

## Review

GABA<sub>A</sub> receptors and alcohol

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## ARTICLE INFO

Available online 14 March 2008

## Keywords:

Alcohol  
GABA  
Receptor

## ABSTRACT

There is substantial evidence that GABAergic neurotransmission is important for many behavioral actions of ethanol and there are reports spanning more than 30 years of literature showing that low to moderate (3–30 mM) concentrations of ethanol enhance GABAergic neurotransmission. A key question is which GABA receptor subunits are sensitive to low concentrations of ethanol in vivo and in vitro. Recent evidence points to a role for extrasynaptic receptors. Another question is which behavioral actions of alcohol result from enhancement of GABAergic neurotransmission. Some clues are beginning to emerge from studies of knock-out and knock-in mice and from genetic analysis of human alcoholics. These approaches are converging on a role for GABAergic actions in regulating alcohol consumption and, perhaps, the development of alcoholism.

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## 1. Introduction

Despite the fact that alcohol has been used and misused for hundreds of years, the mechanism of action of this simple molecule remains the subject of study. Alcohol use results in diverse behavioral effects, including intoxication, cognitive impairment, motor incoordination, tolerance and dependence, and these effects are likely due to its actions on multiple brain proteins (Davies, 2003; Folesa et al., 2006; Harris, 1999; Krystal et al., 2006). One of the most likely targets of ethanol in the central nervous system (CNS) is the GABA<sub>A</sub> receptor, a member of the ligand-gated ion channel superfamily of receptors. Gamma-aminobutyric acid (GABA), the neurotransmitter that activates GABA<sub>A</sub> receptors, is the major inhibitory neurotransmitter in the adult CNS (Barnard et al., 1998). In an adult neuron, activation of GABA<sub>A</sub> receptors by GABA results in an influx of chloride ions, which results in hyperpolarization of the cell. Along with alcohol, GABA<sub>A</sub> receptors are a

target for benzodiazepines, barbiturates, neurosteroids and volatile and intravenous anesthetics. These drugs enhance GABA<sub>A</sub> receptor function to cause anesthesia, sedation, hypnosis and anxiolysis.

There are three types of GABA receptors. GABA<sub>A</sub> and GABA<sub>C</sub> receptors are ligand-gated, while GABA<sub>B</sub> receptors are G protein-coupled receptors. The ionotropic GABA<sub>A</sub> and GABA<sub>C</sub> receptors are composed of five subunits, which surround a central chloride pore. GABA receptors subunits are heterogeneous, allowing for tremendous receptor diversity. GABA<sub>A</sub> receptor subunits include  $\alpha 1$ – $\alpha 6$ ,  $\beta 1$ – $\beta 3$ ,  $\gamma 1$ – $\gamma 3$ ,  $\delta$ ,  $\epsilon$ ,  $\theta$ , and  $\pi$ , and GABA<sub>C</sub> receptors are composed of the  $\rho 1$ – $\rho 3$  subunits. The most common CNS GABA<sub>A</sub> receptor composition is  $\alpha 1\beta 2\gamma 2s$ , consisting of two  $\alpha 1$ , two  $\beta 2$  and one  $\gamma 2s$  subunit. Detailed overviews of GABA<sub>A</sub> receptor structure, diversity, post-translational processing and modifications, associated diseases, pharmacology, distribution and biophysical properties have been reviewed previously (Ashcroft, 2000; Burt and Kamatchi, 1991; Macdonald and Olsen, 1994; Olsen and Tobin, 1990; Tyndale et al., 1995; Whiting et al., 1999).

GABA<sub>A</sub> receptors were first examined as a target of alcohol action in the 1980s. Drugs that increase GABAergic function, such as uptake inhibitors and GABA agonists, enhance the behavioral actions of ethanol. Meanwhile, drugs that decrease GABAergic function, such as

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receptor antagonists and synthesis inhibitors, reduce ethanol behaviors. Selectively bred long-sleep and short-sleep mice that differ in genetic sensitivity to ethanol were also found to differ in their behavioral sensitivities to GABAergic drugs (Martz et al., 1983). Intoxicating concentrations (5–50 mM) of ethanol were shown to enhance the function of GABA<sub>A</sub> receptors using chloride flux assays (Allan and Harris, 1986; Suzdak et al., 1986; Ticku et al., 1986). This literature is discussed in detail in reviews (Deitrich et al., 1989; Harris, 1999; Mihic and Harris, 1995).

Electrophysiological studies have measured ethanol potentiation in primary cultures including rat dorsal root ganglion neurons (Nakahiro et al., 1991; Nishio and Narahashi, 1990) and chick embryo cerebral cortical neurons (Reynolds and Prasad, 1991). Ethanol, at concentrations ranging from 1 to 50 mM, potentiated GABA<sub>A</sub> responses of acutely dissociated neurons from rat neocortical slices and primary neuronal cultures from chick, mouse and rat brain (Reynolds et al., 1992). GABA<sub>A</sub>-activated chloride currents were also shown to be potentiated by ethanol in cultured mouse hippocampal and cortical neurons (Aguayo, 1990; Reynolds and Prasad, 1991). At the single channel level, ethanol enhanced the frequency of GABA-mediated channel opening events, mean open time, open time percentage, frequency of opening bursts, and mean burst duration (Tatebayashi et al., 1998). Studies of ethanol modulation of GABAergic neurotransmission in slice recordings have been recently reviewed (Weiner and Valenzuela, 2006).

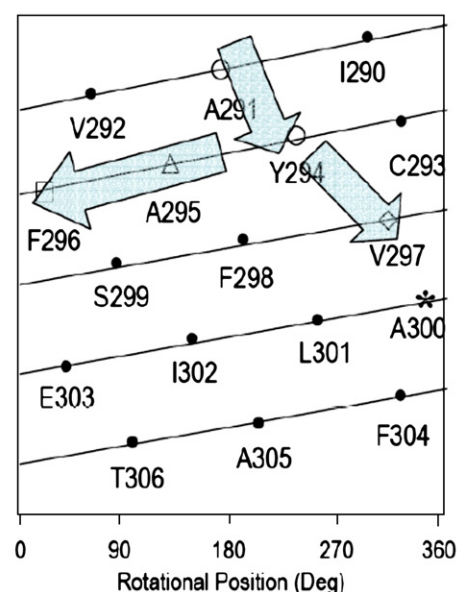
## 2. Alcohol binding sites

Like GABA<sub>A</sub> receptors, glycine receptor currents are enhanced by volatile anesthetics and alcohols (Mascia et al., 1996a,b). Glycine receptors are a simpler model for study, since the  $\alpha 1$  subunit can be homomERICALLY expressed in *Xenopus laevis* oocytes with properties similar to heteromeric receptors (Taleb and Betz, 1994). Although GABA  $\rho 1$  receptors are evolutionarily related to glycine and GABA<sub>A</sub> receptors, homomeric  $\rho 1$  receptors are inhibited by ethanol (Mihic and Harris, 1996). Taking advantage of this difference in pharmacology and the significant sequence homology between the two receptors, chimeric receptor constructs were created that combined sections of the GABA  $\rho 1$  and glycine receptors. These chimeras were used to identify a small region of amino acids required for enhancement of GABA<sub>A</sub> and glycine receptor function (Mihic et al., 1997). Two amino acids in the GABA<sub>A</sub> receptor transmembrane segments (TM) 2 (S270) and 3 (A291) were shown to be critical for allosteric modulation of GABA and glycine receptors by alcohols and volatile anesthetics (Mihic et al., 1997). Replacing either of these two amino acids with the aligned amino acid from the GABA  $\rho 1$  receptor resulted in loss of ethanol potentiation (Mihic et al., 1997). This study provided critical evidence that alcohols and volatile anesthetics specifically acted upon ion channels to alter channel function. Mutation of the aligned TM2 site in the  $\beta 1$  subunit also diminished the ethanol potentiation of GABA<sub>A</sub> receptors (Ueno et al., 1999).

The potency of alcohols increases with carbon chain length, up to a “cut-off” point when drug potency no longer increases. The amino acid volumes at the TM2 and TM3 positions are able to affect the alcohol cut-off in glycine receptors and GABA  $\rho 1$  receptors, which indicates that the binding pocket has a finite size (Wick et al., 1998). While substitutions of smaller amino acids increases the cut-off point to include longer chain alcohols, substitutions of these critical residues with larger amino acids lowers the cut-off size, indicating that the binding cavity is smaller (Wick et al., 1998). Mutation of TM2 and TM3 amino acids to larger amino acids in  $\alpha 2$  and  $\beta 1$  subunits resulted in constitutive GABA<sub>A</sub> receptor activity (Findlay et al., 2000; Ueno et al., 2000, 1999). Using volatile anesthetics of different shapes and molecular sizes together with mutagenesis to manipulate amino acids side chain length, the volume of the alcohol and volatile anesthetic binding site in the GABA<sub>A</sub> receptor was estimated to be between 250 and 370 Å<sup>3</sup> (Jenkins et al.,

2001). Additionally, a third amino acid in TM1, L232, was shown to contribute to the boundary of the amphipathic drug binding cavity in the GABA<sub>A</sub> receptor (Jenkins et al., 2001).

The substituted cysteine accessibility method (SCAM) couples site-directed mutagenesis and biochemical probing with methanethiosulfonate (MTS) reagents in a recombinant expression system (Karlin and Akabas, 1998). Specific positions in a receptor can be mutated to cysteine and probed with MTS reagents. Reaction with a substituted cysteine occurs in the presence of water when the cysteine is ionized. MTS reagents can be used as tools to explore changes in the local environment of specific positions in receptors under different conditions. For example, the SCAM can be used to identify movements of amino acids in the presence and absence of agonist or drug molecules. In electrophysiological experiments, reaction is measured by a change in current after exposure to MTS reagents. The key question of whether S270 was important to the action of alcohols and anesthetics and part of a binding pocket in the GABA<sub>A</sub> receptor was addressed using MTS reagents. The S270C mutant was covalently labeled by either an alkane thiol anesthetic or varieties of methanethiosulfonate compounds, and receptor function was irreversibly enhanced (Mascia et al., 2000). The usual ability of octanol, enflurane and isoflurane to enhance the receptor function was lost following upon occupation of the site, indicating that the action of alcohols and anesthetics stems from binding at a single binding pocket (Mascia et al., 2000). Using the SCAM, the GABA<sub>A</sub> receptor amino acid, A291, was shown to be surrounded by a water-filled cavity, which expanded in the presence of alcohol (Jung et al., 2005), shown in Fig. 1. This position was demonstrated to be a critical site for alcohol binding and alcohol-induced conformational changes, since



- Reactive with MTS in the resting-, alcohol- and GABA-bound states
- △ Reactive with MTS in the alcohol-bound states
- Reactive with MTS in the alcohol- and GABA-bound states
- ◇ Reactive with MTS in the GABA-bound states
- No effect

**Fig. 1.** A helical net representation illustrating the change in GABA-induced currents before and after treatment of MTS reagents on TM3 single Cys substitution mutants in the absence and presence of GABA or alcohols. I290 is positioned at the extracellular end, and T306 is the most intracellular cysteine mutant that was tested in TM3. Each symbol indicates MTS reactivity for the different functional states. The arrows illustrate the expansion of the water-filled cavity. This figure was previously published (Jung, 2005).

mutation of the site prevented alcohol-induced conformational changes from occurring (Jung and Harris, 2006).

Further discussion of the effects of alcohol action on GABA receptors composed of defined subunits can be found in the following review articles (Mihic, 1999; Yamakura et al., 2001). While the GABA<sub>A</sub> receptor  $\alpha 1$ ,  $\alpha 2$ ,  $\rho 1$  and  $\beta 1$  subunits have been the subject of study, many other subunit combinations have only recently been tested for their ethanol responses. For example, it was reported that  $\alpha 4\beta 2\delta$  GABA<sub>A</sub> receptors expressed in *Xenopus* oocytes are very sensitive to alcohol, with a concentration of 1 mM ethanol significantly enhancing GABAergic currents (Sundstrom-Poromaa et al., 2002). Both  $\alpha 6$  and  $\alpha 4$ , when combined with  $\beta 3$  and  $\delta$ -containing subunits were associated with ethanol enhancement of function, which showed a threshold sensitivity at 3 mM and a progressive increase up to 300 mM (Wallner et al., 2003, 2006a). The  $\alpha 4\beta 2\delta$  GABA<sub>A</sub> receptors shown to be sensitive to 1 mM ethanol by Sundstrom-Poromaa et al. (2002) were not sensitive to concentrations of ethanol below 30 mM in the Wallner et al. (2003) study. In addition, other investigators were not able to obtain effects of low (1–30 mM) concentrations of ethanol on these receptors (Borghese et al., 2006a; Yamashita et al., 2006). Because GABA receptors containing the  $\delta$  subunit are found in the extrasynaptic regions and are responsible for tonic inhibition, a model has emerged with ethanol selectively enhancing tonic, rather than synaptic, GABAergic transmission. However, as noted above, this model is controversial and a detailed discussion of the issues is beyond the scope of this review, but differences in results among laboratories was addressed by eight research groups in a special issue of *Alcohol* in 2007 (Volume 41).

The studies of putative alcohol binding sites between transmembrane regions discussed above used large concentrations of ethanol (50–200 mM). A key question is whether GABA receptors have other sites of action for low concentrations (e.g., 3–20 mM) of ethanol. Recent studies proposed that ethanol competes with a benzodiazepine, RO 15-4513, for binding to GABA receptors containing the  $\delta$  subunits and that this represents a novel, high affinity ethanol binding site (Wallner et al., 2006b; Hancher et al., 2006). However, Korpi et al. (2007) and Mehta et al. (2007) were not able to confirm this finding. Thus, the mechanism for actions of low concentrations of ethanol on GABA receptors remains controversial.

GABA<sub>A</sub> receptor subunit combinations have not been exhausted, and there may be more to learn about ethanol action on the GABAergic system by testing additional subunit combinations, including the  $\pi$ ,  $\epsilon$ ,  $\gamma 1$ ,  $\gamma 3$ ,  $\theta$ , or  $\rho 1$ – $3$  subunits. For instance, the  $\rho$  subunits, which are expressed in the brain and spinal cord, may coassemble with other subunits (Pan and Qian, 2005); however, to date the  $\rho 1$  subunit has only been studied when expressed homomERICALLY. Although, the untested receptor subtypes may be rare, or at least restricted to specific brain regions, experiments on new subtypes could provide new insight into ethanol action on GABA<sub>A</sub> receptors.

### 3. Transgenic, knock-out and knock-in mouse models

Transgenic and knock-out (null mutant) mice have been developed and used to study how GABA<sub>A</sub> receptor subunit composition influences the pharmacological and behavioral effects of ethanol and other drugs. Thus far, mice have been developed that individually knock-out the  $\alpha 1$ ,  $\alpha 2$ ,  $\alpha 5$ ,  $\alpha 6$ ,  $\beta 2$ ,  $\beta 3$ ,  $\gamma 2S+L$ ,  $\gamma 2L$  and  $\delta$  GABA<sub>A</sub> receptor subunits. These null mutant mice have been examined to identify subunit-specific changes in the behavioral effects of alcohol and understand the role of specific proteins in drug action. Additionally, transgenic lines of mice that overexpress either the  $\gamma 2L$  or the  $\gamma 2S$  subunits have been developed. These actions of ethanol of GABA<sub>A</sub> receptor subunit knock-out and transgenic mice were recently reviewed (Boehm et al., 2004) and Crabbe et al. (2006) recently published an extensive review on the genetic modifications of 93 alcohol-related genes in mice, including gene overexpression, gene

knock-outs and gene knock-ins. It is difficult to draw generalized conclusions from all the GABA knock-out mice because the same behaviors are not measured in all mutants, but the most pervasive finding is that knock-out of GABA receptor subunits decreases alcohol consumption in the continuous two-bottle choice test (see Crabbe et al., 2006). However, it is important to note that genetic deletion of key neuronal proteins, such as GABA receptors, may lead to compensatory changes in gene expression and brain function. The adaptive changes have been studied for the GABA  $\alpha 1$  subunit knock-out mice and found to be quite extensive (Ponomarev et al., 2006).

Knock-in mice are potentially more powerful than knock-out mice because they allow for examination of specific point mutations that alter only a single aspect of receptor function. Meanwhile, the mutated receptor functions normally in all other aspects, and compensation effects of other receptors and subunits are less likely to occur. Since GABA<sub>A</sub> receptors have multiple subunits and subtypes, this technique is particularly useful in studying the effects of a drug on a specific receptor subunit.

As an example, the use of knock-in mice has led to exciting developments in the understanding of benzodiazepine action on GABA<sub>A</sub> receptors in the CNS. Using mutagenesis and a recombinant expression system, Wieland et al. (1992) identified a single histidine residue in the GABA<sub>A</sub> receptor  $\alpha 1$  subunit at position 101 was critical for high affinity binding of benzodiazepines. While the  $\alpha 1$  subunit conferred high affinity benzodiazepine binding when co-expressed with  $\beta 2$  and  $\gamma 2$  subunits,  $\alpha 6\beta 2\gamma 2$  receptors showed negligible benzodiazepine binding affinity (Wieland et al., 1992). When  $\alpha 1(H101)$  was replaced with arginine, the residue present at the aligned site in  $\alpha 6$  subunit, the  $\alpha 1(H101R)$  decreased the binding affinity for benzodiazepines. Stemming from this study, knock-in mice were created that mutated the histidine to arginine at position 101 in the  $\alpha 1$  subunit, which abolished the amnestic and sedative properties of diazepam (Rudolph et al., 1999; Valenzuela et al., 1995) and partially removed the anticonvulsant properties of the drug (Rudolph et al., 1999). Meanwhile, the  $\alpha 1(H101R)$  mice had normal GABA responses and showed no change in the anxiolytic, myorelaxant, motor-impairing and ethanol-potentiating effects when compared to the wild-type (Rudolph et al., 1999). Ethanol potentiation of the GABA<sub>A</sub> receptor is therefore due to action at a different site on the subunit, or a non-mutated receptor subunit. Following these studies, the  $\alpha 2$  subunit was shown to be responsible for the anxiolytic properties of benzodiazepines, since the anxiolytic action of diazepam was absent in  $\alpha 2(H101R)$  knock-in mice (Vicini et al., 2001). There is also evidence that the  $\alpha 3$  subunit is sufficient to be responsible for the anxiolytic properties of benzodiazepines (Atack et al., 2005; Dias et al., 2005). The  $\alpha 2(H101R)$  knock-in mouse was also used to show that the  $\alpha 2$  subunit is mainly responsible for the myorelaxant activity of diazepam, with the  $\alpha 3$  subunit possibly playing a role in response to high doses of diazepam (Jurd et al., 2003). Furthermore,  $\alpha 5(H105R)$  mice failed to display any sedative tolerance to diazepam (van Rijnsoever et al., 2004). This use of mutagenesis and expression studies culminated in the design of knock-in mice and has led new understandings of pharmacology based upon specificity for GABA<sub>A</sub> receptor subunits and subtypes.

A similar approach is being used to study and understand the effects of alcohol on GABA<sub>A</sub> receptors, as well as other ligand-gated ion channels. Thus far, two viable knock-in GABA<sub>A</sub> receptor mutant mouse lines have been produced. Following identification of S270 as a critical mediator of anesthetic and alcohol action (Mihic et al., 1997), a number of other mutations in the GABA<sub>A</sub> receptor  $\alpha 2$  subunit at S270 were shown to decreased alcohol potentiation and increase sensitivity to GABA (Findlay et al., 2001; Ueno et al., 1999). A gain-of-function GABA<sub>A</sub> receptor  $\alpha 1(S270H)$  mutant mouse line was developed, and while they were resistant to the anesthetic isoflurane, the mice displayed synaptic and behavioral abnormalities (Homanics et al., 2005).

In order to create a mouse with GABA sensitivity like the wild-type receptor, a second mutation was introduced in the  $\alpha 1$  subunit at L277A. The  $\alpha 1(S270H/L277A)$  double mutant had near-normal GABA sensitivity



when expressed in HEK cells and in *Xenopus laevis* oocytes, but showed a faster deactivation in HEK293 cells (Borghese et al., 2006b). Potentiation by isoflurane and ethanol was greatly decreased in the S270H/L277A receptors. A viable, homozygous  $\alpha 1$ (S270H/L277A) knock-in mouse line was manufactured, which showed no overt abnormalities, except hyperactivity (Borghese et al., 2006b). The mutant receptors were less sensitive to ethanol potentiation in hippocampal slice recordings (Werner et al., 2006). Mutant  $\alpha 1$ (S270H/L277A) mice showed more rapid recovery from the motor-impairing effects of ethanol and displayed increased anxiolytic effects of ethanol in comparison to wild-type mice (Werner et al., 2006). Meanwhile, there were no differences in ethanol-induced hypnosis, locomotor stimulation or cognitive impairment. Ethanol preference and consumption were comparable to the wild-type mice (Werner et al., 2006).

This study demonstrates that GABAergic synapses containing the  $\alpha 1$  subunit are important for specific ethanol-induced behavioral effects. Since there are multiple GABA<sub>A</sub> receptor subtypes, different subunits may account for distinct ethanol-induced behavioral effects in the same manner as for benzodiazepine action. Individual GABA<sub>A</sub> receptor subunits have not yet been definitively linked with specific behavioral actions. Coupled with the knowledge derived from knock-out studies of different GABA<sub>A</sub> receptor subunits, future studies on knock-in mice may provide additional understanding of ethanol action on these receptors.

#### 4. Genetic linkage

A number of research groups, including the Collaborative Study on the Genetics of Alcoholism (COGA), have studied human allelic variation and have collected detailed phenotypic data on individuals in families with multiple alcoholics in order to identify genes that increase the risk for alcoholism. Several GABA receptor clusters have emerged from these studies. The COGA, and other groups, identified a region of chromosome 4p that was associated with alcoholism, which includes a cluster of four GABA<sub>A</sub> receptor subunit-encoding genes for the  $\gamma 1$ ,  $\alpha 2$ ,  $\alpha 4$  and  $\beta 1$  subunits (Reich, 1996; Reich et al., 1998). A second cluster of GABA<sub>A</sub> receptor genes on chromosome 5 have also been associated with alcoholism. This GABA<sub>A</sub> receptor cluster encodes genes for the  $\beta 2$ ,  $\alpha 6$ ,  $\alpha 1$ , and  $\gamma 2$  subunits. The third GABA<sub>A</sub> receptor gene cluster on chromosome 15q is also associated with alcoholism. This cluster contains the genes for the GABA<sub>A</sub> receptor  $\alpha 5$ ,  $\beta 3$  and  $\gamma 3$  subunits (Dick et al., 2004). These results are reviewed in detail in an accompanying article.

#### 5. Conclusions

There is substantial evidence that GABAergic neurotransmission is important for many behavioral actions of ethanol, but several key questions remain. As noted above, there are reports spanning more than 30 years of literature showing that moderate (3–30 mM) concentrations of ethanol enhance GABAergic neurotransmission. However, there are also many reports showing no effect of moderate or even large concentrations of ethanol in vivo and in vitro. Thus, a key question is whether there are specific GABA receptor subunits that are sensitive to low concentrations of ethanol in vivo and in vitro. Another possibility (for which there is also evidence – Weiner and Valenzuela, 2006) is that ethanol acts presynaptically to increase the release of GABA, and certain receptors (e.g., extrasynaptic) are particularly sensitive to this spillover of GABA resulting in enhanced GABAergic function. Another key question is which behavioral actions of alcohol require enhanced GABAergic neurotransmission? Some clues are beginning to emerge from studies of knock-out and knock-in mice and, intriguingly, from genetic analysis of human alcoholics. Both of these approaches point to a role for GABAergic actions of alcohol in regulating consumption and, perhaps, the development of alcoholism. Given these results, is it feasible to target this system to treat alcoholism? Medications that block

the GABA<sub>A</sub> receptor are riddled with unwanted and severe side effects, such as convulsions. In order to therapeutically target the GABA<sub>A</sub> receptor safely and effectively, it is necessary to have a better understanding of receptor function, presynaptic and postsynaptic ethanol effects, the alcohol binding site and the subunit-specific effects of alcohol.

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