyn and tyrosine kinase: Recent studies have shown that *fyn* kinase is involved in ethanol-induced changes in $GABA_A$ receptor function. For example, over-expression of *fyn* kinase reduces sedative-hypnotic sensitivity to ethanol (Boehm et al. 2004a). Furthermore, *fyn* kinase knockout mice are less sensitive to the sedative-hypnotic effects of etomidate (an intravenous anesthetic, selective for β2- and/or β3-$GABA_A$ receptors; see Jurd et al. 2003) suggesting that *fyn* kinase alters $GABA_A$ receptor function by acting on $GABA_A$ receptor β2 and β3 subunits (Boehm et al. 2004c). Similarly, *in vitro* and *in vivo* studies have implicated tyrosine kinase in ethanol-mediated alterations of $GABA_A$ receptor function. Ethanol exposure increases tyrosine kinase phosphorylation of the $GABA_A$ receptor subunits α1, β2, and γ2 in mouse cultured cortical neurons (Marutha Ravindran and Ticku 2006) and rat cerebral cortex (Marutha Ravindran et al. 2007). In addition, tyrosine kinase phosphorylation of $GABA_A$ receptor γ2 subunit regulates $GABA_A$ receptor surface expression and function (Kittler et al. 2008). Hence, it appears that multiple kinases are involved in ethanol-mediated alteration in $GABA_A$ receptor function. Therefore, studies of the interactions of $GABA_A$ receptors with these kinases will lead to a better understanding of ethanol actions on $GABA_A$ receptors.

Dephosphorylation: The phosphorylation state of a given protein is governed by the balance between protein kinases that transfer phosphate from adenosine triphosphate (ATP) to the protein (phosphorylation) and protein phosphatase that catalyzes the reverse reaction (dephosphorylation). It is now widely acknowledged that the regulation of protein phosphorylation requires coordinated control of both kinases and phosphatases. The effect of ethanol exposure on protein phosphatase expression and activity is still not known. However, phosphatase inhibitors alter the function of $GABA_A$ receptors by reducing $GABA_A$ receptor rundown (Huang and Dillon 1998). In addition, blockade of phosphatase activity during preparation of cerebral cortical synaptoneurosomes decreases muscimol-induced Cl^- uptake (Kumar et al. 2005). Hence, the balance between phosphatase and kinase activity is important for $GABA_A$ receptor function. Further studies on the effects of ethanol on protein phosphatase activity are warranted.

Ethanol-induced elevation of neuroactive steroids—Neuroactive steroids are endogenous neuromodulators, synthesized *de novo* in the brain as well as adrenal glands, ovaries, and testes (see Biggio and Purdy 2001 for review). Among these compounds, the 3α, 5α- and 3α,5β-reduced metabolites of progesterone (Majewska et al. 1986; Morrow et al. 1987), deoxycorticosterone (Majewska et al. 1986; Morrow et al. 1987), dihydroepiandrosterone (DHEA) (Frye et al. 1996; Kaminski et al. 2005; Park-Chung et al. 1999), and testosterone (Kaminski et al. 2005, 2006) enhance GABAergic neurotransmission. Their systemic administration induces anxiolytic, anticonvulsant, sedative-hypnotic, and cognitive-impairing effects, similar to other $GABA_A$ receptor positive modulators and ethanol (see Morrow et al. 2006 for review).

Systemic administration of moderate doses of ethanol (1–2.5 g/kg) increases brain and plasma levels of (3α,5α)-3-hydroxypregnan-20-one (3α,5α-THP), (3α,5α)-3,21-dihydroxypregnan-20-one (3α,5α-THDOC), and their precursors in rodents (Barbaccia et al. 1999; Gabriel et al. 2004; Khisti et al. 2005; Korneyev et al. 1993; Morrow et al. 1998; 1999; O'Dell et al. 2004; Serra et al. 2003; VanDoren et al. 2000). The ethanol-induced increase in neuroactive steroids is mediated by the hypothalamic–pituitary–adrenal (HPA) axis, since it is no longer observed immediately following adrenalectomy/gonadectomy in rats (Khisti et al. 2003; O'Dell et al. 2004; Porcu et al. 2004). However, ethanol can increase neuroactive steroids

in hippocampal slices from both intact (Sanna et al. 2004) and adrenalectomized/gonadectomized rats (Follesa et al. 2006) and this finding is associated with an enhancement of GABAergic inhibition that can be blocked by the neuroactive steroid biosynthesis inhibitor finasteride (Sanna et al. 2004).

Ethanol-induced elevations in neuroactive steroids reach physiologically relevant concentrations that are capable of enhancing GABAergic transmission. A large body of evidence from multiple laboratories suggests that ethanol-induced elevations of GABAergic neuroactive steroids contribute to several behavioral effects of ethanol in rodents. Neuroactive steroids have been shown to modulate ethanol's anticonvulsant effects (VanDoren et al. 2000), sedation (Khisti et al. 2003), impairment of spatial memory (Matthews et al. 2002; Morrow et al. 2001), anxiolytic-like (Hirani et al. 2005), antidepressant-like (Hirani et al. 2002), and pro-aggressive (Fish et al. 2001) actions. Most of these behavioral responses are prevented by pretreatment with finasteride and/or by prior adrenalectomy (Hirani et al. 2002; 2005; VanDoren et al. 2000). The sedative-hypnotic effect of ethanol is partially blocked by adrenalectomy (Khisti et al. 2003). Importantly, administration of 5α-dihydroprogesterone, the immediate precursor of 3α,5α-THP, to adrenalectomized rats restores effects of ethanol, showing that brain synthesis of neuroactive steroids can modulate effects of ethanol (Khisti et al. 2003). However, neuroactive steroids do not appear to influence the motor incoordinating effects of ethanol, since neither finasteride administration nor adrenalectomy diminish these actions (Khisti et al. 2004). Taken together, these studies suggest that elevations in neuroactive steroids influence many of the GABAergic effects of ethanol *in vivo* and contribute to sensitivity to the behavioral effects of ethanol.

Acute application of ethanol (50 and 100 mM) to freshly isolated rat hippocampal slices increases tissue levels of 3α,5α-THP (Sanna et al. 2004). In addition, application of progesterone (the 3α,5α-THP precursor), CB34 (a peripheral benzodiazepine receptor agonist), or γ-hydroxybutyric acid, all of which promote steroidogenesis, also increases tissue levels of 3α,5α-THP. The increase in 3α,5α-THP induced by ethanol, progesterone, CB34, or γ-hydroxybutyric acid is prevented by pretreatment with the neuroactive steroid inhibitor finasteride. Furthermore, this increase in 3α,5α-THP levels is capable of potentiating $GABA_A$ receptor function, measured by recordings of spontaneous mIPSCs. Ethanol increases mIPSC amplitude and frequency in a time-and concentration-dependent manner: the effect is observed within the first 3 min, then it is reduced at 10 min and reappears after 30 min. In contrast, the decay time is increased 30 min after ethanol exposure. However, pretreatment with finasteride inhibits the increase in mIPSC amplitude and decay time constant at 30 min, but has no effect on mIPSC frequency, suggesting that the presynaptic action of ethanol is not mediated by an increase in neuroactive steroid content (Sanna et al. 2004).

Presynaptic GABA release—Many GABAergic effects of ethanol likely result from the ability of ethanol to enhance presynaptic GABA release in spinal cord (Ziskind-Conhaim et al. 2003), amygdala (Roberto et al. 2003; 2004), hippocampus (Ariwodola and Weiner 2004), and cerebellum (Criswell and Breese 2005) An ethanol-induced increase in GABA release would be expected to modulate all subtypes of $GABA_A$ receptors and could clearly contribute to the behavioral effects of ethanol mediated by $GABA_A$ receptors in brain. This effect of ethanol exhibits regional and neuronal specificity (Criswell et al. 2008), suggesting that it may contribute to heterogeneity of ethanol interactions. Moreover, chronic exposure to GABA alters $GABA_A$ receptor α1 subunit expression (Calkin and Barnes 1994; Montpied et al. 1991a), internalization (Tehrani and Barnes 1997), and decreases $GABA_A$ receptor-mediated inhibition (Roca et al. 1990), similar to the effects of ethanol on $GABA_A$ receptor α1 subunit expression (Kumar et al. 2003). Thus, ethanol-induced increases in GABA release likely contribute to ethanol-induced changes in $GABA_A$ receptor surface expression that appear to be involved in ethanol tolerance, dependence, and withdrawal.

Chronic administration of ethanol

Behavioral effects

Chronic ethanol exposure results in CNS hyperexcitability during and following withdrawal from ethanol exposure. These effects include a heightened risk for seizures, increased anxiety, hyperalgesia, and disruptions in sleep states. While the exact molecular mechanisms attributable to each of these effects are not fully understood, an overwhelming amount of evidence points to $GABA_A$ receptor involvement (see Grobin et al. 1998 for review).

Genetically modified mouse models have contributed to our understanding of the relationship between $GABA_A$ receptor subtype expression and CNS hyperexcitability. Recent work in the ethanol-insensitive (S270H, L277A) α1-$GABA_A$ receptor knockin animals indicates that α1 subunits play a significant role in CNS excitability. Male knockin mice display enhanced ethanol withdrawal-related handling-induced convulsions (HICs) (Werner et al. 2009). Additionally, a number of mortalities were observed in the knockin mice that were attributable to seizure severity, whereas mortalities were not observed following ethanol exposure in control mice. However, knockin mice exhibited spontaneous seizures that may have kindled ethanol-withdrawal seizure susceptibility. Interestingly, α1-$GABA_A$ receptor knockout mice had increased seizure susceptibility to bicuculline as well as more severe seizures and death (Kralic et al. 2002a). In other studies, α1-$GABA_A$ receptor knockout mice did not differ in withdrawal-related HICs, but these mice consumed less ethanol prior to withdrawal than wildtype control mice, possibly masking a difference between mouse lines (Blednov et al. 2003b). Studies have also assessed the role of β subunits in ethanol withdrawal seizure severity. β2-$GABA_A$ receptor knockout mice had increased HICs after chronic ethanol (Blednov et al. 2003b); however, knockouts had higher ethanol intake than controls. In other studies, β3 asparagine265 to methionine (N265M)-$GABA_A$ receptor knockin mice had enhanced withdrawal following chronic ethanol exposure (Sanchis-Segura et al. 2007). Withdrawal from acute high doses of ethanol has also been used to assess the role of specific $GABA_A$ receptor subtypes. The expression of γ2 subunits, which are involved in phasic inhibition, has also been thought to play a role. Work has shown that allelic variation in *gabrg2*, the gene encoding for the γ2 subunit, was correlated with withdrawal-related behavior (Hood and Buck 2000). However, γ2L-$GABA_A$ receptor knockouts had similar withdrawal-related HICs (Homanics et al. 1999a). In studies of subunits involved in tonic inhibition, δ-$GABA_A$ receptor knockout mice displayed decreased ethanol withdrawal HICs than controls; however, this effect was dependent on background strain of the mice (Mihalek et al. 2001). Interestingly, studies in α6-$GABA_A$ receptor knockout mice (which pairs with δ in the cerebellum) did not differ in ethanol withdrawal-related HICs (Homanics et al. 1997). Finally, withdrawal from acute high doses of ethanol has also been used to assess the role of specific $GABA_A$ receptor subtypes. For instance, γ2-$GABA_A$ receptor transgenic mice did not differ from controls (Wick et al. 2000) in sensitivity to acute withdrawal HICs (Boehm et al. 2004b).

Apart from genetically modified mouse models, other studies have also implicated $GABA_A$ receptors in ethanol dependence. For instance, pentylenetetrazole-kindled animals displayed ethanol withdrawal behavior similar to animals exposed to repeated cycles of ethanol withdrawal (Davidson et al. 1999). Moreover, animals subjected to repeated withdrawals exhibit faster pentylenetetrazole-kindled seizure activity compared to controls and animals experiencing only a single ethanol withdrawal (Ripley et al. 2002). Additionally, while lorazepam could decrease HIC severity in repeated withdrawals, this intervention led to an exacerbated final withdrawal compared to animals that did not receive lorazepam during multiple withdrawals from ethanol (Becker and Veatch 2002). These results hint that this interactive effect between benzodiazepines and ethanol may involve $GABA_A$ receptors. Studies with benzodiazepines have also implicated $GABA_A$ receptors in ethanol withdrawal-related hyper-algesic effects. Diazepam reversed ethanol withdrawal hyperalgesia, but this

effect could not be reversed by flumazenil (Gatch 1999). Studies have also shown that flumazenil reduces seizure severity (Buck et al. 1991b) and reverses the anxiogenic-like effects (Moy et al. 1997) associated with ethanol withdrawal. While known as a benzodiazepine antagonist, flumazenil has also been shown to have some partial agonist activity (see File and Pellow 1986 for review) and the effects of partial agonists are enhanced following chronic ethanol exposure (Liang et al. 2004). These studies, as well as those involving genetically modified mice, suggest that specific $GABA_A$ receptor subtypes and/or regulation of these receptors contribute to ethanol dependence and further implicate $GABA_A$ receptors as potential therapeutic targets for treating alcoholism.

Functional adaptations

Prolonged ethanol consumption and repeated ethanol withdrawals produce many adaptations of $GABA_A$ receptor function (Table 2). For instance, ethanol exposures result in the development of tolerance to many of the GABAergic effects of ethanol including the sedative, motor incoordinating, and acute cognitive-impairing effects of ethanol (Le et al. 1986;Silvers et al. 2003c). Withdrawal from ethanol produces marked increases in CNS excitability that form a criterion for ethanol dependence (Becker et al. 1997;Devaud et al. 1996;McCown and Breese 1990;Moy et al. 2000). Substantial evidence suggests that these behavioral and neural adaptations involve marked alterations in the pharmacological properties of $GABA_A$ receptors (Table 2).

Alterations in $GABA_A$ receptor subunit expression, localization, and trafficking

The development of ethanol dependence is associated with alterations in many of the functional properties of $GABA_A$ receptors throughout the brain which is attributed to altered expression or composition of $GABA_A$ receptors on the cell surface. Chronic ethanol administration differentially alters the expression of distinct $GABA_A$ receptor subunit mRNA and peptide levels in various brain regions (Table 3). The levels of $GABA_A$ receptor α1, α2, and α3 subunit mRNA/peptides are reduced in the cerebral cortex while α4, β1, β2, β3, γ1, and γ2 subunit mRNA/peptide levels are increased in cerebral cortex following chronic ethanol exposure. Since $GABA_A$ receptor α1 subunits are the most abundant subunit in the cerebral cortex, the reduction in the expression of this subunit is likely to have significant functional consequences. $GABA_A$ receptor α4 subunit expression measured by immunohistochemical labeling in brain slices is sparse across most brain regions except the thalamus, dentate gyrus, and striatum (Bencsits et al. 1999;Chandra et al. 2006;Pirker et al. 2000). However, selective modulation of α4 subunit expression in the hippocampus, using antisense oligonucleotides, has been shown to significantly alter the functional properties of $GABA_A$ receptors and modulate steroid withdrawal excitability (Moran et al. 1998;Smith et al. 1998). Therefore, despite the relatively low expression of α4 subunit peptide, modulation of α4 subunit expression clearly influences $GABA_A$ receptor function and GABA-mediated behaviors *in vivo*. Recombinant expression and homologous gene deletion studies have shown that functional properties are regulated by the subunit composition of $GABA_A$ receptors. For example, recombinant $GABA_A$ receptors with α4β2γ2 subunits respond to GABA and benzodiazepine agonists with lower efficacy than α1β2γ2 receptors (Whittemore et al. 1996). Moreover, homologous genetic deletion of α1 subunits dramatically alters the pharmacological properties of $GABA_A$ receptors, reducing the potency and efficacy of GABA and of the benzodiazepine diazepam (Kralic et al. 2002a,b). Hence, alterations in $GABA_A$ receptor subunit expression appear critically important in regulation of $GABA_A$ receptor function following chronic ethanol administration.

The regulation of various $GABA_A$ receptor subunits by ethanol differs across brain regions (Grobin et al. 2000). For example, chronic ethanol consumption for 14 days increases $GABA_A$ receptor α4 subunit peptide expression in the cerebral cortex and the hypothalamus (Devaud et al. 1997), decreases α4 subunit peptide levels in the amygdala and nucleus

accumbens (Papadeas et al. 2001), and does not alter α4 subunit peptide levels in the hippocampus or ventral tegmental area (Matthews et al. 1998; Papadeas et al. 2001). However, repeated ethanol withdrawals or longer ethanol exposure increases α4 subunit peptide expression in the hippocampus (Cagetti et al. 2003; Matthews et al. 1998) and these effects are associated with alterations in the pharmacological responses of $GABA_A$ receptors to benzodiazepine agonists and inverse agonists (Cagetti et al. 2003). Furthermore, in cerebellum, α6 subunit mRNA and peptide levels are increased while α1 subunit peptide and mRNA are decreased following chronic ethanol administration (Mhatre et al. 1993; Morrow et al. 1992). Therefore, it is clear that adaptation in $GABA_A$ receptor subunits expression caused by chronic ethanol administration is not universal across brain regions. The mechanisms that underlie regional differences in the regulation of $GABA_A$ receptors by ethanol are unclear but may involve mechanisms to be described in the following sections.

Alterations in synaptic localization of receptors—Given the important role of synaptic vs. extrasynaptic $GABA_A$ receptors in acute ethanol effects, it is important to understand the role of these receptor populations following chronic ethanol exposure. Cagetti et al. (2003) found changes in synaptic $GABA_A$ receptor function in the hippocampus of rats that had undergone chronic intermittent ethanol (CIE) treatment followed by 2 days of withdrawal. CIE treatment decreases the frequency, rise time, amplitude, and decay time of mIPSCs in pyramidal neurons from CA1, an overall change in $GABA_A$ receptor function that is consistent with increased neuronal excitability. Interestingly, CIE eliminated the effects of diazepam on the mIPSCs and increased mIPSC sensitivity to the α4 subunit-selective benzodiazepine Ro15-4513. These pharmacological changes are consistent with a decreased expression of α1-$GABA_A$ receptors and an increased expression of α4-$GABA_A$ receptors in the synapse. Brief exposure (48–72 h) to the neuroactive steroid 3α,5α-THP may cause a similar effect since there is a decrease in the decay time of mIPSCs recorded in hippocampal CA1 pyramidal cells (Hsu et al. 2003). The decrease in mIPSC decay time was blocked by infusion of α4 subunit antisense oligonucleotides into the hippocampus, suggesting that this change in synaptic $GABA_A$ receptor kinetics is due to an increase in synaptic α4 subunit expression. Additionally, tonic current is also modified after chronic ethanol exposure. Specifically, the effects of various $GABA_A$ receptor modulators on tonic currents are reduced or ablated after CIE treatment (Liang et al. 2004). Indeed, $GABA_A$ receptor ligands with selectivity for α4-$GABA_A$ receptors exerted decreased effects on extrasynaptic currents as well as increased effects on synaptic currents. Electron microscopy revealed an increase in synaptic localization of α4, but not δ subunits on the dentate granule cells of CIE rats (Liang et al. 2006). Taken together, these studies show that translocation of α4-$GABA_A$ receptors from an extrasynaptic to synaptic localization may represent a mechanism of $GABA_A$ receptor adaptation to ethanol exposure.

Endocytosis and trafficking—Several mechanisms for $GABA_A$ receptor adaptation following chronic ethanol exposure have been proposed that include alterations in gene expression, post-translational modification, synaptic localization, intracellular signaling, and neuroactive steroid responses to ethanol. The expression of $GABA_A$ receptors involves a highly regulated process of synthesis, assembly, endocytosis, and recycling or degradation (see Fig. 1). Golgi-derived vesicles provide newly synthesized receptors to the cell surface, whereas clathrin-coated vesicles mediate endocytosis of surface receptors that are ultimately degraded or recycled back to the cell surface (Lodish et al. 1996). Alterations in the expression and composition of various $GABA_A$ receptors could result from selective endocytosis, recycling, and/or trafficking of newly synthesized receptors to the cell surface. Recent studies have shown the importance of $GABA_A$ receptor trafficking on the surface expression of receptors following ethanol exposure (Kumar et al. 2004). Altered $GABA_A$ receptor subunit composition and expression on the cell surface following ethanol exposure is thought to contribute to the development of ethanol dependence. Therefore, investigation of the mechanisms for altered

$GABA_A$ receptor trafficking following ethanol exposure may provide novel targets for drug development.

Chronic ethanol exposure selectively increases the internalization of α1-$GABA_A$ receptors into clathrin-coated vesicles of the cerebral cortex with a corresponding decrease in these receptors in the synaptic fraction (Kumar et al. 2003). In contrast, there is no change in the internalization of α4-$GABA_A$ receptors into clathrin-coated vesicles although there is a significant increase in α4 subunit peptide in the synaptic fraction following chronic ethanol exposure. Hence, the regulation of intracellular trafficking following chronic ethanol exposure appears to alter the subtypes of $GABA_A$ receptors on the cell surface. This may account for alterations in the pharmacological properties of $GABA_A$ receptors that have been observed. Internalization of $GABA_A$ receptors following chronic ethanol administration is mediated by clathrin and the adaptor complex (AP) since the association of adaptin-α and clathrin with α1-$GABA_A$ receptors in the intracellular fraction was increased following chronic ethanol administration (Kumar et al. 2003). $GABA_A$ receptor β2 and/or γ2 subunits are required for recognition of the receptor by the AP-2 that precedes clathrin-dependent endocytosis (Herring et al. 2003; Kittler et al. 2005, 2008). Therefore, it is possible that PKC-mediated phosphorylation of $GABA_A$ receptor subunits, receptor-associated proteins, and/or AP-2 following chronic ethanol administration alters the recognition and endocytosis of $GABA_A$ receptors. Indeed, chronic ethanol consumption results in increased expression of α4-, β2-, and β3-$GABA_A$ receptor subunits in the cerebral cortex and all of these subunits contain consensus phosphorylation sites for PKC (Macdonald 1995; Mohler et al. 1996; Wisden et al. 1991). In contrast, α1-, α2-, and α3-$GABA_A$ receptor subunits are decreased in the cortex and these subunits do not contain consensus phosphorylation sites for PKC. Hence, it is likely that phosphorylation of $GABA_A$ receptor subunits can prevent internalization by blocking AP-2 binding. A recent study has shown that a single dose of ethanol can increase the internalization of $GABA_A$ receptor α4 and δ subunits (Liang et al. 2007). However, this study did not investigate the mechanism of $GABA_A$ receptor internalization. Therefore, investigation of $GABA_A$ receptor endocytosis and trafficking following ethanol exposure is essential for understanding the mechanisms of ethanol adaptations.

Trafficking of $GABA_A$ receptors can be regulated by many protein kinases, including PKC. Chronic ethanol consumption decreases association of PKCγ with α1-$GABA_A$ receptors and decreases expression of the α1 subunit at the cell surface. In contrast, chronic ethanol exposure increases association of PKCγ with α4-$GABA_A$ receptors and increases expression of α4 at the cell surface (Kumar et al. 2002). However, in the hippocampus, the association of PKCγ with $GABA_A$ receptors is not altered and there is no alteration of α1 subunit expression following chronic ethanol exposure (Kumar et al 2004). Therefore, it appears that association of PKCγ with $GABA_A$ receptors and phosphorylation of subunits may influence the trafficking of receptors *in vivo* following chronic ethanol administration. The increased association of PKCγ with α4-$GABA_A$ receptors may phosphorylate $GABA_A$ receptor subunits and prevent recognition of the receptor by AP-2, thus preventing its internalization. Indeed, it has been shown that phosphorylation of $GABA_A$ receptor subunits reduces the binding of receptors with AP-2 and subsequent internalization (Kittler et al. 2005, 2008). In addition, reduced PKC-dependent $GABA_A$ receptor phosphorylation enhances receptor binding to the AP-2 and promotes their endocytosis (Terunuma et al. 2008). Taken together, it appears that PKC-mediated phosphorylation plays an important role in $GABA_A$ receptor cell surface expression following chronic ethanol exposure.

$GABA_A$ receptor subunits contain phosphorylation sites for many protein kinases including PKC, PKA, and *fyn* kinase. Phosphorylation can alter receptor function directly by changing its conformation and/or indirectly by altering receptor expression. Since PKA and *fyn* kinase alter $GABA_A$ receptor function, it is possible that these protein kinases are also involved in

trafficking of $GABA_A$ receptors. For example, chronic activation of PKA in cerebellar granule cells increases cell surface expression of $GABA_A$ receptor α1 subunit (Ives et al. 2002). In addition, ethanol exposure alters expression and translocation of PKA (Diamond and Gordon 1994; Newton and Messing 2006; Pandey 1998). Hence, it is likely that PKA is also involved in trafficking of $GABA_A$ receptors following ethanol exposure. However, to date, the role of these protein kinases has not yet been studied in trafficking of $GABA_A$ receptors, especially following ethanol exposure. Future studies will determine the specific role of various protein kinases in $GABA_A$ receptor trafficking and resolve the precise mechanisms that account for alterations in $GABA_A$ receptor function and expression following chronic ethanol administration.

Alterations in ethanol-induced neuroactive steroid responses

Chronic ethanol consumption in rats results in blunted elevation of cerebral cortical 3α,5α-THP (Morrow et al. 2001) and plasma and brain deoxycorticosterone levels following acute ethanol challenge (Khisti et al. 2005) compared to pair-fed control rats. These findings suggest that there is tolerance to ethanol-induced increases in neuroactive steroid levels. The loss of ethanol-induced increases in neuroactive steroids may contribute to ethanol tolerance since the elevations of neuroactive steroids are required for anxiolytic, sedative, anticonvulsant, and cognitive-impairing effects of ethanol. Furthermore, since decreases in brain neuroactive steroid levels are concomitant with decreases in plasma neuroactive steroid levels, it is likely that the observed decreases in 3α,5α-THP and deoxycorticosterone levels are dependent on blunted HPA axis activity. It is well known that chronic stress results in adaptation of the HPA axis leading to decreases in stress-induced changes in the levels of corticosterone in rats (Spencer and McEwen 1990). This blunting of the HPA axis is associated with a reduction in corticotrophin releasing factor and adrenocorticotropin releasing hormone elevations following ethanol challenge (Lee et al. 2001). Furthermore, repeated ethanol withdrawal also leads to blunting of withdrawal-induced elevations in corticosterone levels (Borlikova et al. 2006). Thus, chronic ethanol administration induces adaptations of the HPA axis to promote loss of stress-induced increases in GABAergic neuroactive steroids that contribute to the regulation of homeostasis as well as ethanol sensitivity.

Chronic ethanol administration to rodents and humans produces tolerance to ethanol and cross-tolerance to benzodiazepines and barbiturates. In contrast, ethanol-dependent rats are sensitized to the anticonvulsant effects of 3α,5α-THP and 3α,5α-THDOC (Cagetti et al. 2004; Devaud et al. 1996; 1998). $GABA_A$ receptor sensitivity to 3α,5α-THP and 3α,5α-THDOC is enhanced in the cortex, but sensitivity to alphaxalone is reduced in the hippocampus (Cagetti et al. 2004) of ethanol-dependent rats, likely due to adaptations in $GABA_A$ receptor expression described above. Since ethanol-dependent rats are sensitized to anticonvulsant actions of neuroactive steroids, this class of compounds may be useful during ethanol withdrawal. Indeed, neuroactive steroid therapy may have advantages over benzodiazepine therapy since benzodiazepines exhibit cross-tolerance with ethanol. Further studies are needed to explore this possibility.

Cross-talk with other neurotransmitter systems

As our understanding of cellular signaling pathways has advanced, it has become clear that intracellular communication between receptors is critical for the coordinated function of cells. There are multiple receptors expressed in every cell and their signals pass through a common set of downstream effectors distinguished by multiple isoforms with slightly different specificities and activities. The coupling among these pathways causes interactions among the signals sent by the different classes of receptors. Recent molecular pharmacology studies have demonstrated that ethanol acts on several neurotransmitter systems in the brain such as GABA, NMDA, glycine, 5-hydroxytryptamine (5-HT; serotonin) 3, and nicotinic acetylcholine

receptors as well as L-type Ca^{2+} channels and G protein-coupled receptors. Following the direct actions of ethanol on receptors in the brain, a second wave of indirect effects is initiated that subsequently leads to the typical acute behavioral effects of ethanol, ranging from disinhibition to sedation and hypnosis, with increasing concentrations of ethanol. Therefore, a clear understanding of the interactions between neurotransmitter receptors and their signaling pathways is critical for understanding the mechanisms that underlie ethanol tolerance and dependence.

Chronic ethanol exposure up-regulates NMDA receptor function and down-regulates $GABA_A$ receptor function (see Crews et al. 1996 for review). Since many neurons contain both NMDA and $GABA_A$ receptors and ethanol modulates both NMDA and $GABA_A$ receptors, interactions between these receptors may play an important role in $GABA_A$ receptor adaptation following ethanol administration. For example, administration of dizocilpine (a potent non-competitive antagonist of the NMDA receptor) to rats, like ethanol, increases membrane expression of $GABA_A$ receptor α4 subunits in hippocampus (Matthews et al. 2000b). The effect on $GABA_A$ receptors following NMDA receptor activation is mediated by intracellular Ca^{2+}, since the Ca^{2+} chelator 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid (BAPTA) blocks the down-regulation of $GABA_A$ receptors following NMDA receptor activation (Robello et al. 1997). In addition, the application of glutamate in hippocampal cultures leads to translocation of PKCα and PKCγ to the plasma membrane and cytoplasmic organelles, respectively, while PKCε localization remains unaltered (Buchner et al. 1999; Etoh et al. 1991). Therefore, interactions of NMDA and $GABA_A$ receptors via intracellular signaling pathways may play a vital role in $GABA_A$ receptor adaptation following chronic ethanol administration. In addition, both NMDA and $GABA_A$ receptor activation, as well as ethanol administration, alter brain-derived neurotrophic factor (BDNF) expression in neurons (MacLennan et al. 1995; Obrietan et al. 2002; Tapia-Arancibia et al. 2001; Zafra et al. 1991). BDNF has been shown to alter $GABA_A$ receptor function and expression. For example, addition of BDNF in cortical cell cultures increases internalization of $GABA_A$ receptor α2, β2, β3, and γ2 subunits (Brünig et al. 2001). In contrast, chronic application of BDNF increases expression of $GABA_A$ receptor α6 subunit in cerebellar granular cells that is blocked by concomitant application of ethanol (Ericson et al. 2003). Therefore, it is possible that ethanol-induced altered trafficking and expression of $GABA_A$ receptors are partially mediated by BDNF. Future studies on interactions of BDNF and $GABA_A$ receptor following chronic ethanol exposure are needed to clarify the role of BDNF on ethanol-induced adaptations of $GABA_A$ receptors.

Likewise, serotonin receptors, a primary target of ethanol action, can modulate $GABA_A$ receptor function and expression via PKC-mediated pathways. Activation of 5-HT2 receptors results in inhibition of $GABA_A$ receptor-mediated currents that can be blocked by the Ca^{2+} chelator BAPTA and the receptor for activated C-kinase (RACK) inhibitory peptide (RACK1-rVI) (Feng et al. 2001). In addition, even though 5-HT3 is considered to be an excitatory receptor, it is often expressed on inhibitory GABAergic interneurons, thus activation of 5-HT3 receptors by ethanol may contribute to some of the inhibitory actions of ethanol via increased release of GABA (Lovinger 1999). Furthermore, activation of 5-HT3 receptors is also known to increase the release of dopamine and glutamate (Lovinger 1999). Hence, it is clear that activation of serotonin receptors can alter $GABA_A$ receptor function and expression. In addition, recent studies have concluded that dopamine and β-adrenergic receptors can also modulate $GABA_A$ receptors via PKA-mediated pathways (Flores-Hernandez et al. 2000). Taken together, it is obvious that receptor cross-talk plays a vital role in modulation of $GABA_A$ receptor function and expression following ethanol exposure.

Recent evidence indicates that many other neurotransmitter and cytokines may be involved in ethanol-mediated alteration in $GABA_A$ receptor function and expression. For example, the

mGluR5 antagonist 2-methyl-6-(2-phenyl-ethynyl)pyridine (MPEP) modulates the GABAergic effects of ethanol (Besheer and Hodge 2005). Furthermore, the mGluR5 receptor selective agonist (R,S)-2-chloro-5-hydroxyphenylglycine (CHPG) enhances GABA-gated currents via post-synaptic effects in amacrine cells (Hoffpauir and Gleason 2002). Likewise, cytokines may be involved in ethanol-mediated alterations in $GABA_A$ receptor function and expression. Tumor necrosis factor α (TNF-α) concentrations in the brain are elevated by ethanol administration (Crews et al. 2006; Kiefer et al. 2002) and this may contribute to a decrease in $GABA_A$ receptor α1 subunit expression (Stellwagen et al. 2005) following chronic ethanol exposure. Understanding of the mechanisms of ethanol dependence has been complicated by the multiple actions of ethanol at various neurotransmitters and/or intracellular signaling pathways. Hence, understanding of these interactions between neurotransmitter systems is essential to the development of novel therapeutic targets for alcoholism.

Studies in non-human primates

In the past 10 years, there have been new studies on the effects of ethanol administration to non-human primates that have also investigated effects on $GABA_A$ receptor function and plasticity. Cynomolgus macaques (*Macaca fascicularis*) are a powerful model to study the effects of long-term ethanol exposure as they will freely self-administer intoxicating quantities of ethanol with drinking patterns that mimic human alcoholics (Grant et al. 2008b; Vivian et al. 2001). Long-term ethanol self-administration by cynomolgus macaques alters the pharmacological and functional properties of $GABA_A$ receptors by decreasing GABA potency, but not efficacy, in the basolateral amygdala neurons. These alterations are associated with decreased α2 and α3 subunit mRNA expression and with a trend for a decrease in α1 subunit mRNA expression. No significant effect of gender or significant interaction between gender and ethanol exposure is observed for any of the subunits examined. Furthermore, the mean expression levels for both the α2 and the α3 subunits were significantly correlated with the total amount of ethanol consumed (Floyd et al. 2004). In the same cohort of cynomolgus macaques, Hemby et al. (2006) found significant decreases in mRNA expression for: α2, α4, β1, β3, γ1, γ2, and γ3 in orbitofrontal cortex; β1, β2, γ1, and δ in dorsolateral prefrontal cortex; but no changes in anterior cingulate cortex. In addition, Anderson et al. (2007) showed that long-term ethanol self-administration reduces the sensitivity of amygdala $GABA_A$ receptors to the benzodiazepine flunitrazepam and decreases mRNA expression of β1 and γ2 subunits. Furthermore, there are significant effects of ethanol drinking on the γ2 subunit gene in males but not females; however, there was no correlation between subunit mRNA expression and total ethanol intake, suggesting that this gender adaptation is not related to drinking. Interestingly, $GABA_A$ receptor density, measured by [^{3}H]Ro15–4513 binding, does not differ in the parietal and temporal cortex, cerebellum, or hippocampus after long-term ethanol self-administration in these cynomolgus macaques (Sullivan et al. 2005). Overall, the effects of ethanol in cynomolgus macaques differ from rat models in several aspects and suggest that studies in non-human primates provide unique insights that may have more relevance for human alcoholism.

Alcohol tolerance mechanisms

Although many people consume ethanol, the fact that only a select number of individuals develop alcoholism remains enigmatic. Tolerance is thought to play a major role in the progression towards alcoholism and may therefore contribute to risk for the disease. Tolerance is defined as a reduced response to a constant amount of ethanol, such that a greater amount is needed to obtain the same effect. Tolerance is complex because it can involve multiple behaviors that respond to ethanol exposure in different ways. It can also be segregated as either innate or acquired (i.e., requiring a previous ethanol exposure), with the latter being further broken down into acute, rapid, and chronic tolerance, based on the temporal relationship

between ethanol exposures and their duration (Kalant et al. 1971). Acute tolerance is characterized by adaptations that occur immediately during exposure to ethanol. Rapid tolerance is typically defined as tolerance manifested after a single exposure has been eliminated from the system. Chronic tolerance involves various forms of multiple or continual ethanol exposure paradigms. $GABA_A$ receptors are implicated in tolerance to ethanol's effects. For instance, in humans, sons of alcoholics exhibit decreased sensitivity to diazepam in eye-movement tasks (Cowley et al. 1994), indicative of reduced sensitivity to GABAergic stimulation. In animal studies, a considerable amount of work has been done to assess the role of $GABA_A$ receptors in innate and acquired tolerance to ethanol, as described below.

Innate tolerance

Various studies using $GABA_A$ receptor modulators in inbred, selectively bred, and recombinant inbred strains that differ in ethanol responses have correlated $GABA_A$ receptor action to innate ethanol tolerance. For instance, mice selectively bred for differences in diazepam responses also differed in ethanol responses. Mice that were more resistant to diazepam were less ataxic to ethanol, compared to diazepam-sensitive mice (Gallaher and Gionet 1988). The $GABA_A$ receptor agonist taurine may also be involved in different ethanol-related responses in Sardinian alcohol-preferring (sP) rats versus non-preferring (sNP) rats. Recent microdialysate studies indicate that sP rats had reduced ethanol-related taurine release compared to sNP rats (Quertemont et al. 2000). Notably, alcohol-preferring rats were less sensitive to the sedative-hypnotic effects of ethanol (Kurtz et al. 1996); therefore, it is possible that differences in ethanol-related behaviors in these animals may be related to differences in endogenous $GABA_A$ receptor ligands like taurine or responses to other $GABA_A$ receptor modulators. Indeed, extrasynaptic receptors involved in tonic inhibition have recently been shown to be sensitive to low concentrations of taurine (Jia et al. 2008a). However, sP and sNP rats do not differ in their sedative-hypnotic responses to other benzo-diazepines and barbiturates (Colombo et al. 2000), but sP rats are more sensitive to the sedative-hypnotic effects of γ-hydroxybutyric acid (Colombo et al. 1998). It is possible that neuroactive steroids may also be involved in innate responses in these animals. 3α,5α-THP and 3α,5α-THDOC are increased in sP vs. sNP rats (Barbaccia et al. 1999). In other selectively bred animals, withdrawal seizure-prone mice are not only found to be more sensitive to the anxiolytic effects of ethanol than withdrawal seizure-resistant mice but they are also more sensitive to the anxiolytic effects of pentobarbital (Atkins et al. 2000). In other studies, FAST & SLOW mice, selectively bred for differences in locomotor stimulatory effects of ethanol, also differ in responses to the convulsant effects of $GABA_A$ receptor modulators, and to the locomotor-stimulant effects of benzodiazepines (Shen et al. 1998). Aside from selectively bred animals, inbred strains of mice also suggest a role for $GABA_A$ receptors. C57BL/6J mice are not only less sensitive to the sedative-hypnotic effects of ethanol than 129/SvJ mice but are also less sensitive to the GABA agonist propofol (Homanics et al. 1999b). Interestingly, the same study indicated that C57BL/6J mice are more sensitive to zolpidem and midazolam, but not Ro15-4513 and pentobarbital. Besides pharmacologic modifiers, molecular biological techniques have been used to assess the role of specific $GABA_A$ receptor subunits in innate tolerance. Using a coding polymorphism difference in C57BL/6J and DBA/2J mice, different lines of BXD recombinant inbred strains were used to determine whether variations in the gene for the $GABA_A$ receptor γ2 subunit correlated with ethanol-related behaviors. BXD lines with γ2 of DBA/2J origin were less ataxic on the screen test, but had greater ethanol-induced hypothermia (Hood and Buck 2000). A trend was also noted for sedative-hypnotic effects as well as a second motor ataxia test.

Acquired tolerance

Apart from innate tolerance, various inbred, selectively bred, and recombinant inbred rodent strains, as well as genetically modified mouse models, have been used to assess acquired

ethanol tolerance. Acute functional tolerance (AFT) is a commonly used assay to assess acute tolerance. AFT is defined as greater sensitivity to ethanol effects during the rising phase of the blood ethanol curve than equivalent ethanol concentrations on the falling phase. However, because accurately assessing blood ethanol concentrations on the rising phase is difficult due to the immediate effects of ethanol, AFT is commonly assessed by measuring the ability of a subject to exhibit a specific ethanol-related behavior (e.g., motor coordination, loss of righting reflex) during successive ethanol exposures. Blood ethanol measurements are taken upon the appearance of each behavior and the difference in blood ethanol level on the successive measurements is indicative of acute tolerance.

AFT to the motor-impairing effects of ethanol is associated with a reduction in the ability of ethanol to enhance muscimol-stimulated Cl^- uptake in cerebellar microsacs (Wallace et al. 2007). Studies in $GABA_A$ receptor genetically modified models have shown that AFT to motor incoordination is decreased in γ2S- and γ2L-$GABA_A$ receptor transgenics (Wick et al. 2000), but does not differ in α1-, α5-, α6-, γ2L-, or δ-$GABA_A$ receptor knockout mice (Boehm et al. 2004b; Homanics et al. 1998, 1999a; Kralic et al. 2003; Mihalek et al. 2001). However, recent work in α1-$GABA_A$ receptor (S270H, L277A) knockin mice suggests that α1-$GABA_A$ receptors are involved in AFT to ethanol (Werner et al. 2009), since these mice display decreased AFT to motor coordination.

$GABA_A$ receptor involvement in acute, rapid, and chronic ethanol tolerance has also been assessed in various animal models. For instance, selectively bred mice that differ in diazepam sensitivity also differ in rapid tolerance development. Mice that are more resistant to diazepam's effects also develop more rapid tolerance than diazepam-sensitive mice (Gallaher and Gionet 1988). Inbred long-and short-sleep mice display differences in rapid tolerance. Long-sleep mice that display a greater response to ethanol's sedative-hypnotic effects also develop greater rapid tolerance (Radcliffe et al. 2005). Indeed, these mice differ in $GABA_A$ receptor inhibitory post-synaptic responses (Proctor et al. 2004), suggesting the involvement of different GABAergic responses to ethanol. BXD mice have also been used to assess tolerance. Assessment of the parental strains, C57BL/6J and DBA/2J, indicates that although DBA/2J mice were more sensitive to ethanol's motor ataxic effects, they also develop greater AFT (Gallaher et al. 1996). Subsequent tests show quantitative trait loci (QTL) for ethanol tolerance on chromosomes 9 and 11. While these studies did not identify a role for $GABA_A$ receptors in tolerance, it is possible that subunits from the $GABA_A$ receptor subunit gene cluster on chromosome 11 may be involved. Other studies show that mice selectively bred for ethanol withdrawal severity do not differ in tolerance to ethanol's hypothermic or sedative-hypnotic effects (Crabbe and Kosobud 1986), although these mice exhibit differences in $GABA_A$ receptor mRNA subunit expression (Keir and Morrow 1994) as well as differential changes in subunit expression after chronic ethanol (Buck et al. 1991a). Together, these results support the notion that various genes contribute to ethanol-related responses and that tolerance to specific ethanol-related behaviors may be independent of withdrawal.

Genetically modified mice have also been used to assess the role of $GABA_A$ receptors in chronic tolerance. Recent work suggests that α1-$GABA_A$ receptors are involved in chronic tolerance to ethanol's motor ataxic effects. $GABA_A$ receptor α1 (S270H, L277A) knockin mice that are initially less sensitive to ethanol's motor ataxic effects display decreased tolerance after repeated ethanol exposure (Werner et al. 2009). Furthermore, β3-$GABA_A$ receptors appear to play a role in chronic tolerance. β3 (N265M) knockin mice display enhanced tolerance to ethanol's sedative-hypnotic effects compared to controls (Sanchis-Segura et al. 2007). However, α6- and δ-$GABA_A$ receptor knockout mice do not differ from controls in chronic tolerance to ethanol's sedative-hypnotic effects (Homanics et al. 1998; Mihalek et al. 2001). Studies have also been carried out in genetically modified mouse models of genes known to indirectly influence $GABA_A$ receptor function. Work has shown that PKCγ knockout mice

did not develop chronic tolerance to ethanol's sedative-hypnotic or hypothermic effects, but this response was strain dependent (Bowers et al. 1999). PKCε knockout mice are resistant to tolerance to motor-impairing effects of ethanol (Wallace et al. 2007). Further studies are needed to establish the role of other $GABA_A$ receptor subunits and other $GABA_A$ receptor-associated proteins in AFT as well as acute and chronic tolerance to other behavioral measures.

Tolerance and altered $GABA_A$ receptor function and regulation

Recent work has suggested that alterations in $GABA_A$ receptor expression may underlie acquired ethanol tolerance. Studies indicate that a single high ethanol exposure results in alterations in $GABA_A$ receptor subunit expression. One hour following ethanol exposure, α4 and δ surface expression is decreased in the hippocampal CA1 region, but no difference in total subunit levels is found (Liang et al. 2007). This decrease in cell surface extrasynaptic receptors is associated with a decrease in ethanol-enhanced tonic current. Furthermore, a significant enhancement of surface expression of α4 and γ2 subunit proteins, as well as a decrease in α1 and δ subunits, was noted 2 days following ethanol exposure. Additionally, the ethanol-induced increase in α4 subunit expression requires the binding of heat shock factor 1 at a downstream site (Pignataro et al. 2007). These adaptive changes are accompanied by an increase in mIPSC response to ethanol and decreased response to diazepam (Liang et al. 2007), suggesting that the observed changes in receptor expression might underlie altered sensitivity to ethanol. Furthermore, chronic ethanol exposure altered the recovery of baseline spontaneous neural activity of medial septal/diagonal band of Broca neurons following GABA microiontophoresis (Matthews et al. 2000a). These studies suggest that the observed changes in receptor expression following chronic ethanol exposure might underlie altered sensitivity to ethanol and GABA. Interestingly, the mIPSC effects were transient, and responses returned to normal after 2 weeks. It is possible that these transient changes contribute to ethanol tolerance. Tolerance may be restricted to selected brain regions or cell populations. Tolerance to ethanol's effects in the CA3 region of neonatal rats does not occur in slices exposed to ethanol for a prolonged period (Galindo and Valenzuela 2006). Finally, inhibition of synaptic $GABA_A$ receptors results in the incorporation of receptors diffusing from peri- and extrasynaptic sites (Thomas et al. 2005), independent of receptors arising from intracellular stores. Overall, while not pinpointing the exact role of $GABA_A$ receptors in ethanol action, these studies lend support to a possible role of changes in $GABA_A$ receptor subtype expression contributing to tolerance to various ethanol-related behaviors.

Adolescence

Adolescence (often defined as 12 to 20 years of age in humans) is a developmental period highlighted by continued neurobiological development accompanied by changes in personality. The neurobiological alterations include changes in neuronal networks, brain size, and receptor number and compositions, while changes in personality include increased risk choices and experimentation with drugs, including ethanol. In fact, increased drug use, particularly ethanol, is a commonly noted factor at this developmental stage in humans (Kim et al. 2008; Lim et al. 2007). The development of animal models to better understand the impact of ethanol exposure during adolescence has rapidly proceeded in the last decade. The need for animal models is obvious due to ethical and legal restrictions limiting ethanol research in human adolescence. The terms adolescence and puberty are often used interchangeably. While their timing overlaps, the terms are not synonymous (Sisk and Foster 2004; Spear 2000). Puberty refers to gonadal maturation, while adolescence refers to a gradual period of maturation of social and cognitive behaviors (Powell 2006; Sisk and Foster 2004). The precise timing of adolescence in rodents is difficult to determine as there is no specific event signaling the beginning and end of this developmental period and the boundaries vary depending on genetic differences, gender, nutrition, and other environmental periods (Spear 2007). In rats, a

conservative estimate of adolescence spans the period between postnatal days (PD) 28 and 42 (Spear 2007), but some harbingers of female adolescent development can begin as early as PD 20 and continue through PD 55 in males (Spear 2000, 2007). Studies investigating the effect of ethanol in adolescent rats have used the period between PD 28 and 60 (Smith 2003), while in mice, adolescence has been characterized as the period from PD 22 to 60 (Laviola et al. 2003).

Behavioral sensitivity

It has become clear that ethanol produces different behavioral responses in adolescence compared to adults. Specifically, age modulates ethanol effects on motor incoordination, anxiety, and cognitive function. Researchers have also shown that sensitivity to ethanol-induced ataxia increases during ontogeny (Land and Spear 2004; Smith 2003; Spear and Varlinskaya 2005). Specifically, adolescent rats exhibit less severe motor impairment following ethanol exposure (Spear and Varlinskaya 2005; White et al. 2002a). Throughout development, a natural increase in sensitivity to the motor-impairing effects of ethanol between adolescence and adulthood emerges (Silveri and Spear 1998).

Besides motor ataxia, anxiety has been shown to play a role in the recurrence and initiation of drinking behavior. Given the role of the $GABA_A$ receptor system in anxiety, it is reasonable to surmise that the adolescent developmental period may be characterized by changes in the anxiolytic effects of ethanol. Recent studies report attenuated withdrawal-related anxiety and a lessened anxiolytic effect of ethanol in adolescent rats as measured in a social interaction test (Doremus et al. 2003; Varlinskaya and Spear 2002, 2004). Interestingly, early adolescent C57BL/6J mice show enhanced anxiety and greater anxiolytic response to ethanol on the elevated plus maze (Hefner and Holmes 2007). In addition to potential species differences, such contrary results are also likely confounded by different baseline levels of social interaction and plus maze behavior between adolescence and adults. However, it is possible that lessened anxiogenesis and increased anxiolysis during adolescence may also be a reflection of the developmental state of the $GABA_A$ receptor system that underlies anxiety and may be a contributing factor to problem drinking. Salimov et al. (1996) reported that ethanol consumption during the adolescent period results in lower responsivity to novelty-induced anxiety and stress. Again, these outcomes are predictive of a greater risk for problem drinking as a result of ethanol exposure during the adolescent developmental period.

Ethanol impairs cognition, executive function, and attentional processes that involve several brain regions. Among the constellation of cognitive impairments produced by ethanol, learning and memory, which is hippocampal dependent, is particularly vulnerable to ethanol-induced impairments (see Matthews and Silvers 2004 for review). However, the magnitude of these cognitive impairments is age dependent. For example, early studies reported that acute ethanol administration to adolescent rats produced greater learning and memory deficits compared to adults (Land and Spear 2004; Markwiese et al. 1998) and this differential effect was dose dependent (Acheson et al. 2001). However, the opposite effect (adolescents are less sensitive than adults to the effects of ethanol) has also been reported (Land and Spear 2004; Rajendran and Spear 2004). Regardless of whether adolescents are more or less sensitive to ethanol-induced cognitive impairments, it is clear that ethanol affects the hippocampus of adolescents differently than adults. Although the neurobiological mechanisms underlying this difference are not yet elucidated, it is known that ethanol more potently attenuates hippocampal long-term potentiation in brain slices from adolescent rats compared to adult rats (Pyapali et al. 1999). In addition, moderate ethanol doses (30 mM) enhance $GABA_A$ receptor-mediated inhibitory tonic currents more potently in the dentate gyrus of adolescent rats compared to adult rats (Fleming et al. 2007). These results demonstrate that innate differences, including differences in $GABA_A$ receptors, exist in the hippocampus between adolescent and adult

animals that likely underlie differential sensitivity to ethanol-induced impairments in cognition.

Developmental alterations in $GABA_A$ receptor expression

Given that adolescent subjects differ from adults on many ethanol-related behaviors, it is possible that the spatial and temporal changes in $GABA_A$ receptor subunit expression throughout the developmental process may contribute to their phenotypic differences. Each subunit exhibits unique developmental expression patterns based on regions and age, suggesting that the expression and function of $GABA_A$ receptors during development may differ from those found in adults (Henschel et al. 2008). $GABA_A$ receptor subunit expression of α1, α2, α3, α5, and γ2 changes throughout development, reaching adult-like levels during the third month of life (Jenkins and Simmons 2006). Immunoreactivity of α1 subunit is low at birth and restricted to a few brain regions, with levels increasing during the first postnatal weeks (Fritschy et al. 1994). In contrast, α2 subunit distribution is widespread in the superficial layers (I–IV) of the neocortex (Yu et al. 2006) at birth. These levels decrease in some regions shortly after the appearance of the α1 subunit and co-expression occurs briefly, with α1 eventually replacing α2 subunit (Fritschy et al. 1994; Henschel et al. 2008). High levels of α3 subunit are found within the frontal and perirhinal cortex, with up-regulation occurring from PD 10 to 30; the α5 subunit has high expression throughout the brain, especially in the deep cortical layers (Yu et al. 2006). The initial increases in α1, α2, α3, and γ2 found during the early postnatal period change during development, and decrease until PD 60, while α5 levels decrease from the high levels found in early development throughout the aging process (Yu et al. 2006). Prominent throughout development, β2 and β3 immunoreactivity shows consistent levels in both the neonatal and adult brain (Fritschy et al. 1994). In humans, a similar increase in α1 and γ2 expression occurs with age in the temporal lobe, hippocampus, and basal ganglia (Kanaumi et al. 2006).

Consistent with developmental shifts in $GABA_A$ receptor expression within specific brain regions, the cerebellum displays levels of α6 subunit expression that increase with age and expression of α1, γ2, β2, β3, and δ subunits that decreases with age (Gutiérrez et al. 1997). Within the hippocampus, consistently high levels of γ2 and α5 subunit expression are found across development, the α1 subunit shows decreasing levels through development and aging (Yu et al. 2006), and levels of the δ subunit mRNA increase throughout development, with the highest levels being found in adults (Laurie et al. 1992). Increasing levels of the δ subunit mRNA across development are interesting, given the proposed link of δ-$GABA_A$ receptors to low-dose ethanol sensitivity (Choi et al. 2008). Considering that adolescents are less sensitive to ethanol's motor ataxic effects compared to adults, and δ subunit mRNA levels are lower in adolescents compared to adults, it is tantalizing to speculate that this subunit is a critical mediator underlying the changes in ethanol's effect on $GABA_A$ receptors across development. Overall, because $GABA_A$ receptor subunit expression differs across development, it is likely that changes in $GABA_A$ receptor stoichiometry underlie many of the developmental specific effects of ethanol previously reviewed. Furthermore, chronic ethanol exposure alters $GABA_A$ receptor mRNA and protein expression. As such, it is predicted that chronic ethanol exposure during adolescence, when $GABA_A$ receptors are changing, should also alter $GABA_A$ receptor protein expression in a manner that differs from the changes found following adult ethanol exposure.

Ethanol intake and chronic ethanol exposure

Adolescent rats consume more ethanol than adults (Doremus et al. 2005). Indeed, adolescents have been shown to consume two to three times more ethanol than adults relative to body weight (Lancaster et al. 1996). Some variation in adolescent ethanol self-administration has been found in consumption which may reflect methodological differences. When consumption

is examined in a 24-h continuous access paradigm, adolescents exhibit a greater increase in ethanol licking behavior over a 4-week period, compared to adults (Bell et al. 2006). However, when voluntary ethanol self-administration is examined, adolescents consumed significantly more ethanol when measured as g/kg body weight (Vetter et al. 2007). When the consumption off the floor paradigm is used, adolescents show high levels of consumption that decrease gradually into adulthood (Truxell et al. 2007). Overall, adolescents consume more ethanol than adults; this difference is particularly evident when consumption is calculated relative to body weight rather than proportion of overall consumption (Nance 1983). Complicating interpretation of these results, rates of ethanol metabolism vary with age. Adolescents have been observed to have faster rates of ethanol elimination than adults (Brasser and Spear 2002; Hollstedt et al. 1977); however, these results are not consistently found (Kelly et al. 1987; Silveri and Spear 2000). Additionally, these results have not been found to account for the attenuated ethanol sensitivity shown by adolescents to many of the acute effects of ethanol (Silveri and Spear 2000). The reported high levels of adolescent consumption may reflect age-related changes in both the rewarding and aversive properties of ethanol (Vetter et al. 2007).

The subjective properties of a drug play a large part in consumption, abuse, and addiction patterns. That is to say, the subjective experience of greater reward or less aversion as it relates to a drug will lead to a greater risk of abuse. Assessment of the motivational properties of ethanol has included the use of ethanol-induced conditioned place preference and ethanol-induced conditioned taste aversion (CTA) (Chester and Cunningham 1999; Chester et al. 1998). Interestingly, although not without inconsistencies, GABAergic involvement has been implicated in both of these tasks. For example, adult mice exposed to ethanol (via inhalation) during the adolescent period show an attenuated ethanol-induced CTA (Chester and Cunningham 1999; Smith 1989). Additionally, a chronic intermittent pattern of exposure, resulting in multiple withdrawals during adolescence, exacerbates this attenuated CTA (Diaz-Granados and Graham 2007; Graham and Diaz-Granados 2006). When subjects were administered diaze-pam during the withdrawal periods in order to assess the role of possible $GABA_A$ receptor withdrawal-related perturbations, the attenuation of the ethanol-induced CTA was not dissimilar between diazepam-treated and control animals, suggesting that $GABA_A$ receptor perturbations during withdrawal were not playing a role in the attenuation of CTA. However, adult mice that were exposed to only diazepam during the adolescent period showed the same attenuated CTA as adult mice exposed to only ethanol during adolescence. Thus, the rewarding and aversive properties of ethanol, and therefore ethanol self-administration, in adulthood are related to the interaction of these drugs with a developmentally dynamic GABAergic system.

Chronic exposure to ethanol during adolescence has also revealed long-lasting changes in hippocampal function and GABAergic neuroactive steroids. For example, CIE exposure to high dose ethanol during adolescence does not impair spatial learning during the exposure (Silvers et al. 2003a) but does produce cognitive tolerance to ethanol's memory impairing effects when animals are tested as young adults (Silvers et al. 2003b, 2006). However, the cognitive tolerance is not permanent (Silvers et al. 2006) and may in fact lead to sensitization of ethanol's effect on memory following a long delay (White et al. 2000). Mirroring the cognitive tolerance, CIE exposure during adolescence blunts ethanol-induced inhibition of hippocampal pyramidal neurons, and this effect lasts into young adulthood (Tokunaga et al. 2006). Furthermore, chronic intermittent ethanol during adolescence also reduces hippocampal 3α,5α-THP levels in response to an ethanol challenge (Silvers et al. 2006) and this reduced neuroactive steroid response directly mirrors the reduced cognitive and neurophysiological effect following ethanol challenge. Therefore, it is likely that the prolonged cognitive and neurophysiological tolerance found following chronic ethanol exposure during adolescence is due to GABAergic mechanisms such as altered neuroactive steroid levels. Chronic ethanol exposure has also altered other ethanol-related behaviors in adolescents. Although sensitivity

to the motor-impairing effects of ethanol increases during development, repeated adolescent binge drinking episodes prevent the emergence of this normal developmental change (White et al. 2002b). Additional studies have also revealed a persistent tolerance to the sedative effects of ethanol, resulting in continued attenuation of the animal's loss of righting reflex (Silveri and Spear 1998; Silvers et al. 2003b).

However, few studies have investigated whether chronic ethanol administration during adolescence alters $GABA_A$ receptor structure or function. In fact, only three studies directly compare the two age groups. Specifically, adolescent and adult rats do not differ on bicuculline-induced seizure threshold (Wills et al. 2008), but differ on pentylenetetrazole-induced seizures (Acheson et al. 1999) following chronic ethanol. However, chronic ethanol exposure for 1 month differentially altered $GABA_A$ receptor function in adult vs. adolescents as measured by 3α,5α-THDOC potentiated Cl^- flux (Grobin et al. 2001). These limited studies suggest that perhaps chronic ethanol might differentially alter $GABA_A$ receptor function when animals are exposed during adolescence as compared to adulthood. However, additional work is clearly needed on this topic.

Human alcoholics

Do the effects of chronic ethanol exposure in animal studies translate to humans? Behavioral adaptations to ethanol in human beings mimic those observed in animal models. Further, GABAergic neurotransmission is important for many behavioral actions of ethanol and several studies in human alcoholics have highlighted changes in $GABA_A$ receptor function and plasticity that resemble the adaptations found in animal models of ethanol dependence. For instance, human alcoholics exhibit ethanol tolerance and benzodiazepine and barbiturate cross-tolerance (Volkow et al. 1993; Woo and Greenblatt 1979). Early studies conducted in post-mortem human brains have suggested the involvement of $GABA_A$ receptors: [^{3}H]muscimol binding density is greater in alcoholic cerebral cortex (Tran et al. 1981) and superior frontal gyrus of non-cirrhotic alcoholics (Dodd et al. 1992) compared to healthy controls. Increases, decreases, or no changes in benzodiazepine binding have been reported in several studies of postmortem human alcoholics (Dodd 1995; Dodd et al. 1992; Freund and Ballinger 1988).

In vivo neuroimaging studies have shown that 1–6 months abstinent male ethanol-dependent subjects have reduced binding of tracers for the GABA/benzodiazepine receptor in the frontal cortex, measured by [^{11}C]-flumazenil positron emission tomography (PET, Gilman et al. 1996) or [^{123}I]-iomazenil single photon emission computed tomography (SPECT, Abi-Dargham et al. 1998; Lingford-Hughes et al. 1998). A trend for a reduced [^{123}I]-iomazenil binding was reported in ethanol-dependent women withdrawn from ethanol with benzodiazepines and abstinent from ethanol for at least 3 months (Lingford-Hughes et al. 2000). Using a [2-deoxy-2[^{18}F]-fluoro-D-glucose] PET study, Volkow et al. (1997) found that the inhibitory response to a lorazepam challenge was blunted in the orbitofrontal cortex and cingulate gyrus in 8–11 weeks (during detoxification) abstinent ethanol-dependent subjects, suggesting reduced GABA/benzodiazepine receptor function. However, this study did not report withdrawal intensity. In contrast, after 1 week of abstinence from ethanol dependence, higher [^{123}I]-iomazenil binding to GABA/benzodiazepine receptors was observed in the parietal, frontal, cingulated, temporal, insular, and occipital cortex of non-smoking (but not smoking) ethanol-dependent subjects. Furthermore, the GABA/benzodiazepine receptor availability in the cerebellum and occipital lobe at 1 week of abstinence was correlated with the severity of ethanol withdrawal determined in the subjects every 6 h immediately after admission to the clinic (Staley et al. 2005). Using [^{11}C]-flumazenil PET to measure the pharmacokinetics and pharmacodynamics of midazolam, Lingford-Hughes et al. (2005) showed that ethanol dependence was associated with decreased midazolam-induced time asleep in the absence of reduced benzodiazepine receptor occupancy in ethanol-dependent

patients abstinent for at least 6 weeks. The authors hypothesized that this reduced sensitivity in ethanol dependence may reflect a difference in the subunit profile of the $GABA_A$ receptors, either as part of the vulnerability to, or as a consequence of their ethanol dependence. More recently, Taylor et al. (2008) found that $GABA_A$ receptor function, assayed by midazolam-induced slow saccadic eye movement and sedation, was not affected in ethanol-dependent patients after 2 months of abstinence.

These changes in $GABA_A$ receptor function are accompanied by changes in receptor plasticity. Several studies reported changes in $GABA_A$ receptor subunit expression in post-mortem brains from human alcoholics, although results are often controversial. Lewohl et al. (1997) showed that $GABA_A$ receptor α1 subunit mRNA expression was elevated in human alcoholic superior frontal cortex, compared with non-alcoholic subjects, while α2 and α3 mRNA did not change. Further, α1 protein expression was greater in the superior frontal compared to the motor cortex of cirrhotic alcoholics, but no such difference was observed in controls or uncomplicated alcoholics. In contrast, α3 peptide expression was lower in superior frontal than in motor cortex of uncomplicated alcoholics (Lewohl et al. 2001). However, Mitsuyama et al. (1998) found no difference in α1 and α4 subunits mRNA expression in frontal cortex between controls and alcoholics, but β3 mRNA expression was elevated in non-cirrhotic alcoholics. These changes were not accompanied by changes in α1, α4, or β2/3 protein expression between alcoholics and controls. Others have reported no differences in mRNA expression for any β subunit between alcoholics and controls (Buckley and Dodd 2004).

With the development of more sophisticated fine-mapping techniques, studies on $GABA_A$ receptor subunit expression have been associated to specific genotypes. Thus, Dodd and colleagues reported that the GABRB2 gene, which encodes for the β2 subunit, did not influence the β2 subunit expression; rather, expression of β3 subunit was increased in controls with GABRB2-1,2 genotype, but not in alcoholics with the same genotype (Dodd et al. 2004). Furthermore, aldehyde dehydrogenase ADH1C, D2 dopamine receptor DRD2B, excitatory amino acid transporter 2 EAAT2, and apolipoprotein E APOE genotypes modulate $GABA_A$ receptor β subunit expression in superior frontal cortex toward a less-effective form of the receptor (Dodd et al. 2004, 2006). Alleles associated with alcoholism also appear to reduce the β3:β2 ratios in alcoholic's superior frontal cortex. $GABA_A$ receptors with the β2 subunit are less efficacious at mediating chloride flux in response to any GABA concentration, so this subunit switching would have the effect of attenuating $GABA_A$ receptor-mediated inhibition and may make this brain region more susceptible to the neuropathological consequences of chronic alcoholism (Buckley et al. 2006). More recently, Haughey et al. (2008) reported that levels of α2 subunit mRNA and protein in prefrontal cortex differed between subjects with GABRA2 (the gene encoding the α2 subunit) genotypes, with higher levels of α2 subunit expression in prefrontal cortex of subjects with the AA genotype compared to subjects with the AG genotype (SNP rs279858); however, there was no difference between ethanol-dependent and control subjects.

Polymorphisms in the genes encoding the $GABA_A$ receptor subunits have been linked to ethanol response and risk for alcoholism in humans. Genes encoding the $GABA_A$ receptor subunits are clustered in several chromosomal regions including 4p13–q11 (α2, α4, β1, and γ1), 5q34–q35 (α1, α6, β2, and γ2), 15q11–q13 (α5, β3, and γ3), and Xq28 (α3, β4, and ε1) (Enoch 2008). Results from the Collaborative Study on the Genetics of Alcoholism (COGA) found an increased allele sharing among ethanol-dependent individuals in a region on chromosome 4p that includes a cluster of four genes: GABRA2, GABRA4, GABRB1, and GABRG1, encoding for α2, α4, β1, and γ1 subunits, respectively (Reich 1996; Reich et al. 1998). Since then, several studies have shown that GABRA2 is a key gene affecting the risk for alcoholism. Edenberg et al. (2004) reported that single nucleotide polymorphisms (SNPs) in the GABRA2 gene, but not other members in the gene cluster, were associated with both

ethanol dependence and changes in β-frequency electroencephalogram (EEG). Brain oscillations measured by EEG are a key phenotype in ethanol dependence: alcoholics and the offspring of male alcoholics have increased power in the β-frequency band (13–18 Hz) of the EEG (Costa and Bauer 1997). The association of GABRA2 markers with ethanol dependence was subsequently replicated in several independent studies in different populations (Agrawal et al. 2006; Bauer et al. 2007; Covault et al. 2004; Enoch et al. 2006; Fehr et al. 2006; Lappalainen et al. 2005; Soyka et al. 2008). Within these studies, strong association of the GABRA2 gene was found in European ancestry alcoholics without co-morbid drug dependence or major depression (Covault et al. 2004), although results from the COGA study reported the opposite (Agrawal et al. 2006). The association of GABRA2 with alcoholism was linked to anxiety scores (Enoch et al. 2006) and to severity of ethanol withdrawal (Fehr et al. 2006; Soyka et al. 2008), thus potentially influencing treatment outcome (Soyka et al. 2008). Indeed, Bauer et al. (2007) found that genetic vulnerability at the GABRA2 gene influenced the ability of different psychosocial treatments to reduce ethanol use.

Sensitivity to the acute effects of ethanol has also been linked to the GABRA2 gene (Haughey et al. 2008; Pierucci-Lagha et al. 2005). Interestingly, individuals homozygous for the A-allele at GABRA2 reported greater stimulant, sedative, and gastrointestinal subjective effects of acute ethanol, compared with subjects with one or more G-alleles (the G-allele is overrepresented among ethanol-dependent subjects; Covault et al. 2004). Administration of the neuroactive steroid biosynthesis inhibitor finasteride antagonized some of the behavioral effects of ethanol particularly among individuals homozygous for the A-allele, suggesting that neuroactive steroids may be mediating the subjective effects of ethanol, like in rodent studies (Pierucci-Lagha et al. 2005). The genetic association of ethanol dependence with GABRA2 is of significant functional interest as animal studies have identified the α2 subunit as the primary α subunit in limbic regions (McKernan and Whiting 1996). The α2 subunit mediates the anxiolytic effect of benzodiazepines and barbiturates (Dixon et al. 2008; Rudolph et al. 1999) as well as the sedative-hypnotic, but not the sedative, effects of combined exposure to ethanol and benzodiazepines in genetically modified mouse studies (Tauber et al. 2003).

More recent reports suggest that the association of the GABRA2 gene with alcoholism may be complicated by moderate linkage disequilibrium of GABRA2 markers with a functional genetic variation in the adjacent GABRG1 gene (Covault et al. 2008; Drgon et al. 2006; Ittiwut et al. 2008). Indeed, Covault et al. (2008) reported that markers in the GABRG1 region on chromosome 4 had the strongest association with ethanol dependence in the European American population. The γ1 subunit-containing $GABA_A$ receptors show increased sensitivity to neuroactive steroids compared to the γ2 subunit-containing $GABA_A$ receptors (Puia et al. 1993). This subunit is increased in ethanol-dependent rats (Devaud et al. 1995) that are sensitized to the anticonvulsant effects of GABAergic neuroactive steroids (Devaud et al. 1996). Altered sensitivity to neuroactive steroids may affect ethanol sensitivity and therefore modulate risk for ethanol dependence (Morrow et al. 2006). In addition to GABRA2, other genes that encode for the $GABA_A$ receptor subunits have been associated with ethanol dependence. Thus, on chromosome 4, association of the GABRB1 gene with alcoholism was reported by several groups (Long et al. 1998; Parsian and Zhang 1999; Reich et al. 1998; Song et al. 2003).

Results for markers in the $GABA_A$ receptor gene cluster on chromosome 5 are still controversial, with associations reported in some samples, but not others (Dick et al. 2005; Sander et al. 1999; Song et al. 2003). Thus, a positive association between ethanol dependence and SNPs at the GABRG2 gene, encoding for the γ2 subunit, was observed in Scottish (Loh et al. 1999), Finnish (Radel et al. 2005), and Japanese populations (Loh et al. 2000). Association with the GABRB2 gene, encoding for the β2 subunit, was reported in Scottish (Loh et al. 1999), Finnish, and Southwestern Native American (Radel et al. 2005) populations. The

GABRA1 gene, encoding for the α1 subunit, was associated with several drinking behavior phenotypes, including history of blackouts, age at first drunkenness, level of response to ethanol, and ethanol dependence (Dick et al. 2006). Associations with the GABRA6, encoding for the α6 subunit, have been found in Scottish (Loh et al. 1999) and Finnish (Radel et al. 2005) populations. Furthermore, sons of alcoholics with the proline385 to serine polymorphism at GABRA6 were less sensitive to the effects of ethanol and this polymorphism was a predictor for the development of alcoholism (Schuckit et al. 1999). In the same subjects, this polymorphism was also associated with reduced sensitivity to benzodiazepines (Iwata et al. 1999) and to the sedative effects of ethanol (Hu et al. 2005).

Finally, fine mapping of a $GABA_A$ receptor gene cluster on chromosome 15 reported modest evidence of haplotypic association to ethanol dependence for the GABRG3 gene, encoding the γ3 subunit (Dick et al. 2004). Moreover, Song et al. (2003) showed evidence for imprinting association, with paternal, but not maternal, transmission of GABRA5 and GABRB3 genes, encoding the α5 and β3 subunits, being associated with alcoholism.

Therapeutic implications for alcoholism

The discovery of new mechanisms of ethanol actions on GABAergic transmission over the past 10 years opens new opportunities to intervene in the effects of ethanol on different aspects of brain function. It is now possible to inhibit effects of ethanol by blockade of α4-$GABA_A$ receptors or by inhibition of neuroactive steroid synthesis. The blockade of ethanol actions may prevent ethanol-induced toxicity and/or facilitate increased consumption due to the loss of ethanol sensitivity. Likewise, it is also possible to enhance ethanol sensitivity by targeting α4-$GABA_A$ receptors with selective agonists or promoting neuroactive steroid synthesis. While there are many advances in the molecular realm, it remains unclear whether it is advantageous or disadvantageous to manipulate ethanol actions in humans. Future studies are clearly needed to elucidate the therapeutic relevance of the advances of the last 10 years.

Future directions—unanswered questions

The advances of the past 10 years raise many new questions to explore. The identification of ethanol binding sites on various $GABA_A$ receptor subtypes is yet unclear. The role of protein kinases and phosphatases in ethanol actions are limited to too few $GABA_A$ receptor subtypes. The coordinated actions of ethanol on multiple non-GABAergic targets that indirectly influence GABAergic neurotransmission seem too complex to untangle with physiological approaches. There is a lot of work to do.

Following chronic ethanol administration, there are specific adaptations of $GABA_A$ receptors that appear to mediate increased CNS excitability. These mechanisms could be targeted for therapeutic benefits. However, there are many unanswered questions in this realm as well. What is the significance of changes in α2-, α3-, β2-, β3-, and γ1-$GABA_A$ receptors? Can we modulate PKC and PKA interactions with $GABA_A$ receptors selectively? Would effects of these protein kinases on other ion channels interfere with therapeutic strategies for modulation of $GABA_A$ receptors? Can we target specific phosphorylation sites on specific proteins? Would this interfere with the actions of other endogenous modulators?

Standing back to widen our perspective, must we ask if our animal models are adequate for understanding the effects of ethanol on humans? Can we begin to model the genetic diversity of humans, the neurobiology, endocrinology, and enhanced complexity of the human being? Can we apply the knowledge gained from laboratory animals to develop treatments for ethanol addiction, toxicity, and dependence? History would argue the answer is yes, so our challenge is to choose the most important questions and persevere in the complexity of the problem to make contributions to science and society.

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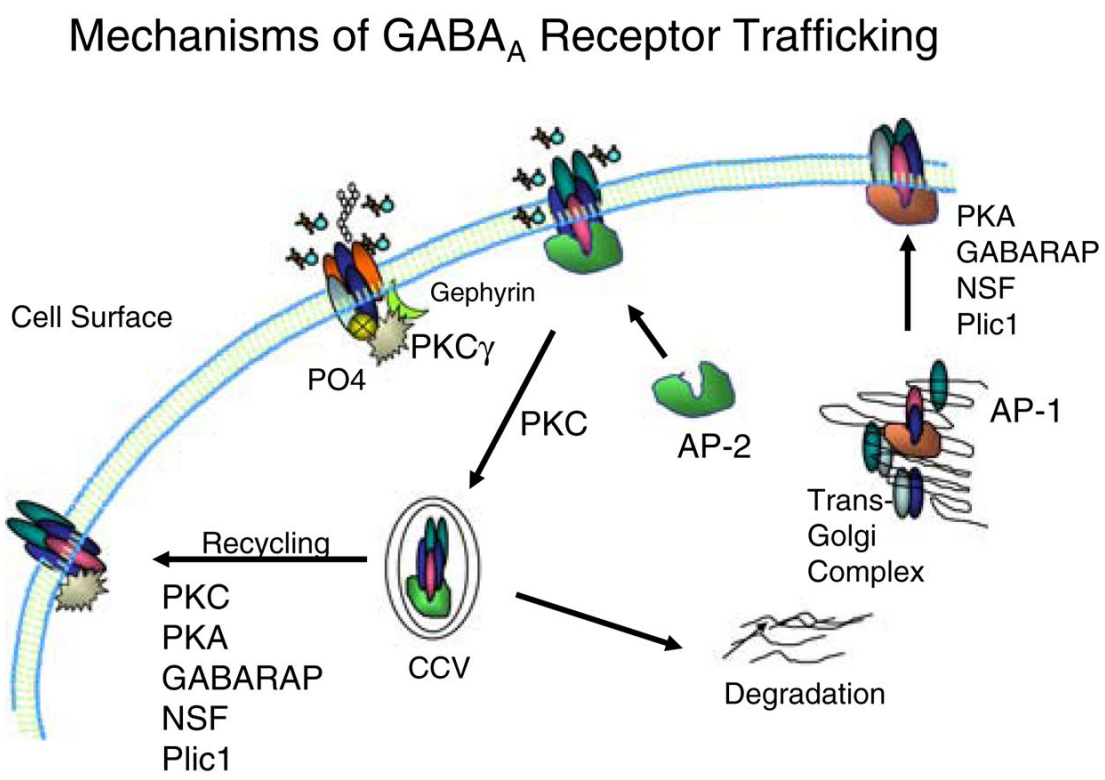

Fig. 1.

Mechanisms of $GABA_A$ receptor endocytosis and recycling. Endocytosis by clathrin-coated vesicles requires adaptor complex-2 (AP-2) and clathrin binding to the receptor. AP-2 is specific for endocytosis of surface proteins including $GABA_A$ receptors. First, AP-2 recognizes and binds to $GABA_A$ receptors, then clathrin binds the complex and the receptor is internalized. Phosphorylation of $GABA_A$ receptors can prevent AP-2 binding and subsequent internalization. Internalized receptors may be recycled back to the cell surface or degraded depending upon undefined intracellular signals. Protein kinases can alter many steps in the receptor trafficking process. For example, PKC phosphorylation can alter recycling of receptors, whereas PKA can increase surface expression of $GABA_A$ receptors. Adaptor complex-1 (AP-1) transports newly synthesized receptors from the trans-Golgi reticulum to the cell surface. Many proteins are required for intracellular trafficking of $GABA_A$ receptors. For example, $GABA_A$ receptor-associated protein (GABARAP) is involved in trafficking of $GABA_A$ receptors from the endoplasmic reticulum/Golgi complex towards the plasma membrane and possibly also in vesicle-mediated cycling between the plasma membrane and cytoplasmic pool of receptors. *N*-ethylmaleimide-sensitive factor (NSF) interacts with GABARAP and influences intracellular trafficking of $GABA_A$ receptors. Gephyrin reduces the diffusion of $GABA_A$ receptors from the membrane and facilitates their clustering at synapses. Protein linking integrin-associated protein to cytoskeleton-1 (Plic-1) is also involved in $GABA_A$ receptor trafficking and facilitates surface expression of the receptors (see Chen and Olsen 2007 for review)

Psychopharmacology (Berl). Author manuscript; available in PMC 2010 February 1.

Table 1

Acute ethanol effects on extrasynaptic receptors and tonic inhibition

Experimental system	Brain region/cell type	Ethanol concentration[a]	Experimental considerations	Recombinant $GABA_A$ receptor(s)	Reference
Recombinant recordings					
	X. laevis oocytes	1 mM		α4β2δ	(Sundstrom-Poromaa et al. 2002)
		3 mM		α4/6β3δ	(Wallner et al. 2003)
		3 mM		α6β3δ	(Hanchar et al. 2006)
		3 mM		α4β3δ (blocked by Ro15-4513)	(Wallner et al. 2006)
		30 mM		α4/6β2δ	(Wallner et al. 2003)
		100 mM		α4β3δ	(Borghese et al. 2006a, b)
		No effect (30 mM)		Concatenated α1β3δ	(Kaur et al. 2009)
	L(tk-) fibroblasts	3–100 mM		α4β3δ (transfected with PKCδ)	(Choi et al. 2008)
		300 mM		α4β3δ	(Borghese et al. 2006a, b)
	CHO cells	No effect (10–100 mM)		α4/6β2δ, α6β3δ	(Yamashita et al. 2006)
Slice recordings					
	Hippocampal CA1 pyramidal cells	1–3 mM	Progesterone withdrawal		(Smith et al. 2004) (Sundstrom-Poromaa et al. 2002)
		50 mM			(Liang et al. 2007)
		No effect (30 mM)			(Wei et al. 2004)
	Dentate gyrus granule cells	30 mM			(Wei et al. 2004)
		30 mM			(Glykys et al. 2007)
		30 mM	↑ tonic current in adolescents; ↑ basal tonic current in adults		(Fleming et al. 2007)
		30 mM	Not observed in PKCδ KOs		(Choi et al. 2008)
		50 mM			(Liang et al. 2008)
		No effect (30 mM)			(Borghese et al. 2006a, b)
	Molecular layer interneurons	20 mM	α1 co-localized with δ; observed in α4 KOs		(Glykys et al. 2007)
		30 mM	Not observed in PKCδ KOs		(Choi et al. 2008)
	Thalamus:				
	Ventrobasal neurons	50 mM			(Jia et al. 2008a)
	Relay neurons	30 mM	Not observed in PKCδ KOs		(Choi et al. 2008)

Experimental system	Brain region/cell type	Ethanol concentration[a]	Experimental considerations	Recombinant $GABA_A$ receptor(s)	Reference
	Cerebellar granule cells	10 mM	↑ ethanol enhancement with $R_{100}Q$ mutation in outbred rats		(Hanchar et al. 2005)
		20 mM	↑ spontaneous IPSCs		(Carta et al. 2004)
		25 mM	↑ spontaneous IPSCs; no ethanol enhancement with $R_{100}Q$ mutation in outbred rats		(Botta et al. 2007a)
		40 mM	↑ spontaneous IPSCs; No ethanol enhancement with $R_{100}Q$ mutation in outbred rats		(Botta et al. 2007b)
		50 mM	↑ spontaneous IPSCs; No ethanol enhancement with $R_{100}Q$ mutation in inbred AT and ANT rats		(Valenzuela et al. 2005)
		100 mM			(Casagrande et al. 2007)
		No effect (30 mM)			(Yamashita et al. 2006)
Ro15-4513 binding					
	HEK293T cell	1–30 mM		α6β3δ	(Hanchar et al. 2006)
		No effect (1–100 mM)		α4/6β3δ	(Korpi et al. 2007)
	Cerebellum	1–30 mM			(Hanchar et al. 2006)
	Cerebral cortex, cerebellum	500 mM			(Mehta et al. 2007)
		No effect (1–50 mM)			
	C57BL/6 sections	No effect (1–100 mM)			(Korpi et al. 2007)

↑, increased. Abbreviations: *ANT* alcohol non-tolerant; *AT* alcohol tolerant; *CHO* Chinese hamster ovary; *HEK* human embryonic kidney; *IPSCs* inhibitory post-synaptic currents; *KO* knockout; *PKCδ* protein kinase C δ

[a] Lowest effective ethanol concentration

Table 2

Effects of chronic ethanol exposure on $GABA_A$ receptor function in brain

$GABA_A$ receptor activity	**Alteration**	**Reference**
Cl^- channel function	↓ Cl^- flux[a,b]	(Kang et al. 1996; Morrow et al. 1988; Sanna et al. 1993; Ticku and Burch 1980)
	↔ Cl^- flux[c,d]	(Allan and Harris 1987; Tremwel et al. 1994)
	↓ mIPSC area[c]	(Cagetti et al. 2003; Liang et al. 2004; Liang et al. 2006, 2007)
	↓ Tonic current[c]	(Liang et al. 2007)
Ethanol-enhanced Cl^- channel function	↓ Cl^- flux[d]	(Allan and Harris 1987; Morrow et al. 1988)
	↑ mIPSC enhancement[c]	(Liang et al. 2006, 2007)
	↓ Tonic current enhancement[c]	(Liang et al. 2006, 2007)
TBPS binding	↑ Binding[a]	(Sanna et al. 1993)
Barbiturate modulation	↓ Pentobarbital-mediated Cl^-flux[a]	(Morrow et al. 1988)
Benzodiazepine modulation	↓ Flunitrazepam Cl^- Flux[d]	(Buck and Harris 1990; Sanna et al. 1993)
	↓ Diazepam-enhanced mIPSCs[c]	(Cagetti et al. 2003)
	↓ Diazepam-enhanced tonic current[c]	(Liang et al. 2004)
	↓ Zolpidem-enhanced mIPSCs[c]	(Liang et al. 2004)
	↓ Zolpidem-enhanced tonic current[c]	(Liang et al. 2004)
Ro15-4513 modulation	↑ Cl^- flux inhibition[a]	(Buck and Harris 1990)
	↑ Binding[c,d]	(Mhatre et al. 1988; Cagetti et al. 2003)
	↑ mIPSCs enhancement[c]	(Cagetti et al. 2003)
	↓ Tonic current enhancement[c]	(Liang et al. 2004)
THIP modulation	↓ Enhancement of tonic current[c]	(Liang et al. 2004)
	↑ mIPSCs[c]	(Liang et al. 2004)
Neuroactive steroid modulation	↑ 3α,5α-THP Cl^- flux[a]	(Devaud et al. 1996)
	↑ 3α,5α-THDOC Cl^- flux[a]	(Devaud et al. 1996)
	↓ Alphaxalone mIPSC enhancement[c]	(Cagetti et al. 2003)

↑ Increased, ↓ decreased, ⇓ abolished, ↔ no change

Abbreviations: *3α,5α-THP* (3α,5α)-3-hydroxypregnan-20-one; *3α,5α-THDOC* (3α,5α)-3,21-dihydroxypregnan-20-one; *TBPS t*-butylbicyclo-phosphorothionate; *THIP* 4,5,6,7-tetrahydroisothiazolo-[5,4-c]pyridine-3-ol

[a] Cerebral cortex

[b] Cerebellum

[c] Hippocampus

[d] Cerebral cortex, hippocampus, and cerebellum

Table 3

Effects of chronic ethanol administration on $GABA_A$ receptor subunit expression in cerebral cortex, hippocampus, and cerebellum

Subunit		Alteration	Reference
α1	mRNA	$\downarrow^{a,b,c}$	(Charlton et al. 1997; Devaud et al. 1995; Liang et al. 2007; Mhatre and Ticku 1992; Montpied et al. 1991b; Morrow et al. 1992)
		$\leftrightarrow^{b}$	(Petrie et al. 2001)
	Peptide	$\downarrow^{a,b,c}$	(Cagetti et al. 2003; Charlton et al. 1997; Devaud et al. 1997; Kumar et al. 2002; Marutha Ravindran et al. 2007; Mhatre et al. 1993; Werner et al. 2009)
		$\leftrightarrow^{c}$	(Marutha Ravindran et al. 2007; Matthews et al. 1998)
α2	mRNA	$\downarrow^{a}$	(Mhatre and Ticku 1992,1994b; Montpied et al. 1991b)
		$\leftrightarrow^{c}$	(Cagetti et al. 2003)
	Peptide	$\downarrow^{a,b}$	(Marutha Ravindran et al. 2007; Mhatre et al. 1993; Mhatre and Ticku 1994b)
		$\leftrightarrow^{c}$	(Marutha Ravindran et al. 2007; Matthews et al. 1998)
α3	mRNA	$\downarrow^{a}$	(Mhatre and Ticku 1994b)
		$\leftrightarrow^{a}$	(Mhatre and Ticku 1992; Montpied et al. 1991b)
	Peptide	$\downarrow^{a}$	(Mhatre et al. 1993; Mhatre and Ticku 1994b)
		$\leftrightarrow^{c}$	(Matthews et al. 1998)
α4	mRNA	$\uparrow^{a,c}$	(Devaud et al. 1995; Mahmoudi et al. 1997)
		$\leftrightarrow^{c}$	(Petrie et al. 2001)
	Peptide	$\uparrow^{a,c}$	(Cagetti et al. 2003; Devaud et al. 1997; Kumar et al. 2002; Liang et al. 2007; Marutha Ravindran et al. 2007; Matthews et al. 1998)
α5	mRNA	$\uparrow^{a}$	(Charlton et al. 1997)
		$\downarrow^{a}$	(Mhatre and Ticku 1992)
		$\leftrightarrow^{a,c}$	(Devaud et al. 1995; Mahmoudi et al. 1997; Petrie et al. 2001)
	Peptide	$\downarrow^{a}$	(Charlton et al. 1997)
		$\leftrightarrow^{c}$	(Liang et al. 2007)
α6	mRNA	$\uparrow^{b}$	(Morrow et al. 1992)
		$\leftrightarrow^{b}$	(Petrie et al. 2001)
	Peptide	$\uparrow^{d}$	(Marutha Ravindran et al. 2007)
β1	mRNA	$\uparrow^{a,b}$	(Mhatre and Ticku 1992, 1994a)
		$\leftrightarrow^{a}$	(Devaud et al. 1995)

Subunit		Alteration	Reference
β2	mRNA	↑[a]	(Mhatre and Ticku 1994a)
		↔[a]	(Devaud et al. 1995)
	Peptide	↑[a,b]	(Devaud et al. 1997; Marutha Ravindran et al. 2007)
		↔[c]	(Marutha Ravindran et al. 2007; Matthews et al. 1998)
β3	mRNA	↑[a]	(Mhatre and Ticku 1994a)
		↔[a]	(Devaud et al. 1995)
	Peptide	↑[a]	(Devaud et al. 1997)
		↔[c]	(Matthews et al. 1998)
γ1	mRNA	↑[a,c]	(Cagetti et al. 2003; Devaud et al. 1995)
		↔[c]	(Petrie et al. 2001)
	Peptide	↑[a]	(Devaud et al. 1997)
γ2	mRNA	↔[b,c]	(Petrie et al. 2001)
	Peptide	↑[c]	(Cagetti et al. 2003; Liang et al. 2007; Marutha Ravindran et al. 2007)
		↔[a,b,c]	(Devaud et al. 1997; Marutha Ravindran et al. 2007; Matthews et al. 1998)
γ2S	mRNA	↑[a,c]	(Cagetti et al. 2003; Devaud et al. 1995)
γ2L	mRNA	↔[a,c]	(Cagetti et al. 2003; Devaud et al. 1995)
γ3	mRNA	↔[a]	(Devaud et al. 1995)
δ	mRNA	↔[a,c]	(Devaud et al. 1995; Petrie et al. 2001)
	Peptide	↓[c]	(Cagetti et al. 2003; Liang et al. 2007)

↑ Increased; ↓ decreased; ↔ no change

[a] Cerebral cortex

[b] Cerebellum

[c] Hippocampus