ALCOHOL MODULATION OF $GABA_A$ RECEPTORS: SUBUNIT REQUIREMENTS AND STRUCTURE-ACTIVITY RELATIONSHIPS

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by

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ABSTRACT

Alcohols are believed to exert their behavioural effects at least in part by a selective enhancement of γ-aminobutyric acid type A ($GABA_A$) receptor activity. However, the mechanism(s) by which ethanol and other alcohols facilitate $GABA_A$ receptor function have remained elusive. Historically, alcohols have been thought to act by partitioning into membrane lipids to cause changes in the function of membrane proteins. However, more recent evidence suggests that alcohols interact selectively with hydrophobic regions of specific membrane proteins or phospholipids to alter their function. If a drug-protein interaction is involved in the effects of alcohols on $GABA_A$ receptor function, it was hypothesized that stereoisomers of a simple straight-chain alcohol might show differential effects on receptor function. Alternatively, there may be differences in the effects of closely related alcohols that are not predicted by differences in lipid solubility, and/or a significant influence of the subunit composition of the $GABA_A$ receptor itself. To test these hypotheses concerning the mechanisms of action of alcohols, cloned subunits of human $GABA_A$ receptors were expressed in *Xenopus laevis* oocytes, and the two-electrode voltage-clamp technique was used to quantify the membrane current response to GABA in the presence and absence of different alcohols.

1-butanol and 2-butanol differentially potentiated GABA responses in both $\alpha_1\beta_2\gamma_{2L}$ and $\alpha_2\beta_2\gamma_{2L}$ receptor constructs, with 2-butanol being significantly more potent than 1-butanol as a modulator of $GABA_A$ receptor function. However, 2-butanol has a lower membrane/buffer partition coefficient, and is therefore less lipid soluble than 1-butanol.

Thus, the structure of the alcohol, rather than simple lipid solubility, was more important as a predictor of modulation of $GABA_A$ receptor function. In contrast, the stereoisomers (R)-2-butanol and (S)-2-butanol did not differ in their modulation of receptor function. 1-butanol and 2-butanol were also significantly more potent as modulators at $\alpha_2\beta_2\gamma_{2L}$ compared to $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptor constructs. Thus, the subunit composition of the receptor protein also influences modulation of receptor function by alcohols. The γ_{2L} subunit, which is reported to be necessary for ethanol to potentiate $GABA_A$ receptor activity, was not required for 1-butanol or 2-butanol to increase GABA-mediated responses. Low concentrations (20 mM) of ethanol potentiated $GABA_A$ receptor function at $\alpha_1\beta_2\gamma_{2L}$ receptors. Ethanol potentiation of $GABA_A$ receptor function was blocked by 0.5 μM Ro15-4513, a benzodiazepine receptor partial inverse agonist. However, Ro15-4513 at concentrations up to 5 μM did not block potentiation of $GABA_A$ receptor activity by butanol, heptanol, or the non-volatile anesthetic propofol.

Differential effects of alcohols on $GABA_A$ receptor function in response to changes in subunit composition or structure of the alcohol suggest specific interactions of these agents with the receptor complex. In addition, it appears that longer-chain ($\geq$ 4 carbons) alcohols act at a different site and/or induce a different conformational change in the receptor compared to ethanol. In conclusion, these data support the idea that alcohols have specific interactions with the $GABA_A$ receptor, rather than simply disordering membrane lipids. In addition, there may be at least two distinct mechanisms and/or sites by which alcohols can act to modulate activity at the $GABA_A$ receptor.

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1.1. $GABA_A$ receptor/chloride channel complex

γ-aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the mammalian central nervous system (CNS) (Tanelian *et al.*, 1993). There are two classes of GABA receptors, namely $GABA_A$ receptors and $GABA_B$ receptors. $GABA_A$ receptors are ligand-gated ion channels and activation of these receptors by GABA is responsible for fast inhibitory postsynaptic potentials. $GABA_B$ receptors are coupled to calcium or potassium channels via G-proteins and mediate slower and longer lasting synaptic inhibition. The $GABA_A$ receptor is the most widely studied of the two classes of receptor families and appears to be a primary target for the action of sedatives and anesthetics, including barbiturates, benzodiazepines and volatile general anesthetics (Macdonald *et al.*, 1985; Mody *et al.*, 1991; Nakahiro *et al.*, 1989; Twyman *et al.*, 1989). Tanelian *et al.* (1993) extensively reviewed $GABA_A$ receptors as a site for anesthetic drug action, including ethanol. As well, specific classes of neurosteroids have been found to positively and negatively modulate $GABA_A$ receptor function (Zaman *et al.*, 1992; reviewed in Majewska, 1992).

1.1.1. $GABA_A$ receptor structure

The $GABA_A$ receptor/chloride ion channel complex is a heteropentameric glycoprotein of about 275 kDa in size (Macdonald and Olsen, 1994). It is made up of

different combinations of five glycoprotein subunits which come together to form the ion channel complex. Binding affinities and channel kinetics are determined by the subunit composition of the receptor (Tanelian *et al.*, 1993). The exact subunit composition and/or stoichiometry of native $GABA_A$ receptors is not known at this time.

More than 15 $GABA_A$ receptor subunits have been characterized by molecular cloning and these subunits have been separated into five families (α, β, γ, δ and ρ). There is 30-40% sequence homology among the five families. Each subunit family has multiple subtypes with about 70-80% amino acid sequence identity among them. These subtypes have been designated $\alpha_{1\text{-}6}$, $\beta_{1\text{-}4}$, $\gamma_{1\text{-}3}$, δ and $\rho_{1\text{-}2}$ (Macdonald and Olsen, 1994). Two of the subunit subtypes, γ_2 and β_4, have RNA splice variants to create further diversity. The γ_{2L} splice variant of the γ_2 subunit contains an eight amino acid insert, which is not present on γ_{2S}, that has a consensus sequence for phosphorylation by protein kinase C. This insert, along with receptor phosphorylation, may be necessary for ethanol modulation of $GABA_A$ receptor function (Wafford and Whiting, 1992).

Each subunit subtype consists of an amino-terminal on the extracellular surface, with putative N-glycosylation sites, four transmembrane-spanning regions (M1-M4), and an intracellular cytoplasmic loop between M3 and M4 which can be modified by phosphorylation (Macdonald and Olsen, 1994). M2 is thought to line the inside of the channel where Cl^- selectively passes through the opening (Tanelian *et al.*, 1993).

It is likely that different combinations of subunit subtypes exist in different populations of neurons. For example, using *in situ* hybridization techniques, α, β, γ and

δ mRNAs show different regional distributions and are sometimes altered during different stages of development. There are also species differences in the distribution of $GABA_A$ receptor subunits in different regions of the brain (Tanelian *et al.*, 1993). While there is evidence for particular $GABA_A$ receptor subunits coming together to form functional $GABA_A$ receptors *in vivo*, the exact stoichiometry of these subunits is not known. With the use of molecular biological and electrophysiological techniques, various $GABA_A$ receptor subunit combinations have been expressed in *Xenopus* oocytes and other non-neuronal cell lines to investigate the physiological and pharmacological properties of different receptor subunit assemblies.

1.1.2. $GABA_A$ receptor isoforms in the brain

Single, double or triple combinations of $GABA_A$ receptor subunits can produce different functional $GABA_A$ isoforms (Macdonald and Olsen, 1994). *In situ* hybridization techniques can be used to localize $GABA_A$ receptor gene products or mRNA, while $GABA_A$ subtype-specific antibodies can also be used to determine $GABA_A$ subunit subtype composition. Miralles *et al.* (1994) mapped out the distribution of γ_{2S} and γ_{2L} mRNAs and proteins in the rat brain using both these techniques. They showed that these two splice variants of the γ_2 subtype show both different and overlapping distribution patterns in the rat brain. Similarly, Gutiérrez *et al.* (1994) found colocalization of γ_{2S} and γ_{2L} subunits in some brain areas (eg. molecular layer of the cerebellum) and differential distribution patterns in others (eg. hippocampus and cerebellar Purkinje cells). The

functional significance of this is not known. Poulter *et al.* (1993) suggest that γ_{2S} is the predominant embryonic γ_2 subunit isoform and that $GABA_A$ receptor activation may be important during neurodifferentiation and synaptogenesis.

Several groups have shown, using subunit-specific antibodies, that α_1, β_2 and γ_2 frequently co-localize in the same receptor complex throughout the brain (Miralles *et al.*, 1994). Gao and Fritschy (1994) demonstrated that staining for the α_1 subunit corresponds to the presence of $GABA_A$ receptors. They reported that this is supported by biochemical and immunohistochemical evidence that there is a frequent association of the α_1 subunit with the β_2, β_3 and γ_2 subunits in native $GABA_A$ receptors. Fritschy *et al.* (1994) showed that $GABA_A$ receptors present in neonatal and adult brain differ in subunit composition, with $\alpha_2\beta_{2,3}\gamma_2$ probably being expressed by most neurons in neonatal brain and $\alpha_1\beta_{2,3}\gamma_2$ being most prevalent in adult brain. They suggest replacement of $GABA_A$ receptors takes place in a majority of neurons during development. Persohn *et al.* (1992) point out that α_1, β_2 and γ_2 transcripts were the most abundant and ubiquitous in the rat brain and that a combination of these three subunits is likely to code for native receptors in rat brain. According to Wisden *et al.* (1992), α_1 and β_2 mRNAs are the most widely codistributed in the brain and γ_2 mRNA often colocalizes with them. They also state that "classical" $GABA_A$ electrophysiological responses can be demonstrated with this triplet combination. They point out that expression studies on recombinant receptors show that the α subunit is responsible for the major pharmacological differences with respect to benzodiazepine binding on these $\alpha\beta\gamma$ combinations, but that the γ_2 subunit is required for potentiation

of GABA by benzodiazepines. Finally, Laurie *et al.* (1992) concluded from their studies on the cerebellum and olfactory bulb that $\alpha_1\beta_2\gamma_2$ was a prominent receptor isoform in these brain regions. The receptor isoforms that I will be concentrating on, $\alpha_1\beta_2\gamma_{2L}$, $\alpha_2\beta_2\gamma_{2L}$ and $\alpha_2\beta_2$, are putatively major isoforms which occur in the CNS. Any effect of alcohols on them would be significant for brain function.

1.2. Pharmacological modulation of $GABA_A$ receptor function

The $GABA_A$ receptor contains specific binding sites for GABA, barbiturates, benzodiazepines, and the anesthetic steroids as well as picrotoxin (Macdonald and Olsen, 1994). GABA concentration-response curves are sigmoidal and generally have Hill coefficients of about two, suggesting that two GABA molecules must bind for full activation of the receptor. $GABA_A$ receptors are not only activated by GABA, but also by structural analogs of GABA including muscimol, 4,5,6,7-tetrahydroisoxazolopyridin-3-ol (THIP) and isoguvacine (Macdonald and Olsen, 1994). The competitive antagonist, bicuculline, is selective for the $GABA_A$ receptor. Picrotoxin, a non-competitive $GABA_A$ receptor antagonist, acts by blocking the Cl^- channel. $GABA_A$ receptor channel modulation by GABA and other anesthetic compounds has been reviewed by Tanelian *et al.* (1993).

Sedative-hypnotic and anesthetic drugs can affect GABAergic inhibition in a number of ways. Some directly activate the receptor, some enhance GABA binding to the receptor, while others act indirectly to influence opening of the Cl^- channel.

Anesthetics may act by more than one mechanism. Much of the evidence for the involvement of the $GABA_A$ receptor channel complex in sedation and hypnosis comes from genetic studies. For example, mice bred for short or long duration of ethanol-induced sleeping time also showed differences in barbiturate and benzodiazepine-induced sleeping time, suggesting that there may be a common site underlying the hypnotic actions of these drugs (Tanelian *et al.*, 1993).

As mentioned earlier, different modulatory agents use different mechanisms to enhance GABAergic inhibition. The benzodiazepines increase binding of endogenously released GABA, whereas barbiturates modify the $GABA_A$ receptor Cl^- channel such that it stays open longer after the binding of GABA. Whereas there are distinct sites of action for benzodiazepines, steroids and barbiturates, it is not clear whether there are distinct binding sites for volatile anesthetics and alcohols. Indeed, the cellular and molecular mechanisms of action of ethanol are not clear and numerous sites of action have been proposed. In addition to the anesthetic compounds mentioned above, $GABA_A$ receptor function can also be regulated by ions, such as Ca^{2+}, as well as phosphorylation of intracellular regulatory sites on the receptor (Tanelian *et al.*, 1993).

1.2.1. GABA and anesthesia

The effects of several distinct classes of anesthetics on $GABA_A$ receptor function was reviewed in some detail by Tanelian *et al.* (1993). It is known that anesthetics can influence GABAergic inhibition by a number of different mechanisms. Some agents

directly activate the receptor, some facilitate binding of GABA to the receptor, some enhance coupling between agonist binding and receptor activation, whereas others directly influence Cl^- channel opening. Many of these anesthetic compounds act by more than one mechanism, and it is necessary to look at multiple mechanisms of action to understand anesthetic effects at the $GABA_A$ receptor-channel complex.

1.2.1.1. Anesthetic modulation of the $GABA_A$ receptor

Mihic *et al.* (1994b) used the *Xenopus* oocyte expression system to study the actions of anesthetics on $GABA_A$ receptors *in vitro* and also provided evidence that the $GABA_A$ receptor complex is a major mediator of anesthetic action *in vivo*. Mihic *et al.* (1994a) used anesthetic concentrations of ethanol (50-400 mM) and butanol (1-20 mM) and observed potentiation of $GABA_A$ receptor-activated Cl^- currents (I_{GABA}) in a variety of $GABA_A$ receptor constructs. Propofol is a sedative-hypnotic drug which has become widely used as an intravenous anesthetic for the induction and maintenance of general anesthesia (Smith *et al.*, 1994). Recent electrophysiological studies have provided evidence that clinically relevant concentrations (i.e. 10-50 μM) of the drug can increase inhibition by GABA (Collins, 1988; Hales and Lambert, 1991a; Hales and Lambert, 1991b; Orser *et al.*, 1994; Yamamura *et al.*, 1991). The direct actions of general anesthetics, including propofol, on the $GABA_A$ receptor, were studied by Sanna *et al.* (1995). They expressed a functional homomeric Cl^- channel made up of the β_1 $GABA_A$ receptor subunit in *Xenopus* oocytes. This homomeric recombinant receptor was directly

activated by GABA, propofol and pentobarbital, but was insensitive to bicuculline. Moreover, the steroid anesthetic alphaxalone could not directly activate homomeric β_1 receptors, but did potentiate currents induced by GABA. In addition, they found that propofol and pentobarbital potentiated currents induced by GABA at heteromeric receptors at concentrations that had little or no direct action at these receptors. From their results, they concluded that the potentiation of GABA action produced by propofol, pentobarbital and alphaxalone occurs at a different site than the direct channel activating properties of these anesthetic compounds.

There is also evidence that $GABA_A$ receptors are modulated by volatile anesthetics. Moody *et al.* (1994) demonstrated significant differences in potencies of (+)- and (-)- isoflurane stereoisomers on $GABA_A$ receptor function. They suggested from this, that proteins rather than lipids are the primary sites of anesthetic action. Pharmacologically relevant concentrations of volatile anesthetics act as positive modulators at $GABA_A$ receptors (Moody *et al.* 1994). They increase Cl^- flux and enhance binding of other positive modulators such as benzodiazepine agonists (Nakao *et al.*, 1991; Harris *et al.*, 1993).

Other compounds, including barbiturates, benzodiazepines, and ethanol may have anesthetic effects as a result of modulation of the $GABA_A$ receptor-channel complex. Tanelian *et al.* (1993) conclude from their review that actions on the $GABA_A$ receptor complex account for the dominant CNS depressant effects of several of these distinct classes of anesthetics.

1.2.1.2. Ethanol modulation of the $GABA_A$ receptor

There are a number of inconsistencies in the literature concerning the action of ethanol on $GABA_A$ receptor function and several groups have attempted to determine the actual site(s) and mechanism(s) of action of this compound. Studies have been carried out *in vivo* and *in vitro* using a variety of behavioural/genetic, neurochemical, and electrophysiological techniques.

Suzdak *et al.* (1986) demonstrated that ethanol (20-100 mM) enhanced $GABA_A$ receptor-mediated uptake of Cl^- into isolated brain vesicles or synaptoneurosomes *in vitro*. Similarly, Glowa *et al.* (1989) observed ethanol potentiation of $GABA_A$ receptor-mediated Cl^- ion flux *in vitro* using synaptoneurosomes. They also observed the anxiolytic and intoxicating properties of ethanol *in vivo* and noted that the effects of ethanol *in vitro* occurred at concentrations similar to those associated with ethanol's behavioural effects. In addition, Harris and Allan (1988) demonstrated that low concentrations of ethanol enhance muscimol-dependent Cl^- uptake by brain membranes from mice. The concentration of ethanol that they used (i.e. 10-20 mM) is associated with sedation and mild intoxication *in vivo*. Similarly, Allan *et al.* (1988) found that 10-30 mM ethanol enhanced muscimol-stimulated Cl^- flux in membranes of ethanol-sensitive mice (LS) and rats (HAS). Their results suggested that genetic differences in ethanol sensitivity are related to differences in $GABA_A$ receptor channel sensitivity to ethanol. They did observe a correlation between behavioural effects of ethanol and the effects of ethanol on Cl^- flux in the cerebellum and cortex. In addition, they saw little or no effect

on Cl^- flux with anesthetic ethanol concentrations (50-100 mM), suggesting that the effects of ethanol on the $GABA_A$ receptor are more important at lower concentrations.

Cellular electrophysiology has also been used to study ethanol's effects. From their study using transfected cells from the mouse, Harris *et al.* (1995) suggested the existence of ethanol sensitive and insensitive $GABA_A$ receptors which may differ in subunit composition. They reported that the γ_{2L} subunit was necessary for ethanol to potentiate I_{GABA} and that this potentiation was greatest with 10 mM ethanol. Interestingly, though, there was considerable variation among the cells with respect to their ethanol sensitivity, depending on whether or not the cells were grown on polylysine coverslips. If the cells were grown on coverslips coated with polylysine, ethanol potentiated GABA responses in these cells, while no effect was observed in cells that were grown on uncoated coverslips. They concluded, therefore, that although necessary, the γ_{2L} subunit is not sufficient for ethanol potentiation of I_{GABA}. They proposed that some post-translational modifications may be taking place in the cells which may be important for ethanol sensitivity and that differences in culture conditions or cell cycle could play a part in the observed cell variation.

Other studies have also observed ethanol modulation of $GABA_A$ receptor function. Ethanol (25-200 mM) enhanced I_{GABA} in some cultured mouse neurons from the hippocampus (Aguayo and Pancetti, 1994). Aguayo and Pancetti (1994) also observed potentiation using higher ethanol concentrations (425 and 850 mM). From these results, they suggested that ethanol may be acting at several sites, with low ethanol

concentrations activating a protein such as phosphatase or inactivating a kinase, and higher ethanol concentrations interacting with the membrane lipid domain. Their proposed mechanism of action for ethanol was that ethanol may potentiate I_{GABA} by facilitating dephosphorylation. Low ethanol concentrations (1-50 mM) potentiated I_{GABA} in some of the cells from different brain regions of the chick, mouse, and rat (Reynolds *et al.*, 1992). Potentiation by ethanol of the $GABA_A$ response was greatest when lower concentrations of GABA were used which is consistent with other studies. As well, there was wide variation among animals in the same species with respect to ethanol's ability to potentiate GABA in cells of the cerebral cortex (Reynolds *et al.*, 1992). Palmer *et al.* (1990) studied the effects of ethanol on single human central neurons. They transplanted human cerebral and cerebellar cortical tissue into the anterior eye chamber of rats and allowed it to develop over several months. They identified two populations of neurons, those more ethanol-sensitive and those less sensitive to ethanol, and found that ethanol depressed action potential discharge in a reversible, dose-dependent manner. Ethanol concentrations used were within the range eliciting behavioural signs of intoxication in humans. In rat brain slice preparations, 80 mM ethanol enhanced I_{GABA} in cortex and intermediate lateral and medial septum, but not in CA1 of the hippocampus (Soldo *et al.* 1994). In this study, it was suggested that there is more than one mechanism of action for ethanol potentiation of the $GABA_A$ response. Ethanol may cause a change in Cl^- conductance, alter the sensitivity of the $GABA_A$ receptor such that more channels would be activated by the same amount of GABA, or shift the Cl^- equilibrium potential altering

the amount of current flowing through the ion channel without changing conductance (Soldo *et al.* 1994). In contrast, Weiner *et al.* (1994) found potentiation of synaptic $GABA_A$ responses in hippocampal CA1 neurons. In this study, the effect of ethanol was influenced by G-protein activation. That is, intracellular perfusion with GTPγS, which would activate G-proteins irreversibly, enhanced ethanol potentiation of synaptic $GABA_A$ responses.

Low ethanol concentrations have also been observed to enhance $GABA_A$ responses in a number of studies using the *Xenopus* oocyte expression system. Wafford and Whiting (1992) injected bovine mRNAs for $\alpha_1\beta_1\gamma_{2L}$ and $\alpha_1\beta_1\gamma_{2S}$ receptor combinations into *Xenopus* oocytes and found that ethanol (20 mM) only potentiated receptors containing the γ_{2L} subunit and not those containing γ_{2S}. They went on to alter the eight amino acid consensus phosphorylation sequence contained in the γ_{2L} subunit and eliminated the potentiating effects of ethanol. Since benzodiazepine potentiation was unaffected by any of the mutations, they suggested a specific role of this eight amino acid insert and its consensus phosphorylation site for protein kinase C in ethanol potentiation. They concluded therefore, that phosphorylation of the consensus site on the γ_{2L} subunit is necessary for ethanol potentiation of I_{GABA}. An *in vitro* study carried out in *Xenopus* oocytes using mRNAs from brains of long sleep (LS) and short sleep (SS) mice demonstrated ethanol potentiation of GABA responses in a dose-dependent manner up to 60 mM, in oocytes injected with LS mRNA, but inhibition of GABA responses by ethanol in those injected with SS mRNA (Wafford *et al.* 1990). LS and SS mice are

selectively bred for their marked difference in ethanol sensitivity.

Ethanol potentiation observed in these studies by Wafford and Whiting (1992) and Wafford *et al.* (1990) represents a low-dose effect of ethanol according to Mihic *et al.* (1994a). They proposed a distinct high-dose or anesthetic effect of ethanol as well, and suggested that the two effects of ethanol on the $GABA_A$ receptor likely have different mechanisms of action. They tested anesthetic concentrations of ethanol (50-400 mM) and butanol (1-20 mM) for their effects on I_{GABA} in *Xenopus* oocytes. They did observe greater ethanol potentiation with lower concentrations of GABA and found that the effect of ethanol (100-300 mM) on I_{GABA} was not affected whether the receptor construct contained γ_{2S} or γ_{2L}.

Other studies have failed to observe this ethanol potentiation of I_{GABA}. Sigel *et al.* (1993) did not observe ethanol enhancement of the GABA response with 20 mM ethanol in many batches of oocytes expressing various rat recombinant subunit combinations having the γ_{2S} or γ_{2L} splice variant. They also tested ethanol concentrations up to 100 mM and observed a very small potentiation (<20 %) of I_{GABA} with 100 mM in all subunit combinations tested. Possible reasons for this discrepancy with other groups were suggested. There may be sequence differences in subunits across different species, differences in experimental conditions, and/or post-translational modifications which may be taking place in different batches of oocytes once the receptor protein has been assembled.

1.3. Regional action of ethanol and specific receptor constructs

Enhancement of GABA responses by ethanol varies with brain region. The cerebellum appears especially sensitive to ethanol, whereas the hippocampus appears relatively insensitive to the effects of ethanol (Wafford and Whiting, 1992). According to Soldo *et al.* (1994), ethanol may differentially modulate $GABA_A$ receptors in different regions of the brain because of differences in receptor subunit composition in various brain regions. This is supported by an *in vivo* study carried out by Criswell *et al.* (1993), in which binding of zolpidem (a type 1 benzodiazepine agonist) was high in areas where ethanol enhanced I_{GABA}, suggesting that ethanol affects specific $GABA_A$ receptors in particular brain regions. They hypothesize that a specific $GABA_A$ receptor that binds zolpidem is also sensitive to ethanol and is composed of $\alpha_1\beta_2\gamma_2$ subunits. The fact that mRNA for γ_{2L} and γ_{2S} was found in both ethanol-sensitive and ethanol-insensitive brain regions, suggests that the γ_{2L} subunit alone is not sufficient for ethanol's actions at the $GABA_A$ receptor which is consistent with what other groups have reported. Instead, Criswell *et al.* (1993) suggest that the combination of $GABA_A$ receptor subunits is important for ethanol sensitivity. They also reported from other studies, that chronic ethanol had an effect on specific $GABA_A$ receptor subunits (i.e. reduced $GABA_A$ receptor α_1 and α_2 subunits and increased α_6 subunit mRNA levels). This further supports the idea that ethanol has an effect on specific regions of the brain.

1.4. Ethanol and Ro15-4513

Ro15-4513, a benzodiazepine receptor partial inverse agonist, has been suggested to be an alcohol antagonist. Evidence for this comes from a number of studies. This compound blocks the effects of ethanol on Cl^- flux (Glowa *et al.*, 1989; Suzdak *et al.*, 1986; reviewed in Tanelian *et al.* 1993). In addition, behavioural studies show that Ro15-4513 antagonizes many of the effects of ethanol such as sedation, anticonflict and intoxication (Deacon *et al.*, 1990; Glowa *et al.*, 1989; Suzdak *et al.*, 1986; reviewed in Tanelian *et al.*, 1993). In a review by Grant (1994), it was reported that Ro15-4513 consistently blocked the effects of ethanol *in vitro* but only blocked some of the behavioural effects of ethanol, and that these effects varied whether ethanol treatment was chronic or acute. According to Grant (1994), the ability of Ro15-4513 to block some of the behavioural effects of ethanol may be an additive interaction rather than a pharmacological antagonism at the same receptor.

Reynolds *et al.* (1992) demonstrated that Ro15-4513 (100 nM) blocked ethanol potentiation of I_{GABA} in cultured rat cerebral cortical neurons. Ethanol's effects were also found to be antagonized by Ro15-4513 *in vitro* at human central neurons (Palmer *et al.*, 1990). In an *in vivo* study, Palmer *et al.* (1988) found that Ro15-4513 antagonized the electrophysiological effects of locally applied ethanol on Purkinje neurons in the cerebellum. In *Xenopus* oocytes, Ro15-4513 (1 μM) had no direct antagonistic effects on GABA current, but did antagonize ethanol's potentiating effect on $GABA_A$ receptors expressed from LS mouse whole brain mRNA (Wafford *et al.* 1990). From these Ro15-

4513 studies, it has been suggested that the ability of ethanol to enhance I_{GABA} appears to involve the benzodiazepine binding site, in particular, an increased efficiency of coupling between the benzodiazepine binding site and the Cl^- channel.

1.5. Ethanol modulation at other receptors/ion channels

The $GABA_A$ receptor-channel complex does not appear to be the only site which influences genetic sensitivity to ethanol (Allan *et al.* 1988). In fact, the selective effects of ethanol at a number of other ionotropic receptors, including NMDA and 5-HT_3, has been extensively reviewed by Grant (1994). Grant (1994) stated that it is evident from the literature that ethanol is not a specific receptor ligand and that it alters a number of neurochemical processes by acting at different classes of receptors.

1.5.1. NMDA receptor

Several groups have shown using biochemical techniques (Hoffman *et al.*, 1989a, b; Dildy and Leslie, 1989), brain slices (Gonzales and Woodward, 1990; Woodward and Gonzales, 1990), *in vivo* studies (Simson *et al.*, 1991), and behavioural methods (Grant *et al.*, 1991; Grant and Colombo, 1992; Sanger, 1993), that ethanol inhibits the function of NMDA receptors. Lovinger *et al.* (1989) showed ethanol inhibition of NMDA receptor function in hippocampal neurons. This inhibition by ethanol (5-50 mM) increased in a concentration-dependent manner. Ethanol also inhibited NMDA receptor-mediated responses in cortical and hippocampal membranes of the mouse (Snell *et al.*,

1993). Another group showed that a homomeric NMDA receptor was sensitive to ethanol blockade in *Xenopus* oocytes (Treistman *et al.*, 1993). However, no agreement concerning ethanol's mechanism of action at this receptor has been reached. It may be possible that ethanol is acting at only a single NMDA receptor subtype (reviewed in Grant, 1994). However, there is evidence for the NMDA receptor involvement in ethanol intoxication and withdrawal. In a study by Grant *et al.* (1990), it was demonstrated that an up-regulation of NMDA receptors in the hippocampus following chronic ethanol treatment may mediate seizures associated with ethanol withdrawal in dependent animals. Furthermore, ethanol may produce an interactive effect at $GABA_A$ and NMDA receptor complexes, which may be responsible for some of its behavioural effects (Grant, 1994). For example, following chronic ethanol treatment, GABAergic neurotransmission is decreased, whereas NMDA receptor activity is increased (Lovinger, 1993). This down-regulation of $GABA_A$ receptor function may contribute to the induction of ethanol withdrawal seizures.

1.5.2. The 5-HT_3 receptor

The 5-HT_3 receptor is believed to regulate behavioural and physiological effects of ethanol, such as tolerance, ethanol consumption, anxiolytic effects and temperature regulation (Sellers *et al.*, 1992). Recently, there was evidence for a direct interaction between ethanol and the 5-HT_3 receptor using patch-clamp techniques. Ethanol (25-100 mM) potentiated the effects of 5-HT at the 5-HT_3 receptor and this potentiation was

blocked by a specific 5-HT_3 antagonist (Lovinger, 1991; Lovinger and White, 1991). Potentiation of the recombinant 5-HT_3 receptor-mediated ionic current was also demonstrated in a human embryonic kidney cell line (Lovinger and Zhou, 1994). Machu and Harris (1994) observed ethanol potentiation of 5-HT_3-mediated current, with greater potentiation at lower concentrations of 5-HT. Butanol also enhanced 5-HT_3 receptor function with a greater degree of potency. However, as is the case for other ligand-gated ion channels, the mechanism(s) of action at this receptor is not known.

1.5.3. G protein-coupled receptors

Sanna *et al.* (1994) recently reported that ethanol (25-200 mM) inhibited both 5-HT and ACh at 5-HT_{1C} and M_1 cholinergic receptors respectively. They suggested that ethanol inhibition of 5-HT_{1C} receptors requires protein kinase C-mediated phosphorylation. Ethanol may activate PKC responsible for receptor phosphorylation, which in turn, results in inhibition. Another possibility is that ethanol may act directly on the receptor protein, but to do so the receptor must be phosphorylated. These two hypotheses are similar to those proposed for ethanol effects on ligand-gated ion channels. In fact, G-protein-coupled receptors may be as sensitive to ethanol as are ligand-gated ion channels (Sanna *et al.* 1994). It is possible that ethanol interacts directly with the effector systems associated with G proteins (Grant, 1994).

1.6. Site of alcohol action: membrane vs. protein

For a long time, it was believed that alcohols and anesthetics had their action on the membrane lipids of neurons in the CNS. Hence, the lipid theory of alcohol action emerged which held that the action of alcohols on neuronal proteins was secondary to the action of alcohols on the perturbation of membrane lipids (Peoples and Weight, 1995). As well, according to the lipid theory, as carbon chain length increases in n-alcohols, the lipid solubility of the alcohol increases (i.e. increased membrane/buffer partition coefficient), and the potency of the alcohol for disordering membrane lipids increases exponentially with this increased lipid solubility (McCreery and Hunt, 1978; Lyon *et al.*, 1981). However, as the number of C atoms increases from 6-8, a cutoff effect for alcohol intoxication occurs, even though the potency for disordering the membrane lipids continues to increase (McCreery and Hunt, 1978; Lyon *et al.*, 1981; Franks and Lieb, 1986; Huidobro-Toro *et al.*, 1987). In fact, different cutoff effects in alcohol potency have been observed with receptors including ATP-gated ion channels (Li *et al.*, 1994), 5-HT_3 receptor-ion channels (Fan and Weight, 1994), $GABA_A$ receptors (Nakahiro *et al.*, 1991; Peoples and Weight, 1994), and NMDA receptor complexes (Peoples and Weight, 1995). Li *et al.* (1994) demonstrated a cutoff effect for inhibition of ATP-gated ion channels by a series of aliphatic and halogenated alcohols. They observed increased inhibition from 1-3 carbon atoms. Peoples and Weight (1995) also observed a cutoff effect for inhibition of NMDA receptor function by a series of straight-chain alcohols. Both of these groups suggested a direct interaction of the alcohols with a hydrophobic

pocket on the receptor protein. They speculated that when the volume of the alcohol exceeds the volume of the hydrophobic pocket, the alcohol is probably not able to bind, and therefore can not exert its effect.

1.7. *Xenopus* oocyte expression system

The *Xenopus* oocyte as a model system was reviewed by Snutch (1988). Electrophysiologic studies using this model system, have provided insight into the mechanisms underlying modulation of $GABA_A$ receptor function by alcohols and other anesthetic agents.

The injection of mRNA into *Xenopus* oocytes results in the functional expression of various neurotransmitter receptors and voltage-gated ion channels. Before injection, oocytes are defolliculated to eliminate endogenous receptors for catecholamines, gonadotropins, purinergic agonists, and acetylcholine, as well as GTP-binding proteins and adenylate cyclase that mediate these responses (Snutch 1988). The mRNA to be injected is isolated from a neuronal source such as the brain or prepared *in vitro* from cloned DNA, and nanogram amounts are injected into individual oocytes. Receptors and ion channels which are expressed are characterized using electrophysiological techniques, typically the two-electrode voltage-clamp.

Dascal (1987) described the *Xenopus* oocyte as a useful model for studying molecular mechanisms of second messenger-mediated neurotransmitter responses and concluded that the oocyte may become the model of choice for studying signal

transduction. At present, this expression system is used by many groups to study both neurotransmitter and voltage-gated ion channels. For example, Sanna *et al.* (1994) studied the function of 5-HT_{1C} and muscarinic M_1 cholinergic receptors, both G protein-coupled receptors, after injection of whole mouse brain mRNA into *Xenopus* oocytes. Since oocytes express many endogenous Ca^{2+}-activated Cl^- channels, they are convenient to study metabotropic receptors which are linked to a common second messenger system which releases Ca^{2+} from internal stores. According to Sanna *et al.* (1994), these G protein-coupled receptors (5-HT_{1C} and M_1) may be as sensitive to ethanol as are ligand-gated ion channels.

Knoflach *et al.* (1992) injected cRNAs into *Xenopus* oocytes to determine structure-activity relationships of recombinant $GABA_A$ receptors, and compared the results they obtained in oocytes with those from transfected cells. They found that the $\alpha_3\beta_1\gamma_2$ subunit combination produced similar GABA sensitivities in oocytes and transfected cells, but that modulation by flunitrazepam of the GABA response was much weaker in oocytes. This might suggest some difference in post-translational processing or assembly of subunits in different expression systems, making it difficult to compare functional properties of recombinant and native receptors (Knoflach *et al.*, 1992). Ebert *et al.* (1994) expressed recombinant human $GABA_A$ receptors in *Xenopus* oocytes to investigate the pharmacology of $GABA_A$ agonists on various α, β, γ subunit combinations. Wafford *et al.* (1993) used the same expression system to compare the functional role of different γ subunits and obtained results which were similar to those

reported in transfected cells. Properties of different α subunits on the $GABA_A$ receptor were investigated using oocytes by Levitan *et al.* (1988). They reported that expressed receptors shared many properties with native $GABA_A$ receptors, but were not identical. Hadingham *et al.* (1993b) expressed recombinant human $GABA_A$ receptors containing different β subunits in *Xenopus* oocytes to determine the influence of the β subunit on $GABA_A$ receptor pharmacology.

Xenopus oocytes have also been used to study modulation of the $GABA_A$ receptor by ethanol and other alcohols. Sigel *et al.* (1993) expressed various $GABA_A$ receptor subunit combinations having the γ_{2S} or γ_{2L} splice variant isolated from rat brain in *Xenopus* oocytes to determine the effects of ethanol on I_{GABA}. Wafford *et al.* (1990) injected mRNAs for the $GABA_A$ receptor as well as NMDA receptor from brains of LS and SS mice into *Xenopus* oocytes to investigate the molecular basis for the difference in ethanol sensitivity between these two strains. As well, anesthetic concentrations of ethanol and butanol were tested for their effects on I_{GABA} in *Xenopus* oocytes injected with human cDNAs (Mihic *et al.*, 1994a).

A number of studies have described reasons why the *Xenopus* oocyte expression system is a useful model for studying the physiological and pharmacological properties of various receptors and ion channels. *Xenopus* oocytes consistently synthesize and assemble a variety of neurotransmitter receptors and voltage-gated ion channels after injection of foreign mRNA and are capable of undergoing electrophysiological recordings which are used to elucidate structure-activity relationships (Snutch, 1988). Expression of

several different ion channels and receptors can be seen by injecting mRNA isolated from whole rat brain, whereas injecting mRNA for a particular receptor or ion channel allows the study of a specific receptor construct in isolation (Snutch, 1988). The large size of the oocyte allows the microdissection of single cells, penetration with two electrodes during voltage-clamp or other electrophysiological recordings, and the injection of substances into the cells, thereby allowing regulation or control of cell contents (Dascal, 1987). Mihic *et al.* (1994a) described the *Xenopus* oocyte as a useful model system for studying the modulation of $GABA_A$ receptors by various drugs using recombinant $GABA_A$ receptor subunit combinations. They suggested that there is excellent agreement among studies carried out using the *Xenopus* oocyte expression system as well as between studies using oocyte models and other systems including whole cell preparations, cultured cell lines, or brain slices. Also, the oocyte allows one to look at the direct actions of various drugs on specific ion channels without the interference of neighbouring cells or competing processes, which is often the case in neuronal preparations (Machu and Harris, 1994). They suggested that investigation of these cloned receptor constructs may provide insight into structure-activity relationships and influence of subunit requirements for the actions of various drugs including alcohols and anesthetics. The *Xenopus* oocyte model is useful for studying the actions of anesthetics on mammalian $GABA_A$ receptors (Mihic *et al.*, 1994b). According to Mihic *et al.* (1994b), other studies have reported that modulators of $GABA_A$ receptor function, including benzodiazepines, barbiturates, steroids, and zinc, appear to have similar actions in oocytes as they do in other systems.

They also suggested that the compounds they studied in oocytes acted similarly in spinal cord preparations in the rat. Finally, Sigel and Baur (1988) reported that voltage- and ligand-gated current responses expressed in the oocyte remained stable over a long time period.

Despite the apparent usefulness of the *Xenopus* oocyte as a model system, there are also certain limitations. The oocyte may only selectively express some receptor subtypes or ion channels in mRNA preparations containing multiple mRNAs, perhaps due to post-translational modification carried out by the oocyte (Snutch, 1988). Sometimes, post-translational modifications proceed incorrectly (Dascal, 1987). As well, different batches of oocytes and different RNA preparations contribute to the diversity of results obtained. Finally, an obvious concern is whether or not results on the function of an ion channel or receptor synthesized in oocytes are comparable to the electrophysiological properties of these receptors or ion channels *in vivo*.

Apart from some limitations, it would appear that the oocyte is an excellent model for studying the physiological and pharmacological properties of ion channels, including those regulated by second messengers. This model system has been used extensively by several groups to study pharmacological profiles of various receptor constructs using recombinant cDNAs or cRNAs from a number of different species.

1.8. Rationale and hypotheses

The purpose of this study was to look at alcohol modulation of $GABA_A$ receptors,

in particular, to assess subunit requirements and structure-activity relationships for this modulation by various alcohols. It has long been believed that alcohols and anesthetics produced their effect by disordering or "fluidizing" membranes of neurons in the CNS. However, more recent evidence has shifted the emphasis to the idea that specific membrane proteins or lipid-protein domains are selectively sensitive to alcohol. Since findings in the literature are so controversial with regards to ethanol potentiation of I_{GABA}, we set out to study the effects of ethanol on I_{GABA}, comparing pharmacological profiles of different receptor constructs. For comparison, the effects of 1- and 2-butanol on I_{GABA} using $\alpha_1\beta_2\gamma_{2L}$ and $\alpha_2\beta_2\gamma_{2L}$ receptor constructs were examined. If there is a direct action of alcohols at the $GABA_A$ receptor, then one might expect that stereoisomers of the simplest chiral alcohol, 2-butanol, would vary in their potency as modulators of $GABA_A$ receptor function. Using this premise, we decided to compare the effects of the two stereoisomers of 2-butanol on $GABA_A$ receptor function. In addition, while it is believed that the γ_{2L} subunit is required for ethanol to potentiate I_{GABA}, it remained to be determined whether the same was true of butanol. Furthermore, to help in our understanding of the mechanism of action of alcohols at the $GABA_A$ receptor, we set out to examine the effects of Ro15-4513, reportedly an ethanol antagonist, to determine if this compound altered the ability of different alcohols to modulate $GABA_A$ receptor function.

The pharmacological properties of different receptor subunit combinations were characterized using the *Xenopus* oocyte as our model system and the two-electrode

voltage-clamp recording technique. For these experiments, human cloned RNAs for $GABA_A$ receptor subunits were made from cloned DNAs. In addition, it was necessary to characterize the oocyte model and to get the oocytes to express $GABA_A$ receptors. The following hypotheses were tested:

1) Ethanol potentiates I_{GABA} and hence enhances $GABA_A$ receptor function.

2) 2-butanol is more potent than 1-butanol at each receptor construct.

3) There is a difference between the effects of the stereoisomers of 2-butanol on $GABA_A$ receptor function.

4) The γ_{2L} subunit is necessary for the simplest chiral alcohol to potentiate I_{GABA}.

5) Ro15-4513 blocks ethanol potentiation of I_{GABA} as well as the potentiation of GABA by other simple alcohols.

II. General methods

2.1. Maxi plasmid preparation by alkali lysis

2.1.1. Transfection of cDNAs

cDNAs for human α_1, α_2, β_2 and γ_{2L} subunits of the $GABA_A$ receptor were obtained from Dr. Paul Whiting (Merck, Sharp and Dohme Research Laboratories). Each subunit was cloned into the pCDM8 vector (invitrogen). cDNA stocks of the various $GABA_A$ receptor subunits were resuspended in Tris EDTA (TE) to a final concentration of 1 μg/μl. An aliquot of the stock was further diluted with TE to make a final concentration of 5 ng/μl. At this time, the stocks of competent cells were removed from the -70°C freezer and placed on ice. The cells used to take up the human cDNA for GABA receptor subunits were the *E. coli* strain MC1061/p3 (Invitrogen).

To transfect the plasmids, 10 μl of 5 ng/μl cDNA was mixed with 100 μl of competent cells. The mixture was left on ice for 20-30 minutes. After this time, the cDNA and cell mixture was heat shocked at 42°C for 50 seconds. Immediately after heat shocking, prewarmed LB at 37°C was added as quickly as possible to a final volume of 1 ml. The tube was then placed horizontally on a shaker for 45 minutes at 37°C to allow proper aeration and the cDNA to be taken up. The suspension was spun down at 6000 rpm for 10 seconds, the supernatant discarded and the pellet resuspended in 100 μl sterile LB at room temperature. The microorganisms were then plated aseptically on agar plates already prepared with antibiotics (tetracycline and ampicillin) with 2%, 10% and 88%

of the 100 μl suspension divided into 3 separate plates. The plates were then incubated overnight at 37°C.

The following day, fresh agar plates containing tetracycline and ampicillin were used to restreak a single bacterial colony from one of the three plates for each subunit, so that there was now one plate for each of the subunits. Again, the bacteria were incubated at 37°C overnight.

2.1.2. Maxi plasmid preparation

For each individual subunit, 500 ml of LB was placed into a 2 L conical flask and the media was autoclaved. The broth was then allowed to cool to room temperature and antibiotics were added as follows: 750 μl tetracycline (5 mg/ml) and 250 μl ampicillin (50 mg/ml). At this point, the LB was inoculated with the desired organism. A metal loop was flamed and then cooled by touching the agar and the broth was inoculated with the loop after it had just touched a single bacterial colony. The inoculate was incubated overnight at 37°C with shaking.

After 17-18 hours, when the cultures were well saturated with cells, they were transferred to pre-cooled 250 ml centrifuge buckets and placed on ice for 10-20 minutes. The cells were then centrifuged at 4000 rpm for 10 minutes. The supernatant was drained off and the insides of the buckets were carefully wiped to remove excess medium. For 250 ml cultures, the pellets were suspended in 6.5 ml of solution 1 and transferred to 50 ml polypropylene Oakridge tubes. The tubes were left to stand at room temperature for

5 minutes. Solution 2 was freshly prepared at this time and 13 ml of this solution was added to each tube. The suspensions were mixed gently but thoroughly to ensure complete lysis of the cells and were placed on ice for 5 minutes. Then 6.5 ml of ice cold solution 3 was added to each tube, which were then mixed by swirling end over end. The tubes were again placed on ice for 10 minutes. After this time, the precipitate (protein and chromosomal DNA) was spun down at 12 000 rpm for 30 minutes and the supernatant was poured into fresh 50 ml Oakridge tubes. The supernatant was precipitated with 0.6 volumes (approximately 15 ml) of iso-propanol, mixed well and left to stand at room temperature for 5 minutes. Plasmid DNA precipitate was obtained by centifugation at 8000 rpm for 10 minutes. The supernatant was drained off and discarded and the tubes were inverted on paper towel and allowed a couple of minutes to dry.

Plasmid DNA pellets were resuspended in 3.0 ml of solution 4 and transferred to 15 ml Corex^R centrifuge tubes. If necessary, the pH was adjusted with NaOH to be neutral to alkaline. The volume of each tube was then made up to exactly 4.2 ml. 30 μl of 10 mg/ml RNAase A was added to each tube. CsCl (4.7 g) was added and was completely dissolved by inverting the tubes which were covered with parafilm. Then 500 μl ethidium bromide stock (10 mg/ml) was added. The tubes were centrifuged at 8000 rpm for 10 minutes to remove any precipitate. The supernatant was placed in quickseal ultracentrifuge tubes, balanced and heat sealed. Samples were spun in the Vti vertical rotor at 45 000 rpm at 20°C for $>$ 18 hours.

Tubes were then carefully removed from the rotor. Plasmid DNA was sometimes

visible in ordinary light. To facilitate removal, the plasmid DNA band was visualized by exposure to long-wave ultraviolet light. The top of each tube was punctured with a 1cc syringe tip to allow the entry of air and this was left in place. Plasmid DNA was collected by carefully inserting a 1cc syringe needle just below the plasmid band and withdrawing as much of the band as was possible. This was usually between 0.5 and 1 ml. Once the bands were extracted, they were each placed in 15 ml polypropylene tubes. 2 ml of solution 4 was added to each tube to make up the volume. Then, to remove ethidium bromide, each sample was extracted with an equal volume of butanol (2 ml). The extraction process was repeated 3 times or until no ethidium bromide was extracted into the organic phase. Each time the samples were vortexed for a few seconds, the aqueous phase (bottom) was retained, and the organic phase was discarded. The samples were then placed in a dessicator for a few minutes to get rid of excess b anol.

After the butanol extractions, the samples were extracted twice with an equal volume of phenol:chloroform:isoamylalcohol (ratio 25:24:1) and once with an equal volume of H_2O saturated chloroform. Again, the samples were vortexed each time and in each case, the upper aqueous layer was kept and transferred to clean polypropylene tubes. After the chloroform extraction, however, the the aqueous layer was transferred to a 15 ml Corex^R centrifuge tube. Sodium acetate (3 M; pH 5.2) (1/10 the volume of the sample) was added, followed by 2 volumes of 100% ethanol to precipitate the DNA. The samples were covered with parafilm and placed at -20°C overnight.

The sample containing the precipitated DNA was centrifuged at 15 000 rpm for

30 minutes. The supernatant was removed and the pellet was washed with approximately 5 ml 70% ethanol at -20°C. The pellet was then dried in a dessicator for about 10-30 minutes. A micropipette was used to resuspend the pellet in 500 μl TE. At this stage, a spectrophotometer was used to estimate the concentration of plasmid DNA in each sample. Double stranded DNA generally gives an optical density reading of 1 per 50 μg/ml at 260 nm. Typical yields of plasmid DNA were 800-1600 μg from a 500 ml culture. The dilution factor used was 1 in 500 or 1 μl of sample in 500 μl TE.

2.1.3. Separation of substances by electrophoresis in agarose gel

The purity of the plasmid preparation was checked by running a 1% agarose mini-submarine gel. The agarose gel was measured out and placed in a conical flask to which the required amount of 1 x TBE buffer was added. The solution was placed in the microwave for 2 minutes, after which time, it was checked for any undissolved particles. If any particles were present, the solution was swirled and reheated for 30 seconds until thoroughly dissolved. Care was taken to avoid boiling in the flask. Once the particles were dissolved, the solution was cooled down by gentle swirling to a temperature of about 60°C. At this point, 3.3 μl EtBr (for 50 ml solution) was added and mixed well, avoiding formation of any air bubbles.

Meanwhile, both ends of a clean plastic cast were taped with autoclave tape. The comb was aligned for the formation of wells, which must be parallel to the base. The agarose gel was poured slowly into the cast, which was on a level surface, avoiding air

bubbles. The gel was allowed to polymerize for 20-30 minutes. If the gel was kept a longer time, 1 x TBE buffer was added on top of it to prevent it from drying out. After adding the buffer, the comb was taken out very carefully, without disfiguring the wells formed. The excess buffer was drained off and the autoclave tape was removed. The marker and the samples were now added into the wells carefully with a micropipette. The marker contained: 1 μl of 1 Kb ladder (Gibco), 1 μl 10x Blue Juice and 8 μl dH_2O for DNA samples. Each sample contained: 1 μl sample, 1 μl 10x Blue Juice and 8 μl dH_2O. The samples were made up in separate microcentrifuge tubes.

Once the gel was ready to run, 1 x TBE was placed in a clean electrophoresis chamber and the cast along with the gel was placed in the chamber very slowly until the buffer covered the surface of the gel entirely. The wells were placed close to the cathode (-ve electrode) since cDNA, an electronegative molecule, will migrate towards the anode (+ve electrode). The counter was set at frequency 100 and the setup was allowed to run at a constant voltage of 100 V for 30-60 minutes. After the gel run, the gel was removed from the chamber and was placed under UV light to check for the proper bands. In some cases, the plasmids were linearized with the appropriate restriction enzymes prior to gel electrophoresis. This avoids the problem of supercoiling of plasmid DNA, which can adversely affect migration in the gel.

2.1.4. *In vitro* transcription of RNA

The RiboMAX™ Large Scale RNA Production System was used to produce

milligram quantities of RNA for *in vitro* translation in *Xenopus* oocytes. Before *in vitro* transcription, the DNA templates were linearized to produce RNA of defined length (Promega Technical Bulletin, 1993). The DNA was linearized by digestion with appropriate restriction endonucleases followed by extractions with phenol/chloroform and chloroform and subsequently by ethanol precipitation. It was important to use enzymes which gave blunt ends or 5' overhangs, since 3' overhangs may cause the enzyme to "double back" on the DNA, leading to the synthesis of RNA from the complementary strand as well. However, in some cases, it was necessary to use restriction enzymes that did generate 3' protruding ends, and in these cases, T4 Polymerase was used to fill in the overhang and to give blunt ends to the linearized DNA template before transcription. The restriction enzymes used for each subunit were: Bam HI for human α_1 cDNA, Pst I for human α_2 cDNA, Sca I for human β_2 cDNA and Zho I for human γ_{2L} cDNA.

2.1.4.1. DNA template linearization

1 μl of restriction enzyme was added to 5-10 μl of plasmid cDNA (concentration approximately 2 μg/μl) to linearize the transcription vector containing the gene of interest 3' of the insert. 10x incubation buffer (in which the restriction enzyme has 100% activity), 1.5-2.0 μl, was added to the above mixture, and dH_2O was used to make up the volume so that the concentration of the buffer in the final mix is 1x. In other words, if 2 μl of buffer were used, the final volume was 20 μl. The mixture was then incubated in a 37°C water bath for at least 2 hours and sometimes overnight.

After the incubation period, dH_2O was added to a final volume of 50 μl. The mixture was extracted once with an equal volume of phenol/chloroform and once with an equal volume of chloroform. It was then ethanol precipitated by adding 1/10 the volume of 3 M NaOAc pH 5.2, and twice the volume of 100% EtOH. The mixture was placed at -20°C for 30 minutes, after which time, it was centrifuged at 12 000 rpm for 10 minutes. The supernatant was thrown away and the pellet was washed with cold -20°C 70% EtOH, taking care not to disturb it. The pellet was then dried under a vacuum for 15-30 minutes and resuspended in TE such that the concentration of the plasmid was approximately 1 μg/μl.

2.1.4.2. Conversion of a 3' overhang to a blunt end using T4 DNA polymerase

5 μl 10x T4 DNA polymerase buffer was added to 5 μl cDNA, along with 39.5 μl dH_2O and 0.5 μl T4 polymerase. This mixture was kept at room temperature for 2 minutes, and then 2 μl of deoxy nucleotides (2 mM dNTPs) were added. The mixture was incubated at 37 C for 15 minutes. Following incubation, extraction with an equal volume of phenol/chloroform was carried out along with EtOH precipitation as outlined above.

2.1.4.3. *In vitro* transcription of RNA

Typically, a 50 μl reaction for T7 RNA polymerase was set up at room temperature and reaction components were added in the following order: T7

Transcription 5x Buffer, 10 μl, rNTPs (25 mM ATP, CTP, UTP), 11.25 μl, rGTP (1 mM) + m7G(5')ppp(5')G cap (3 mM), 6.0 μl, linear DNA template (1 μg/μl), 5 μl, Nuclease-Free water, 12.75 μl, enzyme mix, 5 μl. The use of capped RNA is suggested for use in the *Xenopus* oocyte translation system. The G cap analog present at the 5' end of most eukaryotic mRNAs, is important for the binding of translation initiation factors and contributes to mRNA stability (Promega Technical Bulletin, 1993). As well, it is important to dissolve the DNA templates in nuclease-free water before they are added to the reaction. The reaction was gently pipetted up and down to mix and was incubated at 37°C for 2-4 hours.

2.1.4.4. Removal of DNA template following transcription

After performing the *in vitro* transcription, RQ1 RNase-free DNase was added to a concentration of 1 U/μg of template DNA (in this case, 5 μl). The reaction mixture was incubated for 15 minutes at 37°C, and then extracted with 1 volume of phenol:chloroform:isoamyl alcohol (25:24:1) pH 4.5. At this point, it was vortexed for 1 minute and centrifuged at top speed in a microcentrifuge for 2 minutes. The upper, aqueous phase was transferred to a fresh tube and 1 volume of chloroform:isoamyl alcohol (24:1) was added. Again, the mixture was vortexed for 1 minute and centrifuged as above. The upper, aqueous phase was transferred to a fresh tube. Now, the RNA was directly precipitated by adding 1/10 the volume of 3 M NaOAc or KOAc, pH 5.2, and 1 volume of isopropanol or 2.5 volumes of 95% EtOH. After mixing, the reaction

mixture was placed on ice for 2-5 minutes, and was then spun at top speed in a microcentrifuge for 10 minutes. The supernatant was poured off and the pellet was washed with 1 ml of 70% EtOH and dried under vacuum. The RNA sample was then resuspended in TE buffer or nuclease-free water to a volume identical to the transcription reaction, 50 μl, and stored at -70°C.

2.2. Surgery and treatment of oocytes

Oocytes were obtained from *Xenopus laevis* frogs kept individually in large holding containers. Each frog was given a number and a record was kept of the dates the frogs underwent surgery. There was usually a 6 week period between surgeries on any one frog, and no frog had surgery more than once a month. Frogs were anaesthetized in 500 ml of filtered water containing approximately 1.25 g 3-aminobenzoic acid ethyl ester and some crushed ice for about 15-20 minutes. The hind foot pad was pinched to determine whether the frog was anaesthetized. The surgery was performed with the frog on ice. A small incision was made in the skin and abdominal wall with a scalpel and surgical scissors, and a couple lobes of ovary were removed. Oocytes were placed in storage medium containing (in mM): 88 NaCl, 2 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 5 Hepes, 2.4 $NaHCO_3$, 2 pyruvic acid, 0.5 theophylline, 100 U/ml penicillin and 100 μg/ml streptomycin. The pH was 7.4, when adjusted with NaOH, and the solution was sterile filtered.

The oocytes were then manually defolliculated with fine forceps, and were then

placed in large Petri dishes coated with agar. Agar media (1.5%) was prepared by adding 50 ml sterile distilled water to 0.75 g bacteriological agar in a polypropylene tube, and boiling for about 15-30 minutes, or until the agar was dissolved. The media was poured into the Petri dishes and allowed to dry. This agar coating helped prevent sticking of the oocytes to the bottom of the Petri dishes. Storage solution was placed in each of the dishes and was changed on a daily basis.

2.3. Injection of oocytes

The oocytes were injected on the same day as the surgery. Injection needles were beveled to make the impaling of the oocytes smoother so that minimal damage was done to the eggs. The needle was subsequently filled with light mineral oil and was ready for injecting. Selected combinations of cRNAs for $GABA_A$ receptor subunits were prepared, and small aliquots were drawn into the injection needle. The concentration of each subunit was approximately 0.8-1 ng/nl, and small volumes (10-50 nl) were injected into the vegetal pole of oocytes using the Drummond "Nanoject" Automatic Injector (Drummond Scientific Company) loaded with the micropipette. The oocytes were placed singly in wells formed by a plastic mesh grid during the injections, and after injection, were placed in groups of 20-30 oocytes in the freshly prepared agar coated Petri dishes containing storage solution. The oocytes were left at room temperature and usually expressed $GABA_A$ receptors a couple of days after injection. Oocytes were used in electrophysiological recordings for up to two weeks after cRNA injection.

2.4. Electrophysiological recording

Oocytes rested in a well formed by a plastic mesh grid in the recording chamber (bath volume approximately 0.5 ml) with the animal pole facing upwards. They were impaled with two glass electrodes filled with 3 M KCl and clamped at -70 mV using an Oocyte Clamp (Model# OC-725B; Warner Instrument Corp.). If $GABA_A$ currents were very large (in μA range), the holding potential tended to fluctuate, and so the oocyte was clamped at -40 or -50 mV, which was more stable. Currents were continuously plotted on a chart recorder. Oocytes were perfused with a control solution containing (in mM): 88 NaCl, 2 KCl, 1 $CaCl_2$, 1 $MgCl_2$ and 5 Hepes. The pH of the control solution was 7.4, when adjusted with NaOH.

Drugs were dissolved in the control solution, and placed in reservoirs attached to a series of valves, which allowed multiple solutions to be applied to the same oocyte. All solutions were gravity-fed at flow rates of 6-8 ml/min. Drugs were applied for up to a minute or until the peak current amplitude was obtained. Whenever alcohols or other drugs were applied, they were co-applied with GABA. During experiments, the control GABA solution was frequently applied to monitor the peak current response before and after drug application. A washout period was allowed between drug applications, and this time varied depending on the concentration of drug used (3-10 minutes).

2.5. Data analysis and statistics

Data from each experiment were obtained from 4-7 oocytes isolated from at least

two different frogs. GABA concentration-response data were fit to the Hill equation;

$$I = I_{MAX}[C^n/(C^n + EC_{50}^n)]$$

where I and I_{MAX} repesent the current amplitude induced by the GABA concentration, C, and its maximum amplitude, respectively, EC_{50} is the concentration of GABA giving the half-maximal response, and n is the Hill coefficient. A non-linear least squares regression analysis (Inplot4, Graphpad Software Inc.) was used to "fit" to the experimental data. The effects of alcohols on I_{GABA} were compared by plotting the ratio of the test current to the control ($I_{ALCOHOL}/I_{CONTROL}$) versus the concentration of alcohol. Linear regression analysis (Inplot4) was used to get an estimate of the affinity (in mM) of each alcohol for individual receptor constructs. This analysis assumes that different alcohols would have the same efficacy to potentiate $GABA_A$ receptor function. Previous work by other groups (Nakahiro *et al.*, 1991) suggests that different alcohols do have equal efficacies to potentiate $GABA_A$ receptor function, and differ only in potency. Where appropriate, either a paired t-test or ordinary one-way analysis of variance (Instat2, Graphpad Software Inc.) was used to test for statistically significant differences among data sets. Post-hoc tests were done using the Bonferroni test for multiple comparisons. Two sets of data were considered to be significantly different when $p < 0.05$.

2.6. Solutions

2.6.1. Preparation of liquid broth (LB) + 1.5% agar

LB media for agar plates contained 5 g Bacto Tryptone, 2.5 g Bacto yeast extract

and 5 g NaCl in 500 ml distilled water. The pH was adjusted with NaOH to 7.0. Bacteriological agar (Sigma) was added in the amount of 7.5 g (1.5%) to the 1000 ml conical flask containing 500 ml LB and the broth was stirred. The top of the flask was covered with tinfoil and autoclave tape was placed snugly around the neck, holding the foil in place. The LB was then autoclaved and allowed to cool to less than 50°C. Antibiotics were added aseptically near a bunsen flame. Tetracycline, 750 μl (5 mg/ml), and ampicillin, 250 μl (50 mg/ml), were added to 500 ml LB. Stock solutions of these antibiotics were stored at -20°C and tetracycline was covered with tinfoil because of its sensitivity to light. The media was aliquotted (10-15 ml) into 100 mm Petri dishes and allowed to dry overnight in a dark room.

2.6.2. Solutions for maxi plasmid preparation

Four solutions were prepared and used for the plasmid preparation. Solution 1 contained 50 mM glucose, 25 mM Tris pH 8.0 and 10 mM EDTA pH 8.0. Solution 2 contained 0.2 M NaOH and 1% SDS. This solution was made fresh for each preparation just before it was ready to be used. Solution 3 contained 5 M KOAc pH 4.8 and glacial acetic acid (6.26 ml per 100 ml). Solution 4 contained 50 mM Tris pH 8.0 and 1 mM EDTA. Solutions 1, 3 and 4 were stored at room temperature.

2.6.3. 1% agarose mini-submarine gel

Three solutions had to be prepared to separate substances by electrophoresis in

an agarose gel. The first solution was 10x Tris borate EDTA buffer (TBE) containing: 108 g Trisbase, 55 g boric acid (H_3BO_4), 40 ml 0.5 M EDTA pH 8.0 and dH_2O to a final volume of 1 L. This solution was autoclaved. From this solution, a stock of 1 x TBE was prepared when required. The second solution was 10x Blue Juice containing: 0.42% Bromophenol Blue, 0.42% Xylene Cyanol FF and 25% Ficoll (Type 400; Pharmacia) in water. The third solution was 1.0% agarose gel. For 8 samples, 0.5 g agarose gel was used to make 50 ml of solution. For 15 samples, a larger volume of solution was used (approximately 150 ml).

2.7. Sources of chemicals

The following chemicals were used: Gum agar, tris[hydroxymethyl]aminomethane, ethylenediamine tetraacetic acid, dimethylsulfoxide (DMSO), bovine serum albumin, DL-dithiothreitol, caffeine, theophylline, pyruvic acid, 3-aminobenzoic acid ethyl ester, GABA (obtained from Sigma); Bacto Tryptone, Bacto Yeast Extract, sodium dodecyl sulphate, diethyl pyrocarbonate (obtained from BDH); Agarose electrophoresis gel, Bam HI, Pst I, Sca I, Xho I, T4 DNA Polymerase, penicillin, streptomycin (obtained from Gibco); rATP, rUTP, rCTP, rGTP (obtained from Pharmacia); RNAase Inhibitor, $m^7G(5')ppp(5')G$ (obtained from Boehringer Mannheim); Bromophenol Blue (obtained from Bio-Rad); xylene cyanol FF (obtained from Kodak); propofol (obtained from ICI Pharma); Intralipid (obtained from Kabi Pharmacia Canada, Inc.); flurazepam and Ro15-4513 (obtained from Hoffman La-

Roche). All other analytical grade laboratory reagents were obtained from Sigma Chemical Company or Fisher Scientific Company.

III. Results

3.1. $GABA_A$ receptor characterization

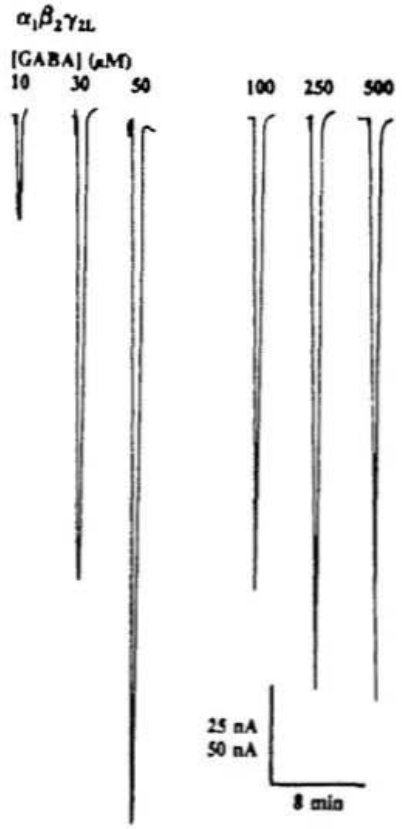
$GABA_A$ receptor subunit cRNAs were transcribed *in vitro* from human cDNAs and were injected into *Xenopus* oocytes in various combinations. Recombinant $GABA_A$ receptors were functionally expressed in the oocytes after 1-2 days. Before the effects of different alcohols were examined, it was necessary to characterize the properties of the $GABA_A$ receptor constructs ($\alpha_1\beta_2\gamma_{2L}$, $\alpha_2\beta_2\gamma_{2L}$, and $\alpha_2\beta_2$) to be used in these experiments. Perfusion with GABA produced concentration-dependent increases in GABA currents for all combinations of $GABA_A$ receptors tested (Figures 1-3). For $\alpha_1\beta_2\gamma_{2L}$, $\alpha_2\beta_2\gamma_{2L}$ and the $\alpha_2\beta_2$ receptors, the respective EC_{50} values were 63 $\pm$ 9 μM, 43 $\pm$ 3 μM and 2.58 $\pm$ 0.08 μM. The corresponding Hill coefficients were 1.26 $\pm$ 0.09, 1.15 $\pm$ 0.26 and 1.01 $\pm$ 0.22.

To characterize the pharmacological properties of the $GABA_A$ receptor, the ability of the $GABA_A$ receptor competitive antagonist, bicuculline, to inhibit GABA current was studied. This antagonist almost completely blocked GABA responses at the $\alpha_1\beta_2\gamma_{2L}$ receptor (Figure 4). As well, to determine allosteric modulation of the GABA response, the effect of flurazepam was examined. Flurazepam potentiated the current induced by 30 μM GABA (Figure 4). The vehicles used to dissolve various drugs (Intralipid, DMSO) had no significant effect on currents activated by GABA.

Figure 1. GABA concentration-response curve determined in *Xenopus* oocytes injected with $\alpha_1\beta_2\gamma_{2L}$ human recombinant cRNA.

A, Membrane currents activated by increasing concentrations of GABA in an oocyte injected with $\alpha_1\beta_2\gamma_{2L}$ cRNA. Calibration is 25 nA for first three traces and 50 nA for last three traces. B, Normalized current amplitude plotted as a function of log [GABA] and fit to the logistic equation (see Methods). Data points in this figure and remaining figures are the mean $\pm$ S.E.M (n = 6).

A


$\alpha_1\beta_2\gamma_{2L}$
[GABA] (μM)
10
30
50
100
250
500
25 nA
50 nA
8 min

B

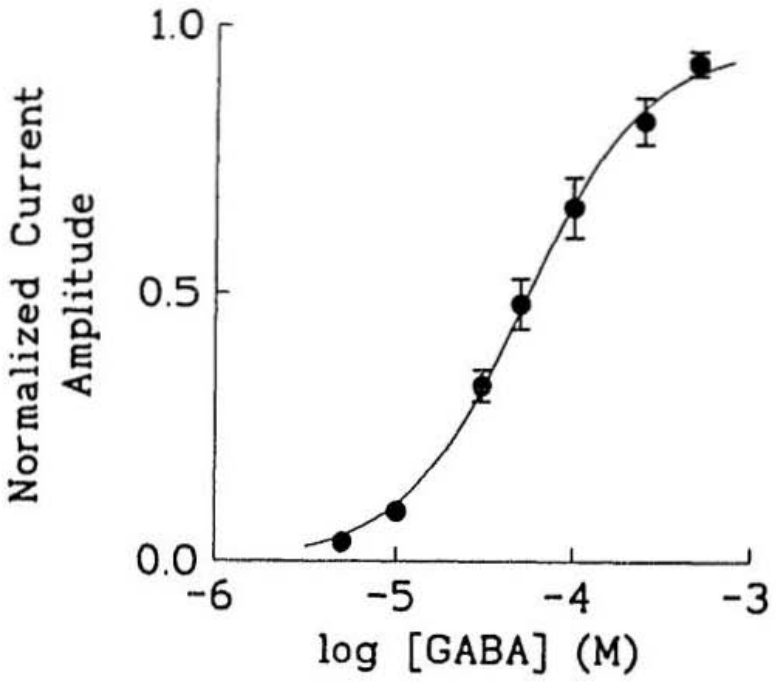
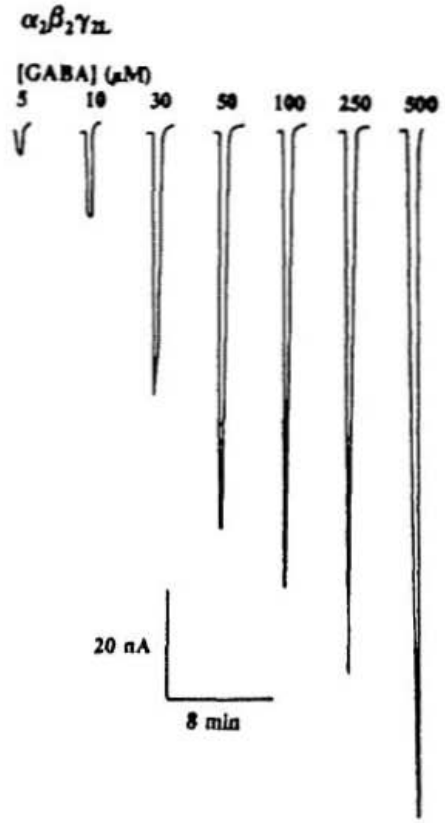

1.0
0.5
0.0
Normalized Current Amplitude
-6
-5
-4
-3
log [GABA] (M)

Figure 2. GABA concentration-response curve determined in *Xenopus* oocytes injected with $\alpha_2\beta_2\gamma_{2L}$ human recombinant cRNA.

A, Membrane currents activated by increasing concentrations of GABA in an oocyte injected with $\alpha_2\beta_2\gamma_{2L}$ cRNA. B, Normalized current amplitude plotted as a function of log [GABA] (n = 4).

A


$\alpha_2\beta_2\gamma_{2L}$
[GABA] (μM)
5
10
30
50
100
250
500
20 nA
8 min

B

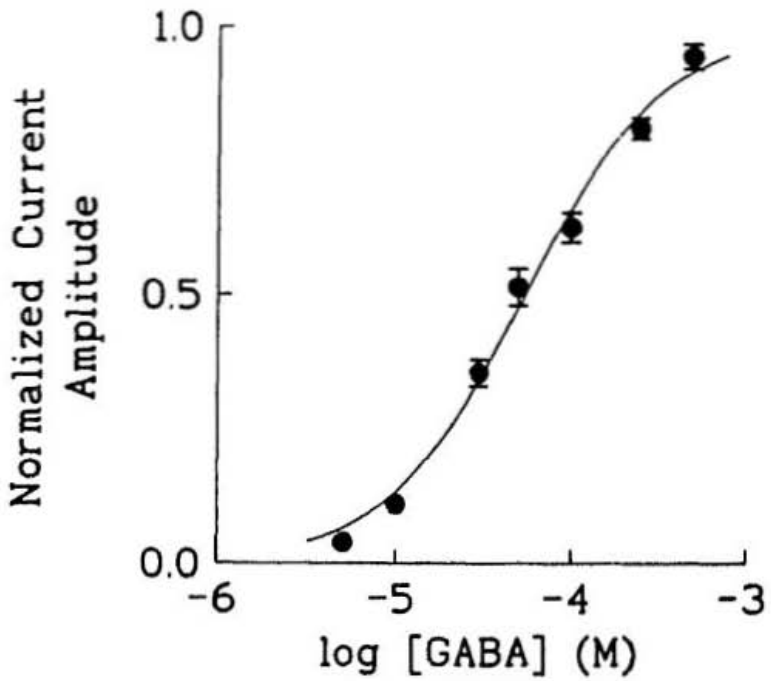

Normalized Current Amplitude
1.0
0.5
0.0
-6
-5
-4
-3
log [GABA] (M)

Figure 3. GABA concentration-response curve determined in *Xenopus* oocytes injected with $\alpha_2\beta_2$ human recombinant cRNA.

Normalized current amplitude plotted as a function of log [GABA] (n = 4).

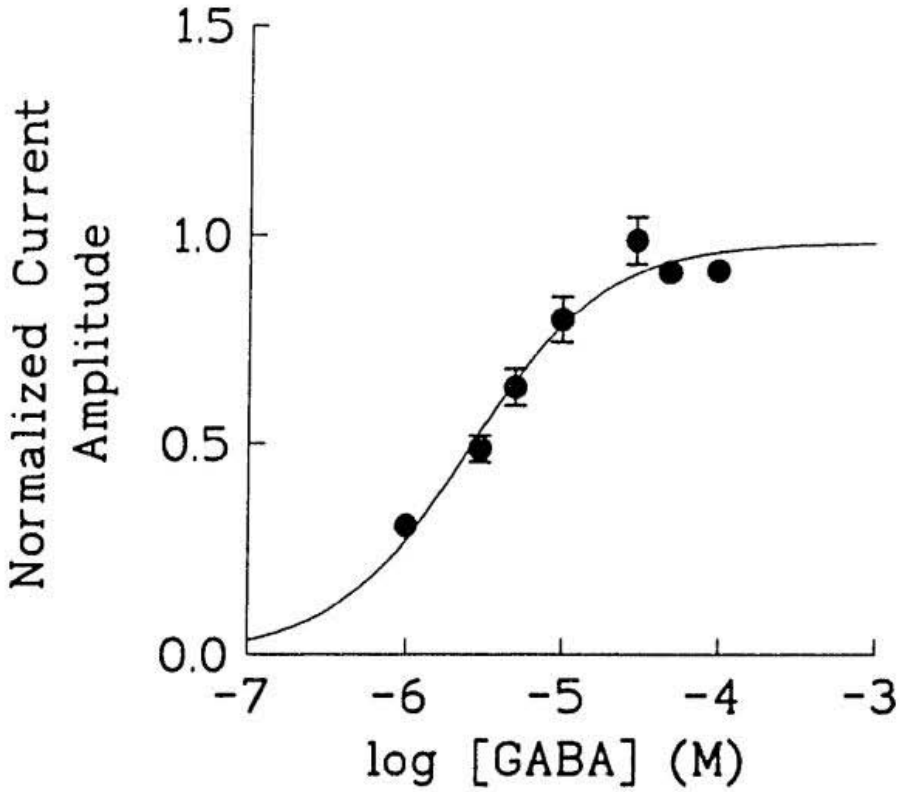
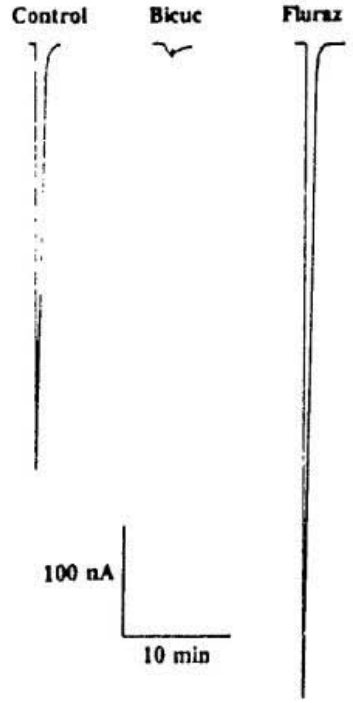

Normalized Current Amplitude
1.5
1.0
0.5
0.0
-7
-6
-5
-4
-3
log [GABA] (M)

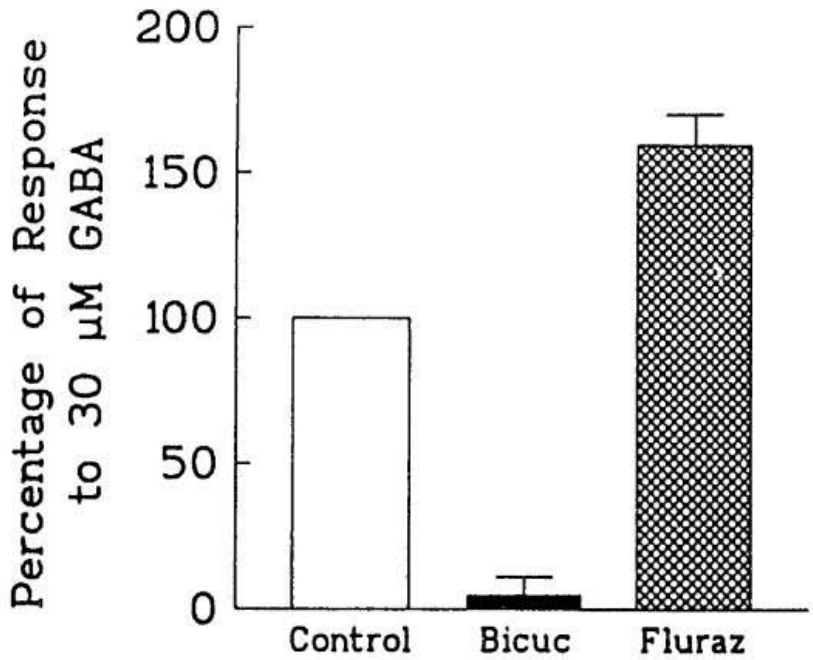
Figure 4. Characterization of GABA-activated Cl^- currents expressed in *Xenopus* oocytes.

A, Representative tracings show the effects of 30 μM bicuculline and 1 μM flurazepam on GABA currents evoked by 30 μM GABA, measured in oocytes expressing $\alpha_1\beta_2\gamma_{2L}$ receptors. B, Cumulative data from 4 separate oocytes. Similar results were obtained in oocytes injected with $\alpha_2\beta_2\gamma_{2L}$ cRNA (data not shown).

A


Control
Bicuc
Fluraz
100 nA
10 min

B


Percentage of Response to 30 µM GABA
200
150
100
50
0
Control
Bicuc
Fluraz

3.2. Effects of butanol on I_{GABA}

3.2.1. 1-butanol and 2-butanol

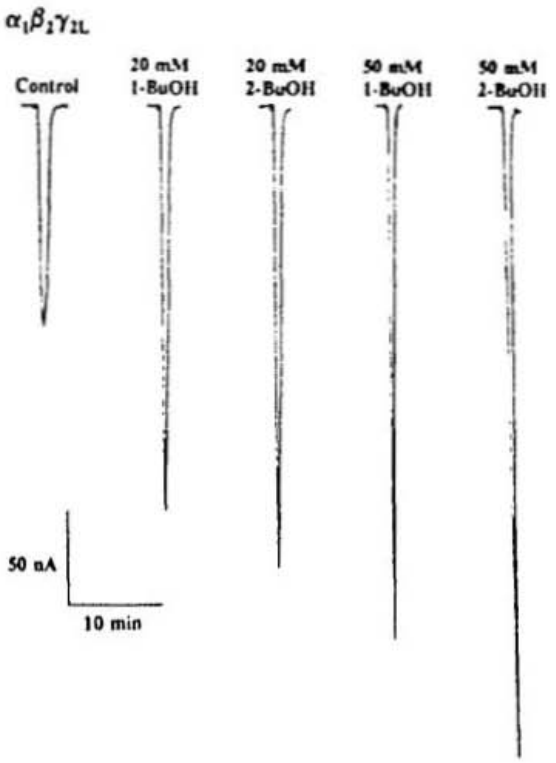
To investigate the influence of subunit composition on the modulation of the $GABA_A$ receptor by alcohol, the effects of 1-butanol and 2-butanol on $GABA_A$-activated Cl^- current were studied using the $\alpha_1\beta_2\gamma_{2L}$ and $\alpha_2\beta_2\gamma_{2L}$ receptor constructs. Concentrations of butanol (5-80 mM) were co-applied with 20 μM GABA by bath perfusion. Butanol at concentrations >100 mM alone produces only negligible currents (<10 nA). Both alcohols potentiated GABA responses in a concentration-dependent manner (Figure 5). However, 2-butanol was more potent than 1-butanol over the entire concentration range examined. Linear regression analysis was performed to compare the potentiating effects of 1- and 2-butanol on GABA currents at the $\alpha_1\beta_2\gamma_{2L}$ receptor (Figure 5). Representative traces show the effects of 20 and 50 mM each of 1- and 2-butanol on membrane currents induced by 20 μM GABA for the $\alpha_2\beta_2\gamma_{2L}$ receptor construct (Figure 6). Again, linear regression analysis was carried out using a range of butanol concentrations (Figure 6). Estimates of the affinity of 1-butanol and 2-butanol for the $\alpha_1\beta_2\gamma_{2L}$ and the $\alpha_2\beta_2\gamma_{2L}$ receptor constructs were calculated as the reciprocal of the slopes of the linear regression (Table 1). The affinity constant for 2-butanol was significantly lower than that for 1-butanol for both receptor constructs.

The effect of 1-butanol on the $GABA_A$-activated Cl^- current at the $\alpha_1\beta_2\gamma_{2L}$ receptor construct was compared to that at the $\alpha_2\beta_2\gamma_{2L}$ receptor construct. Figure 7 illustrates that the degree of potentiation of GABA currents by 1-butanol was significantly larger at the

Figure 5. Potentiation of GABA-activated currents by 1-butanol and 2-butanol in oocytes expressing the $\alpha_1\beta_2\gamma_{2L}$ receptor construct.

A, Representative current tracings show the effects of 20 μM GABA control and GABA plus 1-butanol or 2-butanol. B, Linear regression analysis of potentiation of GABA responses by 1-butanol and 2-butanol. The degree of potentiation of GABA-evoked current ($n = 7$) by butanol is plotted versus the concentration of butanol.

A


$\alpha_1\beta_2\gamma_{2L}$
Control
20 mM 1-BuOH
20 mM 2-BuOH
50 mM 1-BuOH
50 mM 2-BuOH
50 nA
10 min

B

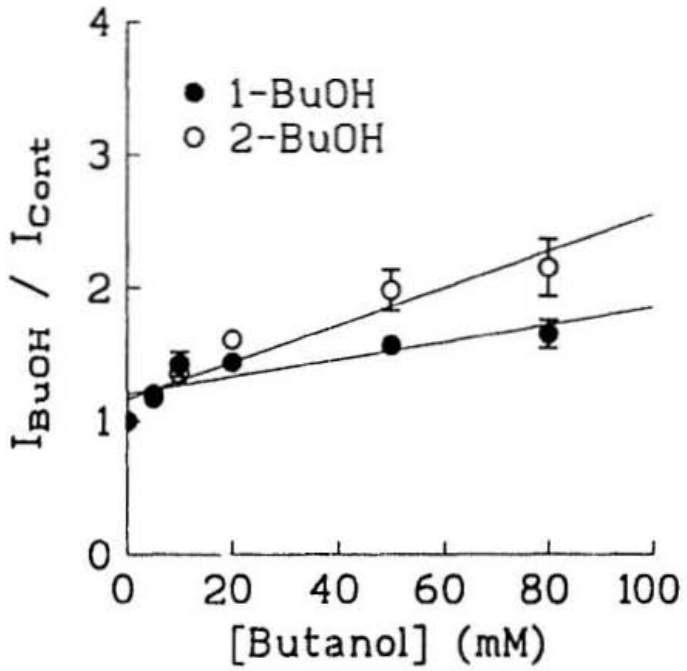
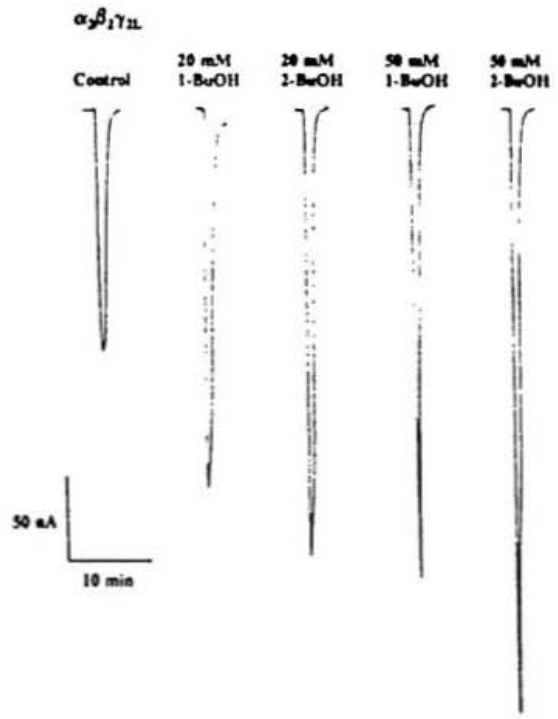

1-BuOH
2-BuOH
I_{BuOH} / I_{Cont}
[Butanol] (mM)
0
1
2
3
4
0
20
40
60
80
100

Figure 6. Potentiation of GABA-activated currents by 1-butanol and 2-butanol in oocytes expressing the $\alpha_2\beta_2\gamma_{2L}$ receptor construct.

A, Representative current tracings show the effects of 20 μM GABA control and GABA plus either 1-butanol or 2-butanol. B, Linear regression analysis of potentiation of GABA responses by 1-butanol and 2-butanol (n = 6).

A


$\alpha_3\beta_2\gamma_{2L}$
Control
20 mM 1-BuOH
20 mM 2-BuOH
50 mM 1-BuOH
50 mM 2-BuOH
50 nA
10 min

B

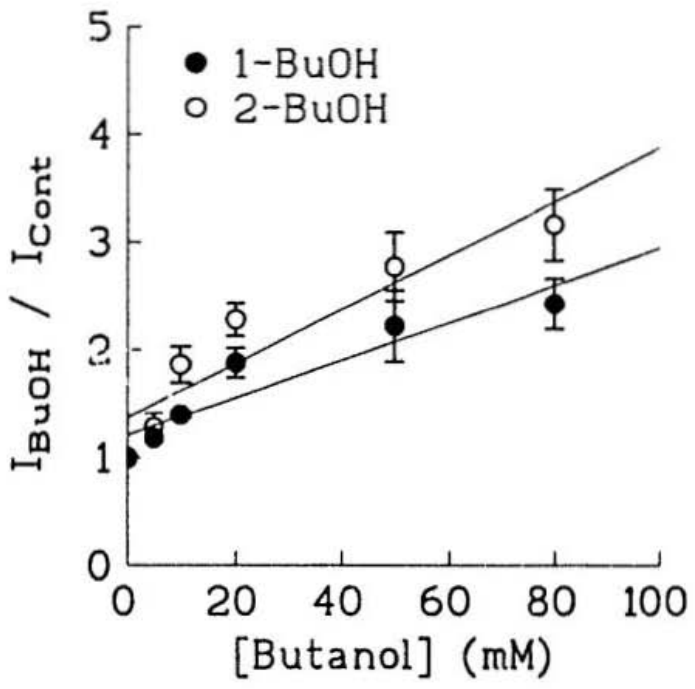

1-BuOH
2-BuOH
I_{BuOH} / I_{Cont}
0
1
2
3
4
5
0
20
40
60
80
100
[Butanol] (mM)

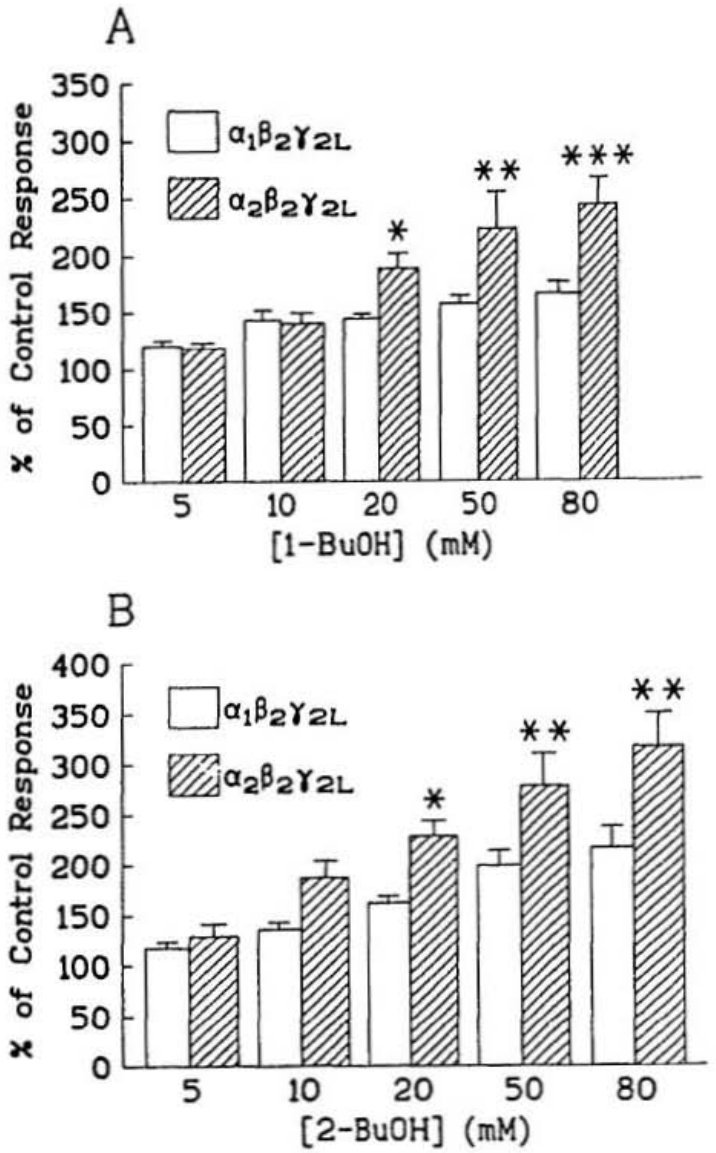
Table 1. Estimates of affinity (mean ± S.E.M.) of 1-butanol and 2-butanol for $\alpha_1\beta_2\gamma_{2L}$ and $\alpha_2\beta_2\gamma_{2L}$ receptor constructs.

Subunit combination	1-butanol	2-butanol
$\alpha_1\beta_2\gamma_{2L}$	204.30 ± 54.76 (7)	119.49 ± 43.33 (7)[a]
$\alpha_2\beta_2\gamma_{2L}$	47.53 ± 8.67 (6)	32.42 ± 5.74 (6)[b]

Currents were elicited by 20 μM GABA. Numbers in parentheses represent number of oocytes. [a], $p = 0.001$ compared to 1-butanol; [b], $p = 0.0072$ compared to 1-butanol (paired t test).

Figure 7. 1-butanol and 2-butanol differentially potentiate GABA responses at the $\alpha_1\beta_2\gamma_{2L}$ and the $\alpha_2\beta_2\gamma_{2L}$ receptor constructs.

Experiments were carried out with 20 μM GABA. A, Degree of potentiation of GABA currents by 1-butanol (n = 3 to 7). *, $p<0.05$, **, $p<0.01$ and ***, $p<0.001$, Bonferroni post-hoc test. B, Degree of potentiation of GABA currents by 2-butanol (n = 3 to 7). *, $p<0.05$ and **, $p<0.01$, Bonferroni post-hoc test.


A
350
300
250
200
150
100
50
0
% of Control Response
α1β2γ2L
α2β2γ2L
*
**
***
5
10
20
50
80
[1-BuOH] (mM)
B
400
350
300
250
200
150
100
50
0
% of Control Response
α1β2γ2L
α2β2γ2L
*
**
**
5
10
20
50
80
[2-BuOH] (mM)

$\alpha_2\beta_2\gamma_{2L}$ receptor construct than the degree of potentiation at the $\alpha_1\beta_2\gamma_{2L}$ receptor with 1-butanol concentrations of 20, 50 and 80 mM. Similarly, 2-butanol showed a greater degree of potentiation of GABA current at the $\alpha_2\beta_2\gamma_{2L}$ receptor compared to the $\alpha_1\beta_2\gamma_{2L}$ receptor, at 20, 50 and 80 mM concentrations of 2-butanol (Figure 7).

3.2.2. Effects of (R)- and (S)-2-butanol

The effects of (R)-2-butanol and (S)-2-butanol on I_{GABA} were compared at $\alpha_1\beta_2\gamma_{2L}$ and $\alpha_2\beta_2\gamma_{2L}$ receptor constructs. Figure 8 shows the linear regression analysis for the stereoisomers of 2-butanol at the $\alpha_1\beta_2\gamma_{2L}$ and the $\alpha_2\beta_2\gamma_{2L}$ receptor constructs. The slopes of the regression lines were not significantly different for each isomer at each receptor, although the slopes differed between receptors (Figure 8, Table 2).

3.2.3. Effects of 1- and 2-butanol in the presence or absence of γ_{2L}

To test the importance of the γ_{2L} subunit in the potentiation of I_{GABA} by butanol, the $\alpha_2\beta_2\gamma_{2L}$ and $\alpha_2\beta_2$ receptor constructs were examined. Butanol potentiation of I_{GABA} occurred both in the presence and absence of the γ_{2L} subunit (Figure 9). However, the $\alpha_2\beta_2\gamma_{2L}$ combination displayed greater enhancement of the GABA response (3 μM) by 1-butanol and 2-butanol than did the $\alpha_2\beta_2$ receptor construct. However, since the $\alpha_2\beta_2\gamma_{2L}$ and $\alpha_2\beta_2$ receptors have very different sensitivities to GABA (EC_{50} = 43 $\pm$ 3 μM and 2.58 $\pm$ 0.08 μM respectively), this is an expected observation. Concentrations of GABA yielding equivalent responses would give a more accurate comparison of the effects of

Figure 8. Comparison of the effects of (R)-2-butanol and (S)-2-butanol on $GABA_A$ receptor-activated currents in oocytes expressing human $\alpha_1\beta_2\gamma_{2L}$ and $\alpha_2\beta_2\gamma_{2L}$ receptor constructs.

A, Linear regression analysis (n = 3) for the $\alpha_1\beta_2\gamma_{2L}$ receptor construct. Currents were elicited by 20 μM GABA. B, Linear regression analysis (n = 5) for the $\alpha_2\beta_2\gamma_{2L}$ receptor construct. Currents were elicited by 3-5 μM GABA.

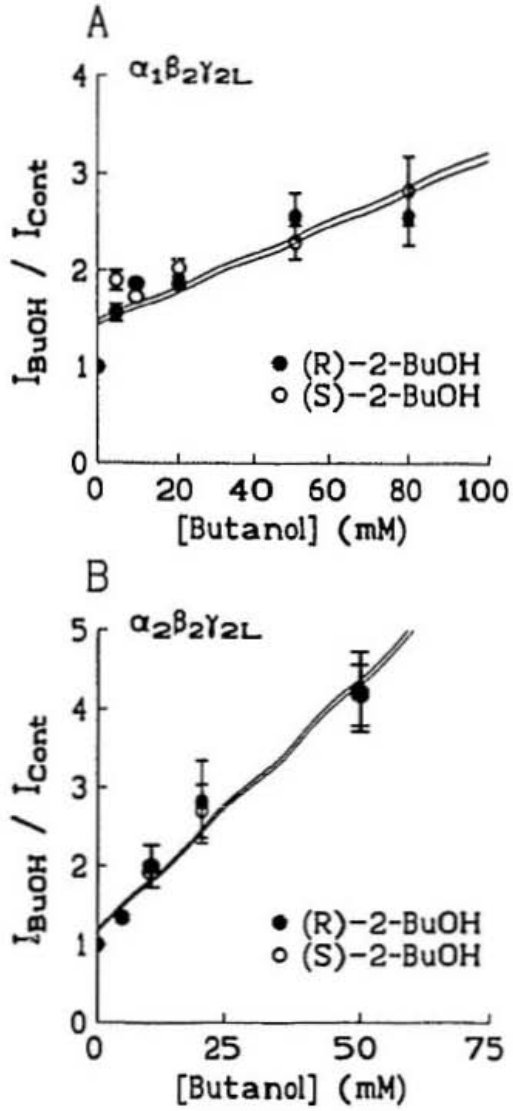
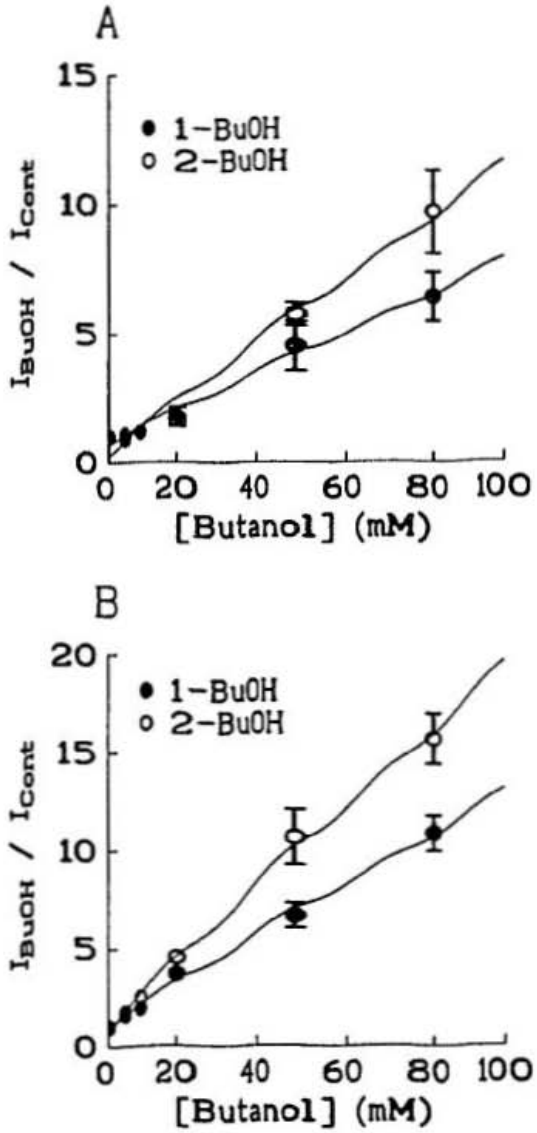

A
$\alpha_1\beta_2\gamma_{2L}$
I_{BuOH} / I_{Cont}
● (R)−2-BuOH
○ (S)−2-BuOH
[Butanol] (mM)
B
$\alpha_2\beta_2\gamma_{2L}$
I_{BuOH} / I_{Cont}
● (R)−2-BuOH
○ (S)−2-BuOH
[Butanol] (mM)

Table 2. Estimates of affinity (mean ± S.E.M.) of (R)-2-butanol and (S)-2-butanol for $\alpha_1\beta_2\gamma_{2L}$ and $\alpha_2\beta_2\gamma_{2L}$ receptor constructs.

Subunit combination	(R)-2-butanol	(S)-2-butanol
$\alpha_1\beta_2\gamma_{2L}$	70.80 ± 22.62 (3)	68.01 ± 19.91 (3)
$\alpha_2\beta_2\gamma_{2L}$	18.54 ± 3.54 (5)	17.36 ± 2.81 (5)

Slopes were obtained from data in Figure 8. Numbers in parentheses represent number of oocytes.

Figure 9. Comparison of the effects of 1-butanol and 2-butanol on $GABA_A$ receptor-activated currents in oocytes expressing human $\alpha_2\beta_2$ and $\alpha_2\beta_2\gamma_{2L}$ receptor constructs. Control currents were obtained with 3 μM GABA. A, Linear regression analysis (n = 4) for the $\alpha_2\beta_2$ receptor construct. B, Linear regression analysis (n = 4) for the $\alpha_2\beta_2\gamma_{2L}$ receptor construct.


A
15
10
5
0
● 1-BuOH
○ 2-BuOH
I_{BuOH} / I_{Cont}
0 20 40 60 80 100
[Butanol] (mM)
B
20
15
10
5
0
● 1-BuOH
○ 2-BuOH
I_{BuOH} / I_{Cont}
0 20 40 60 80 100
[Butanol] (mM)

1- and 2-butanol on I_{GABA} at these two receptor constructs. Estimating the affinity of 1-butanol and 2-butanol for each receptor construct from the slope of the linear regression (Figure 9) revealed that there was a significant difference between 1-butanol and 2-butanol at the $\alpha_2\beta_2\gamma_{2L}$ receptor construct (Table 3), whereas at the $\alpha_2\beta_2$ receptor this was not statistically significant (Table 3).

3.3. Effects of Ro15-4513 with flurazepam, butanol and ethanol

To show the effect of Ro15-4513 on the action of benzodiazepines, flurazepam was co-applied with Ro15-4513 and GABA. Ro15-4513 (500 nM) reduced flurazepam potentiation of GABA current (Figure 10). To compare the effects of Ro15-4513 on alcohol modulation of the GABA response in the same oocyte, butanol was co-applied with GABA and Ro15-4513. Current traces show that Ro15-4513 (500 nM) does not affect 10 or 80 mM 2-butanol potentiation of I_{GABA} (Figure 10). Ethanol potentiation of the GABA response was observed in some, but not all of the oocytes tested. To test the effects of protein kinase C on ethanol potentiation of I_{GABA}, 1,2-oleoylacetylglycerol (OAG) was used to determine if activating endogenous PKC in the oocytes would alter ethanol sensitivity. It was found that OAG (25 μM), which alone produced 50% reduction in GABA response, did not produce any increase in ethanol sensitivity to the $GABA_A$ receptor (data not shown). Hence, differences in ethanol's ability to enhance $GABA_A$ receptor function were not due to differences in PKC dependent phosphorylation. Seasonal variation in the oocytes, however, did appear to play a role in the lack of effect

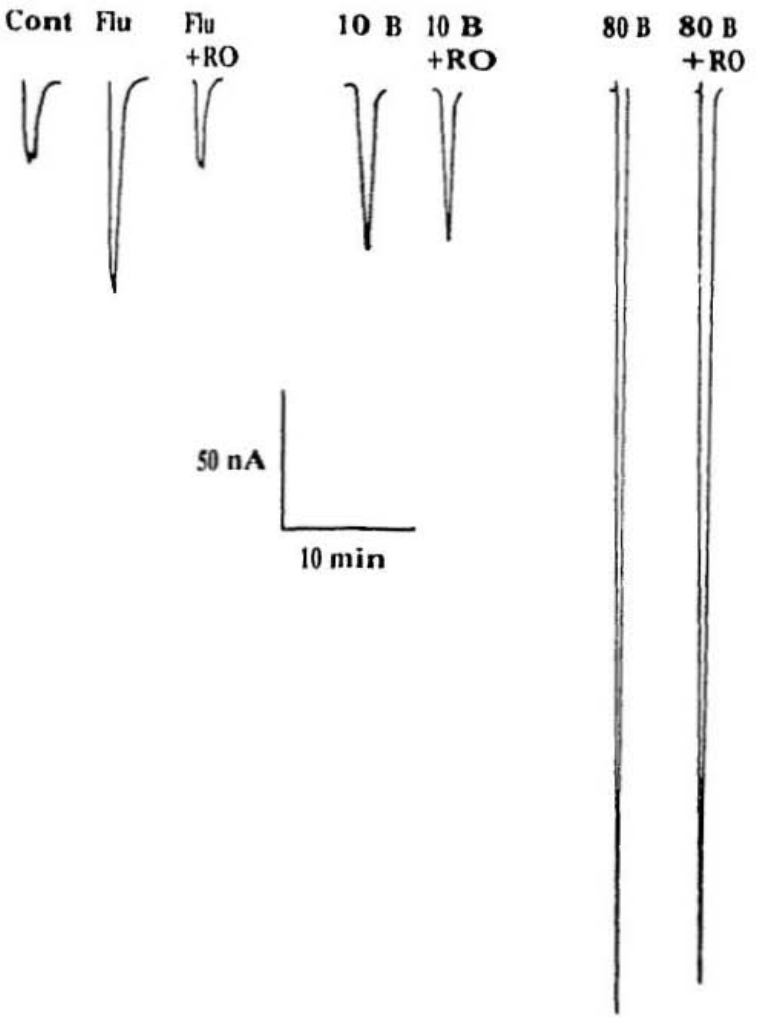
Table 3. Estimates of affinity (mean ± S.E.M.) of 1-butanol and 2-butanol for $\alpha_2\beta_2\gamma_{2L}$ and $\alpha_2\beta_2$ receptor constructs.

Subunit combination	1-butanol	2-butanol
$\alpha_2\beta_2\gamma_{2L}$	8.42 ± 0.76 (4)	5.48 ± 0.52 (4)[a]
$\alpha_2\beta_2$	14.61 ± 1.79 (4)	9.39 ± 1.34 (4)[b]

Numbers in parentheses represent number of oocytes. [a], $p = 0.0072$ compared to 1-butanol; [b], $p = 0.0785$ compared to 1-butanol (paired t test).

Figure 10. Potentiation of GABA-activated Cl^- current by flurazepam and butanol in *Xenopus* oocytes expressing the $\alpha_1\beta_2\gamma_{2L}$ receptor construct.

Representative current tracings show the effects of 5 μM GABA (control), and modulation by flurazepam and Ro15-4513. Ro15-4513 (500 nM) eliminates flurazepam potentiation of the GABA current. By contrast, in the same oocyte, Ro15-4513 does not affect the ability of 10 or 80 mM butanol to potentiate GABA current.


Cont
Flu
Flu
+RO
10 B
10 B
+RO
80 B
80 B
+RO
50 nA
10 min

of ethanol, since oocytes obtained from the same frog at different times of the year showed different sensitivities to ethanol (data not shown). Oocytes showing ethanol potentiation of GABA current were obtained from two donor frogs. As expected, ethanol potentiated the response to GABA. Thus, ethanol at a concentration of 20 mM produced a 34 $\pm$ 8% increase ($p < 0.001$, $n = 4$) in the response to 10 μM GABA at the $\alpha_1\beta_2\gamma_{2L}$ receptor construct.

Current traces produced by the co-application of 100 mM ethanol and 10 μM GABA are shown in Figure 11. Figure 11 also shows the interaction of Ro15-4513 with ethanol. Ro15-4513, a benzodiazepine receptor partial inverse agonist, had little or no effect on control GABA current, but when co-applied with 100 mM ethanol and 10 μM GABA eliminated the ethanol potentiation of GABA current (Figure 11). As a comparison, 10 mM butanol was co-applied with GABA in the same oocyte. Again, Ro15-4513 had no effect on butanol potentiation of the GABA response (Figure 11). Figure 12 combines data from 5 oocytes and demonstrates that Ro15-4513 significantly reduces 100 mM ethanol potentiation of the GABA response but has no effect on the butanol potentiation. Similar results were obtained using 200 mM ethanol and 20 mM butanol in 4 oocytes (Figure 12).

3.4. Effects of Ro15-4513 on other agents

To investigate the site and mechanism of action of Ro15-4513 at the $GABA_A$ receptor, different concentrations of butanol, heptanol and propofol, were co-applied with

Figure 11. Potentiation of GABA-activated Cl^- current by ethanol and butanol in oocytes expressing the $\alpha_1\beta_2\gamma_{2L}$ receptor construct.

Representative current tracings show the effects of 10 μM GABA (control), and modulation by ethanol and Ro15-4513. Ro15-4513 (0.5 μM) eliminates ethanol potentiation of the GABA current. By contrast, in the same oocyte, butanol potentiation of the GABA current is not blocked by Ro15-4513.

$\alpha_1\beta_2\gamma_{2L}$

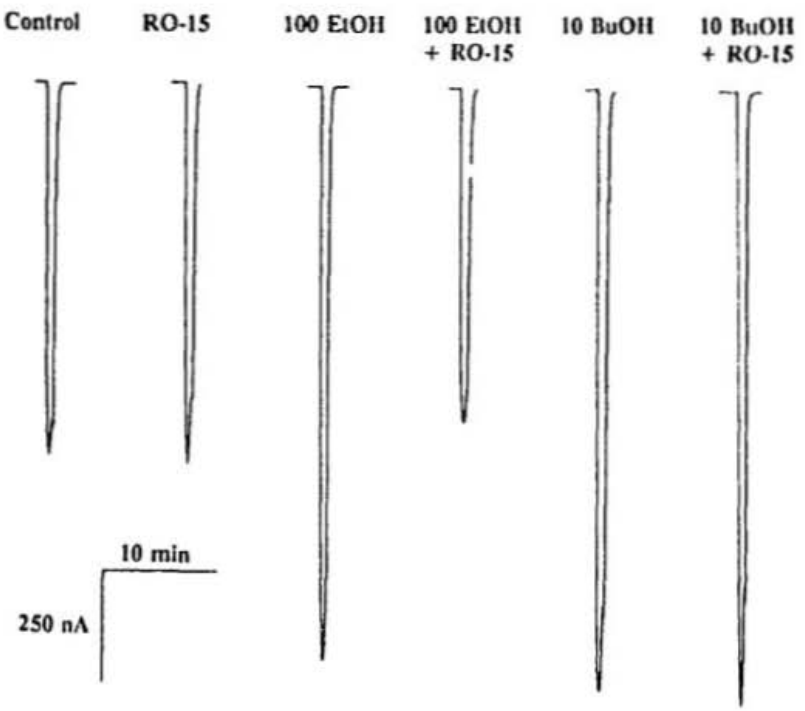
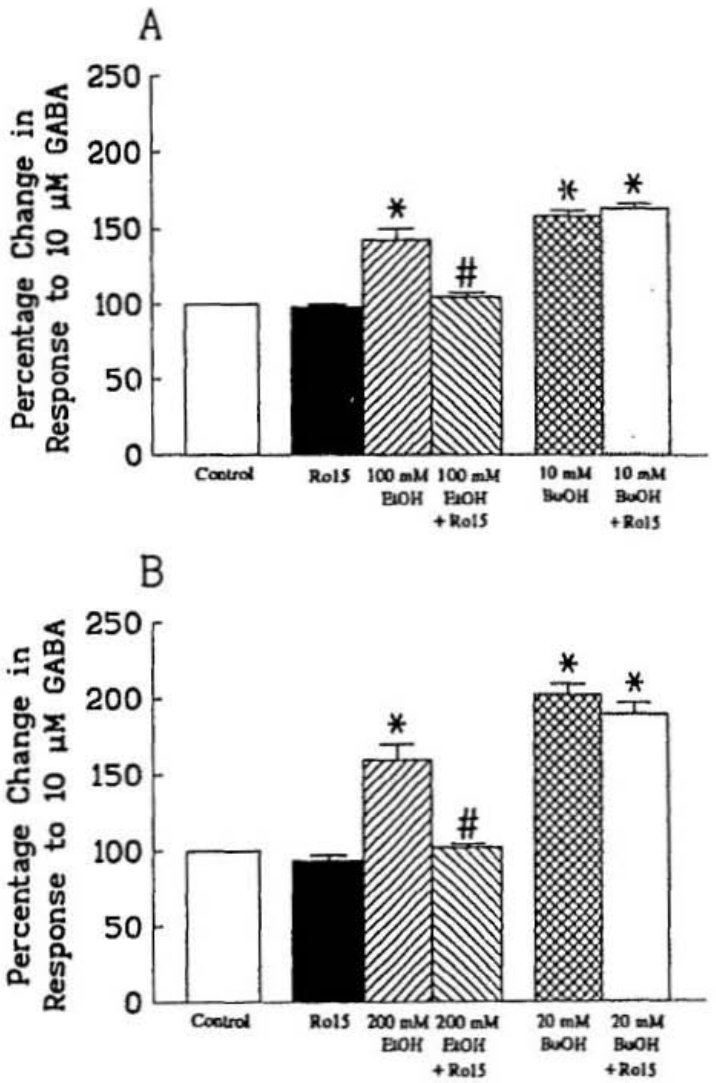


Figure 12. Ro15-4513 reduces ethanol, but not butanol potentiation of GABA currents at the $\alpha_1\beta_2\gamma_{2L}$ receptor construct.

A, Ro15-4513 did not change the control GABA response. However, Ro15-4513 completely blocked potentiation of the GABA response by 100 mM ethanol (#, $p<0.001$ compared to 100 mM ethanol, Bonferroni post-hoc test). In contrast, Ro15-4513 did not affect potentiation induced by 10 mM butanol (*, $p<0.001$ compared to Ro15-4513 alone, $n = 5$, Bonferroni post-hoc test). B, Increasing the concentration of ethanol to 200 mM and butanol to 20 mM yielded the same results as in (A). Significance levels as in (A) ($n = 4$).


A
Percentage Change in Response to 10 µM GABA
250
200
150
100
50
0
*
#
*
*
Control
Ro15
100 mM EtOH
100 mM EtOH + Ro15
10 mM BuOH
10 mM BuOH + Ro15
B
Percentage Change in Response to 10 µM GABA
250
200
150
100
50
0
*
#
*
*
Control
Ro15
200 mM EtOH
200 mM EtOH + Ro15
20 mM BuOH
20 mM BuOH + Ro15

Ro15-4513 and GABA. Figure 13 illustrates that Ro15-4513 (500 nM), when co-applied with increasing concentrations (5-80 mM) of 1- or 2-butanol, had no effect on the ability of these alcohols to potentiate I_{GABA}.

To determine the effect of different concentrations of Ro15-4513 on the GABA response, 500 nM and 5 μM Ro15-4513 were co-applied with 10 μM GABA. The GABA response was not affected by either concentration of Ro15-4513 (Figure 14). Figure 14 also shows that the same concentrations of Ro15-4513, when co-applied with 20 mM butanol or 1 mM heptanol, did not affect potentiation of I_{GABA} by these alcohols. In the presence of increasing concentrations of propofol (0.5-40 μM) and 5 μM GABA, 1 μM Ro15-4513 did not significantly affect I_{GABA} (Figure 15).

Figure 13. Ro15-4513 does not block butanol potentiation of GABA currents at the $\alpha_1\beta_2\gamma_{2L}$ receptor construct.

A, Ro15-4513 (0.5 μM) did not affect potentiation of the GABA current by any of the concentrations of 1-butanol (n = 3). B, Similarly, Ro15-4513 (0.5 μM) did not affect potentiation of the GABA current by any of the concentrations of 2-butanol (n = 3).

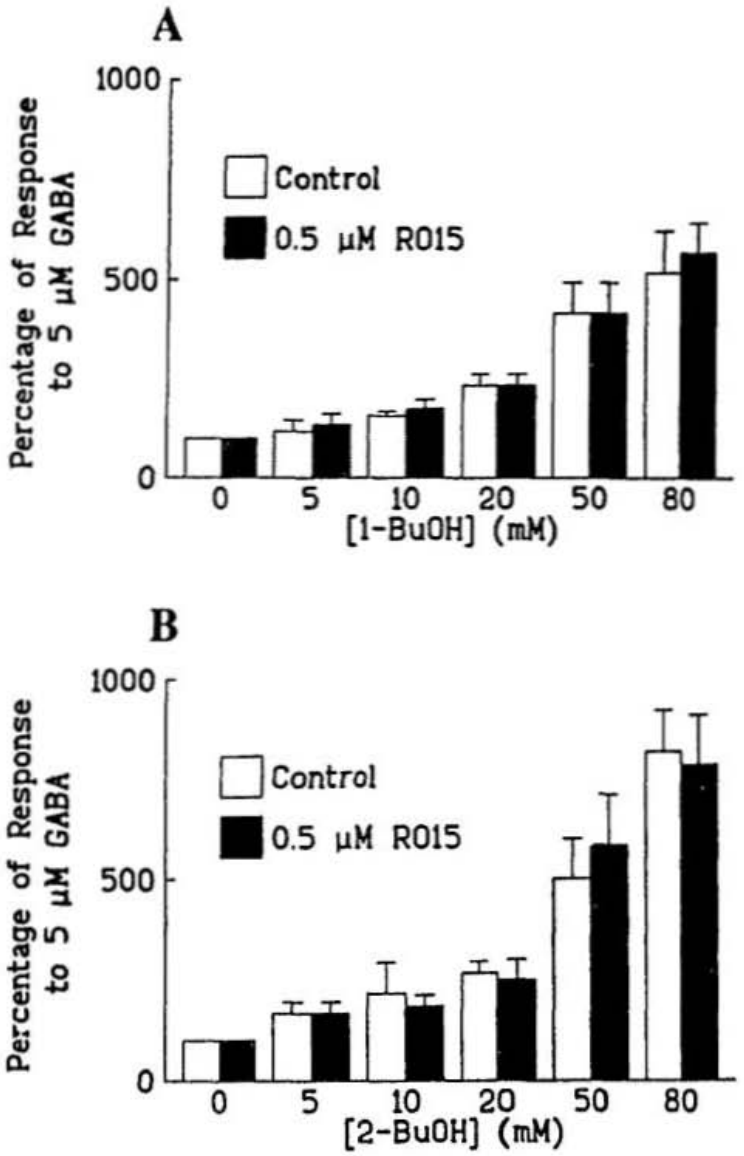

A
1000
500
0
Percentage of Response to 5 µM GABA
Control
0.5 µM RO15
0
5
10
20
50
80
[1-BuOH] (mM)
B
1000
500
0
Percentage of Response to 5 µM GABA
Control
0.5 µM RO15
0
5
10
20
50
80
[2-BuOH] (mM)

Figure 14. Concentrations of Ro15-4513 do not block potentiation of GABA currents by butanol or heptanol at the $\alpha_1\beta_2\gamma_{2L}$ receptor construct.

Ro15-4513 (0.5 and 5 μM) did not change the control GABA response. Similarly, Ro15-4513 did not block potentiation of the GABA response by 20 mM butanol or 1 mM heptanol (n = 4) (The open bar represents potentiation of I_{GABA} by each alcohol, the first hatched bar in each group represents 0.5 μM Ro15-4513 $\pm$ the alcohol and the second hatched bar in each group represents 5 μM Ro15-4513 $\pm$ the alcohol).

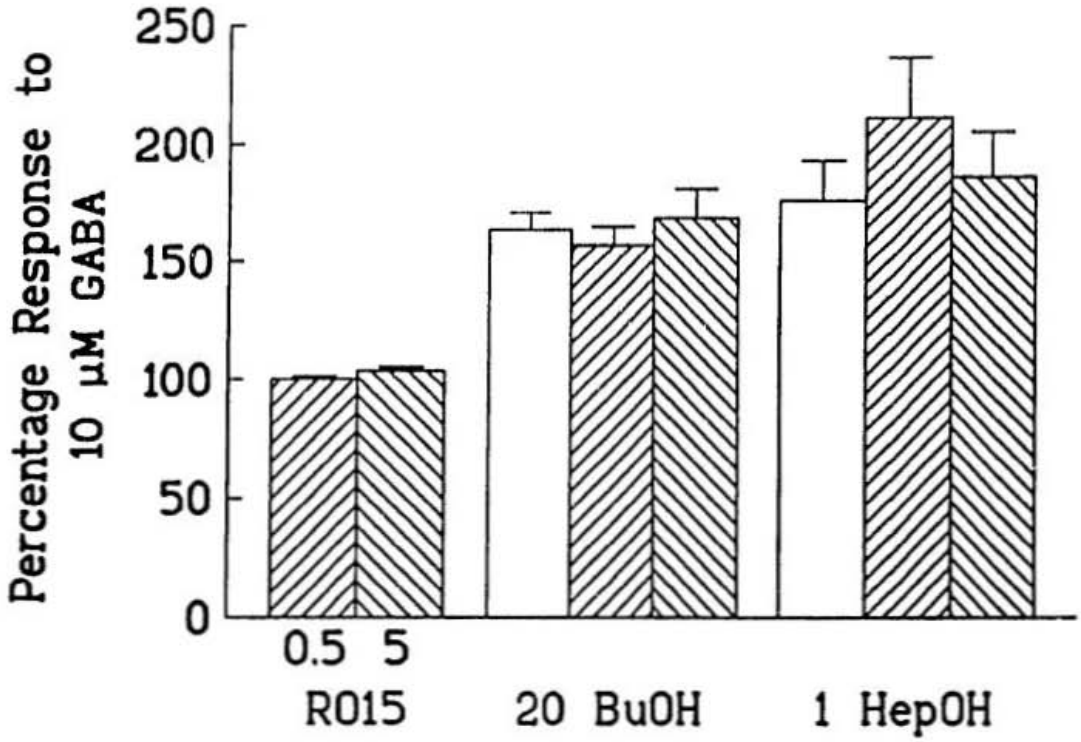
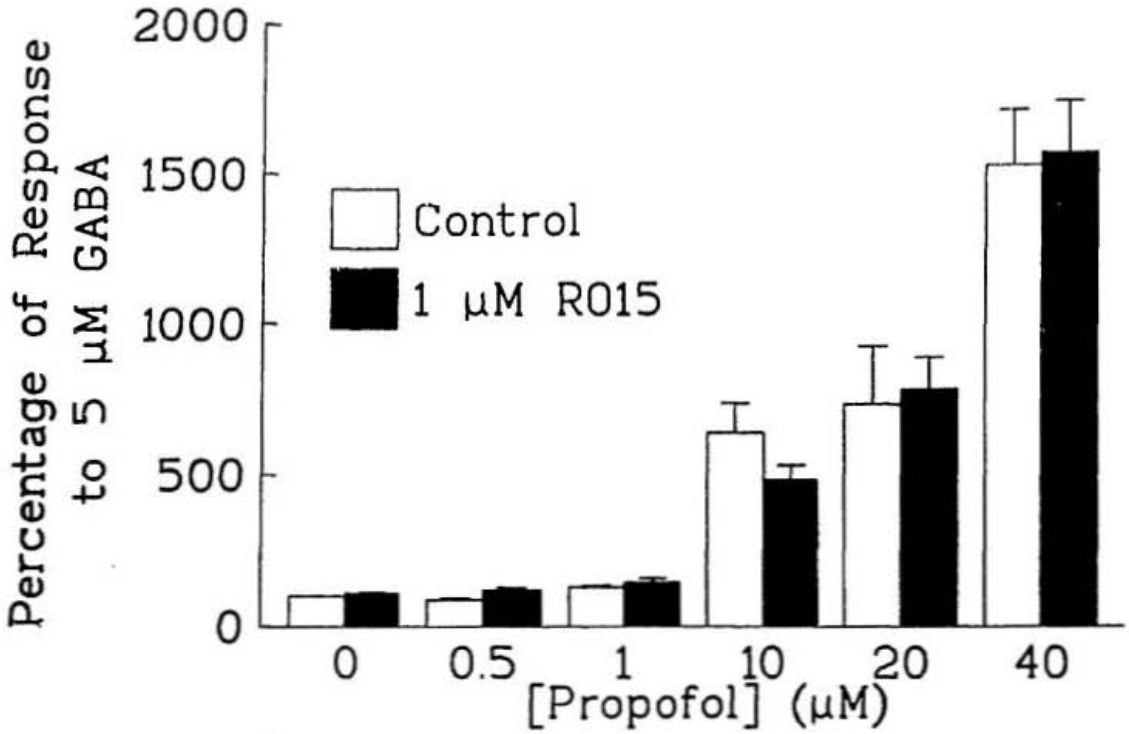

Percentage Response to 10 µM GABA
250
200
150
100
50
0
0.5 5
R015
20 BuOH
1 HepOH

Figure 15. Ro15-4513 does not block propofol potentiation of GABA currents at the $\alpha_1\beta_2\gamma_{2L}$ receptor construct.

Ro15-4513 (1 μM) did not affect potentiation of the GABA current by any of the concentrations of propofol (0.5-40 μM) (n = 3).


Percentage of Response to 5 µM GABA
2000
1500
1000
500
0
Control
1 µM R015
0
0.5
1
10
20
40
[Propofol] (µM)

IV. Discussion

4.1. Characterization of human recombinant $GABA_A$ receptors

$GABA_A$ concentration-response curves showing concentration-dependent increases in GABA currents for $\alpha_1\beta_2\gamma_{2L}$, $\alpha_2\beta_2\gamma_{2L}$ and $\alpha_2\beta_2$ human recombinant receptors, expressed in *Xenopus* oocytes, were similar to what others have reported in different systems. That is, the receptors which were expressed in the oocytes had EC_{50} values and Hill slopes similar to what has been reported for the same receptor constructs (Amin and Weiss, 1993; Levitan *et al.*, 1988). In addition, the GABA current was blocked by bicuculline, a competitive $GABA_A$ antagonist, and was potentiated by flurazepam, a benzodiazepine agonist. This demonstrated accepted pharmacological characteristics for "classical" $GABA_A$ receptors and confirmed the expression of recombinant $GABA_A$ receptors in the *Xenopus* oocytes.

4.2. A two site model of action of alcohols: ethanol versus longer-chain alcohols

As outlined in the introduction, there are distinct sites of action for benzodiazepines, steroids, and barbiturates on the $GABA_A$ receptor channel complex. However, it remains uncertain whether there are distinct binding sites for volatile anesthetics and alcohols. A number of groups have attempted to elucidate the site(s) and mechanism(s) of action of ethanol on the $GABA_A$ receptor. However, there remain a number of inconsistencies pertaining to ethanol's effects on GABA current (Mihic *et al.*,

1994a; Sigel *et al.*, 1993; Wafford and Whiting, 1992).

From a review of the literature, and from the present work, it seems reasonable to hypothesize that alcohols act at two distinct sites and/or have two mechanisms of action at the $GABA_A$ receptor. It appears that longer-chain alcohols ($\geq$4 C) act at a different site than ethanol and/or ethanol induces a conformational change in the $GABA_A$ receptor which differs from the longer-chain alcohols and anesthetics. In the present study, support for a two site model of action of alcohols is based on the observation that butanol enhances I_{GABA} in oocytes expressing $\alpha\beta$ receptors as effectively, if not more effectively, than I_{GABA} in oocytes containing $\alpha\beta\gamma$ receptors. The same is true of barbiturates, which effectively potentiate $GABA_A$ responses in the absence of a γ_2 subunit (Valeyev *et al.*, 1993). In contrast, ethanol does not potentiate I_{GABA} in oocytes expressing the $\alpha\beta$ combination (Wafford *et al.*, 1991; Wafford and Whiting, 1992). It is possible that in the absence of a γ_2 subunit, ethanol can not bind to the receptor. Similarly, the γ_2 subunit is necessary for benzodiazepines to modulate the $GABA_A$ receptor (Macdonald and Olsen, 1994; Wisden *et al.*, 1992).

In a study by Wafford and Whiting (1992), ethanol potentiated the GABA response only in oocytes expressing receptors containing the γ_{2L} subunit and not in oocytes expressing receptors containing the γ_{2S} subunit. They found that altering the eight amino acid insert, present only on the γ_{2L} splice variant, eliminated ethanol potentiation, suggesting a specific role of the insert in ethanol enhancement of $GABA_A$ receptor function. Similarly, Harris *et al.* (1995) reported that the γ_{2L} subunit was necessary for

ethanol to potentiate I_{GABA} in transfected mouse neurons. Although ethanol can potentiate I_{GABA} in oocytes expressing the γ_{2S} construct, much higher concentrations of ethanol are required (Mihic *et al.*, 1994a; Sigel *et al.*, 1993). Clearly, it would seem that potentiation of I_{GABA} by ethanol is not an all or none phenomenon depending on whether γ_{2L} or γ_{2S} is present. It does appear though, that ethanol enhances I_{GABA} more effectively in the presence of γ_{2L} versus γ_{2S}. Perhaps there is a difference in the affinity of ethanol for the $GABA_A$ receptor with γ_{2L} or ethanol may be more effective in inducing a conformational change in the receptor in the presence of a γ_{2L} subunit. Regardless, other findings as well as those from the present study imply some specificity of action of ethanol with the $GABA_A$ receptor protein.

Longer-chain alcohols may act at a site which is associated with barbiturates or anesthetics such as propofol. In contrast, ethanol, because of its physical and chemical properties, acts at a different site which is associated with effects at lower concentrations. In a genetic study, Allan *et al.* (1988) found that 10-30 mM ethanol enhanced muscimol-stimulated Cl^- flux in membranes of ethanol sensitive animals, but that 50-100 mM ethanol had little or no effect on Cl^- flux in membranes of these animals, suggesting that actions for ethanol on the $GABA_A$ receptor are more important at lower ethanol concentrations. Mihic *et al.* (1994a) suggested that the potentiation seen by Wafford *et al.* (1990) and Wafford and Whiting (1992) by ethanol (20 mM), represents a low-dose effect of ethanol requiring the γ_{2L} subunit. From their own data, Mihic *et al.* (1994a) suggested a distinct high-dose or anesthetic effect as well for ethanol and that the two

effects likely have different mechanisms. Similarly, Aguayo and Pancetti (1994) observed ethanol potentiation of I_{GABA} in some neurons with low ethanol concentrations (25-200 mM), while potentiation was observed in all neurons at higher ethanol concentrations (425 and 850 mM). They also suggested that different mechanisms may be responsible for this potentiation of GABA by ethanol and that ethanol may act at several sites. For example, low ethanol concentrations may affect a protein, such as phosphatase or a kinase, whereas higher concentrations of ethanol may exhibit less specificity in other interactions in the membrane lipid domain.

4.2.1. Ro15-4513 selectively blocks the effects of ethanol but not that of other alcohols

Further support for a two site model of action of alcohols is the finding that butanol and longer-chain alcohols are not sensitive to Ro15-4513, whereas the potentiating effects of ethanol are blocked by this compound. In the present study, Ro15-4513 blocked flurazepam as well as ethanol potentiation of GABA current but did not affect the action of other diverse chemical agents. Flurazepam served as a positive control since Ro15-4513 is known to block the potentiating effects of benzodiazepines or GABA current. To compare the effects of Ro15-4513 on ethanol versus longer-chain alcohols, equieffective concentrations of the alcohols were used. In this case, 100 and 200 mM ethanol were roughly equivalent to 10 and 20 mM butanol concentrations. Ro15-4513 completely blocked the potentiating effects of 100 and 200 mM ethanol, but had no effect on the potentiating effects of butanol and heptanol. Even concentrations of

Ro15-4513 ten times greater than that needed to block ethanol had no influence on the response to butanol or heptanol. Since Ro15-4513 selectively blocked ethanol's actions at 100 and 200 mM, this would suggest that ethanol has a selective effect. In my work, I did not see evidence for a low versus high dose effect of ethanol in $\alpha_1\beta_2\gamma_{2L}$ receptors. However, very high concentrations of ethanol (>200 mM) were not used in my experiments.

Ro15-4513, a benzodiazepine receptor partial inverse agonist which acts close to the benzodiazepine site on the $GABA_A$ receptor, has also been suggested to be an alcohol antagonist. Ro15-4513 blocks the effects of ethanol on Cl^- flux (Glowa *et al.*, 1989; Suzdak *et al.*, 1986; reviewed in Tanelian *et al.*, 1993). Reynolds *et al.* (1992) demonstrated that Ro15-4513 (50-100 nM) inhibits the ability of ethanol to enhance I_{GABA} in cultured rat cortical neurons. In an *in vitro* study in human neuronal tissue, Palmer *et al.* (1990) suggested that Ro15-4513 antagonizes ethanol's effects by interacting indirectly with ethanol through some allosteric mechanism. In an *in vivo* study, Palmer *et al.* (1988) demonstrated a noncompetitive antagonism of Ro15-4513 on the effects of ethanol. Wafford *et al.* (1990) showed that 1 μM Ro15-4513 had no direct antagonistic effects on GABA current, but did inhibit ethanol's potentiating effect on I_{GABA} expressed from LS whole brain mouse mRNA in *Xenopus* oocytes.

A number of studies suggest that the ability of ethanol to enhance GABA current involves the benzodiazepine binding site. The ability of ethanol to enhance I_{GABA} may be due to an increased efficiency of coupling between the benzodiazepine site and the Cl^-

channel (Reynolds *et al.*, 1992). Low concentrations of Ro15-4513 can antagonize the effects of ethanol at the $GABA_A$ receptor by binding to benzodiazepine recognition sites associated with the receptor (Glowa *et al.*, 1989). Suzdak *et al.* (1986) suggested an interaction of Ro15-4513 with the benzodiazepine receptor since the effects of Ro15-4513 (*in vitro* and *in vivo*) are completely blocked by benzodiazepine receptor antagonists. In addition, Ro15-4513 blocks the behavioural effects of benzodiazepines, partially antagonizes the behavioural effects of ethanol, but does not block the behavioural effects of barbiturates (Deacon *et al.*, 1990). The effects of Ro15-4513 on behavioural responses to ethanol (including self-administration) can be reversed with the benzodiazepine receptor antagonist Ro15-1788 (Deacon *et al.*, 1990; June *et al.*, 1994), again suggesting that these effects are mediated through the benzodiazepine receptor complex.

It has been proposed that benzodiazepines affect the rate constant involved in the binding of the first GABA molecule to the receptor (Macdonald and Olsen, 1994). In their review, Macdonald and Olsen (1994) stated that several drugs modify the $GABA_A$ receptor at different steps in either the binding or gating processes of $GABA_A$ receptor channel activation. Again, they propose that benzodiazepines affect the affinity of the first GABA binding site for GABA which ultimately causes an increase in the frequency of channel opening. In other words, an increased association rate or a decreased dissociation rate at the first binding site would account for increased open frequencies of the GABA receptor channel. It is possible that ethanol affects the same rate constant as benzodiazepines, but there are no published reports on ethanol's effects on GABA at the

single channel level. In conclusion, it appears that ethanol and benzodiazepines share some part of a mechanism of action or induce some conformational shift which can be blocked by Ro15-4513.

4.3. Variability in ethanol potentiation of I_{GABA}

In this study, ethanol potentiated I_{GABA} in some, but not all of the oocytes tested. In fact, the variability with ethanol appeared to be seasonal. That is, ethanol failed to potentiate GABA currents in oocytes from the same donor frog and injected with the same RNA preparation at different times of the year. It may be that the oocytes process $GABA_A$ subunits in slightly different ways. However, this appeared to be critical only for ethanol since butanol always enhanced I_{GABA}. This idea of seasonal variability may explain some of the discrepancies contained in the literature concerning ethanol's ability to modulate $GABA_A$ receptor function in oocytes. Sigel *et al.* (1993) and Mihic *et al.* (1994a), using the same subunit combinations, were unable to replicate the results of Wafford and Whiting (1992), who demonstrated potentiation of GABA currents with low concentrations of ethanol (10-20 mM). Differences in subunits across species, different experimental conditions, or posttranslational modification of the receptor channel in different batches of oocytes, were offered as possible explanations for the discrepant results (Sigel *et al.*, 1993). Indeed, perhaps some posttranslational modification, such as phosphorylation of the receptor or receptor subunit assembly, is different in oocytes at different times of the year. The $GABA_A$ receptor complex is subject to phosphorylation

via several protein kinases (Tanelian *et al.*, 1993). According to Wafford and Whiting (1992), the γ_{2L} subunit, which has a consensus sequence for phosphorylation by protein kinase C, along with receptor phosphorylation may be necessary for modulation of $GABA_A$ receptor function by ethanol. However, we found that phosphorylation of the receptor by PKC is not sufficient to cause ethanol sensitivity, since treatment of oocytes with OAG, which would activate endogenous PKC in oocytes, did not restore ethanol sensitivity of the receptor complex.

4.4. Further evidence for protein theory of alcohol action at $GABA_A$ receptor

According to the lipid theory of alcohol action, as lipid solubility of the alcohol increases (i.e. increased membrane/buffer partition coefficient), potency of the alcohol for disordering membrane lipids increases exponentially (McCreery and Hunt, 1978; Lyon *et al.*, 1981). In the present study, however, membrane/buffer partition coefficients for 1-butanol and 2-butanol did not predict their potency to potentiate GABA responses. In fact, 2-butanol was more potent in potentiating GABA currents than was 1-butanol even though 2-butanol has a lower membrane/buffer partition coefficient (i.e. 0.815 for 2-butanol compared to 1.52 for 1-butanol). Some groups have observed different cutoff effects in alcohol potency as the number of C atoms increases with different types of receptors (Li *et al.*, 1994; Fan and Weight, 1994; Nakahiro *et al.*, 1991; Peoples and Weight, 1994; Peoples and Weight, 1995). For example, the cutoff for inhibiting NMDA receptor-mediated responses is 6-8 carbon atoms (Peoples and Weight, 1995). However,

these longer-chain alcohols are still able to insert into membranes and disorder membrane lipids (increase fluidity). It has been suggested that alcohols interact directly with hydrophobic pockets on the receptor proteins, and that bigger alcohol molecules are not able to bind to the size-specific hydrophobic pockets and thus can not exert their effects (Li *et al.*, 1994; Peoples and Weight, 1995).

Wood *et al.* (1989) reported relatively small changes in membrane fluidity induced by pharmacologically relevant concentrations of ethanol. Thus, levels of ethanol which are reached in the brain may not be sufficient to produce significant changes in membrane fluidity. In addition, Harris *et al.* (1984) demonstrated that membrane preparations prepared from selected lines of animals (LS versus SS mice) showed similar changes in membrane fluidity when exposed to ethanol *in vitro*. Since ethanol-induced changes in lipid fluidity were almost the same in the two lines of mice, this cannot account for the striking behavioural difference observed in the two lines. Furthermore, there is a strong argument for a specific action of ethanol on the receptor protein versus the membrane lipid since there appear to be relatively stringent requirements for specific subunits and/or posttranslational modifications of the receptor complex.

There was also a difference in the degree of butanol potentiation of GABA current expressed in oocytes containing $\alpha_1\beta_2\gamma_{2L}$ versus $\alpha_2\beta_2\gamma_{2L}$ receptor constructs. In fact, there were significant differences in the receptors with respect to their responses to both 1- and 2-butanol. Current responses with both alcohols were greater in oocytes expressing α_2 containing receptors. These results certainly support the idea of an interaction of alcohols

with the receptor protein. Similarly, effects of benzodiazepines vary depending on subunit composition, that is, whether the α_1 or the α_2 subunit is present (Hadingham *et al.*, 1993a; Puia *et al.*, 1991). Like benzodiazepines, it appears that alcohols vary in their affinities and efficacies depending on the type of α subunit present. Differential effects of modulatory agents in response to changes in subunit composition suggests specific interactions of those agents with the receptor complex.

4.4.1. Effect of stereoisomers of 2-butanol on I_{GABA}

Moody *et al.* (1994) observed significant differences in potencies of (+)- and (-)-isoflurane, a volatile anesthetic, both *in vitro* and *in vivo*. They suggested from their results, that proteins are the primary sites of anesthetic action. In contrast to what was expected, (R)-2-butanol and (S)-2-butanol did not differ in their modulation of $GABA_A$ receptor function. A possible explanation for the observed similarity is that the structural differences that do exist between the two molecules are not great enough to significantly alter drug effects. The stereoisomers are still relatively simple molecules. Even though they are the simplest chiral alcohols, the molecules are not sufficiently bulky or complicated enough to have strict structure-activity relationships. Clearly, there is no strict requirement for these molecules to interact with the $GABA_A$ receptor.

4.5. Future research

The findings in the present study raise a number of new questions which should

certainly be addressed in future to further our understanding of the structure-activity relationships of alcohols on the $GABA_A$ receptor as well as the influence of subunit composition on these alcohols. One additional experiment would be to combine alcohols, such as ethanol and butanol, to determine what effect this combination has on I_{GABA} potentiation. Depending on whether the effect is additive, enhanced or attenuated compared to their individual effects on I_{GABA}, it may be possible to distinguish if there are two closely linked sites on the $GABA_A$ receptor for the binding of different alcohols. Another experiment which should be carried out is to determine what effect methanol has on I_{GABA}, to see if there is a linear relationship between carbon chain length and its effects on the $GABA_A$ receptor. In other words, it would be interesting to see if there is a similarity in the effects of alcohols with just a difference in potency as carbon chain length increases, or if ethanol is in a class by itself as a unique alcohol molecule because of its size and physicochemical properties. In addition, equieffective concentrations of GABA should be used to give a more accurate comparison of the effects of 1- and 2-butanol on I_{GABA} at $\alpha\beta$ versus $\alpha\beta\gamma$ receptor constructs.

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