INTERACTION BETWEEN PROPOFOL AND BENZODIAZEPINE SITE LIGANDS TO POTENTIATE $GABA_A$ RECEPTOR ACTIVATION IN HUMAN RECOMBINANT RECEPTORS EXPRESSED IN *Xenopus* oocytes

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Interaction between propofol and benzodiazepine site ligands to potentiate $GABA_A$ receptor activation in human recombinant receptors expressed in *Xenopus* oocytes

by

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ABSTRACT

Potentiation of inhibitory synaptic transmission mediated by γ-aminobutyric acid (GABA) acting at the GABA type A ($GABA_A$) receptor is increasingly regarded as a primary site of action for anesthetic drugs. $GABA_A$ receptors across different brain regions can have a different protein subunit composition, which influences the pharmacological sensitivity of the receptor complex. The intravenous general anesthetic propofol is frequently combined with benzodiazepines, and there are reports of synergism in the production of anesthesia. However, the nature of this interaction is unknown. The purpose of this study was therefore to examine the interaction between propofol and the benzodiazepine receptor agonist flurazepam at the receptor level. In addition, preliminary studies on the influence of subunit composition on the potentiation of $GABA_A$ receptor function by propofol have been carried out. Two-electrode voltage-clamp recording of GABA-activated membrane currents were performed in *Xenopus laevis* oocytes expressing human recombinant $GABA_A$ receptors.

Propofol was found to produce similar potentiation of GABA (3 μM) receptor-activated currents in receptors composed of $\alpha_1\beta_2\gamma_{2L}$ and $\alpha_2\beta_2\gamma_{2L}$ subunits. Propofol appears to increase the affinity of $GABA_A$ receptors for GABA without changing the maximal current. In contrast, propofol potentiation of GABA currents was influenced by the γ_{2L} subunit. Receptors composed of $\alpha_2\beta_2$ subunits were potentiated to a greater extent than receptors composed of $\alpha_2\beta_2\gamma_{2L}$ subunits. The time course of $GABA_A$ receptor desensitization can be described by a double exponential function. Propofol was found

to reduce receptor desensitization by significantly prolonging the slow time constant of current decay, resulting in a large increase in the steady-state current activated by a high GABA concentration.

The interaction between propofol and flurazepam was examined in receptors composed of $\alpha_x\beta_2\gamma_{2L}$ (x=1 or 2) subunits. Currents activated by GABA (3-30 μM) were potentiated by low concentrations of propofol (0.5-10 μM) plus flurazepam (0.25 and 0.5 μM) to levels which were significantly greater than expected from an additive response. At higher concentrations of propofol (20 μM) or flurazepam (1 μM) the potentiation of GABA receptor-activated currents was not different than expected from an additive response. The benzodiazepine receptor partial inverse agonist RO15-4513 was found to abolish the flurazepam potentiation of GABA-activated membrane currents without affecting the propofol response. Zopiclone, a cyclopyrrolone derivative which is classified as a full agonist at benzodiazepine receptors, did not produce the same greater than additive interaction with propofol. In fact, zopiclone (100 nM) combined with propofol (1-10 μM) produced an enhancement of the GABA current which was significantly less than the expected additive response.

These results suggest that propofol has multiple effects on $GABA_A$ receptor function: An increase in the affinity of the receptor for GABA plus a decrease in receptor desensitization at higher GABA concentrations. The type of α subunit contained in the receptor does not appear to have a major influence on the ability of propofol to modulate $GABA_A$ receptor function. However, propofol was significantly less effective in the presence of a γ_{2L} subunit. Thus similarly to many other modulators, potentiation of

$GABA_A$ receptor function by propofol does exhibit subunit dependence. Synergism observed clinically by the combination of propofol or propofol plus benzodiazepines can be explained by an interaction at the receptor level. In addition to increasing the affinity for GABA, flurazepam increased the affinity of the receptor for propofol, resulting in a greater than expected potentiation of $GABA_A$ receptor function. This interaction between propofol and benzodiazepines was not due to simple potentiation of $GABA_A$ receptor activity, since zopiclone failed to produce the supra-additive effect when combined with propofol.

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DEDICATION

I would like to dedicate this thesis to my father, mother and all the family members, as acknowledgement and token of thanks for their love, continuous support and encouragement throughout all my years, which has brought me this far.

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1.0 Introduction

γ-Aminobutyric acid (GABA) is the most abundant inhibitory neurotransmitter substance in the central nervous system (CNS). Almost 30% of the neuronal cells in brain, mainly the small interneurons are considered to be GABA-ergic, i.e. use GABA as their neurotransmitter substance (9). The most well studied function of synaptically released GABA is to hyperpolarize postsynaptic neurons. GABA also plays an important role as a neurotransmitter and/or paracrine effector to regulate a variety of physiological mechanisms in the periphery (43), but a discussion of these effects is beyond the scope of this thesis. GABA was first identified to be a constituent of the CNS in 1950, though it was first synthesized in 1883. GABA has been found to have major roles, both direct and indirect, in a host of neurological and psychiatric disorders.

A review of the amino acid transmitter shows (14), GABA is synthesized in the presynaptic nerve terminal by α-decarboxylation of L-glutamic acid by the enzyme GAD (glutamic acid decarboxylase). High concentrations of GAD are found in CNS gray matter. A very good correlation has been found between the localization of the enzyme GAD and GABA in the tissue. GAD is known to be tightly bound to its co-factor pyridoxal phosphate, but structural analogs of glutamate, sulfhydryl reagents, thiol compounds, anions and adenine nucleotides can promote dissociation or block association of the co-factor resulting in the inhibition of enzyme activity. GABA is found to be present in high concentrations in brain and spinal cord, but it is also present in trace amounts in peripheral nervous tissues. GABA is mainly metabolized by transamination

in presence of the enzyme GABA-transaminase (GABA-T), which is primarily localized in mitochondria. In brain GABA is also actively taken up into presynaptic nerve endings by a sodium dependent re-uptake mechanism.

The effects of GABA are mediated through a set of receptors which are classified as $GABA_A$ and $GABA_B$ receptors. $GABA_B$ receptors are coupled to G-proteins which decrease calcium or increase potassium conductances. $GABA_B$ receptors are stimulated by baclofen (14).

$GABA_A$ receptors on the other hand are essentially ligand gated chloride channels. $GABA_A$ receptors are competitively blocked by bicuculline. The $GABA_A$ and $GABA_B$ receptors are located both pre- and postsynaptically on most neurons (9). The inhibitory effect of GABA acting at $GABA_A$ receptors in the CNS results from an increase in membrane conductance. The hyperpolarization (and in some cases depolarization) of neurons is produced by an increase in membrane permeability to chloride and bicarbonate ions (14).

1.1 Structure and pharmacology of $GABA_A$ receptors

The $GABA_A$ receptor is included in the superfamily of ligand gated ion channels, which includes the nicotinic acetylcholine receptor, serotonin receptor subtype 3 and glycine receptors. Members of this family have a high degree of similarity in amino-acid sequences (61). $GABA_A$ receptors have a hetero-oligomeric structure composed of peptide subunits which forms an integral ion channel and the extracellular domain of the receptor comprises binding sites for GABA and other pharmacological modulators (77). Within

a very short time (in time scale of microseconds) of release from the nerve terminal, the neurotransmitter GABA binds to the $GABA_A$ receptor protein complex and triggers a conformational change in the receptor structure. The change in configuration opens an aqueous pore across the membrane resulting in the flow of ions along their electrochemical gradient (90).

Electron microscopic studies of isolated porcine cortical $GABA_A$ receptors show a pentameric structure of the receptor protein complex with a central water filled pore which is a characteristic feature of ligand gated ion channels (59). Determination of the molecular structure of the $GABA_A$ receptor was done with photoaffinity labelling by benzodiazepine binding (51) and by solubilization of $GABA_A$ receptor complexes from mammalian brain followed by purification with benzodiazepine affinity chromatography (77). The cloning and sequencing of $GABA_A$ receptor subunits have provided evidence for five different subunit families: α, β, γ, δ and ρ subunits. Most subunits have been found to have multiple subunit isoforms: $\alpha_{1\text{-}6}$, $\beta_{1\text{-}4}$, $\gamma_{1\text{-}3}$ and $\rho_{1\text{-}2}$. There is 30 - 40% sequence identity among the subunit families, whereas the amino acid sequences for subtypes within each family have 70 - 80% homology (51).

Alternative splicing of primary mRNA transcripts also generates subunit isoforms called splice variants. Thus, the γ_2 subunit exists as γ_{2S} and γ_{2L} which differ by 8 amino acid insertion in the intracellular loop between the transmembrane domains M3 and M4 (39). Bovine, rat and human brains possess both forms of the γ_2 subunit as demonstrated by immunocytochemistry. Alternative RNA splicing of the bovine γ_2 subunit results in a long and short variety of the γ_2 subunit (94). Chicken $GABA_A$ receptor β_4 subunit also

has two splice variants, β_4 and β_4', differing in 4 amino acids in the intracellular loop between the M3 and M4 membrane spanning regions (5).

The individual subunits of $GABA_A$ receptors also differ in their molecular weights with α, β and γ being 48-53 kDa, 55-57 kDa and 48 - 50 kDa, respectively. The molecular weight of the purified receptors has been found to be between 240 - 250 kDa, which if compared to the molecular weight of individual subunits is found to be consistent with a pentameric structure (77).

Each subunit of the $GABA_A$ receptor has a prominent N-terminal of about 200 amino acids in the extracellular region, considered to contain ligand binding sites, four putative transmembrane domains (M1 - M4), a short extracellular C-terminal and a cytoplasmic domain of variable length between M3 and M4, which bears consensus sites for phosphorylation (61). Characterization of $GABA_A$ receptors by using radioligands has specified a number of drug binding sites on the receptor complex. In addition to the ligand binding sites, the extracellular N-terminal region also contain sites for glycosylation.

The five protein subunits which presumably forms $GABA_A$ receptors have a circular ring-like arrangement. The individual subunits traverses the lipid bilayer of the neuronal membrane and together forms a cylindrical narrow aqueous pore at the centre. The ion channel is lined by five M2 α-helices and each of them is contributed by an individual subunit. Charged groups at either end of the pore and, the amino acid side chains lining the pore, determine the ion permeability and selectivity (90). Biochemical studies have suggested that each $GABA_A$ receptor contains five protein subunits, but the

actual stoichiometry and the receptor subunit composition in mammalian brain is still unknown. Information is also lacking regarding the subunits which combines to form functional receptors *in situ*.

In recombinantly expressed $GABA_A$ receptors, there is evidence that at least 3 different polypeptides, most commonly $\alpha_x\beta_y\gamma_z$ are needed to form a functional GABA-benzodiazepine receptor complex (50).

$GABA_A$ receptors are the target for a number of pharmacologically and clinically important drugs. Electrophysiological and binding studies suggest that part of the pharmacological action of drugs like sedative-hypnotics, anxiogenic, convulsants, antidepressants, anesthetics, anxiolytics, anticonvulsants and neurosteroids is mediated through $GABA_A$ receptors (74). Many of the drugs in the above mentioned categories do not bind to the GABA binding site but interact indirectly with it by attaching themselves to allosteric sites on the receptor (74). The site for GABA binding is labelled by agonists such as [^{3}H]GABA or [^{3}H]muscimol. Both high and low affinity sites for GABA and other agonists are present on the $GABA_A$ receptor. The low affinity site has some preference for GABA antagonists and can be labelled by specific antagonists like (+)bicuculline or SR 95531 (74). The $GABA_A$ receptor chloride channel can be blocked both by picrotoxin and penicillin. The binding of GABA and other agonists to the ligand binding site is altered by barbiturates, benzodiazepines and steroids (76). Pentobarbital has been shown to increase the number of high affinity sites for GABA (74). There is a separate site for benzodiazepines where agonists like diazepam, flurazepam and antagonists like flumazenil bind (9). Heteromeric $GABA_A$ receptors expressed *in vitro*

bears functional resemblances with the native receptors found in mammalian brains, suggesting the presence of heteromeric receptors *in vivo*. On the other hand, *in vitro* expression of homomeric receptors has also been proved to produce functional ion-channels. This is indicative of the presence of ligand binding sites on individual subunit.

1.2 Pharmacology of $GABA_A$ receptor subunits

Among the subunits which make up the $GABA_A$ receptor, the α subunit plays a major role in imparting specific pharmacological characteristics to the receptor complex. Study of receptor pharmacology is quite often expained by the effects of pharmacological agents in terms of its affinity and efficacy towards specific receptors. Efficacy of a drug is referred to as the maximal effect produced by it at the receptor. Affinity gives a measure of the potency of the drug to bind with the receptor in order to produce a particular level of response. The α subunit has been found to be the major determinant of efficacy for partial GABA agonists like (RS)-dihydromuscimol, piperidine-4-sulfonic acid and 4,5,6,7-tetrahydroisoxazolo[5,4-c] pyridin-3-ol at recombinant $GABA_A$ receptors expressed in *Xenopus* oocytes (19). Again coexpression of α_1 with either α_3 or α_5 along with different β and γ subunits shows that α_1 is mainly responsible for determining the efficacy, whereas the affinity is dependent on the entire combination of subunits. Thus, the pharmacological characteristics of receptors containing two different α subunits are different from those containing a single α subunit (19). To induce large GABA-gated currents, as shown in *Xenopus* oocytes, the presence of at least one α subunit is essential (75). α_5 along with β_1 demonstrates the highest affinity for GABA.

Co-expression of α_4 with β_2 and γ_2 subunits in cultured human embryonic kidney 293 cells produces $GABA_A$ receptor having high binding affinity for muscimol and Ro 15-4513, but not for benzodiazepine agonists. The binding profile of this receptor is similar to $\alpha_6\beta_2\gamma_2$ receptors, but not to $\alpha_1\beta_2\gamma_2$ receptors. This suggests that α_4 and α_6 share similar functional properties (96). The presence of a particular type of α subunit isoform actually helps to determine benzodiazepine sensitivity at $GABA_A$ receptors. Benzodiazepines act in the brain on a specific class of $GABA_A$ receptors often termed benzodiazepine receptors. For the action of benzodiazepine agonists the presence of both α and γ subunits seems to be indispensible. The presence of the γ_2 subunit along with α and β subunits gives rise to a receptor which is positively modulated by benzodiazepines (67). Cells transfected with α_1 and γ_2 subunits produce functional receptors responsive to benzodiazepines and is similar to that found with recombinant $\alpha_1\beta_2\gamma_2$ receptors. This indicates that α and γ subunits have sites important for the action of benzodiazepines (35). The α subunit isoform is a major determining factor in forming type I and II benzodiazepine receptors. $\alpha_1\beta\gamma$ constitutes the type I receptor, whereas type II receptors are heterogenous, containing α_2, α_3 or α_5 subunits. Benzodiazepines exhibit higher affinity for type I rather than type II receptors (66). Type I receptor selective drugs such as Zolpidem and CL 218 872 have high affinity for type I benzodiazepine receptors, whereas type II receptors have much lower affinity for the same drugs (91). The α_5 subunit confers type II benzodiazepine binding, but has lower affinity for zolpidem and Cl 218 872 than that found with type II receptors containing α_2 or α_3 subunits, suggesting that α_5 produces a unique type II benzodiazepine receptor (65).

Type I benzodiazepine receptors are the predominant group in the CNS (66). Full agonists at benzodiazepine type I receptors have negligible action at $\beta_2\gamma_2$ receptors but non-specific benzodiazepine agonists like diazepam produce a significant potentiation at the same receptor. This might be due to the fact that type I benzodiazepines have a binding domain specific for the α_1 subunit, whereas the non-selective drugs might bind to varying sets of interacting domains provided by β_2 and γ_2 subunits (35). Evidence for the presence of allosteric binding sites on α and γ has led to the proposition of location of allosteric benzodiazepine binding site having overlapping domain on the individual subunit proteins (83). The efficacy of benzodiazepines at different receptor subtypes is found to be in the order $\alpha_1\beta_2\gamma_2 > \alpha_1\gamma_2 > \beta_2\gamma_2$ (35). The histidine residue at position 101 on the α_1 subunit is important for the construction of binding domains for benzodiazepine agonists such as diazepam and zolpidem (95).

Cerebellar granule cells possess a novel $GABA_A$ receptor having subunit combination $\alpha_6\beta_2\gamma_2$, containing a unique benzodiazepine site sensitive to antagonists and inverse agonists, but insensitive to agonists such as diazepam. Based on the pharmacological properties, this receptor subtype is considered different from both type I & II benzodiazepine receptors (49). A single amino acid substitution (Arg-100 to glutamine) on the α_6 subunit restores diazepam sensitivity, but the sensitivity to GABA remains unaltered (40).

The presence of a β subunit also plays an important role in determining the action of agonists at the $GABA_A$ receptors. The maximum GABA response increases when the β_2 is present instead of β_1 in combination with the α and γ subunits (18), but the only

exception to this is seen when α_6 subunit is present. The β_3 subunit has a greater sensitivity to GABA than β_1 or β_2 (18,24), when combined with α_1 and γ_1, but sensitivity is increased for β_2 if γ_2 is present. The potency for GABA was found to be in decreasing order for the $\alpha_1\beta_2\gamma_1$, $\alpha_1\beta_1\gamma_3$ and $\alpha_1\beta_2\gamma_2$ receptor subtypes, respectively, but maximum current elicited by GABA was found on the latter combination of receptor (18). Mutational analysis suggests that amino acid residues at position 157, 160, 202 and 205 on the β subunit are essential for activation of the $GABA_A$ receptor channel by GABA and the activation is drastically impaired if they are mutated. Substitution of tyrosine at position 157 and 205 on the β-subunit with phenylalanine or serine decreases sensitivity to GABA and increases the EC_{50}. A greater decrease in GABA sensitivity is found with serine than with phenylalanine substitution suggesting the necessity of aromatic rings for GABA mediated activation of the receptors (1). For the action of benzodiazepines, the presence of α and γ subunits are necessary, but conflicting reports have appeared concerning the influence of the β subunit on benzodiazepine pharmacology (24,66,75).

Another important subunit of $GABA_A$ receptors is the γ_{2L}, which makes a substantial contribution to the pharmacology of the receptor subytpe. The presence of a γ_{2L} subunit in $GABA_A$ receptors along with α_1 and β_1 gives a log-concentration response to GABA with a steeper Hill slope than that obtained with $\alpha_1\beta_1$ alone (33). Hill slope provides a measure of the number of ligand molecules binding to the receptor. Inclusion of the γ_{2L} subunit results in an increase of binding site for GABA within the recetor complex, as is evident from an increase in Hill slope. The potency of GABA at the receptor was also found to decrease with the addition of γ_{2L} (33). Potentiation of GABA-

mediated current by benzodiazepine flunitrazepam is not found at the $\alpha_1\beta_1$ $GABA_A$ receptor and moreover a slight inhibition of the response is noticed too (91). This strongly shows the potentiation of I_{GABA} ($GABA_A$ receptor mediated inward chloride current) is dependent upon the presence of the γ_2 subunit (33, 75).Contrary to these studies, diazepam enhanced I_{GABA} in *Xenopus* oocytes expressing only $\alpha\beta$ subunits (56). This particular benzodiazepine pharmacology is different from other observations and potentiation of GABA-current is elicited non-specifically by agonists, antagonists and inverse agonists at the benzodiazepine site. Benzodiazepines are responsible for increased frequency of channel opening of $GABA_A$ receptor containing the γ_2 subunit. In contrast to benzodiazepines, the effect of pentobarbital is not dependent on the γ_2 subunit (33). Barbiturates on the other hand increase the maximum response to GABA by increasing the mean open time of the channels.

The ligands at the benzodiazepine site have higher efficacy at receptors containing γ_2 compared to the γ_1 subunit. Partial and full inverse agonists for the benzodiazepine site have been found to lose their property and produces slight potentiation of GABA-current at receptors containing the γ_1 subunit along with the α_1 and β_1 subunits (92). The γ_2 subunit is known to be responsible for benzodiazepine potentiation, but homomeric γ_2 receptors have not been found to be sensitive to benzodiazepines (82). Most of the $GABA_A$ receptor subunits form homooligomeric ion channels, although less efficiently and they are insensitive to benzodiazepines (6). A single amino acid residue (threonine-142) on the γ_2 subunit has been found to have a key role in benzodiazepine pharmacology. The antagonists and inverse agonists at the benzodiazepine site behaves

as a partial agonist if threonine-142 is mutated to serine. The affinity for GABA remains unchanged in receptors containing both $\gamma_{2L\text{-}thr}$ and $\gamma_{2L\text{-}ser}$ expressed separately with α_1 and β_2 subunits in HEK 293 cells (53). Non-specific agonists like diazepam, alprazolam or clonazepam produced greater potentiation of I_{GABA} in receptors with mutated subtype than wildtypes and the modulation was strongly dependent upon the concentration of GABA. Benzodiazepine type I selective agonists, such as zolpidem and alpidem produced half maximal potentiation of the GABA response on receptors with mutated subunit, compared to the normal subtype (53).

$GABA_A$ receptors can be modulated functionally by post translational modification of the receptor subunits. This is carried out by glycosylation and phosphorylation (55,85). N-linked glycosylation (32,58,84) and phosphorylation (42,38,44,45,57) plays an important role in receptor regulation and their translational modification depends on the subunit composition of the $GABA_A$ receptor.

1.3 Regional distribution of $GABA_A$ receptor subunits

$GABA_A$ receptors are widely distributed throughout the mammalian CNS. The presence of particular subunit isoforms in specific brain areas has been investigated by immunohistochemical, immunoprecipitation and *in-situ* hybridization techniques. Immunoaffinity chromatography and northern blot analysis are also used as tools to examine expression of different receptor subunits. These different technical tools are employed to determine the subunit combination of receptors in particular brain regions. Study of pharmacological agents on recombinant receptors shows a variety of effects

based upon the receptor subunit combnation. Establishment of receptor composition in specific brain areas will allow development of drugs with more selective and target specific action at the receptor level. Subunit specific antibodies are important research tools to isolate receptors from different regions of brain. Composition of $GABA_A$ receptor varies in different brain regions and immunoprecipitation of these $GABA_A$ receptors from specific brain areas with subunit specific antibodies has helped to partially decipher the subunit composition of the receptors. From different studies it appears that one or more α subunits, one or more β subunits and a γ and/or δ subunit form functional $GABA_A$ receptors *in vivo*. As has been predicted by many researchers, the most common combination might be $\alpha_1\beta_2\gamma_2$ (9). The $\alpha_1\beta_1\gamma_2$ subtype of $GABA_A$ receptor is found to share similar gating properties with neuronal $GABA_A$ receptors (43). Electrophysiological studies have shown that the favoured stoichiometry for recombinant $GABA_A$ receptors appears to be $2\alpha,1\beta,2\gamma$ when α_1, β_2 and γ_2 subunits are expressed (3).

In situ hybridization and immunohistochemical studies show a complicated heterogeneity in the subunit composition of $GABA_A$ receptors in the CNS. The most abundant α subunit isoform found in the adult CNS is α_1. Also *in situ* hybridization points to the fact that α and β subunit RNAs are present in areas of CNS which contain a high density of $GABA_A$ receptors like the hippocampus, cerebral cortex and cerebellum of rat (71). One *in situ* hybridization study of different $GABA_A$ receptor subunits demonstrates (97) co-localization of α_1 and β_2 with the γ_2 subunit mRNA and their primary occurence is found in olfactory bulb, globus pallidus, inferior colliculus, substantia nigra pars reticulata, zona incerta, subthalamic nucleus, medial septum and

cerebellum (97). The α_2 subunit is also evenly distributed across many brain areas, but is absent in some areas of thalamus and cerebellum. Co-localization of α_2 and β_3 mRNAs can be found in areas of amygdala and hypothalamus (97). In much of the forebrain co-distribution of α_4 and δ subunits is found. The predominant form of $GABA_A$ receptor reported in the hippocampal formation is $\alpha_5\beta_1$ along with the γ_2 subunit (97). A wide but uneven distribution can be noticed for the α_3 and α_4 subunits. Primary occurence of α_3 subunit is in the claustrum of basal ganglia, septum, neocortex and a low occurence is seen in olfactory bulb, hippocampus, amygdala and in a few areas of thalamus and hypothalamus. A major distribution of the α_4 subunit is seen in hippocampus and thalamus (97,2). The $GABA_A$ α_5 subunit specific antibody shows low immunoreactivity but is distributed across several neuronal population including cortical interneurons, hippocampal CA3 pyramidal neurons, anterior thalamic reticular nucleus and cerebellar Purkinje cells (86). Neocortex, hippocampus, septum, thalamus and some parts of basal ganglia shows a complex distribution of different α subunits.

Among the β subunit isoforms, β_2 is extensively distributed across the brain. The highest relative specific activity for the β_2 subunit in rat brain is found in the brain stem followed by inferior colliculus, olfactory lobe and cerebellum (50). Similar observation has been noticed with *in situ* hybridization also (97). $\beta 3$ is mainly found in olfactory bulb, cortex, hippocampus and cerebellum but occurence of β_1 is mainly found in hippocampus and rarely in cerebellum (68,97).

The major subtype of the γ subunit is γ_2 which is present in almost all areas of brain, but the pattern of distribution for γ_2 subunit RNA does not necessarily match that

for α and β subunits (67,71). Distribution of γ_1 and γ_3 is fairly restricted in the brain and 80-90% of CNS neurons contain the γ_2 subunit. The primary localization of γ_2 is observed in the mitral cell layer of olfactory bulb, pyriform cortex, parts of neocortex, hippocampus, cerebellar purkinje cells and in some parts of hypothalamus, septum and amygdala (97). Assessment of the distribution pattern of both forms of γ_2 subunit, γ_{2S} and γ_{2L} shows the abundance of latter in layer II of striate cortex and cerebellar Purkinje cells, superior and inferior colliculus, deep cerebellar nuclei, medulla and pons. The regions enriched in γ_{2S} are mitral cells of olfactory bulb, pyramidal neurons of pyriform cortex, layer VI of the neocortex, granule cells of dentate gyrus and pyramidal cells of the hippocampus (22,54). Cerebellar granule cells and some other cortical structures express both γ_{2S} and γ_{2L} in comparable amounts (54). The subunit composition governs the $GABA_A$ receptor pharmacology and difference in regional assembly of $GABA_A$ receptor has some important implications towards it.

1.4 Effect of propofol

Propofol (di-isopropyl phenol) is a potent intravenous anesthetic agent which is chemically unrelated to barbiturates, steroids, imidazole or eugenol agents and is suitable for both inducing and maintaining anesthesia (36). Propofol is well known for its use in the induction of anesthesia in outpatients undergoing ambulatory surgical procedures requiring rapid onset of action, stable operating conditions and rapid recovery from anesthetic effects on cognitive and psychomotor functions (79,41). With propofol, the plasma concentration for sedation is 0.5 - 1.5 μg/ml, while 2 - 6 μg/ml produces

hypnosis (79). A highly lipophilic drug, propofol, has a fast onset of action due to rapid penetration through the blood-brain barrier (37). At both subanesthetic and anesthetic dosages, propofol evenly distributes itself throughout the CNS without any regional preferences (73). Accumulation of propofol is found in brain which is evident from the observation that the concentration of the drug in rat brain after fifteen minutes of propofol infusion is 15.7 $\pm$ 1.9 μg/g which is 3.5 and 8.5 times the drug concentration in whole blood and plasma respectively (73). It is known to be a short acting anesthetic because following drug administration the concentration of propofol in plasma declines rapidly, as it redistributes itself from brain and other highly perfused tissues to less well-perfused tissue sites (37,79).

Two major theories have been advanced to explain the effects of anesthetic agents. It is believed that anesthetics interact with the macromolecular structure of the neuronal membrane by changing physical properties such as fluidity. This suggestion is mainly based on the correlation between the anesthetic potency and lipophilicity of the drug (88). The change in the lipid property is assumed to affect protein structures embedded in the matrix of the cell membrane. The other theory is based on the hypothesis of anesthetic-protein interaction. Anesthetic agents seems to have specific binding sites on receptor protein subunits in the CNS and the interaction causes a conformational change in the receptor structure which leads to altered functional properties (88).

$GABA_A$ receptors, which mediate inhibitory transmission in the CNS, are believed to be a primary site of action of anesthetic agents irrespective of their chemical structures (47,98). General anesthetics have been shown to modify $GABA_A$ receptor mediated

responses (47). A strong correlation exists between the hydrophobicity and anesthetic potency of the chemical compounds (98). However, this may be due to the presence of hydrophobic binding sites on the protein subunits of the receptor. There are probably different ways by which anesthetics mediate their effects through $GABA_A$ receptors. This might involve alteration in agonist binding, receptor-channel coupling or a direct modulation of channel gating (60).

Propofol appears also to interact with $GABA_A$ receptors. In *in vitro* studies with rats, propofol has been found to produce a brief dose-dependent inhibition of pars reticulata neurons of substantia nigra which are known to be inhibited by GABA-mimetic drugs and benzodiazepines (62). The inhibitory effect on pars reticulata neurons was potentiated by diazepam and reversed by picrotoxin and bicuculline and not by the benzodiazepine antagonist Ro 15-1788, thus suggesting that propofol has a GABA-mimetic action. Again, in rat olfactory cortex slices, clinically relevant concentrations of propofol potentiate GABA-mediated transmission (12). The same phenomenon was observed on mouse hippocampal neurons (60), where 2-100 μM of the drug potentiated GABA-activated responses and reduced receptor desensitization. A concentration of 10-1000 μM propofol directly activated and desensitized the receptors, whereas 600-10000 μM propofol produced a non-competitive inhibition of $GABA_A$ receptor responses (60). The effect shown by lower concentrations (2-100 μM) of propofol probably has some pharmacological significance, as the arterial concentration of propofol for loss of consciousness has been found to be between 16.8-22.4 μM (30). Propofol has also been observed to shift the GABA dose-response curve to the left, from mouse hippocampal

neurons, without changing the maximum response (60).

Desensitization of $GABA_A$ receptors depends upon the concentration of GABA, but at the same time it is dependent on other ligands at the receptor site which can cross-desensitize the receptor (60). Cross-desensitization of GABA-mediated responses with propofol is likely, as it has been observed that preincubation with GABA reduces the effect of subsequent propofol application (28). Reduction in the rate of desensitization of GABA responses with low propofol concentrations has been suggested to be due to an allosteric interaction at the $GABA_A$ receptors (60).

Single channel studies of $GABA_A$ receptors have revealed that propofol potentiation of GABA responses results mainly from an increase in channel opening frequency and probability and not due to increase in channel open time or single channel conductance (60). However, a propofol-induced increase in chloride conductance has also been reported (26).

The role of propofol to influence binding characteristics of other drugs to $GABA_A$ receptors in rat cerebral cortex has also been extensively reviewed (62). Propofol was found to enhance GABA binding in cortical membrane preparations and potentiate muscimol-induced stimulation of Cl^- uptake in membrane vesicle preparation. The drug also inhibits t-butylbicyclophosphorothionate (TBPS) binding to unwashed rat cortical membranes (15,62) and it fails to displace flunitrazepam from its binding site, indicating that the binding site for propofol is different from that of the benzodiazepines. Concomitant application of propofol either with alphaxalone or pentobarbitone on rat cerebral cortex membrane produced an additive inhibition of TBPS binding which

provides evidence for separate sites of action of these drugs (15,13). Evidence from drug-binding studies strongly suggests that facilitation of $GABA_A$ receptor function might have relevance in mediating the anesthetic effect of propofol.

The subunit specificity for action of different anesthetics is not very clear, but recently it has been noticed that the presence of β subunit is probably necessary for the positive modulation of the $GABA_A$ receptors by non-volatile anesthetics (31). A recent report has shown that the action of the general anesthetics pentobarbital and propofol is subunit specific (70). In that study, propofol directly activated $\alpha_1\beta_1\gamma_{2S}$, $\alpha_1\beta_1$ and $\beta_1\gamma_{2S}$ $GABA_A$ receptor subtypes but not $\alpha_1\gamma_{2S}$ showing that the presence of the β subunit is required for direct activation of $GABA_A$ receptors by the anesthetic (70). The homomeric β_1 receptor, which is completely insensitive to bicuculline, showed pronounced direct activation with propofol and pentobarbital (70). However, alphaxalone (steroid anesthetic) did not directly activate β_1 homomeric receptors, although it could potentiate the effects of GABA.

Application of propofol to bovine chromaffin cells and rat cortical neurons produces a dose-dependent potentiation of the inward GABA current which is not influenced by the benzodiazepine antagonist flumazenil. This suggests a separate site of action for propofol and benzodiazepines (27). Diazepam but not pentobarbital has also been reported to enhance propofol-induced current on rat hippocampal pyramidal neurons, and this effect is not blocked by RO15-1788 (30). Propofol is increasingly used in clinical practice and benzodiazepines are often used in conjunction with propofol, either as a pre-anesthetic medication to reduce anxiety or as an adjunct for the induction

of anesthesia. In clinical cases propofol has been reported to show synergism with midazolam, a benzodiazepine agonist, though the nature of interaction at the level of $GABA_A$ receptor is not known (72,10).

1.5 Clinical effects of benzodiazepines

The class of chemical compounds classified as imidazobenzopyridine derivatives and sharing a common pharmacological property of sedation and hypnosis are known as benzodiazepines. Drugs included within this category are flurazepam, diazepam, triazolam, flunitrazepam, temazepam and many others. Flurazepam was the first among all benzodiazepines to be marketed selectively as a hypnotic agent (34). All benzodiazepines are lipophilic substances though they vary in their quantitative lipophilicity (20). Diazepam is one of the most lipid soluble substance while lorazepam has less lipid solubility (20). In brain the effects of benzodiazepines are mediated through a specific interaction with $GABA_A$ receptors sensitive to these chemical compounds and are widely distributed on post-synaptic neurons within the CNS (25). Benzodiazepines cause a positive modulation of GABA-induced inward Cl^- current (20). The clinical effect is determined primarily by the dose administered (and thus the degree of receptor occupancy) rather than the particular benzodiazepine (20). All benzodiazepine agonists produce a concentration dependent sedation, drowsiness, performance and memory impairment, amnesia, ataxia, antianxiety and anticonvulsant effects (34). The dosage of different benzodiazepines varies depending upon their potency. The major uses of benzodiazepines are for the treatment of anxiety states and sleep disorders. However,

they are also increasingly used as a pre-anesthetic medication in conjunction with other anesthetic agents. The affinities of different benzodiazepines for $GABA_A$ receptors differs significantly. Like other pharmacological agents, benzodiazepines also cause unwanted side effects like CNS depression including drowsiness, dizziness, fatigue, light-headedness and incoordination. All of these probably represents extension of their pharmacological action (69).

1.6 Merits of the *Xenopus* oocyte expression system

The *Xenopus* oocyte expression system has evolved to be an important and powerful research tool for studying functional properties of cell surface receptors following the translation of exogenous mRNA (80). Oocytes have proven repeatedly to be an efficient *in vitro* system for accurately translating foreign receptor mRNAs, processing the product and effectively inserting it into the oocyte membrane (89). *Xenopus* oocytes are capable of performing a number of post-translational modifications to receptor subunits including glycosylation, phosphorylation and subunit assembly. However, not all the modifications are always seen with all exogenous polypeptides (82), which suggests the possibility that oocytes might be incapable of recognizing all exogenous signals for post translational modification. Transmitter receptors expressed in oocytes display certain properties not found in uninjected oocytes showing that the phenomenon of expressing the brain receptors is dependent on exogenous sources. In oocytes, the size of a particular response mediated by a receptor varies and is probably proportional to the amount of injected mRNA coding for the specific receptor (93).

Functional amino acid receptors (GABA, glycine, NMDA and kainate) are expressed in *Xenopus* oocytes upon injection of rat brain mRNA showing the capacity of oocytes to synthesize and assemble a variety of synaptic neurotransmitter receptors (81). GABA receptors synthesized *in-vitro* possess similar pharmacological and gating properties as found with $GABA_A$ subtypes present in the CNS. They have similar binding sites for benzodiazepines, barbiturates, picrotoxin, bicuculline and other ligands (78,34).

Significant metabolic differences are found between oocytes from different stages (I-VI). The stages of oocyte can have a profound effect on translation of injected messages and processing of proteins (80). Components required for protein synthesis during early embryogenesis are found in full grown oocytes (stage VI) in large reserves. The ability to synthesize proteins varies between different stages of oocytes, the maximum being found in stage VI oocytes. Other than the difference in capacity to synthesize proteins, difference also lies in sizes between various stages of oocytes. Stage V oocytes have a diameter of 1000 - 1200 μM whereas stage VI oocytes have diameter larger than 1200 μM (80).

Oocytes extracted from frogs do not have endogenous $GABA_A$ receptors, but they act as a very good expression system for the receptor proteins formed from the foreign DNA introduced, by subsequent steps of transcription and translation. Ideally, molecular, electrophysiological and pharmacological characterization of a specific ion channel demands its expression in a defined membrane environment where interaction with any other neurotransmitter receptor is absent. Injection of cloned DNA/RNA for particular receptor subunits in oocytes produces receptors and this can be particularly useful for

pharmacological characterization of any receptor combination believed to be present in the CNS. Understanding the molecular nature of synaptic communication is greatly facilitated by these gene probes which encode a host of ion channels and receptors. These probes permit a critical characterization of the structure and function of receptors following expression in *Xenopus* oocytes (81).

A variety of ligand gated ion channels which can be efficiently expressed in oocyte system include $GABA_A$, N-methyl d-aspartate (NMDA), serotonin (5-HT) and acetylcholine receptors. *Xenopus* oocytes perform as a surrogate system for ion channel expression and the receptors so formed after nanogram injection of poly(A)$^+$ RNA can be succesfully characterized with electrophysiological measurements. Electrophysiological recording of ligand-gated ion channels expressed in *Xenopus* oocytes is a sophisticated and widely used technique for examining the pharmacological properties of these proteins. Electrophysiological assay can detect expression of functional receptors after an incubation period of 8-36 hours (81). Standard electrophysiological technique includes the use of two micro-electrode voltage clamp for whole cell recordings. Recording from oocytes has some prominent advantages over neuronal cells. Primarily, the oocytes are much larger in size compared to single neuronal cells which simplifies the whole cell recording and it is also relatively simple to introduce cDNAs or mRNA encoding specific receptor subunits. This permits experiment designed to test the role of specific subunits in imparting the pharmacological properties of the receptors expressed. Secondly, stable, long-lasting (up to several hours) recordings can be obtained, allowing the collection of quantitative as well as qualitative data. The only question that might arise is regarding

the authenticity of expression of all subunit messages injected. Presently, it is not possible to determine whether a homogeneous population of receptors are expressed or if receptor subtypes are formed with different composition and stoichiometry.

1.7 Rationale and objective

Propofol is a widely used short acting, intravenous anesthetic agent which has been found to have an effect on $GABA_A$ receptors. Little information is available regarding the subunit dependence of propofol with regard to modulating $GABA_A$ receptor function. There are a few reports (31,70) which mentioned the importance of the β subunit for propofol's action on the $GABA_A$ receptor. It has also been observed that without the presence of the β subunit propofol fails to directly activate the $GABA_A$ receptor chloride channel. The majority of the work with propofol with respect to its affinity or efficacy has been done on neuronal cells where the subtype of receptors present is not clearly known. Propofol also has effects on other neurotransmitter receptors and ion channels. It is not known if other neurotransmitter receptors that can be modulated by propofol have some effect on the specificity of propofol's action on $GABA_A$ receptor in neurons. Considering from that perspective, use of oocyte expression system is preferred where no other receptors are present except for the ones expressed as intended. There has been no report yet where the efficacy and affinity of propofol have been studied on a defined population of $GABA_A$ receptors. Efficacy of other anesthetic agents such as enflurane has been observed to change with changes in subunit composition of $GABA_A$ receptors. I propose to test the hypothesis that change in subunit

composition of $GABA_A$ receptor will alter the efficacy and/or affinity for propofol.

Benzodiazepines are often combined with propofol as preanesthetic medications either to reduce preoperative stress or to enhance anesthesia. According to clinical reports (72,10), a combination of the benzodiazepine, midazolam, with propofol acts synergistically for the induction of anesthesia. Temazepam was similarly found to alleviate anxiety and significantly increase the cognitive recovery time for patients anesthetized with propofol and alfentanil (4). All reports of synergism between propofol and benzodiazepines come from clinical trials. There is no direct evidence of an interaction at a common receptor, though there are suggestions that the effect might be mediated through $GABA_A$ receptors. I propose to test the hypothesis that there is a significant interaction between the two classes of drugs at $GABA_A$ receptors and that other chemical classes such as cyclopyrrolones which act at or near the benzodiazepine site can also participate in this type of interaction.

To investigate the above hypothesis, I have chosen to employ the *Xenopus* oocyte expression system. The oocyte system can express functional brain receptors, as evident from other research works. All effects of propofol which are found in mammalian cells should therefore be replicated in this model system.

The objectives of this study were to examine:

i) the effect of propofol on GABA-activated responses at $\alpha_1\beta_2\gamma_{2L}$ and $\alpha_2\beta_2\gamma_{2L}$ subtypes of human $GABA_A$ receptors;

ii) if propofol and benzodiazepines act synergistically at the $GABA_A$ receptor and if so, whether at the same site on the $GABA_A$ receptor;

iii) the nature of the interaction of propofol with zopiclone, a cyclopyrrolone derivative known to be a full agonist at the benzodiazepine site on the $GABA_A$ receptor

iv) the role of the γ_{2L} subunit in potentiating $GABA_A$ receptor function with propofol;

v) whether propofol does have any effect on the desensitization of the GABA-mediated receptor responses.

2.0 Methods

2.1 Purification of $GABA_A$ receptor subunit cDNAs by the method of Alkali Lysis

2.1.1 Transfection of selective micro-organism with cDNA

Subunits of the human $GABA_A$ receptor were received as an ethanol precipitate (Merck Sharp & Dohme Research Lab). Each cDNA was dissolved in TE to a final concentration of 1 μg/μL. A 1 μL aliquot of each cDNA stock was then taken into a micro-centrifuge tube and TE was added to a final volume of 200 μL, so as to make a final concentration of 5 ng/μL. The cells (*E. Coli* receiving the transfection (MC1061/P3; Invitrogen Corp.) were kept frozen until used. 50-100 μL of the cell stock was then taken in a micro-centrifuge tube and combined with 5-10 μL of 5 ng/μL cDNA stocks for each subunit. The volume of cDNA was always kept less than or equal to 10% of the cell volume. The tubes were put on ice for 20-30 minutes followed by heat shock at 42°C for 50 seconds. Immediately after the heat shock LB (prewarmed to 37°C) was added to each tube to a final volume of 1 mL. The tubes were shaken for 45 minutes at a temperature of 37°C in a horizontal position to allow proper aeration and the cDNA to be taken up.

After shaking the suspensions were spun down at 6000 rpm for 10 seconds. The supernatants were discarded and the pellets were re-suspended in 100 μL of LB. The micro-organisms were then plated on agar (1.5% Bacto-Agar in liquid broth) with antibiotics (tetracycline - 7.5 μg/mL ; ampicillin - 25 μg/mL). Cells were plated with 2

μL, 10 μL and 88 μL of the cell suspension per plate. The plates were incubated overnight at 37°C.

2.1.2 Maxi plasmid preparation by alkali lysis

Day 1:

Two litre flasks containing 500 mL LB with antibiotics were prepared for individual subunits. A wire loop was flamed, cooled and a single bacterial colony was picked up by gentle touch and transferred to a flask. The innoculates were placed on a shaker overnight at 37°C.

Day 2:

After 17-18 hours of incubation, the cultures were transferred to pre-cooled 250 mL centrifuge buckets, and incubated on ice for 10- 20 minutes. The cells were spun down at 4000 rpm for 10 minutes. The supernatant was drained off and the inside of each individual bucket was wiped carefully to remove excess medium. The pellet from each bucket was suspended in 6.5 mL of Solution 1 and transfered to a 50 mL Oakridge tube. The tubes were allowed to stand at room temperature for 5 minutes. Thirteen ml of freshly prepared Solution 2 was then added to each of the tubes and mixed gently, but thoroughly, to ensure complete lysis of cells. They were placed on ice for another 5 minutes and then 6.5 mL of ice cold Solution 3 was added. At this stage protein and chromosomal DNA precipitated out. The tubes were again placed on ice for another 10

minutes. The precipitated DNAs were then spun down at 12000 rpm for 30 minutes. The supernatants were poured separately into fresh 50 mL Oakridge tubes and precipitated with 15 mL of iso-propanol. They were mixed thoroughly and allowed to stand at room temperature for 5 minutes. At this stage the plasmid DNA precipitated and was spun down at 8000 rpm for 10 minutes. The supernatants were discarded and the pellets were allowed to dry for a couple of minutes by inverting the tube. Each 500 mL culture from a single flask was divided into two 250 mL aliquots and each aliquot was carried through the isolation procedure separately. At this stage the two pellets were pooled together in 3.0 mL of TE buffer and transfered to a 15 mL Corex tube. The pH for individual tubes was checked to ensure that it was neutral to slightly alkaline (adjusted with NaOH if necessary) and the volume was made up to exactly 4.2 mL. 30 μL of RNAase A (10mg/mL) was added to each tube.

To each of the tubes 4.7 g CsCl and 500 μL of ethidium bromide (10mg/mL) was added and dissolved completely. The content of each tube was spun down at 8000 rpm for 10 minutes to separate any precipitate, if present. The clear supernatant from the Corex tubes was transfered to separate Quickseal tubes with a 5 cc syringe and 16 gauge needle. The tubes were heat sealed and checked for leaks by wiping on paper towel while applying gentle pressure. The tubes were put in a vertical VTi 65 rotor and spun in a Beckmann ultra-centrifuge at 45000 RPM at 20°C for 18 - 20 hours.

Day 3:

The tubes were removed carefully from the rotor and kept in the vertical position

so the plasmid band remained stable. The plasmid DNA band was sometimes visible in ordinary light, but was easily visualized as a fluorescent band under long wavelength UV light. Each tube was punctured with a syringe needle (26 gauge) and a 1 cc syringe needle was carefully inserted just below the plasmid band viewed with long wave length UV light. The bands were carefully and very slowly withdrawn and placed into 15 mL poly-propylene tubes. The volume of plasmid DNA obtained varied from 0.5 - 1 mL. The volume of the plasmids was made up to 2 mL with TE buffer. All the samples were repeatedly treated with n-butanol to extract all traces of ethidium bromide. The samples were placed in a dessicator under vacuum for 5-10 minutes to get rid of the excess butanol. The aqueous phase was extracted twice with an equal volume of phenol:chloroform:iso-amyl alcohol (25:24:1) mixture followed by a single extraction with an equal volume of H_2O saturated chloroform. After the final extraction the plasmids were transfered to 15 mL Corex tubes. For precipitating the DNAs, 0.1 volume of 3M sodium acetate (pH 5.2) was added to each tube followed by two volumes of 100% ethanol. The tubes were vortexed mildly, covered with parafilm and kept overnight at -20°C.

Day 4:

The precipitated DNAs were spun down at 15000 rpm for 30 minutes. The supernatant was removed from each of the tubes and the pellet was washed with roughly 5 mL of 70% ethanol kept at -20°C. The tubes were dried under vacuum for approximately 20 - 30 minutes. Once the pellets were dried, they were dissolved in 0.5

mL TE buffer. Approximate concentration for the plasmid DNA was checked by determining the absorbance at 260 nm in a spectrophotometer. Double stranded DNA gives an optical density reading of 1 per 50 μg/mL. The dilution factor for the plasmids was 1:500. Yield ranged from 800 - 1600 μg of plasmid DNA from a 500 mL culture. The purity of the plasmids was checked by running them on a 1% agarose mini-submarine gel, the procedure for which is given below.

2.2 Gel Electrophoresis

Agarose powder was combined with 1X TBE buffer to make a 1% solution and microwaved until dissolved. Ethidium bromide (10 mg/mL) was added (3.3 μL/50 mL) to the luke warm solution.

A comb was aligned for the formation of parallel wells in a clean plastic cast. The agarose gel was poured slowly into the cast, allowed to polymerize for 20 - 30 minutes, and covered with a thin layer of 1X TBE to avoid drying. After addition of TBE, the comb was removed and the remaining TBE drained off. Marker and samples in volumes of 10μL were carefully added to the wells. The composition of marker and samples are given below:

Marker

1 Kb Ladder - 1 μL (Gibco)

10X loading buffer - 1 μL

dH_2O - 8 μL (Diethyl pyrocarbonate [DEPC] treated water for RNA)

Samples

Sample - 1 μL

10X loading buffer - 1 μL

dH_2O - 8 μL (DEPC water for RNA)

The gel was run at 100 V for 20 - 30 minutes depending upon the size of the plasmid. In some cases plasmid DNA was linearized with appropriate restriction enzymes prior to gel electrophoresis. This gives a more accurate estimation of the size of the plasmid DNA obtained.

2.3 Preparation of cRNA from cDNA by *in-vitro* transcription

2.3.1 Linearization of plasmid DNA

5 - 10 μL of cDNA (approximately 2μg/μL) was taken in a micro-centrifuge tube and added to 1.5 - 2.0 μL of 10X incubation buffer, supplied with the restriction enzyme. Incubation buffer was used in which the restriction enzyme for linearization has 100% activity. dH_2O was added to make up the volume so that the concentration of the buffer in the final mix was 1X. Usually 1 μL of the respective restriction enzyme (10 U/μL) was added to linearize the transcription vector containing the gene of interest. 1 unit of enzyme is known to digest 1μg of cDNA at proper temperature and in proper buffer. The volume of enzyme was always kept less than or equal to 10% of the final volume. It was then incubated for at least 2 hours and sometimes overnight at 37°C. After linearization the volume was made to 50μL with dH_2O. The reaction mix was then

extracted once with an equal volume of phenol-chloroform-isoamyl alcohol mixture and once with chloroform separately. The linearized plasmids were then precipitated with ethanol and sodium acetate as described in the maxi plasmid preparation for preparing the cDNAs. Finally the pellet was resuspended in 5 - 10 μL of TE to get the approximate concentration of the plasmid as 1μg/μL. In some cases the restriction enzymes used gave blunt ends or overhangs, either 3' or 5'. For restriction enzymes giving 3' overhangs, an additional step of filling up the 3' overhang with T4 DNA polymerase was followed to give blunt ends, otherwise it would lead to improper transcription.

2.3.2 Filling up of 3' overhang with T4 DNA Polymerase

5 μL of cDNA was taken and added to 5 μL 10X T4 DNA polymerase buffer, 39.5 μL dH_2O and 0.5 μL T4 polymerase. The mixture was kept at room temperature for 2 minutes. To it was added 2μL of the deoxy nucleotides (2 mM dNTP's). The entire mixture was incubated for 15 minutes at 37°C. It was extracted with phenol-chloroform, chloroform and precipitated with ethanol as described above. Finally the plasmid was dissolved in 10 μL of TE. Restriction enzyme Pst I used for linearization of human α_2 cDNA gave 3' overhang and was filled in with T4 polymerase.

2.3.3 In-vitro cold transcription

After the plasmids were linearized, the following reagents were added in order, to separate micro-centrifuge tubes for separate plasmids at room temperature.

10 μL 5X TB

2.5 μL	BSA 2mg/mL
5.0 μL	dithiothreitol (100 mM)
1.0 μL	RNAsin (40U/μL)
8.0 μL	rNTP mix
2.5 μL	7-methyl GTP cap (5 mM)
5.0 μL	rGTP (0.5 mM)
12.5μL	DEPC H_2O
3.0 μL	linearized plasmid DNA (1-3 μg)
0.5 μL	bacteriophage T7 polymerase

The transcription mix was incubated for 1 hour at 37°C. 50 μL DNAase buffer and 1 μL of RNAase free DNAase was then added to the mixture and further incubated for another 20 minutes at 37°C. After incubation, the entire mixture was extracted once with phenol/chloroform and chloroform respectively. Next the cRNA so formed from transcription was precipitated with ethanol at -20°C for at least 2 hours or overnight using potassium acetate (3M pH 4.8). The tube was then centrifuged for 10 minutes at 12000 rpm for the RNA to precipitate and the pellet was carefully washed twice with cold 70% ethanol. The RNA pellet was allowed to dry under vacuum and based on the yield was resuspended in 10-20 μL DEPC treated water. The concentration of RNA was estimated by checking the absorbance at 260 nm in a spectro-photometer. The yield was usually between 8-25 μg of cRNA.

In-vitro transcription was also carried out with RiboMAX™ Large Scale RNA Production System, T7 (Promega Corporation), which gave a fairly high yield of RNA.

2.4 Surgical removal of oocytes and de-folliculation

Oocytes from female *Xenopus laevis* frogs (Xenopus, Inc., Ann Arbor, MI) were removed by surgical procedures. Frogs were individually housed in separate plastic shoe box cages containing filtered tap water. The room was maintained at a temperature of 22°C with a 12 hour alternate light and dark cycle. The frogs were fed 2-3 times a week with a commercially available frog food (Nasco, Fort Atkinson, WI). On the day of surgery individual frogs were brought into the laboratory and were anesthetized before the operation. To anesthetize them, they were transferred separately into small boxes containing 500 ml of ice-cold filtered tap water containing 0.2 - 0.25% 3-aminobenzoic acid ethyl ester (methanesulfonate salt; Sigma Chemical Co.). After some time the frogs were tested for consciousness by lightly pinching their limbs. Anesthetized frogs were placed on their backs on a bed of crushed ice and the abdomen wiped with 70% ethanol. Before the operation all the surgical instruments were also wiped with 70% ethanol. A small incision (1 - 1.5 cm) was made in the abdomen below the diaphragm and above the junction of the hindlegs, either right or left of the midline with a surgical scalpel. Scissors were sometimes used to cut the muscular layer to avoid damaging internal organs. Two to three ovarian sacs containing 200-300 oocytes were removed through the incision with the help of a blunt forcep and cut at their point of junction, so as to minimise bleeding. The oocytes were transferred to a Petri dish containing storage solution (composition mentioned in Appendix I).

Once the oocytes were removed, the incision was stitched with sterile, nonabsorbable, surgical silk suture (Johnson & Johnson Medical Products). The muscular

layer and the skin were stitched separately. After closing the incision, the area was wiped again with EtOH and the frog was then transfered to a small plastic shoe box containing 500 mL of filtered tap water with 5 mL penicillin-streptomycin (penicillin G sodium - 10000 U/mL & streptomycin sulfate - 10 mg/mL; GibcoBRL). After the frog regained consciousness, an additional 500 mL of water was added to the pool. The frog was then kept in the laboratory for an additional 3 days for post-operative observation. Water in the boxes was changed daily and fresh antibiotics were added. This procedure was followed for three days. If afterwards the frog had no complication, it was returned to the Animal Care facility. Consecutive surgeries on the same frog were done on alternate sides of the body with intervals of at least four weeks.

The thecal, epithelial and follicular layers of the oocyte were manually removed using fine forceps (Dumont #5). Stage V-VI oocytes were used for injection and electrophysiological recording. In some cases frogs were injected with 50 U gonadotropin from pregnant mares' serum (Sigma Chemical Co.). Once the oocytes were peeled, they were kept in 1.5% agar coated Petri dishes with storage solution. The peeled oocytes were kept overnight at room temperature (20 - 22°C) and only the healthy oocytes were selected for injection on the next day.

2.5 Micro-injection of oocytes

After preparation of the individual cRNA for each $GABA_A$ receptor subunit, they were mixed together in different combinations of $\alpha_1\beta_2\gamma_{2L}$, $\alpha_2\beta_2\gamma_{2L}$, $\alpha_2\beta_2$ with a final concentration of 0.8 - 1 ng/nL for each subunit. They were stored at a temperature of -

70°C and just before injection the tubes were thawed on ice. The tubes were warmed up at a temperature of 70°C in a thermal cycle (Techne, Model PHC-2) for three minutes and quickly stuck in a bed of crushed ice. Capillary glass tubes (3.5" Drummond# 3-00-203 G/X; Drummond Scientific Company) were pulled on an electrode puller (Narishige Co. Ltd., Model PB-7) with single heater and full weight setting to get glass needles with very fine tips. The glass needles were filled with DEPC treated water and bevelled on a grinder (Narishige Co. Ltd., Model EG-40).

For injection a bevelled glass needle was filled with light mineral oil (Sigma Chemical Co., M-3516) and mounted in the micro-injector (Drummond "Nanoject" Automatic Injector; Drummond Scientific Company). A drop of the cRNA was placed on a glass cover slip and 3-5 μL of the cRNA was drawn in the injection needle. Defolliculated oocytes were placed in an injection chamber (a Petri dish fitted with nylon grid of a size sufficient to fit a single mature oocyte in one square) submerged in oocyte storage solution and the oocytes injected in the vegetal pole with the volume for injection varying between 10-50 nL. After all the oocytes were injected, they were transfered to agar-coated Petri dishes containing storage solution at room temperature.

2.6 Electrophysiological recording from oocytes

After 1-2 days of incubation following cRNA injection, the oocytes usually expressed $GABA_A$ receptors and thus were ready for electrophysiological recording. For recording, an oocyte was placed in a nylon grid (1.5 mm x 1.5 mm) glued to the glass bottom of a flow chamber (Warner Instruments Inc., 8 mm well size) and continuously

perfused with recording solution (storage solution devoid of sodium bicarbonate, pyruvic acid, caffeine/theophylline and antibiotics) at the rate of 6-8 mL/min. The oocyte was completely submerged. Two-electrode voltage clamp recording with an Oocyte Clamp (Model# OC-725B; Warner Instrument Corp.) at -70 mV was carried out using electrodes filled with 3M KCl. The electrodes were pulled on Narishige electrode puller (PB-7) and glass capillary tubes of diameter 1.2 mm (Glass 1BBL W/FIL 1.2 mm, Item# 1B1 20F-4; World Precision Instruments, Inc.) were used for the purpose. The oocyte was impaled with both the electrodes using micro-manipulators. Control (recording) solution was not turned on until the resting membrane potential increased and attained a steady level.

Pharmacological characterization of the receptors was done with a variety of pharmacologically active chemical substances which were applied onto the oocyte membrane in the perfusion chamber after dissolving them in the recording solution. Application of solutions was controlled by a series of manually operated valves connected to the inflow of the recording chamber. Currents activated by GABA, or GABA plus drugs, were recorded on a strip chart recorder (Cole Parmer, Model# 1202-0000). Immediately after application of the drugs for a period of 15-60 seconds, the oocyte was washed thoroughly for a period of 5-15 minutes with the control solution before the next application of drug(s). The control response to GABA was checked periodically during the recording to determine if the response changed over time and to ensure the reversibility of all drug effects. For experiments on desensitization of $GABA_A$ receptors, currents generated by application of 250 μM GABA were acquired and analysed using pClamp suite of programs. The declining phase of the current response was fitted using

a double exponential equation as the deacy can be best described by double xeponential fit compared to single exponential. Fits to single exponential did not give a better fit to the experimental data.

2.7 Analysis of data

Concentration-response relations are shown by sigmoidal curves expressed either as normalized to the maximum response or as a percentage of the control response. Concentration-response data were fitted to the equation:

$$Y = A + \frac{B - A}{1 + (10^{C}/10^{X})^{n}}$$

where X = logarithm of concentration

A = smallest response

B = maximum response

C = logarithm of EC_{50}

n = Hill coefficient

Best fit to the curve was done by the method of non-linear regression analysis. All data were analysed using Inplot (Graphpad Software, Version 4.0).

Studies involving interaction between propofol and flurazepam/zopiclone have been performed in present of fixed concentration of GABA, for each particular experiment. Effects of propofol and benzodiazepine agonists on control GABA response were separately measured at specific $GABA_A$ receptors. Combination of the drugs was applied with the same GABA concentration to find the nature of their interaction at the $GABA_A$ receptor. Data was interpreted by comparing the experimentally observed effect

due to the combination of the drugs with the theoretical additive effect of individual drugs. Arithmatic sum of the effects of individual drugs on the control (GABA) response was considered as the theoretical additive response. The effects on I_{GABA} observed upon concurrent application of propofol and flurazepam/zopiclone has been referred to as the experimental observation in the result section. The two drugs were found to act in synergy if the experimental result was significantly greater then the theoretical additive response or in other word supra-additive, for a particular propofol, flurazepam/zopiclone and GABA concentration. A zero interaction was noticed in cases where the theoretical additive and the experimental data for the drug combination had no significant difference. A negative interaction between the drugs was found where the experimental data was lower than the theoretical additive, for the drug combination.

To test the significance of data, where appropriate , repeated measures analysis of variance or ordinary one way analysis of variance coupled with Newman-Keuls Multiple Comparisons test, or a paired t-test was done. A value of $p<0.05$ was considered to be significant. Instat (Graphpad Software, Version 2.0) was used for statistical analysis.

2.8 Chemicals

The following chemicals were used:

Gum agar, tris[hydroxymethyl]aminomethane, ethylenediamine tetraacetic acid, dimethyl sulfoxide, bovine serum albumin, DL-dithiothreitol, caffeine, theophylline, pyruvic acid, 3-Aminobenzoic Acid Ethyl ester, GABA, zopiclone

(obtained from Sigma Chemical Co.); 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 BRL); 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 either from Sigma Chemical Co. or Fisher Scientific Company.

3.0 Results

3.1 Characterization of $GABA_A$ receptors expressed in *Xenopus* oocytes

After amplification of cloned DNA for specific $GABA_A$ receptor subtypes, cloned RNA was prepared from them by the process of *in vitro* transcription. Recombinant receptors were then expressed in *Xenopus* oocytes following injection with a cRNA mixture containing different subunit combinations. Subunits expressed in the oocyte expression system were $\alpha_1\beta_2\gamma_{2L}$, $\alpha_2\beta_2\gamma_{2L}$ and $\alpha_2\beta_2$. Following cRNA injection, expression of the recombinant receptors in oocytes took 1-3 days and resulted in substantial GABA-activated membrane currents. GABA was applied briefly (0.5-1 min) until the peak response was obtained on oocytes voltage clamped at -70 mV. Oocytes expressing the $\alpha_1\beta_2\gamma_{2L}$ combination showed a concentration- dependent increase of inward current in response to the application of GABA (Fig.1). The sigmoidal concentration-response curve had an EC_{50} for GABA of 58 $\pm$ 9 μM and a Hill slope of 1.30 $\pm$ 0.07 (n=5). Similarly, GABA induced a concentration dependent inward current in oocytes injected with $\alpha_2\beta_2\gamma_{2L}$ $GABA_A$ receptor cRNA (Fig.2). This particular combination of subunits showed an EC_{50} for GABA of 67 $\pm$ 4 μM with a Hill slope of 1.12 $\pm$ 0.05 (n=5).

Pharmacological characterization of the recombinant receptors containing the γ_{2L} subunit was done by using the benzodiazepine agonist flurazepam, which is known to increase the I_{GABA} on receptors containing γ_2 subunits. Application of flurazepam produced a concentration-dependent potentiation of the response to 30 μM GABA (n=4) (Fig.3). Increase in response to different concentrations of GABA (10, 30, 50 and 100

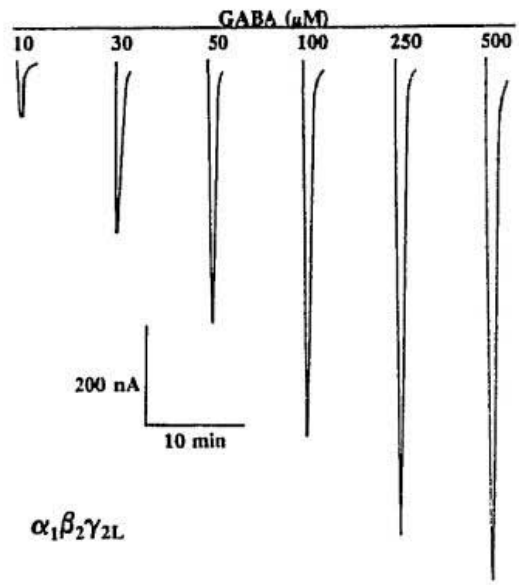
μM) was observed with 0.5 μM flurazepam on receptors composed of both $\alpha_1\beta_2\gamma_{2L}$ and $\alpha_2\beta_2\gamma_{2L}$ subunits expressed in Xenopus oocytes (Fig.4). Flurazepam potentiation of I_{GABA} was found to be the greatest with the lowest concentration of GABA (10 μM). The extent of the potentiation gradually decreased with increasing GABA concentrations.

The voltage-dependence of the inward current produced by the application of 10 μM GABA was done on $\alpha_1\beta_2\gamma_{2L}$ receptor combination. The holding potential was reduced from -70 mV to -20 mV in several steps and at each step the response to GABA was measured. With decrease in the holding potential, the inward current response to GABA decreased and after a certain potential the response reversed to an outward current. The current-voltage relationship is shown in Fig.5. The reversal potential for I_{GABA} was -27.8 $\pm$ 2 mV (n=4) which is similar to other published values.

Bicuculline is a competitive antagonist to GABA on $GABA_A$ receptors and when coapplied at the concentration of 30 μM with 10 μM GABA on human $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors, it produced almost a complete blockade of the response to GABA (n=3) (data not shown).

Figure 1. **Concentration-response of GABA at human $\alpha_1\beta_2\gamma_{2L}$ receptors** a) Current traces showing inward Cl^- current produced with a range of GABA concentrations. b) Plot showing response to GABA (normalized to the maximum response). Data points in this and the remaining figures are expressed as the Mean ± SEM. Data from 5 oocytes.

a


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

b

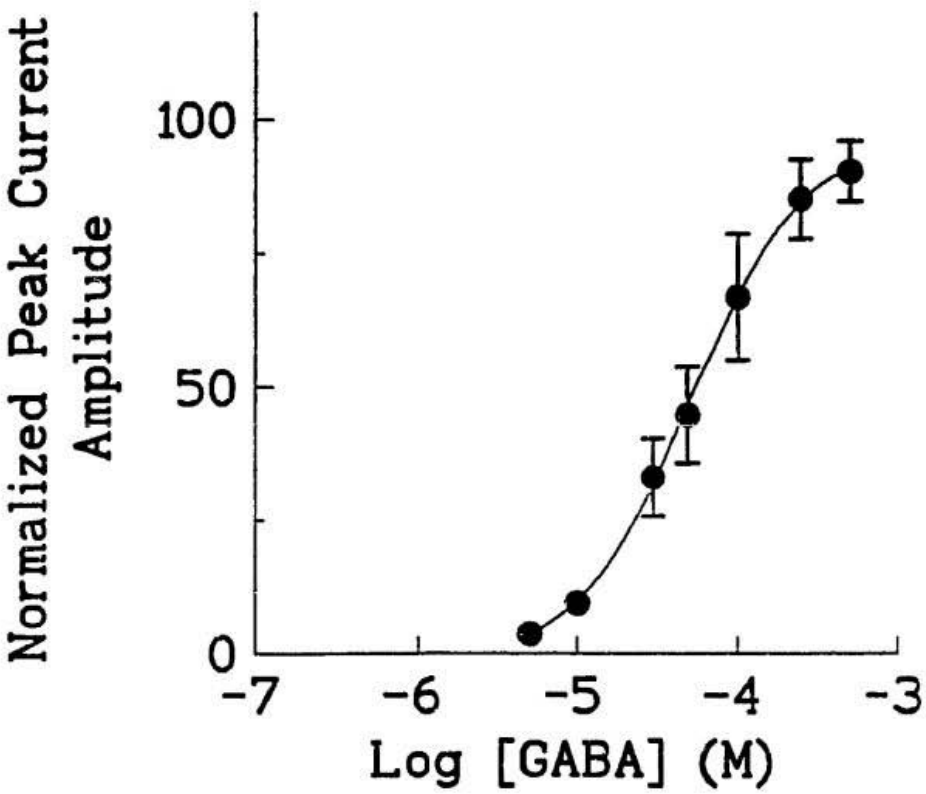
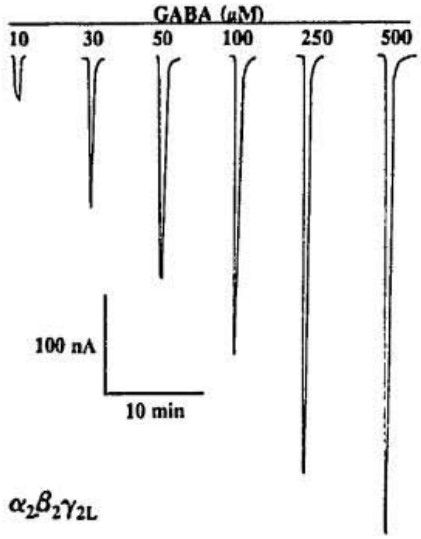
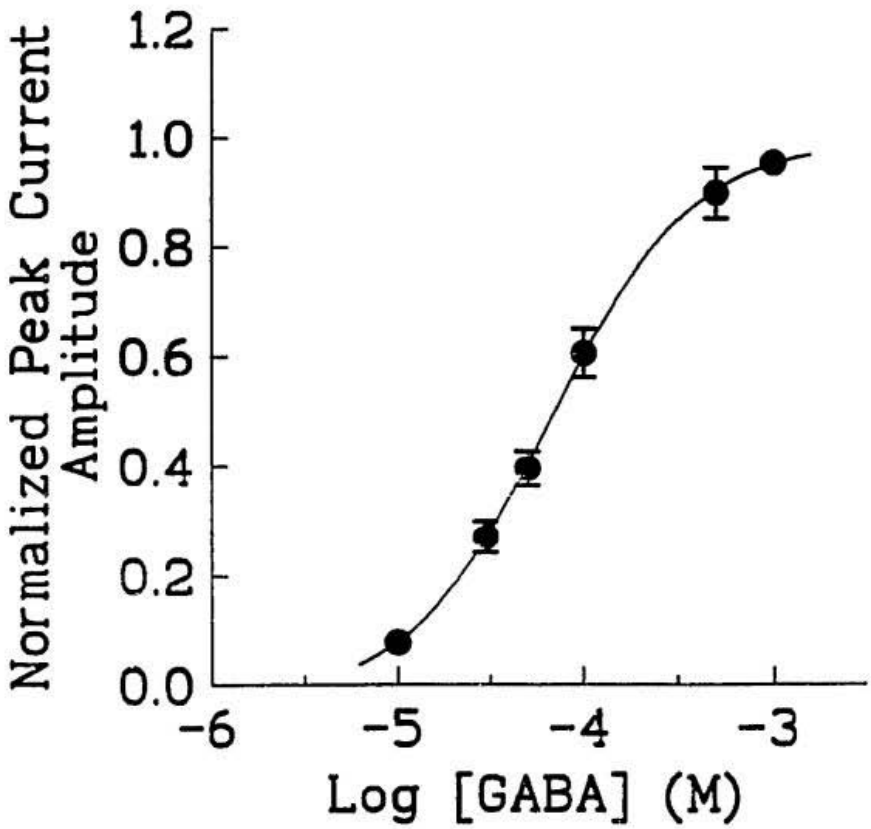

Normalized Peak Current Amplitude
100
50
0
-7
-6
-5
-4
-3
Log [GABA] (M)

Figure 2. **Concentration-response to GABA on human $\alpha_2\beta_2\gamma_{2L}$ receptors** a) Current traces showing inward Cl^- current produced with increasing GABA concentrations. b) Plot showing response to GABA (normalized to the maximum response). Data from 5 oocytes.

a


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

b


Normalized Peak Current Amplitude
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-6
-5
-4
-3
Log [GABA] (M)

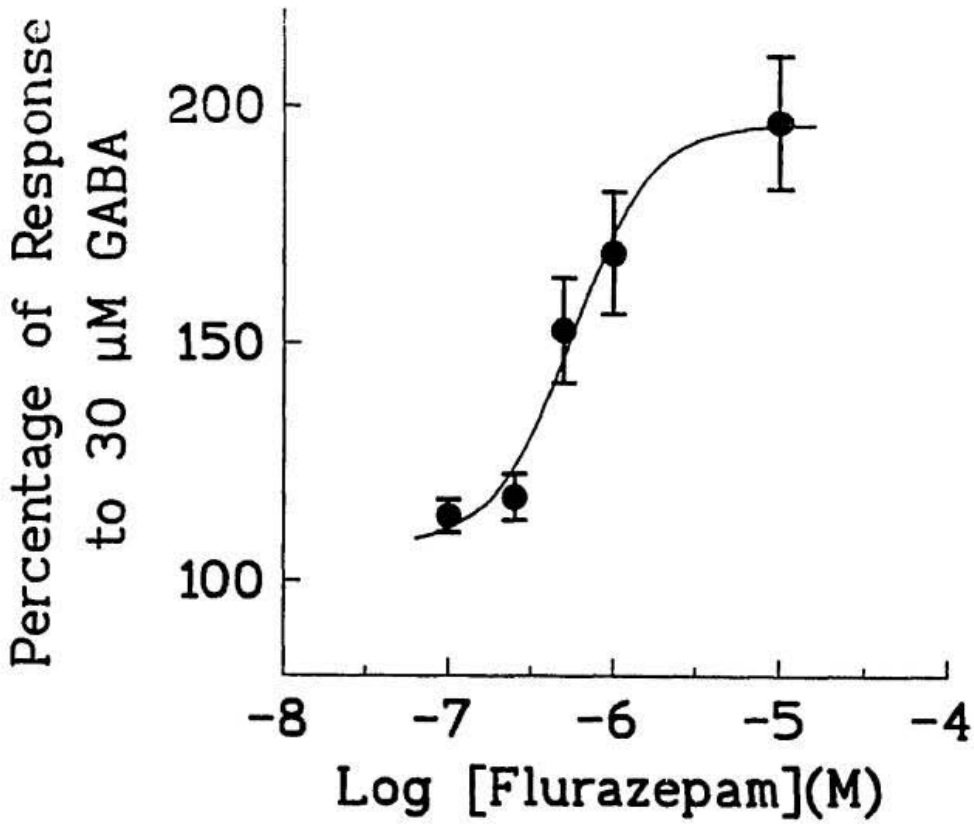
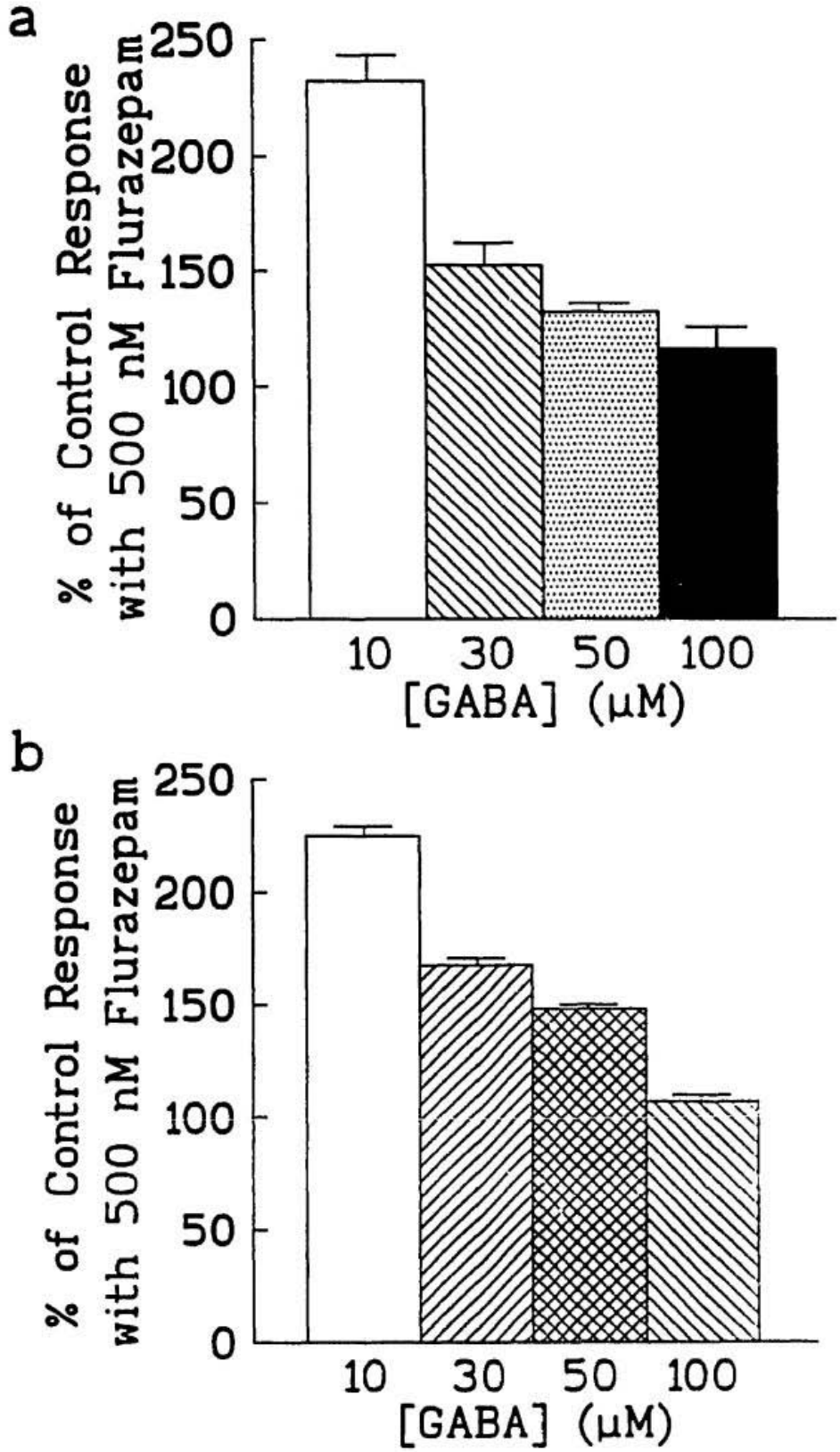


Figure 3. **Flurazepam potentiation of I_{GABA}**
Plot shows enhancement of response to 30 μM GABA with increasing concentrations of flurazepam on human $\alpha_2\beta_2\gamma_{2L}$ $GABA_A$ receptors. Data from 4 oocytes.

Figure 4. **Flurazepam potentiation varies with GABA concentration**
a) Bar graph representing percentage increase in GABA responses with 0.5 μM flurazepam on human $\alpha_1\beta_2\gamma_{2L}$ receptors. Data from 7 oocytes. b) As in (a) with $\alpha_2\beta_2\gamma_{2L}$ $GABA_A$ receptors. Data from 4 to 8 oocytes.


a
% of Control Response with 500 nM Flurazepam
250
200
150
100
50
0
10
30
50
100
[GABA] (μM)
b
% of Control Response with 500 nM Flurazepam
250
200
150
100
50
0
10
30
50
100
[GABA] (μM)

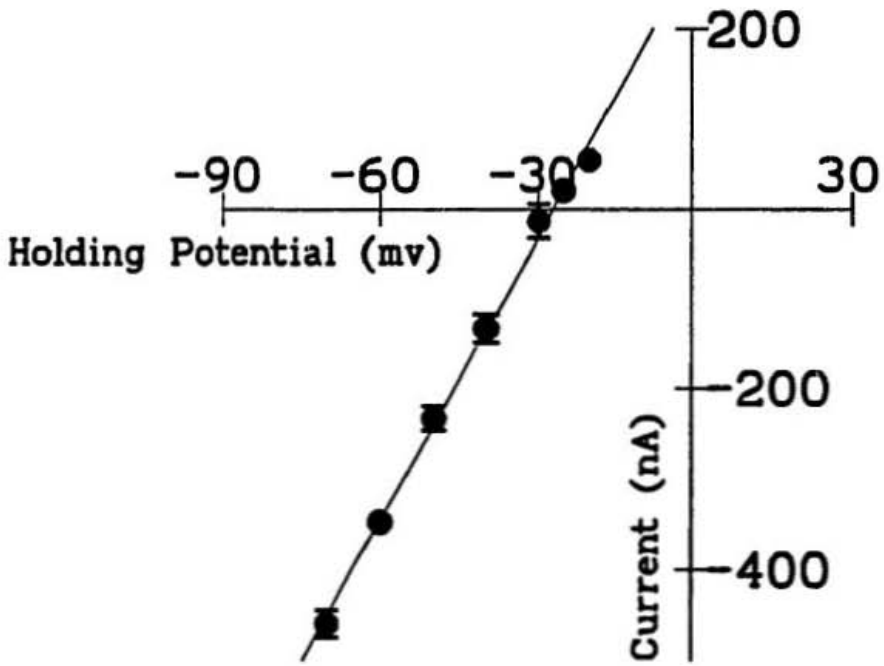


Figure 5. **Current-voltage relationship for GABA-activated current**
The inward current at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors was reduced with the reduction in the holding potential. Reversal potential for current activated by 10 μM GABA is -27.8 $\pm$ 2 mV. Data are from 4 oocytes.

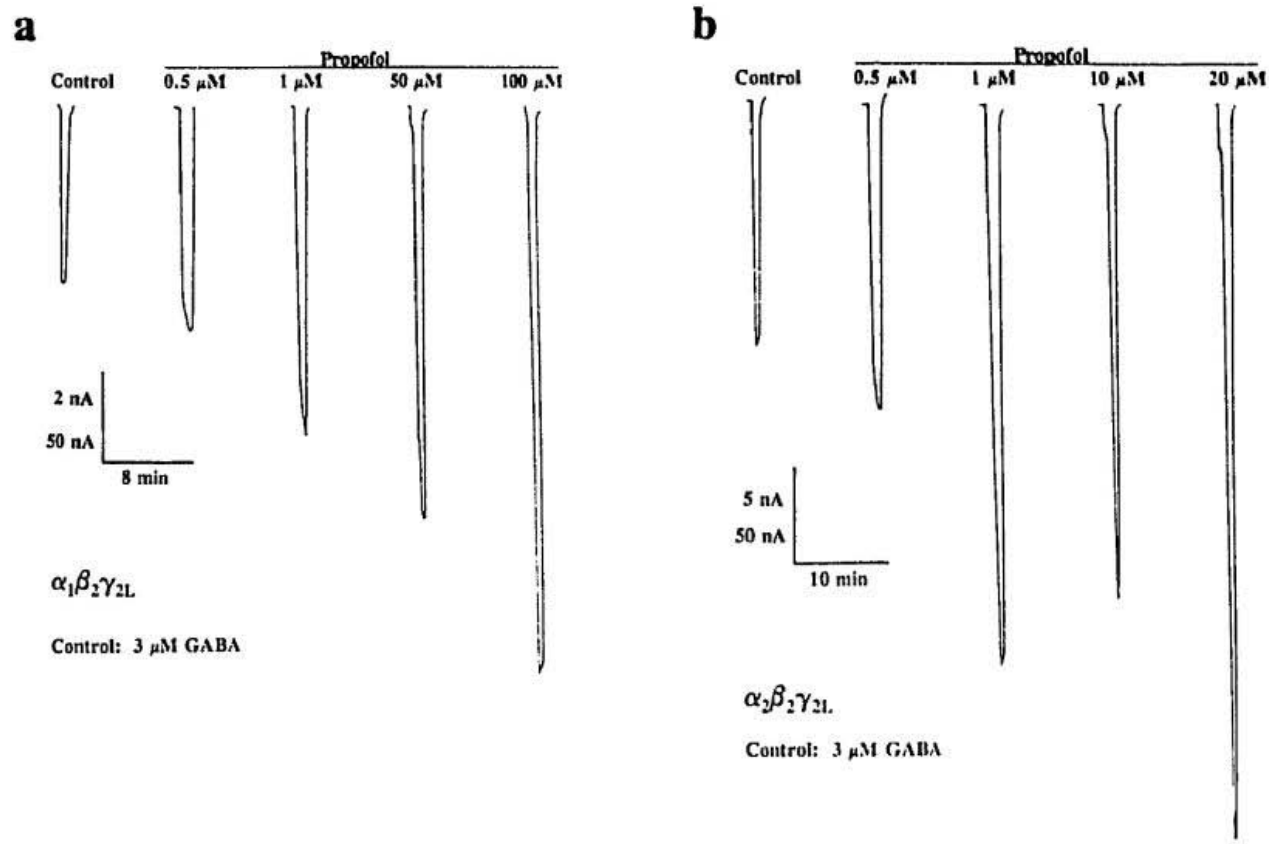
3.2 Propofol and flurazepam interaction at human $GABA_A$ receptors

Application of propofol to oocytes expressing either $\alpha_1\beta_2\gamma_{2l}$ or $\alpha_2\beta_2\gamma_{2l}$ receptors produced a concentration-dependent increase in the response to 3 μM GABA. Propofol was applied to oocytes over a concentration ranging from 0.5 to 100 μM. The vehicle used for dissolving propofol was Intralipid which at a concentration of 0.04 and 0.2% did not have any significant effect on control GABA response (data not shown). The concentration-response relationship for propofol shows a sigmoidal relationship (Fig.6) with a calculated maximum potentiation of 7512% of the 3 μM GABA response at the $\alpha_1\beta_2\gamma_{2L}$ receptor. The EC_{50} for propofol was 26 $\pm$ 6 μM (n=7) with a Hill slope of 1.4 $\pm$ 0.14. Maximum potentiation of the 3 μM GABA response with propofol at $\alpha_2\beta_2\gamma_{2l}$ receptors was 5349% (Fig.6) with an EC_{50} for propofol of 19 $\pm$ 4 μM (n=7) and a Hill slope of 1.3 $\pm$ 0.07. There was no significant difference in the effects of propofol on GABA responses at $\alpha_1\beta_2\gamma_{2l}$ versus $\alpha_2\beta_2\gamma_{2L}$ receptors.

Propofol produced a robust increase in response to GABA when coapplied with 3 μM GABA, but the degree of potentiation was dramatically reduced when higher concentrations of GABA were used. Propofol (0.1-20 μM) was used in conjunction with 10 μM GABA on $\alpha_2\beta_2\gamma_{2L}$ receptor subtype and produced a maximal increase of 949% of the GABA response (Fig.7). The concentration-response relationship showed an EC_{50} for propofol of 6 $\pm$ 1 μM and a Hill slope of 1.28 $\pm$ 0.06 (n=7).

Figure 6. **Effects of Propofol on I_{GABA} from oocytes expressing human recombinant $GABA_A$ receptors**

a) Current traces recorded from an oocyte expressing $\alpha_1\beta_2\gamma_{2L}$ receptors showing potentiation of inward Cl^- current produced by 3 μM GABA with application of propofol (0.5 - 100 μM). Current scale for the left three traces is 2 nA and 50 nA for the last two traces. b) Current traces recorded from an oocyte expressing $\alpha_2\beta_2\gamma_{2L}$ subtype of $GABA_A$ receptor showing potentiation of inward Cl^- current produced by 3 μM GABA. Current scale for the left three traces is 5 nA and 50 nA for the last two traces. c) Concentration response curves for $\alpha_1\beta_2\gamma_{2L}$ (open circles) and $\alpha_2\beta_2\gamma_{2L}$ (closed circles) subtype of human $GABA_A$ receptors. Data from 7 oocytes for each data point.


a
Propofol
Control
0.5 μM
1 μM
50 μM
100 μM
2 nA
50 nA
8 min
$\alpha_1\beta_2\gamma_{2L}$
Control: 3 μM GABA
b
Propofol
Control
0.5 μM
1 μM
10 μM
20 μM
5 nA
50 nA
10 min
$\alpha_2\beta_2\gamma_{2L}$
Control: 3 μM GABA

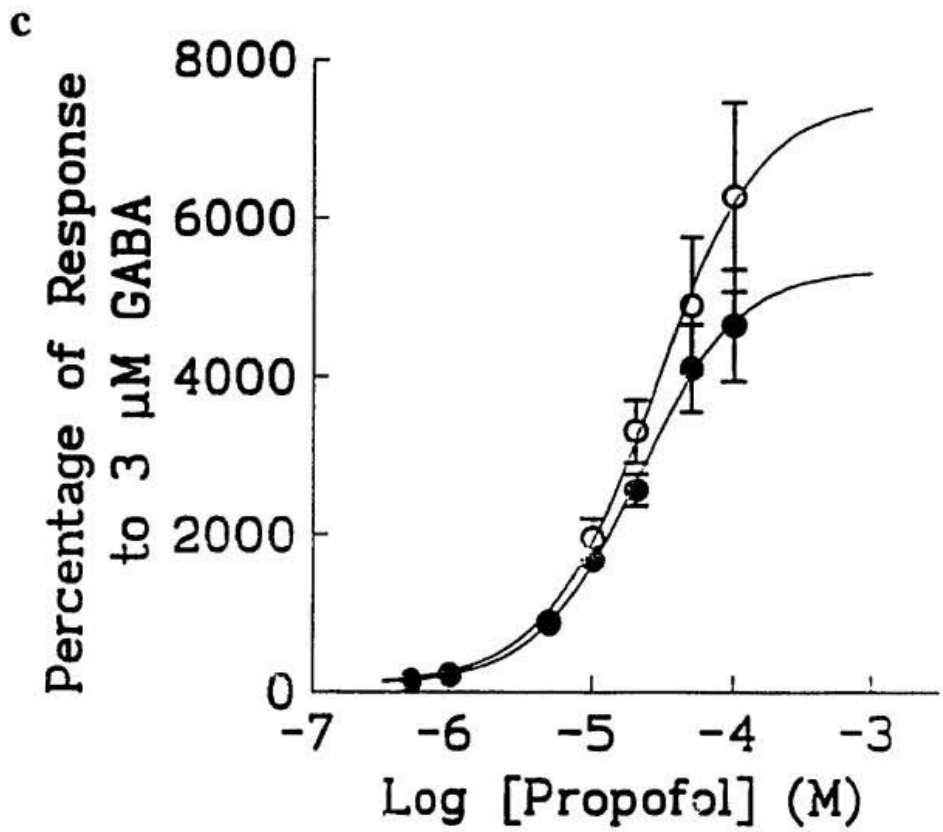
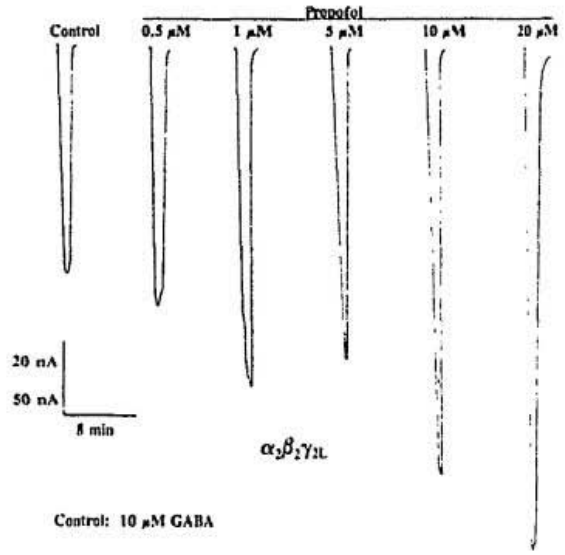

c
Percentage of Response to 3 μM GABA
8000
6000
4000
2000
0
-7
-6
-5
-4
-3
Log [Propofol] (M)

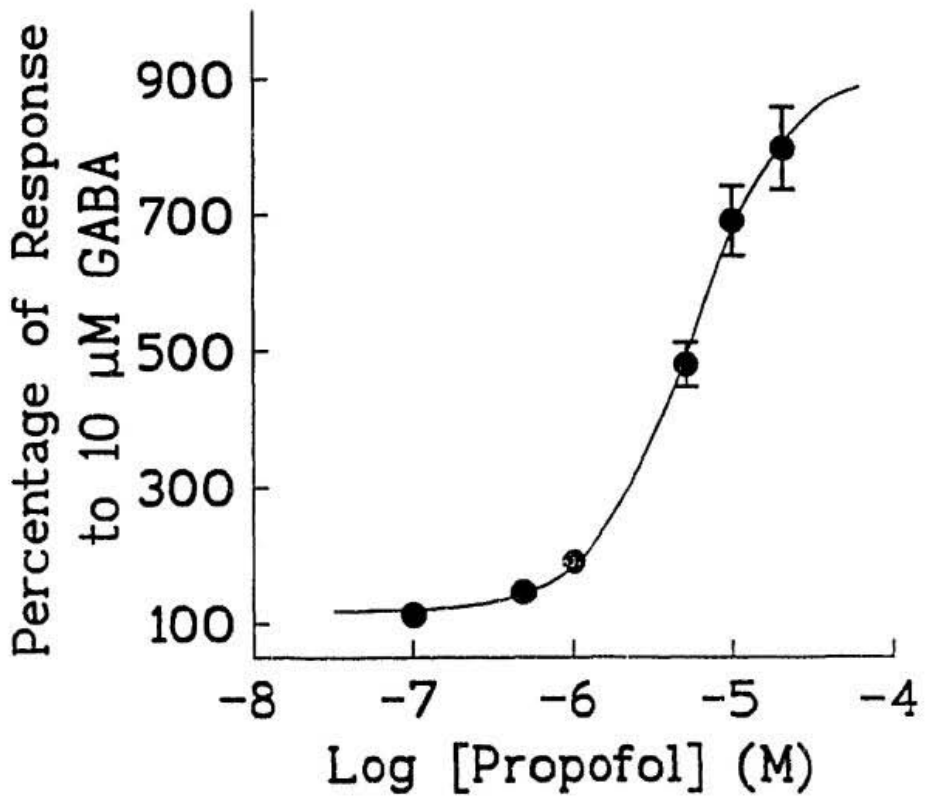
Figure 7. **Propofol enhancement of 10 μM GABA response at $\alpha_2\beta_2\gamma_{2L}$ receptors.**

a) Current traces representing increase in control response with application of propofol. Note that the current scale is 20 nA for control, 0.5 and 1 μM propofol, and 50 nA for 5-20 μM propofol. b) Best fit sigmoidal curve to the concentration dependent increase in GABA response with propofol at human $\alpha_2\beta_2\gamma_{2L}$ $GABA_A$ receptors. Data from 7 oocytes.

a


Propofol
Control
0.5 μM
1 μM
5 μM
10 μM
20 μM
20 nA
50 nA
8 min
$\alpha_2\beta_2\gamma_{2L}$
Control: 10 μM GABA

b


Percentage of Response to 10 μM GABA
900
700
500
300
100
-8
-7
-6
-5
-4
Log [Propofol] (M)

Propofol also changed the affinity of $GABA_A$ receptors to GABA as the concentration-response curve for GABA was shifted to the left for $\alpha_2\beta_2\gamma_{2L}$ receptor subtype with the addition of 10 μM propofol (Fig.8). No significant change in the maximum response was observed with the addition of propofol, but there was a significant change in the EC_{50} value. The EC_{50} for GABA was found to be 45.6 $\pm$ 9 μM and 69.7 $\pm$ 7 μM, with and without propofol respectively ($p < 0.05$). The Hill slope for the curve with GABA alone was 1.12 $\pm$ 0.06 and 1.18 $\pm$ 0.22 with GABA plus propofol. However, propofol (1 μM) did not change either efficacy or affinity of GABA at the same receptor construct (data not shown). The effect of 10 μM propofol on GABA concentration-response at $\alpha_1\beta_2\gamma_{2L}$ receptors could not be evaluated as the data were not properly fit by a sigmoidal relationship.

The interaction between propofol and flurazepam was studied on both $\alpha_1\beta_2\gamma_{2L}$ and $\alpha_2\beta_2\gamma_{2L}$ receptors expressed in oocytes. Propofol and flurazepam individually enhanced I_{GABA} and their individual responses on control GABA-activated current were added and compared to the actual response produced by the coapplication of both of them at a particular $GABA_A$ receptor construct. Propofol (1 and 10 μM) and 0.5 μM flurazepam both separately potentiated the response to 3 μM GABA on human $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors, but when they were combined, a greater than additive effect was obtained (Fig.9).

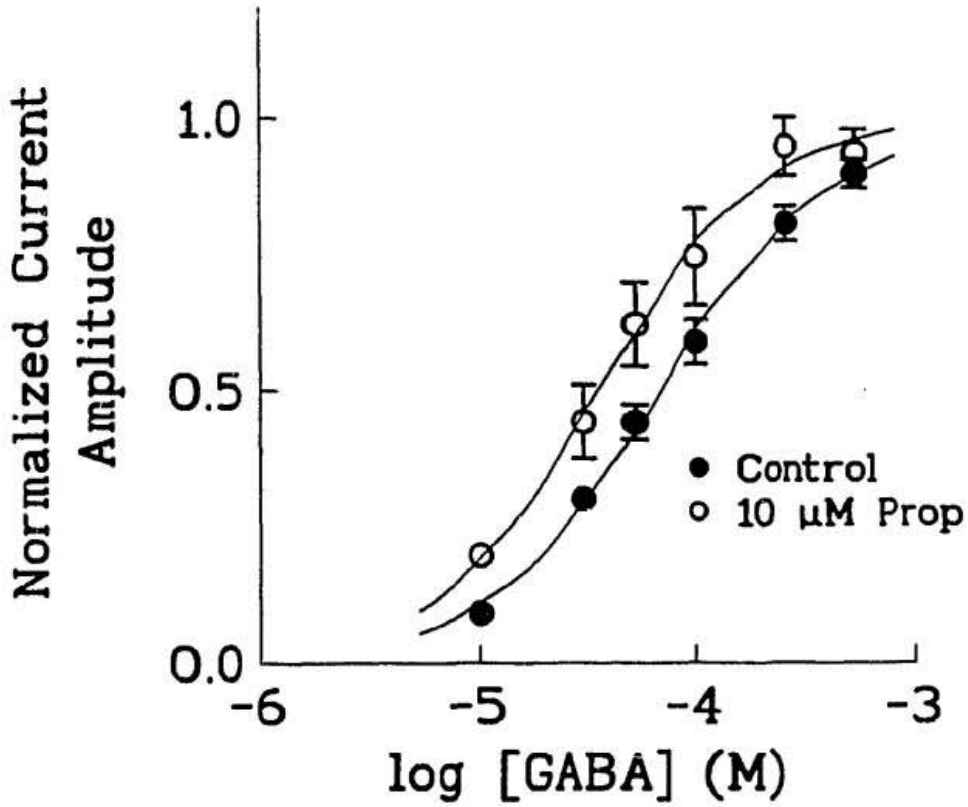
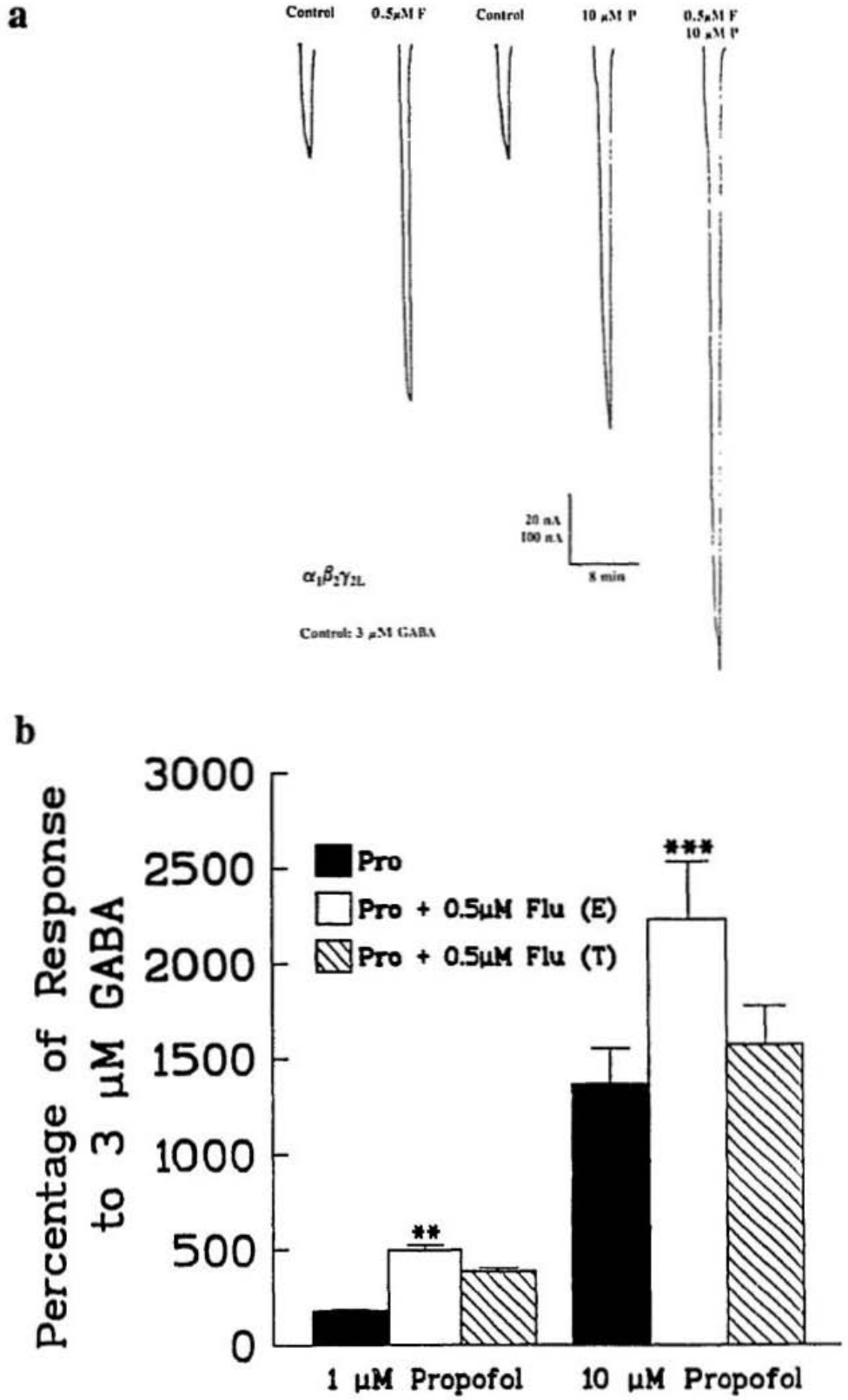


Figure 8. **Effect of propofol on GABA concentration-response relationship** Concentration-response relationship to GABA alone (closed circles) and in presence of 10 μM propofol (open circles) on $\alpha_2\beta_2\gamma_{2L}$. $GABA_A$ receptors. Data normalized to the calculated maximal GABA responses. Data from 5 oocytes.

Figure 9. **Effect of propofol and flurazepam combination on $\alpha_1\beta_2\gamma_{2L}$ receptors**

a) Recording from an oocyte showing increase in control (3 μM GABA) response with 0.5 μM flurazepam and 10 μM propofol separately and also when applied in combination. Current scale for the first three traces from left is 20 nA and 100 nA for the last two. b) Bar graphs showing cumulative data for propofol-flurazepam interaction. Filled bars represent propofol potentiation of response to 3 μM GABA. Open bars denote responses from $GABA_A$ receptors found experimentally (E) to the concurrent application of GABA, propofol and flurazepam, which are significantly different from the calculated additive responses (T) shown by the hatched bars. Flurazepam potentiation of GABA (3 μM) response was 308 $\pm$ 17%. Data from 4 oocytes. **, $p<0.01$ and ***, $p<0.001$.


a
Control
0.5μM F
Control
10 μM P
0.5μM F
10 μM P
20 nA
100 nA
8 min
α1β2γ2L
Control: 3 μM GABA
b
Percentage of Response to 3 μM GABA
3000
2500
2000
1500
1000
500
0
Pro
Pro + 0.5μM Flu (E)
Pro + 0.5μM Flu (T)
**
***
1 μM Propofol
10 μM Propofol

The concentration of propofol also plays an important role in producing the synergistic effect with 0.5 μM flurazepam. On the $\alpha_2\beta_2\gamma_{2L}$ receptor combination, propofol (0.5-10 μM) shows synergy with 0.5 μM flurazepam when coapplied in presence of 3 μM GABA, whereas the effect with 20 μM propofol was not significantly different from the additive effect of the drugs (Fig.10).

Propofol (0.5-100 μM) produced a concentration-dependent potentiation of the inward current response activated by 3 μM GABA at $\alpha_2\beta_2\gamma_{2L}$ receptor. Addition of 0.5 μM flurazepam lowered the maximum facilitation of the GABA-evoked response by propofol and shifted the log concentration-response curve for propofol to the left (Fig.11). Theoretical addition of propofol and flurazepam potentiation of GABA responses was obtained by actual addition of responses to both the drugs. It thus generated a set of data which can also be described by a sigmoidal function. Specific data points for this additive curve have not been shown on the graph. The additive curve has slightly lower maximal response than that obtained with propofol alone in presence of GABA, but EC_{50} values for propofol obtained from both the curves are similar. The EC_{50} for propofol on addition of flurazepam is significantly less compared to that calculated from the additive sigmoidal relation and propofol alone (Table I).

Synergism was observed with propofol and flurazepam at $GABA_A$ receptors after the concentration of GABA was raised too. At 30 μM GABA, the combined effects of propofol (1, 5 and 10 μM) and 0.5 μM flurazepam were found to be significantly different from the theoretical additive response of the drugs on human $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptor subtype (Fig.12). 0.5 and 20 μM propofol failed to demonstrate the synergy at

the above mentioned receptor. A similar phenomenon was observed at $\alpha_2\beta_2\gamma_{2l}$ receptors where only 0.5, 1 and 10 μM propofol showed synergism with 0.5 μM flurazepam and 30 μM GABA, whereas 5 and 20 μM propofol was ineffective in this respect (Fig.13).

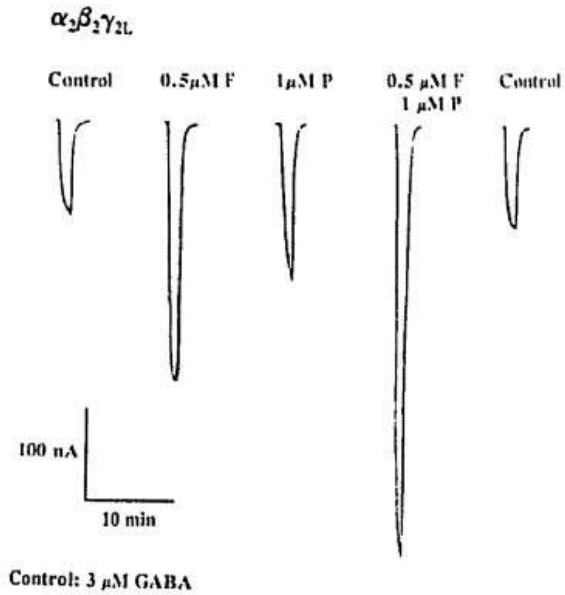
Synergism produced by flurazepam and propofol also varied with the concentration of flurazepam. Different concentrations of flurazepam (0.25-1 μM) coapplied with 5 μM propofol and 10 μM GABA produced different degrees of potentiation. On $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors, 0.25 and 0.5 μM flurazepam produced a synergistic effect with propofol and GABA, whereas 1 μM flurazepam did not (Fig.14).

Ro 15-4513, an inverse agonist at the benzodiazepine site on $GABA_A$ receptors, almost completely blocked the potentiation of I_{GABA} by 1 μM flurazepam, but did not significantly alter the effect of GABA alone or with propofol (Fig.15). Ro 15-4513 (0.5 μM) when coapplied with propofol (5 μM), flurazepam (1 μM) and GABA (10 μM) produced an effect similar to the propofol potentiation of I_{GABA}, thus completely eliminating the component of the response contributed by flurazepam without affecting the response to propofol.

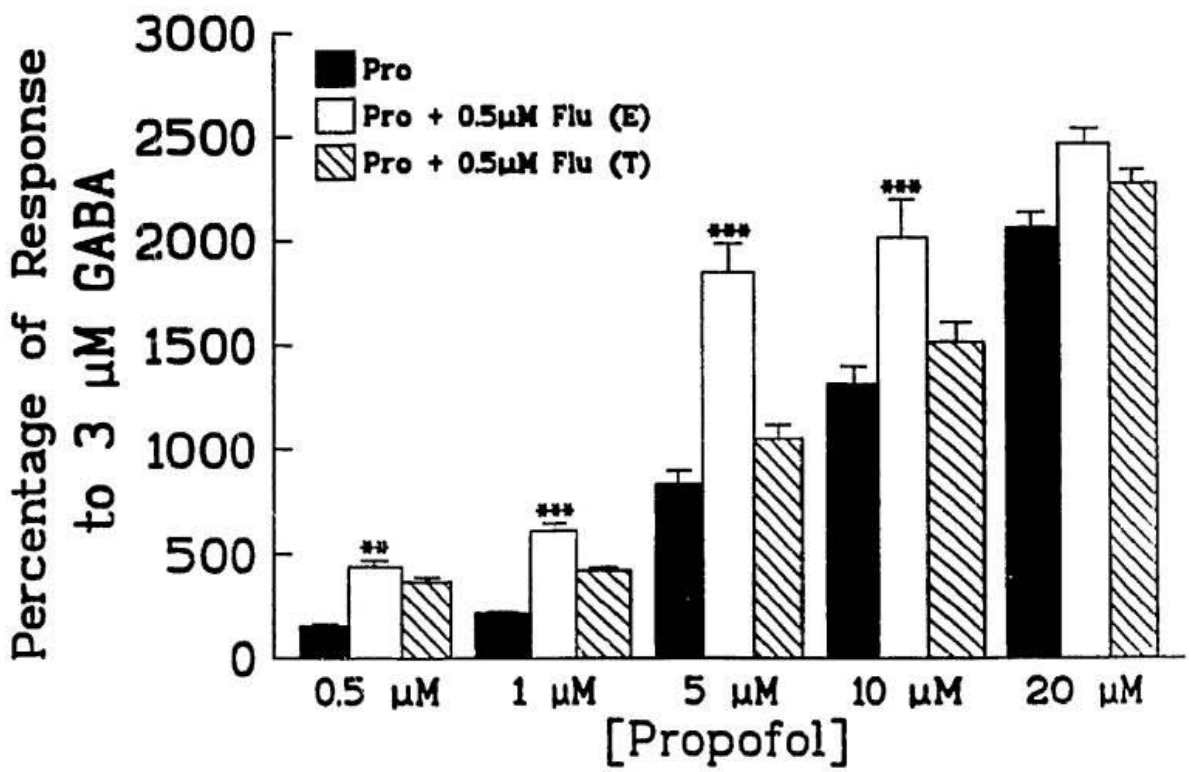
Figure 10. Effect of propofol and flurazepam combination on $\alpha_2\beta_2\gamma_{2L}$ receptors

a) Current traces representing responses to 3 μM GABA with 0.5 μM flurazepam and 1 μM propofol separately and also in combination. b) Synergism due to propofol and flurazepam interaction is found with lower concentrations of propofol (0.5 - 10 μM), but not with 20 μM propofol. Increase in control response to application of propofol is shown by filled bars and experimental result (E) showing its interaction with flurazepam is represented by the open bars. Hatched bars shows theoretical addition (T) of GABA, propofol and flurazepam responses. Enhancement of control GABA (3 μM) current due to flurazepam was 308 $\pm$ 9%. Data from 5 to 9 oocytes. **, $p < 0.01$ and ***, $p < 0.001$.

a


$\alpha_2\beta_2\gamma_{2L}$
Control
0.5μM F
1μM P
0.5 μM F
1 μM P
Control
100 nA
10 min
Control: 3 μM GABA

b


Percentage of Response to 3 μM GABA
3000
2500
2000
1500
1000
500
0
Pro
Pro + 0.5μM Flu (E)
Pro + 0.5μM Flu (T)
**
***
***
***
0.5 μM
1 μM
5 μM
10 μM
20 μM
[Propofol]

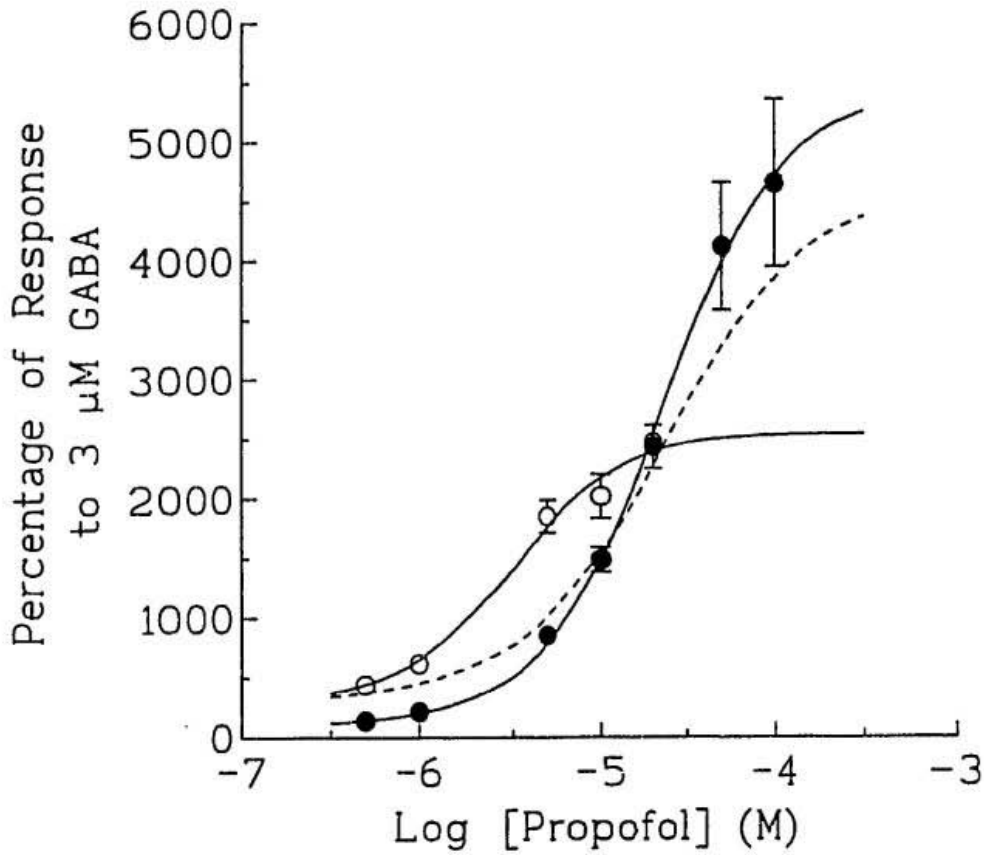


Figure 11. **Effect of flurazepam on propofol induced facilitation of GABA-current in oocytes expressing $\alpha_2\beta_2\gamma_{2L}$ receptors**
Concentration-response relationship for propofol (closed circles) and propofol plus 0.5 μM flurazepam (open circles) in presence of 3 μM GABA. The best-fit sigmoidal curve for the theoretically added responses of propofol, flurazepam and GABA has been shown by the broken line. Data are from 5 to 9 oocytes.

Table 1

Effect of flurazepam on propofol concentration-response relationship data at $\alpha_2\beta_2\gamma_{2L}$ receptor in presence of 3 μM GABA

Propofol generates a sigmoidal concentration-response relationship both in presence of GABA and GABA plus 0.5 μM flurazepam. Best-fit curves are obtained for propofol alone, experimentally observed response for propofol plus 0.5 μM flurazepam (E) and theoretically added responses for propofol and flurazepam (T) in presence of GABA. Data {I_{max}, EC_{50} and Hill slope(n)} from the sigmoidal curves have been compared. Number of observations are indicated in parentheses.

	Propofol	Propofol + Flurazepam (E)	Propofol + Flurazepam (T)
I_{max}	4245 ± 638%	2718 ± 115%	3636 ± 658%
EC_{50}	16 ± 4 μM	4 ± 0.7 μM*	15 ± 5 μM
n	1.55 ± 0.18 (9)	1.56 ± 0.17 (5)	1.54 ± 0.25 (5)

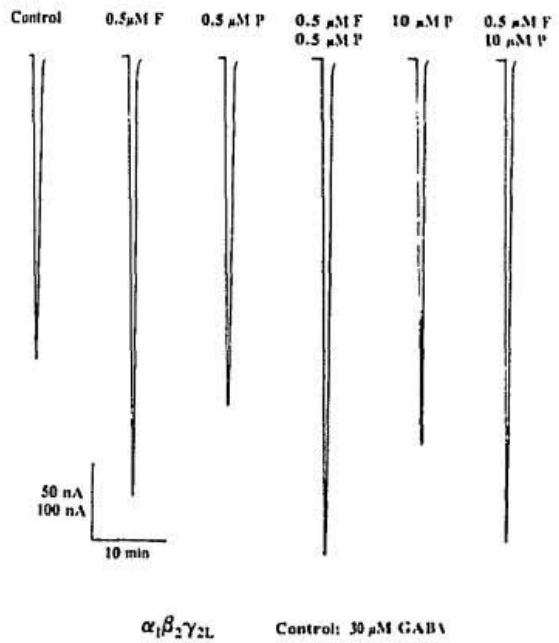
*, $p < 0.01$, compared to both propofol alone and propofol + flurazepam(T)

Figure 12. Effect of 30 μM GABA on propofol and flurazepam interaction on $\alpha_1\beta_2\gamma_{2L}$ receptors

a) Recording from an oocyte showing potentiation of GABA response by flurazepam (0.5 μM) and propofol (0.5 and 10 μM) separately and upon combined application of each concentrations of propofol with flurazepam. Current scale for the first four traces from left is 50 nA and 100 nA for the remaining two traces.

b) Propofol (1, 5 and 10 μM) in presence of 0.5 μM flurazepam produced potentiation (E) significantly greater the theoretical additive response (T). Experimental observations (open bars) for 0.5 and 20 μM propofol were not significantly different from the expected additive results (hatched bars) with flurazepam. Filled bars represent effect of propofol on I_{GABA}. Flurazepam produced 144 $\pm$ 8% enhancement of response to 30 μM GABA. Data calculated as percentage of 30 μM GABA responses (N=4). *, $p<0.05$ and **, $p<0.001$.

a


Control
0.5 μM F
0.5 μM P
0.5 μM F
0.5 μM P
10 μM P
0.5 μM F
10 μM P
50 nA
100 nA
10 min
$\alpha_1\beta_2\gamma_{2L}$
Control: 30 μM GABA

b

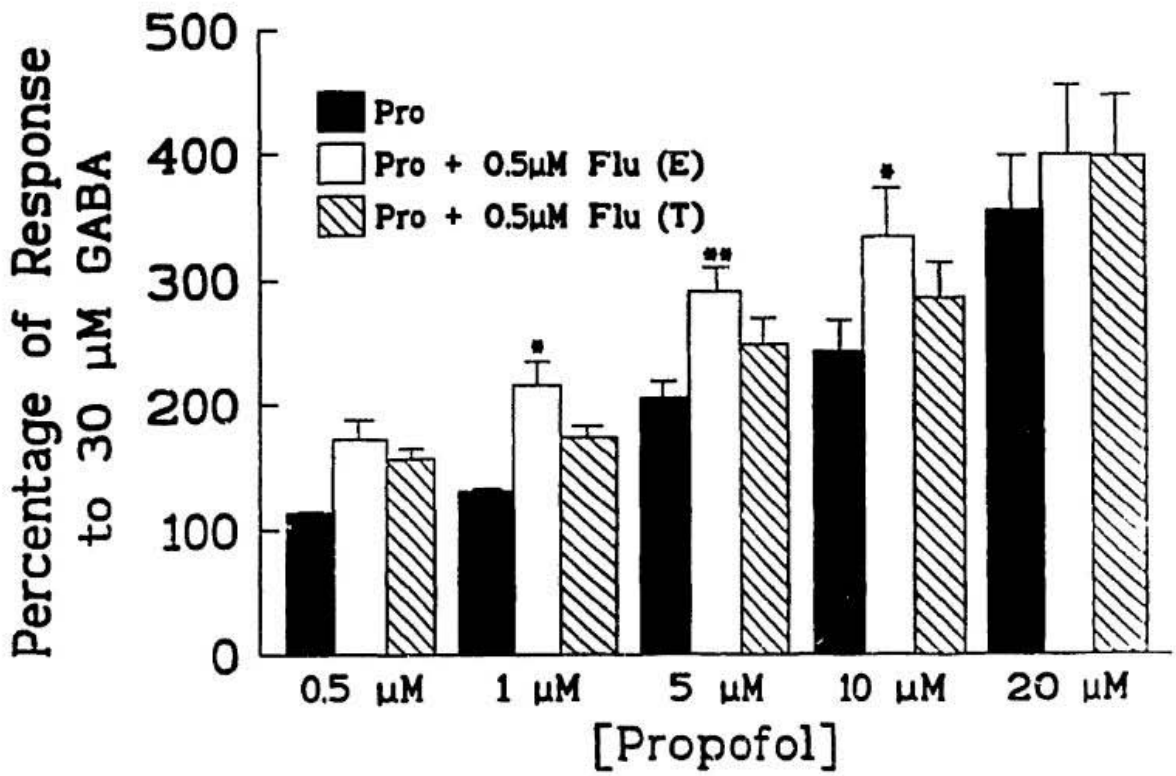
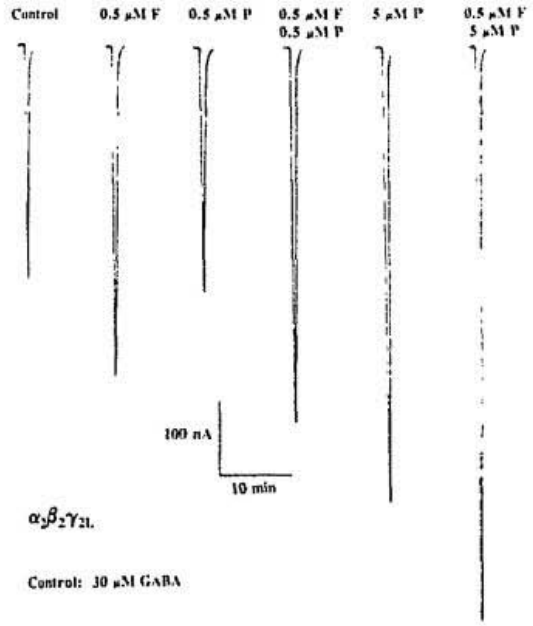

Percentage of Response to 30 μM GABA
500
400
300
200
100
0
Pro
Pro + 0.5μM Flu (E)
Pro + 0.5μM Flu (T)
*
**
*
0.5 μM
1 μM
5 μM
10 μM
20 μM
[Propofol]

Figure 13. Effect of 30 μM GABA on propofol and flurazepam interaction on $\alpha_2\beta_2\gamma_{2L}$ receptors

a) Change in the response to GABA with application of propofol (0.5 and 5 μM) and flurazepam (0.5 μM) separately, as well as in combination, recorded from an individual oocyte. b) Propofol (0.5, 1 and 10 μM) and flurazepam act synergistically at the $GABA_A$ receptors and the experimental findings (E) being shown by open bars has been compared to the theoretical additive (T) responses represented by the hatched bars. 5 and 20 μM propofol plus 0.5 μM flurazepam did not produce effects different than expected additive. Flurazepam increased control response to 30 μM GABA by 148 $\pm$ 3%. Data from 5 to 6 oocytes. *, $p<0.05$, **, $p<0.01$ and ***, $p<0.001$.

a


Control
0.5 μM F
0.5 μM P
0.5 μM F
0.5 μM P
5 μM P
0.5 μM F
5 μM P
100 nA
10 min
$\alpha_2\beta_2\gamma_{2L}$
Control: 30 μM GABA

b

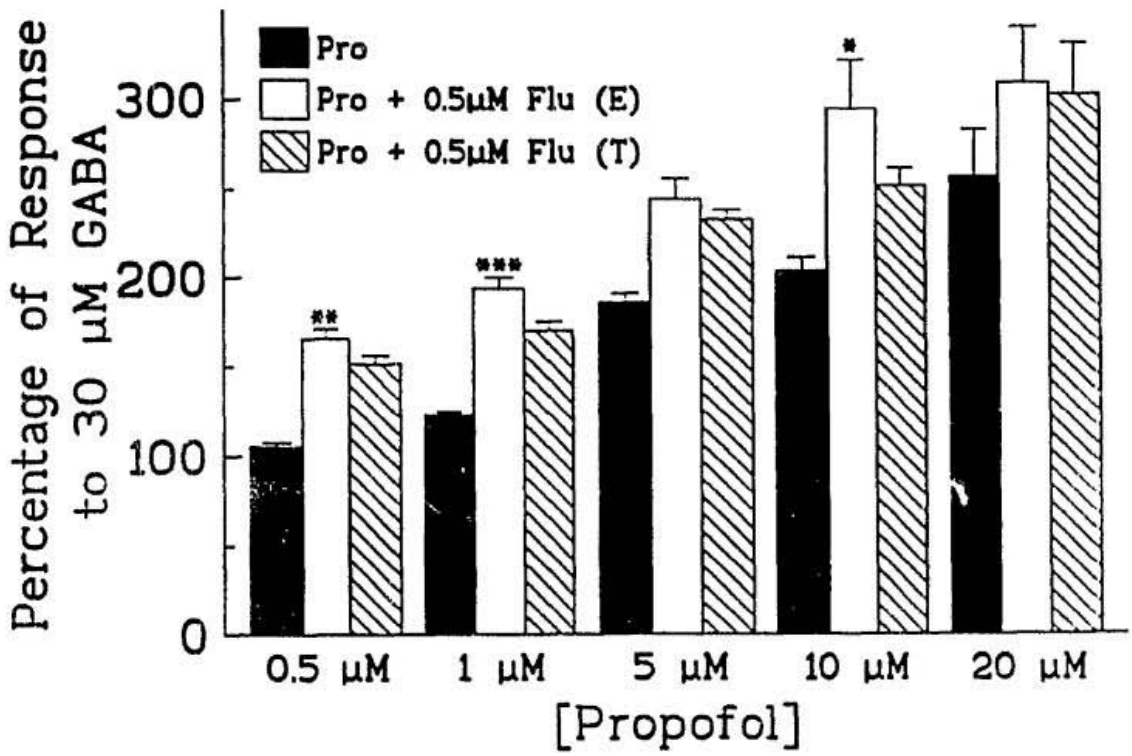
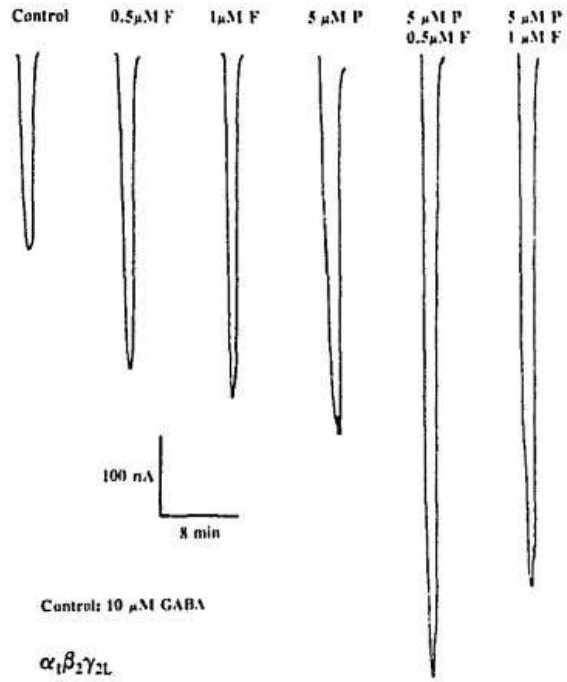

Pro
Pro + 0.5μM Flu (E)
Pro + 0.5μM Flu (T)
Percentage of Response to 30 μM GABA
0
100
200
300
**
***
*
0.5 μM
1 μM
5 μM
10 μM
20 μM
[Propofol]

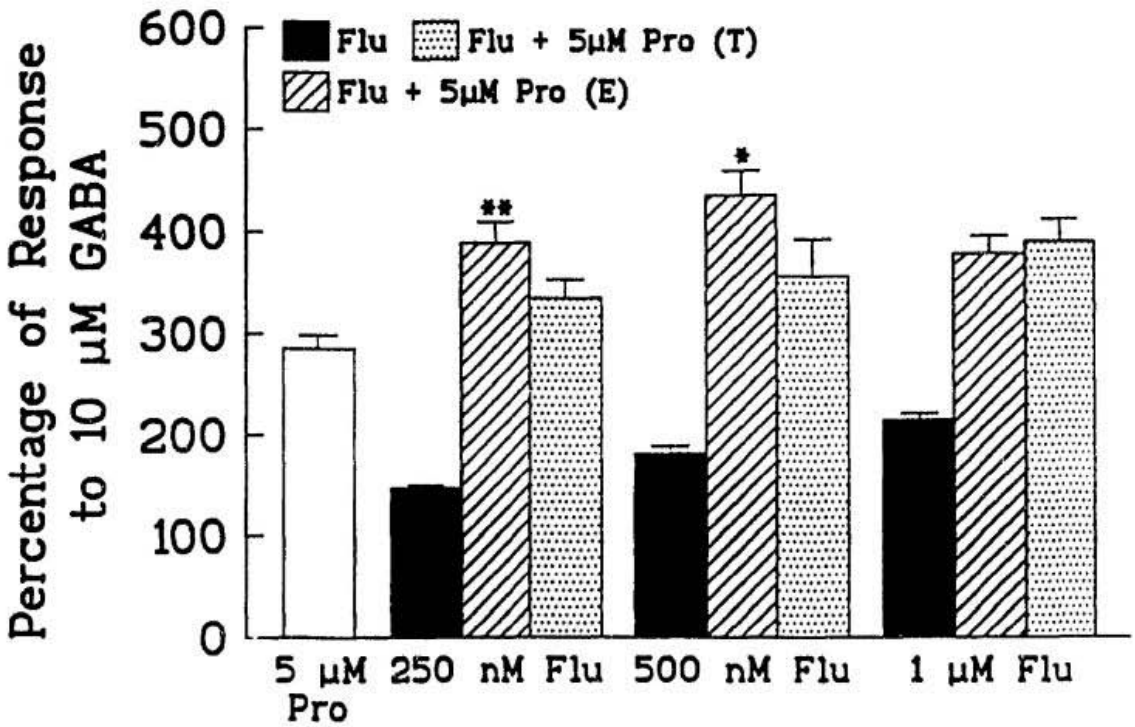
Figure 14. **Effect of flurazepam on propofol modulation of GABA responses**

a) Effect of flurazepam (0.5 and 1 μM) and 5 μM propofol separately on responses to 10 μM GABA and when applied in combination on $\alpha_1\beta_2\gamma_{2L}$ receptors. b) Lower concentrations of flurazepam (0.25 and 0.5 μM), denoted by hatched bars were found to act synergistically in experimental observations (E) with 5 μM propofol at recombinant $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors compared to the speckled bars showing theoretical additive (T) response to the combination of drugs. Solid bars indicate flurazepam potentiation of GABA responses and effect of propofol on control current has been shown by the open bar. Data from 4 or 5 oocytes. *, $p<0.05$ and **, $p<0.01$.

a


Control
0.5μM F
1μM F
5 μM P
5 μM P
0.5μM F
5 μM P
1 μM F
100 nA
8 min
Control: 10 μM GABA
$\alpha_1\beta_2\gamma_{2L}$

b


Percentage of Response to 10 μM GABA
600
500
400
300
200
100
0
Flu
Flu + 5μM Pro (T)
Flu + 5μM Pro (E)
**
*
5 μM Pro
250 nM Flu
500 nM Flu
1 μM Flu

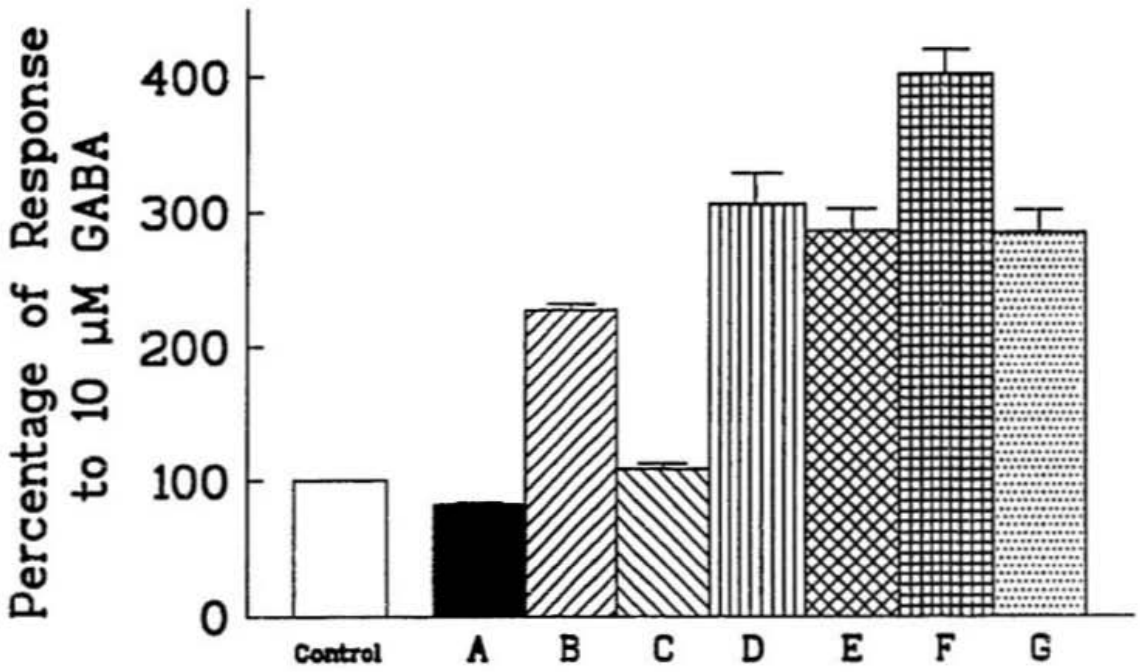


Figure 15. **Effects of Ro 15-4513 on propofol and flurazepam responses**
Ro 15-4513 affects flurazepam but not propofol response in presence of 10 μM GABA on human $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors.
A: Effect of Ro 15-4513 (0.5 μM) on GABA response
B: Flurazepam (1μM) potentiation of GABA response
C: Almost complete elimination of flurazepam (1 μM) response by 0.5 μM Ro 15-4513 in presence of 10 μM GABA
D: Propofol (5 μM) enhancement of I_{GABA}
E: No significant effect of Ro 15-4513 on propofol mediated increase in control GABA response
F: Effect of flurazepam and propofol coapplication on I_{GABA}
G: Ro 15-4513 decreases response to coapplied flurazepam and propofol to the level of propofol alone
Data from 4 oocytes.

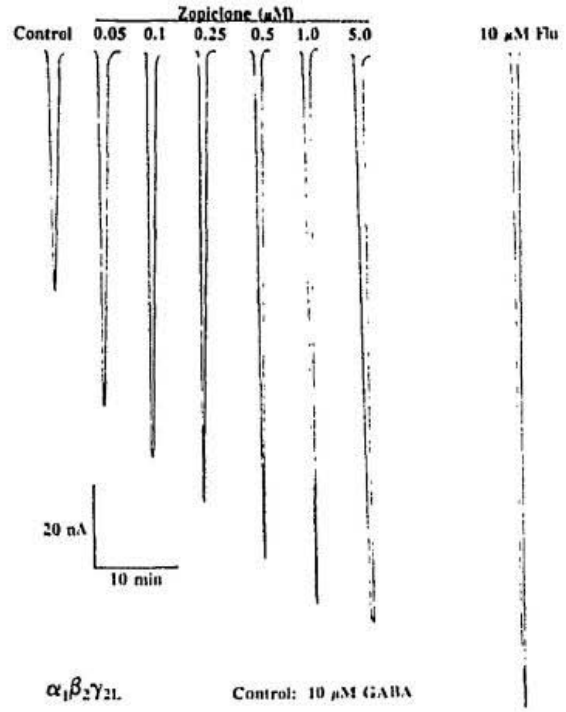
3.3 Interaction between propofol and zopiclone on $GABA_A$ receptors

Cyclopyrrolone derivatives have been found to potentiate the effects of GABA. Zopiclone,included in the class of cyclopyrrrolones and classified as a full agonist at the benzodiazepine site, was tested for its action on inward currents activated by 10 μM GABA on the $\alpha_1\beta_2\gamma_{2L}$ (benzodiazepine type I) receptor. Di-methyl sulfoxide was used as a vehicle to dissolve zopiclone and was found to produce very slight inhibition of GABA current at a concentration of 0.05% (data not shown). Zopiclone (0.05-5 μM) increased the control response to 10 μM GABA with an EC_{50} of 107 $\pm$ 9 nM (Fig.16). The sigmoidal concentration-response relationship almost levelled off at higher zopiclone concentrations with a maximum potentiation of 232 $\pm$ 5% and a Hill slope of 1.26 $\pm$ 0.08 (n=8). Maximum effect of zopiclone was compared to 10 μM flurazepam, a maximally effective concentration of this benzodiazepine agonist. Zopiclone was found to produce equally effective responses as flurazepam. Unlike flurazepam, zopiclone (0.1 μM) when combined with propofol (1 or 10 μM) and 10 μM GABA not only failed to produce an effect greater than the expected additive effect, but actually produced an effect significantly lower than the theoretically additive response (Fig.17).

Figure 16. Effect of zopiclone on GABA-activated current at $\alpha_1\beta_2\gamma_{2L}$ receptors

a) Current traces from an oocyte showing increase in I_{GABA} on application of 0.5 - 5 μM zopiclone and also potentiation of GABA response with 10 μM flurazepam. b) Plot showing concentration-dependent relationship of zopiclone (open circles) to potentiate I_{GABA}. Zopiclone is equally effective in producing a change in the GABA response as flurazepam (10 μM, closed circle). Data from 9 oocytes.

a


Zopiclone (μM)
Control
0.05
0.1
0.25
0.5
1.0
5.0
10 μM Flu
20 nA
10 min
$\alpha_1\beta_2\gamma_{2L}$
Control: 10 μM GABA

b

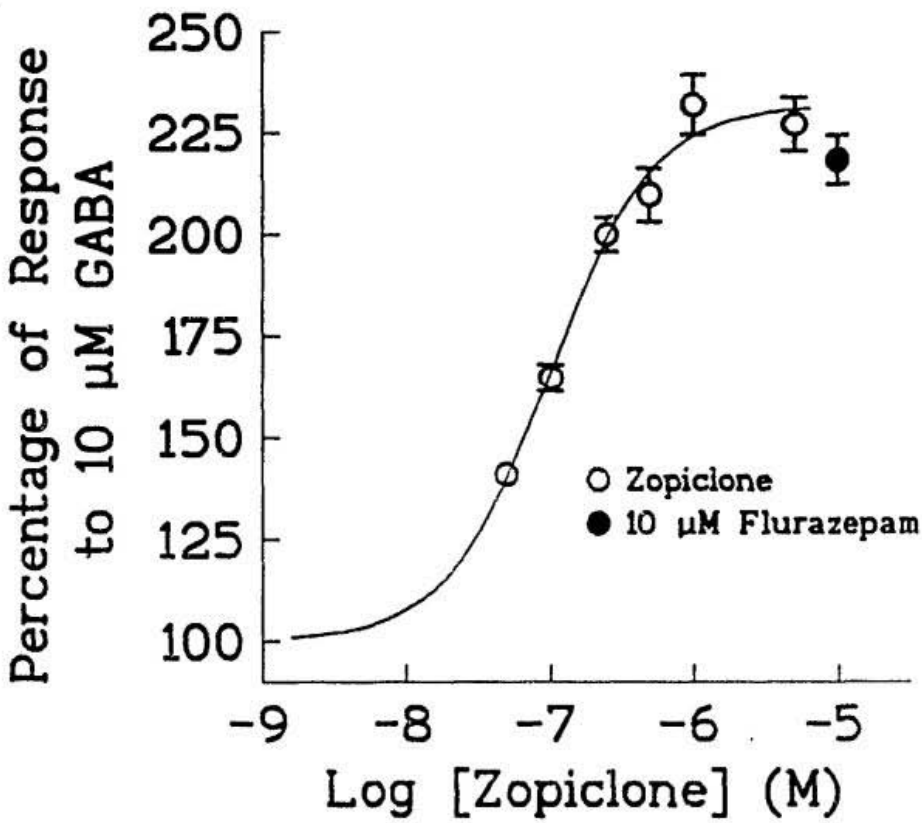
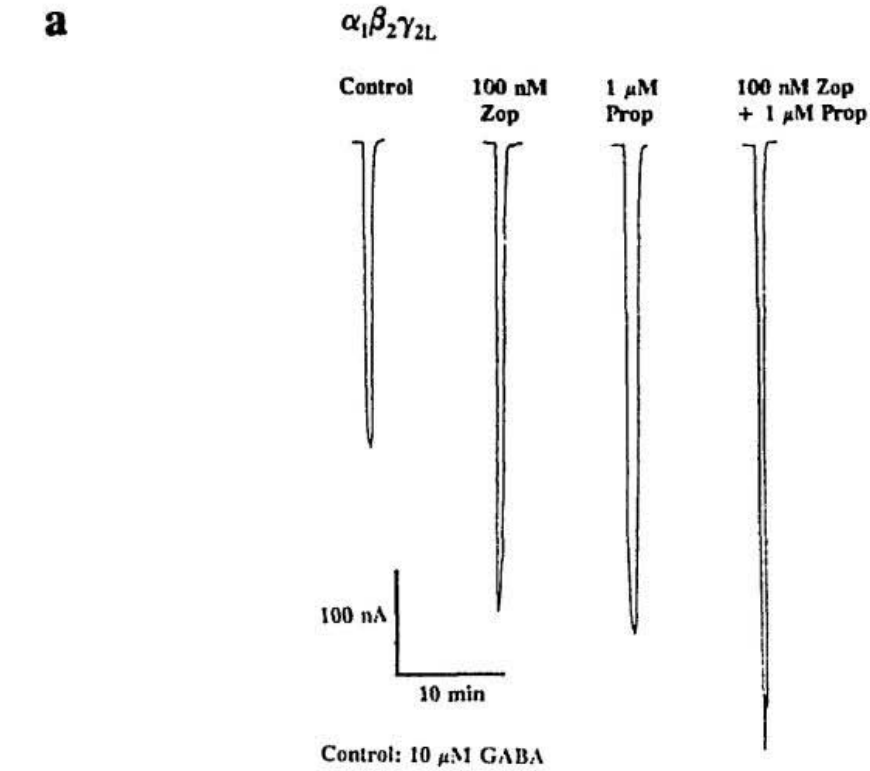
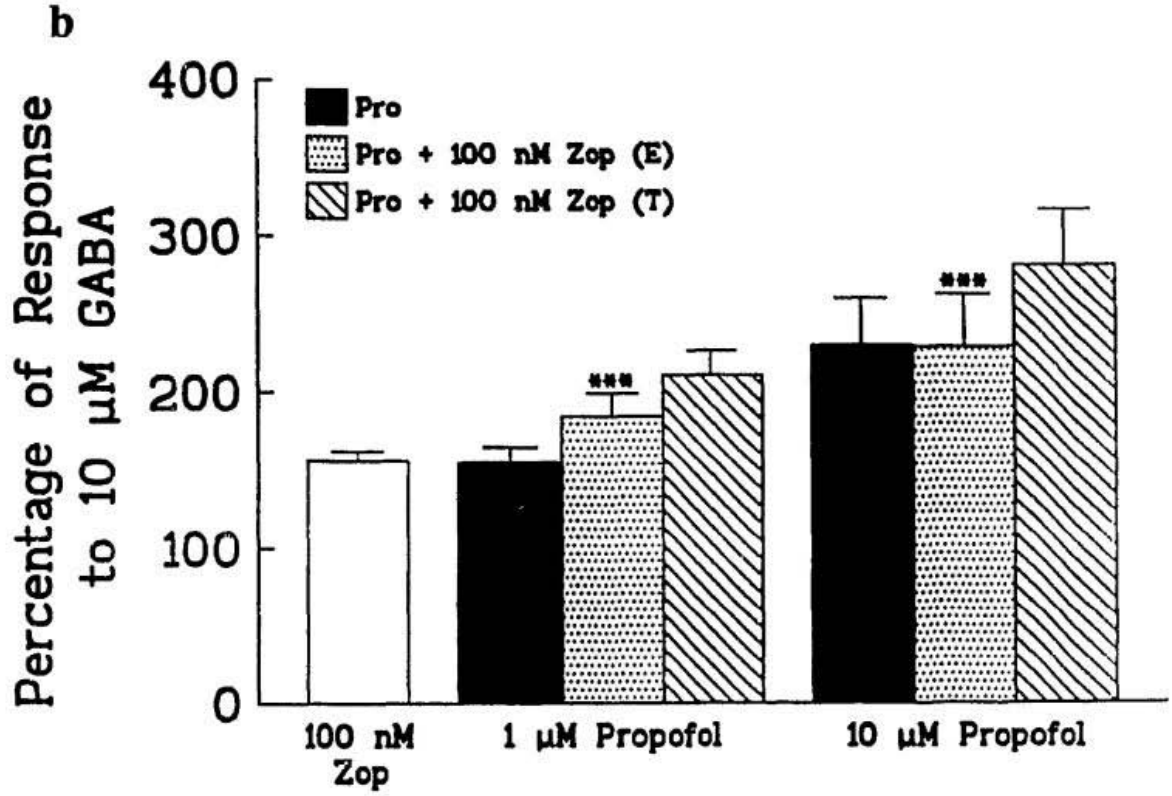

Percentage of Response to 10 μM GABA
250
225
200
175
150
125
100
-9
-8
-7
-6
-5
Log [Zopiclone] (M)
Zopiclone
10 μM Flurazepam

Figure 17. **Negative interaction between propofol and zopiclone on $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptor**

a) Response to GABA in presence of zopiclone and propofol separately and in combination, shown as recorded from an oocyte. b) Change in I_{GABA} on application of zopiclone concurrently with propofol (speckled bars) produced response (E) which was significantly lower than the theoretical additive (T) response of both of the drugs (hatched bars). Filled bars show effect of propofol on inward GABA current and response to 100 nM zopiclone is represented by the open bar. Data from 5 or 6 oocytes. ***, $p<0.001$.


a
$\alpha_1\beta_2\gamma_{2L}$
Control
100 nM Zop
1 μM Prop
100 nM Zop + 1 μM Prop
100 nA
10 min
Control: 10 μM GABA


b
Percentage of Response to 10 μM GABA
400
300
200
100
0
Pro
Pro + 100 nM Zop (E)
Pro + 100 nM Zop (T)
***
***
100 nM Zop
1 μM Propofol
10 μM Propofol

3.4 Effect of propofol on $GABA_A$ receptors with and without γ_{2L} subunit

The response to GABA at the $\alpha_2\beta_2$ combination of human $GABA_A$ receptors were smaller in magnitude compared to the responses found with receptors containing the γ_{2L} subunit. Moreover the response reached a maximum at a lower concentration of GABA compared to the other receptors.The receptor had an EC_{50} of 8 $\pm$ 2 μM for GABA with a Hill slope of 1.23 $\pm$ 0.14 (n=8) (Fig.18). No intermediate concentrations of GABA were used between 1 and 10 μM. For this receptor combination, effects of these intermediate GABA concentrations are important as they are around the median effective concentration and constitutes an important portion of the curve. Inclusion of these GABA concentrations might alter the Hill slope slightly.

Propofol which facilitated the GABA response at $\alpha\beta\gamma$ receptor construct was also found to enhance the GABA-activated inward current at the $\alpha_2\beta_2$ receptor. Unlike propofol, flurazepam which potentiated the response at $GABA_A$ receptors having the γ_{2L} subunit, failed to enhance the effect of GABA at $\alpha_2\beta_2$ receptors (data not shown). The response to 3 μM GABA was increased by propofol (0.5-20 μM) with an EC_{50} of 8 $\pm$ 1 μM (n=6) (Fig.19). The hill slope was 1.78 $\pm$ 0.19.

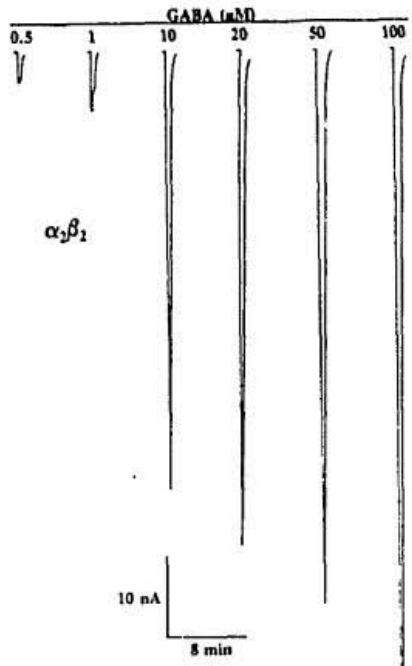
The response to propofol was compared on both $\alpha_2\beta_2\gamma_{2L}$ and $\alpha_2\beta_2$ receptor combinations with equivalent concentrations of GABA. From concentration-responses of GABA at both the receptors, 3 and 30 μM GABA were found to produce a response close to the EC_{30} value at $\alpha_2\beta_2$ and $\alpha_2\beta_2\gamma_{2L}$ respectively. The effect of higher concentrations of propofol (10 and 20 μM) was greater at the $\alpha_2\beta_2$ subtype of receptor than at the $\alpha_2\beta_2\gamma_{2L}$ receptor construct, with equally effective concentrations of GABA for

each receptor type (Fig.20).

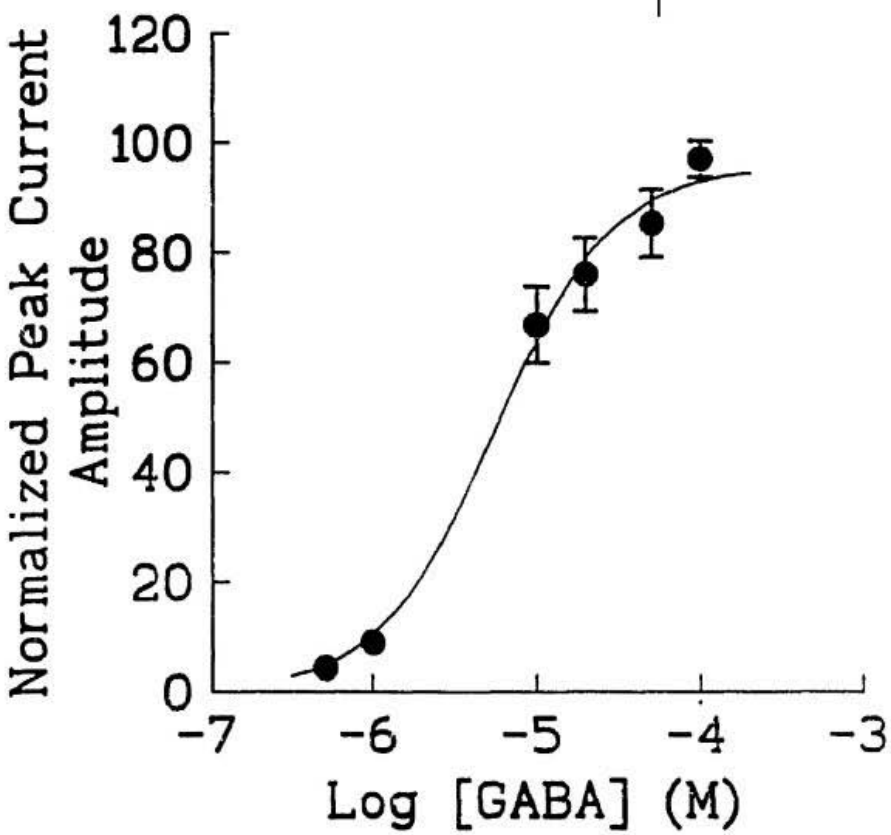
Figure 18. Increase in GABA mediated response on $\alpha_2\beta_2$ combination of $GABA_A$ receptors

a) Raw current traces recorded from an oocyte showing increase in GABA-activated inward current at $\alpha_2\beta_2$ receptors on application of increasing GABA concentrations. b) Cumulative data showing log-concentration response relationship for GABA on human $\alpha_2\beta_2$ subtype of $GABA_A$ receptors. Data from 6 oocytes.

a


GABA (μM)
0.5
1
10
20
50
100
$\alpha_2\beta_2$
10 nA
5 min

b


Normalized Peak Current Amplitude
120
100
80
60
40
20
0
-7
-6
-5
-4
-3
Log [GABA] (M)

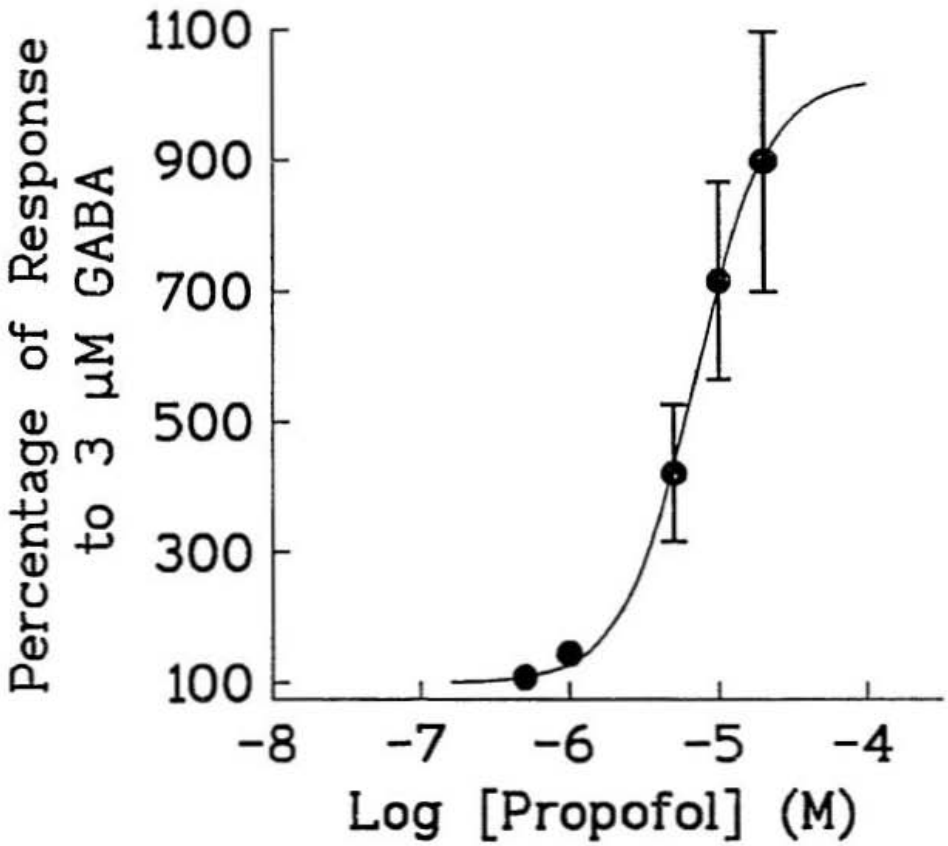


Figure 19. **Facilitation of GABA-activated response on $\alpha_3\beta_2$ receptors by propofol**
Concentration dependent enhancement of response to 3 μM GABA by propofol (0.5-20 μM). Each point is data from 6 oocytes.

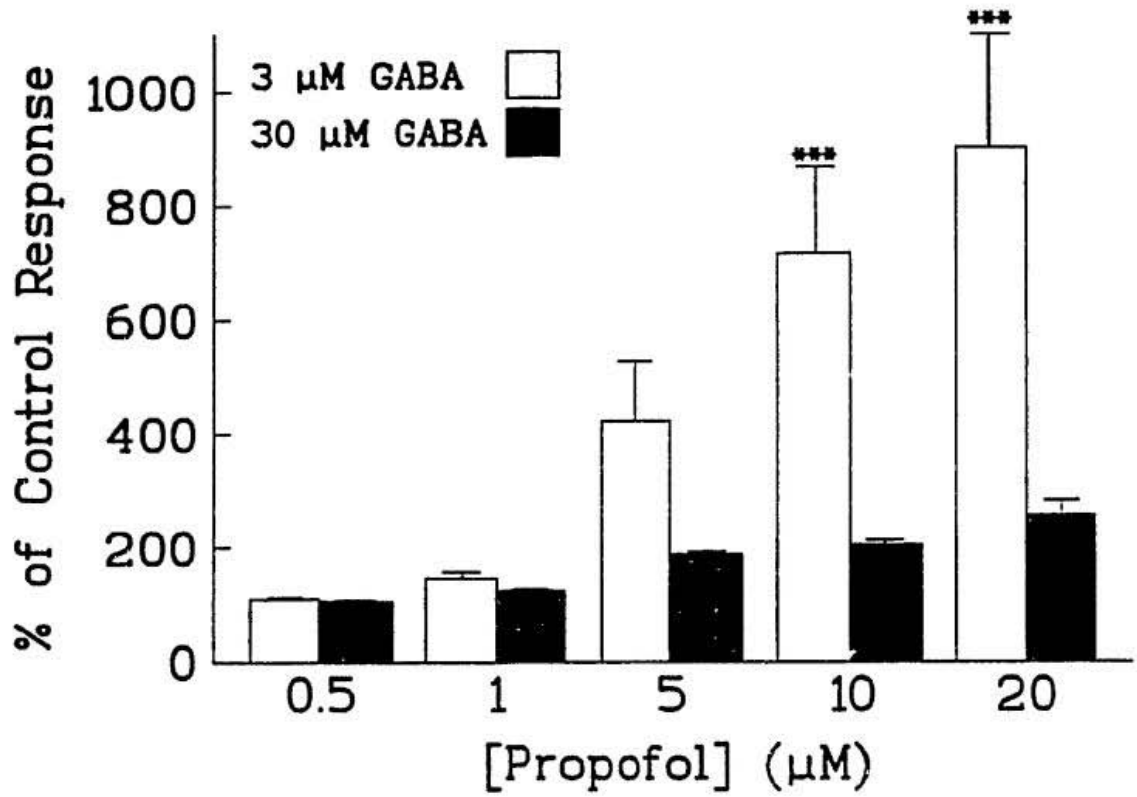


Figure 20. Effect of γ_{2L} subunit on propofol modulation of $GABA_A$ receptor function

Degree of propofol (0.5-20 μM) potentiation at equivalent (EC_{30}) concentrations of GABA on $\alpha_2\beta_2$ (3 μM GABA; open bars) and $\alpha_2\beta_2\gamma_{2L}$ (30 μM GABA; filled bars) receptors. Modulation of GABA response at $\alpha_2\beta_2$ receptors is significantly greater with 10 and 20 μM propofol than at the $\alpha_2\beta_2\gamma_{2L}$ receptor construct. Data from 6 oocytes. ***, $p<0.001$.

3.5 Effect of propofol on $GABA_A$ receptor desensitization

Application of higher concentrations of GABA to $GABA_A$ receptors results in the generation of a transient inward current which desensitizes rapidly and reaches a steady-state current amplitude. 250 μM GABA produced a fast inward current response at the $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptor where decay of the current during the desensitization phase can be fitted by a double exponential equation. Combined application of GABA and propofol did not change the characteristics of the transient current but caused a change in the rate of desensitization and steady-state current amplitude as well. Ratio of steady state to peak current amplitude showed a significant increase after adding 10 μM propofol with GABA (Fig.21). Absolute current amplitude for peak and steady state of the GABA response when compared in the presence and absence of propofol showed clearly that propofol caused a significant increase for both the components (Fig.22). Best fit of the double exponential showed that propofol induced no change in time constant for the first exponential phase, whereas the second phase was significantly increased (Fig.23).

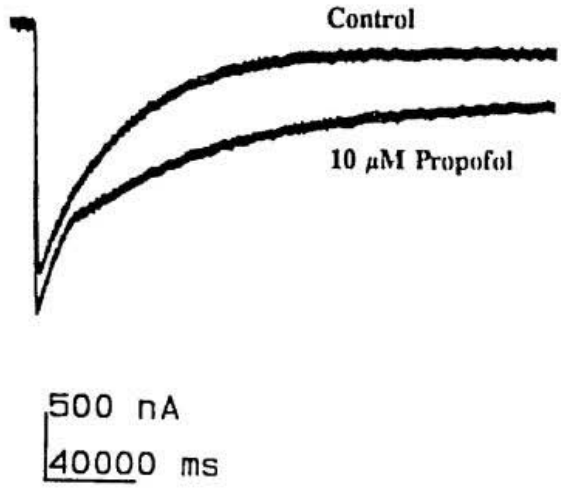
Figure 21. Change in steady state to peak current ratio after combining propofol with GABA

a) Response from an oocyte showing effect of 250 μM GABA on desensitization of the response with and without propofol at the $\alpha_1\beta_2\gamma_{2L}$ receptor construct.

b) Effect of 10 μM propofol on the steady state to peak current amplitude ratio produced by 10 μM GABA at the $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptor. Propofol caused a significance increase in the ratio by increasing the steady state current amplitude. Data from 5 oocytes. **, $p<0.0001$.

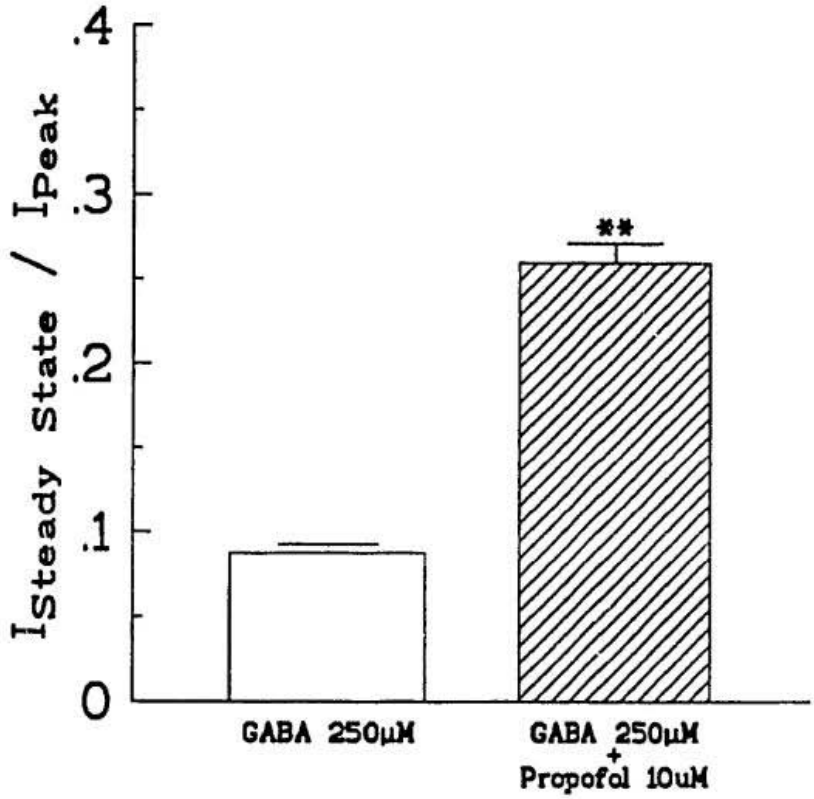
a

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



Control: 250 μM GABA

b



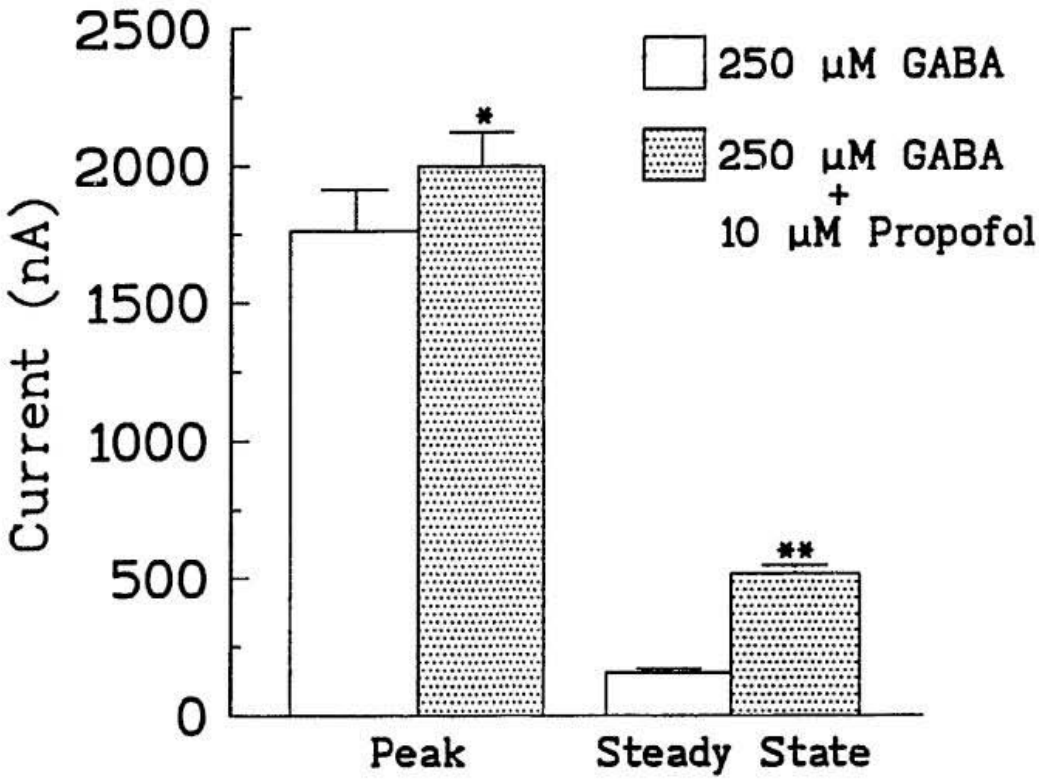


Figure 22. Effect of propofol on GABA-activated peak and steady state current

Application of 10 μM propofol caused significant increase in both peak as well as the steady state current caused by 250 μM GABA at human $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors. Data from 5 oocytes. *, $p<0.02$ and **, $p<0.0001$.

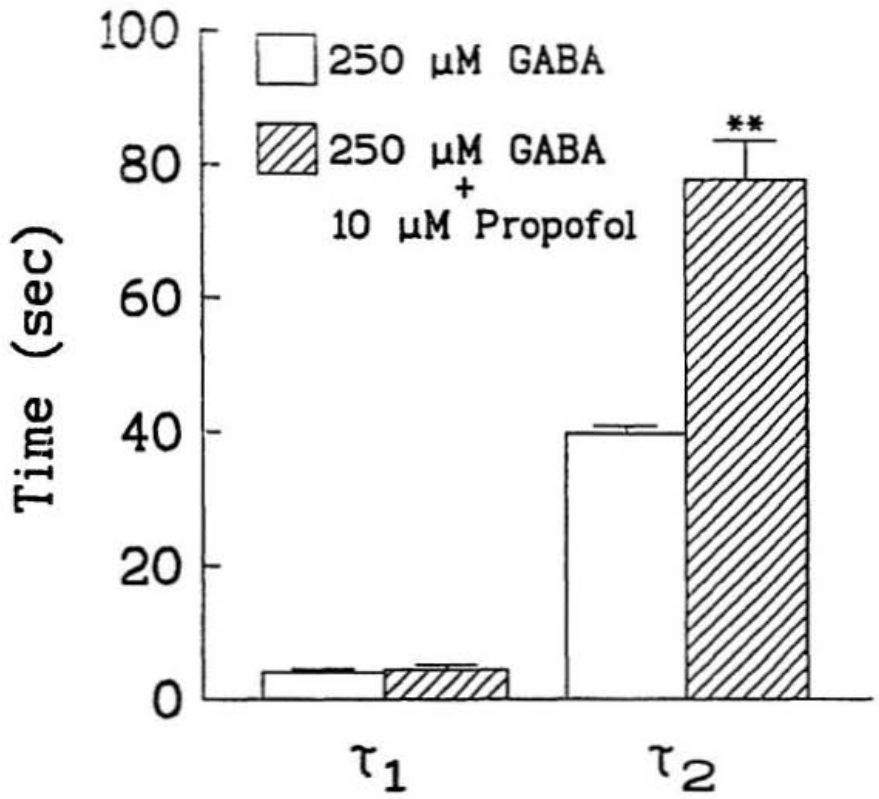


Figure 23. **Effect of propofol on rate of desensitization of GABA responses** Desensitization of GABA-activated current was fitted by a double exponential relationship. Addition of 10 μM propofol with 250 μM GABA did not change the first time constant (τ_1), whereas the second (τ_2) was longer. Data are from 5 oocytes. **, $p<0.003$).

4.0 Discussion

4.1 Characterization of recombinant $GABA_A$ receptors

Recombinant $GABA_A$ receptors expressed in different batches of oocytes coming from different frogs had the subunit combinations of $\alpha_1\beta_2\gamma_{2L}$, $\alpha_2\beta_2\gamma_{2L}$ and $\alpha_2\beta_2$. For all the experiments performed, a steady voltage clamp of oocytes at -70 mV was maintained. It is a widely observed phenomenon that GABA induces a concentration-dependent increase in the receptor response which ultimately reaches a maximum level. GABA at lower concentrations does not produce an apparent receptor desensitization, whereas this becomes quite prominent with increase in the applied GABA concentrations. GABA activated responses at the $\alpha_1\beta_2\gamma_{2L}$ and $\alpha_2\beta_2\gamma_{2L}$ receptor construct had an EC_{50} of 58 $\pm$ 9 μM and 67 $\pm$ 4 μM, respectively. Similar results have been obtained by other groups with $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors expressed in *Xenopus* oocytes where GABA had an EC_{50} of 46 $\pm$ 3.6 μM (1). Benzodiazepine potentiation of the $GABA_A$ receptor response is only observed at receptors having a γ subunit. Oocytes injected with the $\alpha_2\beta_2\gamma_{2L}$ receptor subunit messages were found to produce a concentration-dependent increase of the GABA response on application of flurazepam. Flurazepam potentiation of GABA-activated responses was found to vary with change in GABA concentration at receptors containing the γ_{2L} subunit. Benzodiazepine potentiation of GABA response was seen which verified the expression of the γ_{2L} subunit. The $\alpha_2\beta_2$ subtype of human $GABA_A$ receptor was expressed in oocytes and had an entirely different pharmacological profile compared to the receptors with three subunits. The EC_{50} for GABA at $\alpha_2\beta_2$ receptor construct was 8

$\pm$ 2 μM which was quite low compared to the other two types of receptor. This is similar in findings with other groups, where EC_{50} for GABA at receptors with α_1 and α_2 separately with the β subunit expressed in oocytes was 1.3 $\pm$ 0.2 and 12 $\pm$ 1 μM respectively (46). Moreover, flurazepam (10-100 μM) which potentiated GABA-activated responses on the γ_{2L} containing receptors, failed to facilitate the response at $\alpha_2\beta_2$ receptor. The distinct pharmacological profile of the receptors is consistent with expression of specific receptor subtypes in oocytes. All the recombinant $GABA_A$ receptors produced an inward current in response to GABA and the current-voltage relationship showed a reversal potential of -27 $\pm$ 2 mV, similar to values published by other researchers. The reversal potential for the GABA-evoked current at $\alpha_1\beta_2$ receptors is reported to be -20 mV under similar experimental conditions (11). The reversal potential shows that the currents evoked by GABA were inward chloride currents. Bicuculline is a competitive antagonist at GABA site of $GABA_A$ receptors and it was found to almost completely eliminate responses to GABA at $\alpha_1\beta_2\gamma_{2L}$ receptors. This provides additional evidence that the inward current was mediated through $GABA_A$ receptors.

4.2 Synergism between propofol and flurazepam on the GABA-activated receptor response

Propofol is widely known to act as a positive modulator at the $GABA_A$ receptor site (27,48,30,29,12). Propofol has been found to influence binding of different drugs to rat cortical membrane preparations. It increases binding of [^{3}H]-GABA, reduces

binding of [^{35}S]-TBPS and enhances GABA and muscimol stimulated Cl^- uptake (15,62). Propofol at pharmacologically relevant concentrations potentiate membrane currents activated by exogenous and synaptically released GABA (60). Propofol was found to robustly facilitate the GABA-evoked responses at both the $\alpha_1\beta_2\gamma_{2L}$ and $\alpha_2\beta_2\gamma_{2L}$ subtype of $GABA_A$ receptors. Modulation of response to 3 μM GABA by propofol at both receptor types produced similar EC_{50} values. Propofol enhancement of the GABA response reaches a maximum, which is dependent upon the concentration of GABA employed. Coapplication of propofol with 3 μM GABA produced an enhancement upto 8000% of the control GABA response, whereas the facilitation dropped to 949% when the concentration of GABA was changed to 10 μM, and less than 500% with 30 μM GABA. This is mainly due to the fact that propofol is unable to increase the maximum response that can be obtained by a saturating concentration of GABA at the receptors (Fig.8). With the increase in the concentration of GABA, the control response increases and as a result the degree of modulation by propofol decreases, because the maximum response level remains constant. Although the facilitation of control GABA-evoked current by propofol decreased with increase in GABA concentration, the EC_{50} for propofol at the $\alpha_1\beta_2\gamma_{2L}$ receptor was reduced, when applied with 10 μM GABA, compared to 3 μM GABA. This shows that GABA changes the affinity of propofol towards the $GABA_A$ receptors. Whether GABA changes the efficacy of propofol at the $GABA_A$ receptors, is difficult to predict as the data is insufficient to draw any conclusions.

The maximum response produced by GABA in presence of 10 μM propofol was

not significantly different from that produced by GABA alone (Fig.8). Propofol changed the affinity of $\alpha_2\beta_2\gamma_{2L}$ receptors for GABA but did not change the efficacy which is similar to results with $GABA_A$ receptors on dissociated hippocampal neurons, where 1 and 5 μM propofol lowered the EC_{50} for GABA (29). This probably shows that propofol also changes the affinity of GABA towards the receptors.

Propofol enhances [^{3}H]-flunitrazepam binding to rat brain membranes in a physiological salt solution with an EC_{50} of 23 μM (63). Intravenous administration of propofol produces a dose-dependent inhibition of rat pars reticulata neurons firing which was reversed by picrotoxin and bicuculline and was enhanced by diazepam. However, the benzodiazepine antagonist Ro 15-1788 had no influence on the effect of propofol (62). Moreover, propofol-induced current in acutely dissociated hippocampal neurons was facilitated by diazepam and not by pentobarbital (30). Propofol shows an additive effect on GABA and TBPS binding in rat cerebral cortex with diazepam (13). In clinical studies, propofol and midazolam were found to act in synergy for the induction of anesthesia (72,10). Temazepam also significantly increased the time for cognitive recovery in patients anesthetized with propofol and alfentanil (4).

In the present study, low concentrations of propofol (0.5-10 μM) when coapplied with 0.5 μM flurazepam were found to synergistically potentiate responses to 3 μM GABA at both $\alpha_1\beta_2\gamma_{2L}$ and $\alpha_2\beta_2\gamma_{2L}$ receptors. At the highest concentration (20 μM) of propofol tested, the potentiation of GABA receptor-activated membrane current (I_{GABA}) by the combination was not different than the theoretical additive response. At $\alpha_2\beta_2\gamma_{2L}$ receptor flurazepam produced a significant decrease in the EC_{50} concentration for

propofol without increasing the maximal potentiation of I_{GABA} obtained with propofol. Thus, it appears that flurazepam (and presumably other benzodiazepine receptor agonists), in addition to facilitating I_{GABA} on its own, also increases the affinity of the receptor for propofol, resulting in a greater than expected potentiation of I_{GABA} by lower concentration of propofol. As the concentration of propofol approaches the maximum for potentiation of I_{GABA}, any increase in affinity induced by flurazepam becomes negligible. This idea is further supported by the results obtained with different concentrations of GABA. A greater than additive interaction between propofol and flurazepam was very pronounced when 3 μM GABA was used. However, at 30 μM GABA the difference between the theoretical additive response and the experimental response became smaller and more variable. It is expected that higher concentrations of GABA (50-100 μM), which approach the maximal current amplitude, would also prevent any synergistic interaction between propofol and flurazepam to potentiate I_{GABA}.

The lack of synergy between propofol and flurazepam with higher flurazepam (1 μM) concentration is more difficult to explain. However, it is possible that high concentrations of benzodiazepines might interact with propofol in presence of GABA to produce a less than additive response for both the drugs. Further experiments using higher concentrations of flurazepam (5-20 μM) will be necessary to test the hypothesis that the interaction between propofol and flurazepam is biphasic.

4.3 Zopiclone negatively interacts with propofol on $GABA_A$ receptor-activated response

Zopiclone, classified as a full agonist at the benzodiazepine site, is a potent allosteric modulator of $GABA_A$ receptor function and is known to have hypnotic and anxiolytic properties similar to benzodiazepines. Zopiclone produced a dose-dependent facilitation of GABA response which reached a maximal level comparable to the effect produced by maximally effective flurazepam concentration. Like diazepam, zopiclone also stimulates [^{35}S]-TBPS binding to rat cortical membranes (16) and the extent of the TBPS binding shift gives a measure of efficacy, suggesting zopiclone as a full agonist. Cyclopyrrolones have also been found to inhibit binding of [^{3}H]-benzodiazepines to brain membranes (7). Study of the kinetics of interaction between [^{3}H]-flunitrazepam and [^{3}H]-suriclone with the $GABA_A$ receptor have proposed that benzodiazepines and cyclopyrrolones bind to two distinct, but allosterically coupled sites on the $GABA_A$ receptor (87). It was also suggested that the conformational state of the $GABA_A$ receptor on benzodiazepine binding is different from that of cyclopyrrolone binding. Benzodiazepine binding decreases with increasing temperature (8,87), whereas the binding of cyclopyrrolones remains unaffected. This implies that the former is predominantly enthalpically driven while the latter is driven entropically (17,64). In rat brain membranes photolabelling with flunitrazepam produced inactivation of the benzodiazepine binding site, whereas binding for cyclopyrrolone [^{3}H]-suriclone remained unaffected (16). Irreversible loss of binding sites for benzodiazepine on rat brain membranes was produced by diethylpyrocarbonate (DEPC) treatment but the same

procedure was unable to modify suriclone (a cyclopyrrolone derivative) binding. In the present study, propofol produced an effect in combination with zopiclone which was significantly lower than the additive response This might be due to some overlap and/or interaction between the binding sites of propofol and zopiclone on $GABA_A$ receptors. Alternatively, there may be a negative allosteric interaction between zopiclone and propofol which results in a less than additive response. In either case, these data support the notion that benzodiazepines and cyclopyrrolones modulate $GABA_A$ receptor function via different mechanisms.

4.4 Role of γ_{2L} in propofol potentiation of GABA response

With equivalent concentrations of GABA, enflurane produces significantly greater responses at $\alpha_1\beta_1$ receptors than at $\alpha_1\beta_1\gamma_{2L}$ receptors expressed in *Xenopus* oocytes (47). Propofol similarly produces potentiation of equivalent GABA-activated responses on both $\alpha_2\beta_2$ and $\alpha_2\beta_2\gamma_{2L}$ subtype of human $GABA_A$ receptors. Significant enhancement with 10 and 20 μM propofol at the $\alpha_2\beta_2$ receptor over the $\alpha_2\beta_2\gamma_{2L}$ receptor suggests propofol potentiation of GABA response is not dependent upon the presence of the γ_{2L} subunit. Moreover, it seems that absence of the γ_{2L} subunit helps in more efficient modulation of $GABA_A$ receptor activated response with pharmacologically relevant concentrations of propofol. It is possible that there are higher affinity binding sites for propofol on receptors composed of $\alpha\beta$ compared to $\alpha\beta\gamma_2$ subunits, or $\alpha\beta$ receptor combination might allow greater allosteric modulation between propofol and GABA binding sites. Further experiments will be needed to clarify this point.

4.5 Propofol and benzodiazepine act at different sites on $GABA_A$ receptors

In rat cortical membranes propofol failed to displace flunitrazepam from its binding site indicating the binding site for propofol is different than that for benzodiazepines (15,62). Coapplication of propofol with either alphaxalone or pentobarbital on rat cerebral cortex membranes produced an additive inhibition of TBPS binding, thus providing evidence for separate binding sites of these drugs (13,15). Propofol potentiated I_{GABA} on bovine chromaffin cells and rat cortical neurons and the enhancements were not influenced by benzodiazepine antagonist flumazenil. This is highly suggestive of separate sites of action for propofol and benzodiazepines on $GABA_A$ receptors (27). Ro 15-4513, a partial inverse agonist at the benzodiazepine site, completely eliminates the flurazepam facilitation of the GABA response but has no influence on the modulatory effect of propofol. Combined application of GABA, propofol and flurazepam in presence of Ro 15-4513 totally abolishes the flurazepam component of the response and produces only the propofol enhancement of the $GABA_A$ receptor-activated response. This particular phenomenon is only possible if the sites of action for propofol and benzodiazepine on $GABA_A$ receptor are different. That propofol and benzodiazepines do not share a common binding site is again obvious from the fact that latter has no action on $\alpha\beta$ receptors, whereas, propofol positively modulates the response to GABA, and indeed was more effective as a modulator of GABA responses in the absence of the γ_2 subunit. Other researchers (70) have suggested the importance of the β subunit for the effect of propofol on the $GABA_A$ receptor. They have shown that direct activation of homomeric β_1 GABA receptors with propofol and pentobarbital but not with

the steroid anesthetic alphaxalone. Again, the direct activation of the Cl^- current is more pronounced at β_1 receptors compared to receptors containing α_1 and/or γ_{2S} along with the β_1 subunit. Benzodiazepine pharmacology at $GABA_A$ receptors appears to be influenced primarily by α and γ subunits, whereas the β subunit is not known to have much of effect. The β subunit seems to be important for at least one effect of propofol, the direct activation of the $GABA_A$ receptor chloride channel. A great deal of work remains to be done in order to clarify the role of individual subunits in propofol modulation of $GABA_A$ receptor function.

4.6 Propofol decreases $GABA_A$ receptor desensitization

Inward currents activated by higher concentrations of GABA rapidly reached a peak response followed by a decline to a steady-state level. The time course of receptor mediated current decay can be best described by two exponentials. Addition of propofol with GABA increased peak current amplitude, but had a much more dramatic influence on the steady-state current. Propofol coapplied with GABA did not alter the early, fast time constant of current inactivation (Fig.23; 60), but significantly prolonged the slow time constant of decay. At higher GABA concentrations, propofol did not alter the peak inward current response. It is possible that the peak GABA response after application of high GABA concentration is underestimated due to the desensitization occuring during the onset of the response. Co-application of propofol and GABA has been reported to increase the frequency of $GABA_A$ receptor channel opening (60) which is possible if propofol interacts with the GABA recognition site to allosterically modulate the receptor-

channel complex. Propofol has also been found to increase the steady-state current amplitude of I_{GABA} (60). This suggests that propofol may shift the equilibrium of the receptor population from desensitized non-desensitized states. This finally enhances receptor mediated inward current response during the steady-state.

At synapses, following an action potential the synaptically released GABA is present in a saturating concentration. The concentration then decreases due to reuptake of GABA into the presynaptic nerve terminal and metabolism by GABA-T. Propofol changes the time course for current decay of synaptically evoked GABA responses (60), but is probably not capable of changing the maximum response to GABA. Potentiation of GABA evoked responses can be seen with propofol, but the magnitude is inversely proportional to the concentration of GABA employed. In synaptic events, during the decay phase of the GABA response, the effect of propofol would be expected to increase as the concentration of GABA steadily declines. Propofol would also reduce the rate of decay of the GABA-evoked response, thus increasing the decay time of the inhibitory current. An apparent increase in the decay time of the receptor response could also occur if extrasynaptic receptors, not normally activated by synaptically released GABA because of the limits of diffusion, become activated in the presence of propofol. Propofol when coapplied with GABA could increase the response at extrasynaptic receptors, possible by direct activation of the receptors or by increasing the response to subsaturating concentration of GABA which reaches those receptors. Alternatively, there may be a subset of "silent" receptors which are not activated even in the presence of high concentration of GABA. These receptors may be activated by propofol which would

result in an increased current amplitude during the steady-state response. If this is true, then the peak current would be expected to increase during the combined application of the drugs, but synaptic studies suggest that the peak amplitude remains the same while the decay time and current increases. The former argument is thus favoured to explain this particular effect of propofol and hence the increase in decay current is thus probably due to reduced desensitization or involvement of extrasynaptic receptors. These two effects of propofol would be expected to significantly enhance the inhibitory effect of GABA at the synapses. Although the specific action of propofol at synapses is not known, the ultimate outcome is the prolongation of the inhibitory synaptic events.

4.7 Conclusions

Propofol and flurazepam, which have independent effects at $GABA_A$ receptors, were found to produce a greater than additive potentiation of $GABA_A$ receptor function. This effect could be attributed to a flurazepam-induced increase in the affinity of $GABA_A$ receptors for propofol. The combination of propofol with zopiclone resulted in a less than additive potentiation of GABA receptor-activated membrane current, suggesting that benzodiazepines and cyclopyrrolones have distinct sites and/or mechanisms to modulate $GABA_A$ receptor function. Propofol and benzodiazepines do not act at a common site on the $GABA_A$ receptor since RO 15-4513, which completely blocks the effect of flurazepam, did not alter the potentiating effect of propofol. Both the efficacy and affinity for propofol at the $GABA_A$ receptors changed depending upon the presence and absence of the γ_{2L} subunit along with the α and β subunits. However, no significant change in

the effect of propofol was found with the change in the α subunit isoform of the $GABA_A$ receptor. Propofol also was found to decrease $GABA_A$ receptor desensitization, which could be due to changes in desensitization kinetics.

4.8 Future directions

The nature of interaction between propofol and benzodiazepine site ligands has been examined and it was found that a synergistic interaction between propofol and flurazepam can be seen at the $GABA_A$ receptor level. Further work needs to be done with other benzodiazepines, most notably midazolam which is used clinically, to determine if this type of synergistic interaction is true between propofol and other benzodiazepines.

Zopiclone, which is chemically unrelated to the benzodiazepines, produces a negative interaction at the $GABA_A$ receptor when combined with propofol. Though equivalent degrees of potentiation were obtained with different concentrations of zopiclone and flurazepam, these drugs produced entirely different responses when combined with propofol at the $GABA_A$ receptor. To ensure that there is true negative allosteric interaction between the sites of action of zopiclone and benzodiazepine, experiments must be done with both lower (50 nM) and higher (500 nM) concentrations of zopiclone. There might be a possibility of finding some different kind of interaction between propofol and lower concentration of zopiclone. This is suggested because of the loss of synergism between propofol and a higher concentration of flurazepam (1 μM). Concentrations of zopiclone used for the present study are equivalent to the EC_{50} value and so it might be a good idea to establish the fact of negative interaction with both

lower and higher concentrations of the drug.

To find out if there is any physico-chemical interaction between propofol and benzodiazepines which produces the observed synergism, the effect of combining the two drugs can be tested on the $\alpha\beta$ subtype of $GABA_A$ receptor. This will be the appropriate receptor combination to test this idea as benzodiazepines are known to have no effect on $\alpha\beta$ receptors, whereas propofol modulates the GABA response on this receptor.

Subunit dependence of propofol has been studied by changing the α subunit of $GABA_A$ receptor and the action of propofol alone or in combination with other drugs seemed to be independent of the α subunit isoforms. The presence of different α subunit isoforms are known to impart different benzodiazepine pharmacology to the $GABA_A$ receptor. Though there is no major difference between α_1 and α_2 subunits on the ability of propofol to modulate GABA currents, additional α subunit isoforms can be looked at, in combination with the β and γ subunits, to find out the effect of this subunit, for certain, on propofols action.

Presently, reports indicate that the action of propofol is dependent on the β subunit. To test contribution of the β subunit, expression of $\alpha\gamma_2$ subtype is necessary to compare the effect of propofol with both $\alpha\beta$ and $\alpha\beta\gamma_2$ $GABA_A$ receptor subtypes. Change in the β subunit isoform is needed to find its effect on the propofol modulation of GABA-activated receptor response. If potency, affinity or efficacy varies with the change or elimination of the β subunit, then it can be suggested that the binding of propofol is dependent upon the presence of the β subunit isoform. The β subunit might be particularly important for reduced desensitization of $GABA_A$ receptors induced by

propofol. To find out the importance of β subunit in desensitization, experiments should be designed to test the desensitization kinetics of GABA response with propofol on both $\alpha\gamma_2$ and $\alpha\beta\gamma_2$ receptors having different β subunit isoforms. This will give an idea regarding the specific β subunit involved, if at all, in the binding of propofol to the receptor and influencing desensitization.

BIBLIOGRAPHY

1. Amin, J., & Weiss, D.S. (1993), $GABA_A$ receptor needs two homologous domains of β-subunit for activation of GABA but not by pentobarbital, *Nature*, **366**, 565-569.

2. Araki, T., & Tohyama, M. (1992), Region-specific expression of $GABA_A$ receptor $\alpha 3$ and $\alpha 4$ subunits mRNAs in the rat brain, *Brain Res. Mol. Brain Res.*, **12 (4)**, 293-314.

3. Backus, K.H., Arigoni, M., Drescher, U., Scheurer, L., Malherbe, P., Mohler, H., & Benson, J.A. (1993), Stoichiometry of a recombinant $GABA_A$ receptor deduced from mutation-induced rectification, *Molecular Neuroscience*, **5**, 285-288.

4. Bailie, R., Christmas, L., Price, N., Restall, J., Simpson, P., & Wesnes, K. (1989), Effects of temazepam premedication on cognitive recovery following alfentanil-propofol anaesthesia, *Br. J. Anaesth.*, **63**, 68-75.

5. Bateson, A.N., Lasham, A., & Darlison, M.G. (1991), γ-Aminobutyric $Acid_A$ receptor heterogeneity is increased by alternative splicing of a novel β-subunit gene transcript, *J. Neurochem.*, **56**, 1437-1440.

6. Blair, L.A.C., Letivan, E.S., Marshall, J., Dionne, V.E., and Barnard, E.A. (1988), Single subunits of the $GABA_A$ receptor form ion channels with properties of the native receptor, *Science*, **242**, 577-579.

7. Blanchard, J.C., Boireau, A., Garret, C. et al. (1979), In vitro and in vivo inhibition by zopiclone of benzodiazepine binding to rodent brain receptors, *Life Sci.*, **24**, 2417-2420.

8. Blanchard, J.C., Zundel, Z.L., & Julou, L. (1983), Difference between cyclopyrrolones (suriclone and zopiclone) and benzodiazepine binding to rat hippocampus photolabelled membranes, *Biochem. Pharmacol.*, **32**, 3651-3653.

9. Burt, D.R. (1994), $GABA_A$ receptor activated chloride channels. *Chloride channels: from plants to human disease. Current topics in membranes and transport* (W.G. Guggino, ed.), Academic Press, New York.

10. Caba, F., Echevarria, M., Morales, R., Guerrero, J.A., & Rodriguez, R. (1993), Synergism of midazolam and propofol in the induction of anesthesia, *Rev. Esp. Anestesiol. Reanim.*, **40 (2)**, 69-71.

11. Cestari, I.N., Albuquerque, E.X., & Burt, D.R. (1993), A PCR shortcut to oocyte expression, *Biotechniques*, **14 (3)**, 404-407.

12. Collins, G.G.S. (1988), Effects of the anesthetic 2,6-diisopropylphenol on synaptic transmission in the rat olfactory cortex slice, *Br. J. Pharmacol.*, **95**, 939-949.

13. Concas, A., Santoro, G., Serra, M., Sanna, E., & Biggio, G. (1991), Neurochemical action of the general anesthetic propofol on the chloride ion channel coupled with $GABA_A$ receptors, *Brain Research*, **542**, 225-232.

14. Concas, A., Santoro, G., Mascia, M.P., Serra, M., Sanna, E., & Biggio, G. (1990), The general anesthetic propofol enhances the function of γ-aminobutyric acid-coupled chloride channel in the rat cerebral cortex, *J. Neurochem.*, **55**, 2135-2138.

15. Cooper J.R., Bloom, F.E., & Roth R.H. (1986), Amino-acid transmitters, *The biochemical basis of neuropharmacology*, **5th edition**, Oxford University Press, 124-172.

16. Doble, A., Canton, T., Piot, O., Zundel, J.L., Stutzmann, J.M., Cotrel, C., & Blanchard, J.C. (1992), The pharmacology of cyclopyrrolone derivatives acting at the $GABA_A$/benzodiazepine receptor, *GABAergic Synaptic Transmission* (edited by G. Biggio, A. Concas & E. Costa), Raven Press, New York.

17. Doble, A. (1983), Comparative thermodynamics of benzodiazepine receptor ligand interactions in rat neuronal membranes, *J. Neurochem.*, **45**, 240-243.

18. Ducic, I., Caruncho, H.J., Zhu, W.J., Vicini, S., & Costa, E. (1995), γ-aminobutyric acid gating of Cl^- channels in recombinant $GABA_A$ receptors, *J. Pharmacol. Exp. Ther.*, **272 (1)**, 438-445.

19. Ebert, B., Wafford, K.A., Whiting, P.J., Krogsgaard, L.P., & Kemp, J.A. (1994), Molecular pharmacology of γ-aminobutyric acid type A receptor agonists and partial agonists in oocytes injected with different α, β and γ receptor subunit combinations, *Mol. Pharmacol.*, **46 (5)**, 957-963.

20. Greenblatt, D.J., Miller, M.G., & Shader, R.I. (1990), Neurochemical and pharmacokinetic correlates of the clinical action of benzodiazepine hypnotic drugs, *The American Journal of Medicine*, **88 (Suppl 3A)**, 18S-24S.

21. Gunderson, C.B., Miledi, R., & Parker, I. (1983), Voltage-operated channels induced by foreign messenger RNA in *xenopus* oocytes, *Proc. R. Soc. London*, **220**, 131-140.

22. Gutierrez, A., Khan, Z.U., & De-Blas, A.L. (1994), Immunocytochemical localization of γ2 short and γ2 long subunits of the $GABA_A$ receptor in the rat brain, *J. Neurosci.*, **14 (11 Pt 2)**, 7168-7179.

23. Hadingham, K.L., Harkness, P.C., McKernan, R.M., Quirk, K., Le-Bourdelles, B., Horne, A.L., Kemp, J.A., Barnard, E.A., Ragan, C.I., & Whiting, P.J. (1992), Stable expression of mammalian type A γ-aminobutyric acid receptors in mouse cells: demonstration of functional assembly of benzodiazepine-responsive sites, *Proc. Natl. Acad. Sci. USA*, **89 (14),** 6378-6382.

24. Hadingham, K.L., Wingrove, P.B., Wafford, K.A., Bain, C., Kemp. J.A., Palmer, K.J., Wilson, A.W., Sikela, J.M., Ragan, C.I., & Whiting, P.J. (1993), Role of the β subunit in determining the pharmacology of human γ-aminobutyric acid type A receptors, The American Society for Pharmacology and Experimental Therapeutics, *Molecular Pharmacology*, **44,** 1211-1218.

25. Haefely, W., Kyburz, E., Gerecke, M., & Mohler, H. (1985), Recent advances in the molecular pharmacology of benzodiazepine receptors and in the structure-activity relationships of their agonists and antagonists, *Adv. Drug Res.*, **14,** 165-322.

26. Hales, T.G., & Lambert, J.J. (1992), The actions of propofol on inhibitory amino acid receptors of bovine adrenomedullary chromaffin cells and rodent central neurones, *Br. J. Pharmacol.*, **104,** 621-628.

27. Hales, T.G., & Lambert J.J. (1991), The actions of propofol on inhibitory amino acid receptors of bovine adrenomedullary chromaffin cells and rodent central neurones, *Br. J. Pharmacol.*, **104,** 619-628.

28. Hara, M., Yoshihisa, K., & Ikemoto, Y. (1993), Propofol activates $GABA_A$ receptor - chloride ionophore complex in dissociated hippocampal pyramidal neurons of the rat, *Anesthesiology*, **79,** 781-788.

29. Hara, M., Kai, Y., & Ikemoto, Y. (1994), Enhancement by propofol of the γ-Aminobutyric $Acid_A$ response in dissociated hippocampal neurons of the rat, *Anesthesiology*, **81,** 988-994.

30. Hara, M., Kai, Y., & Ikemoto, Y. (1993), Propofol activates $GABA_A$ receptor-chloride ionophore complex in dissociated hippocampal pyramidal neurons of the rat, *Anesthesiology*, **79,** 781-788.

31. Harris, B.D., Wong, G., Moody, E.J., & Skolnick, P. (1995), Different subunit requirements for volatile and nonvolatile anesthetics at γ-aminobutyric acid type A receptors, *Molecular Pharmacology*, **47,** 363-367.

32. Hastings, G.A., Buller, A.L., Kirkness, E.F., & Fraser, C.M. (1992), Mutagenesis of N-linked glycosylation sites of the $GABA_A$/Benzodiazepine receptor α1 subunit decreases the level of RO 15-1788 binding in transfected 293 cells, *Soc. Neurosci. Abstr.*, **18,** 262.

33. Horne, A.L., Harkness, P.C., Hadingham, K.L., Whiting, P., & Kemp, J.A. (1993), The influence of the γ2L subunit on the modulation of responses to $GABA_A$ receptor activation, *Br. J. Pharmacol*, **108**, 711-716.

34. Houamed, K.M., et al. (1984), Expression of functional GABA, glycine and glutamate receptors in *xenopus* oocytes injected with rat brain mRNA, *Nature*, **310**, 318-321.

35. Im, H.K., Im, W.B., Hamilton, B.J., Carter, D.B., & Vonvoigtlander P.F. (1993), Potentiation of γ-aminobutyric acid-induced chloride currents by various benzodiazepine site agonists with the α1γ2, β2γ2 and α1β2γ2 subtypes of cloned γ-aminobutyric acid type A receptors, *Molecular Pharmacology*, **44**, 866-870.

36. James, R., & Glen, J.B. (1980), Synthesis, biological evaluation, and preliminary structure-activity considerations of a series of alkylphenols as intravenous anesthetic agents, *J. Med. Chem.*, **23**, 1350-1357.

37. Kanto, J., & Gepts, E. (1989), Pharmacokinetic implications for the clinical use of propofol, *Clin. Pharmacokinet.*, **17 (5)**, 308-326.

38. Kellenberger, S., Malherbe, P., & Sigel, E. (1992), Function of the α1β2γ2S γ-aminobutyric acid type A receptor is modulated by protein kinase C via multiple phosphorylation sites, *The Journal of Biological Chemistry*, **267 (36)**, 25660-25663.

39. Kofuji, P., Wang, J.B., Moss, S.J., Huganir, R.L., & Burt, D.R. (1991), Generation of two forms of the γ-Aminobutyric $Acid_A$ receptor γ2-subunit in mice by alternative splicing, *J. Neurochem.*, **56**, 713-715.

40. Korpi, E.R., & Seeburg, P.H. (1993), Natural mutation of $GABA_A$ receptor α_6 subunit alters benzodiazepine affinity but not allosteric GABA effects, *European J. Pharmacol.*, **247**, 23-27.

41. Korttila, K., Nuotto, E.J., Lichtor, J.L., Ostman, P.L., Apfelbaum, J., & Rupani, G. (1992), Clinical recovery and psychomotor function after brief anesthesia with propofol or thiopental, *Anesthesiology*, **76**, 676-681.

42. Krishek, B.J., Xie, X., Blackstone, C., Huganir, R.L., Moss, S.J., & Smart, T.G. (1994), Regulation of $GABA_A$ receptor function by protein kinase C phosphorylation, *Neuron*, **12**, 1081-1095.

43. Krogsgaard, L.P., Frolund, B., Jorgensen, F.S., & Schousboe, A. (1994), $GABA_A$ receptor agonists, partial agonists, and antagonists. Design and therapeutic prospects, *J. Med. Chem.*, **37 (16)**, 2489-2505.

44. Leidenheimer, N.J., McQuilkin, S.J., Hahner, L.D., Whiting, P., & Harris, R.A. (1992), Activation of protein kinase C selectively inhibits the γ-Aminobutyric $Acid_A$ receptor: role of desensitization, *Molecular Pharmacology*, **41,** 1116-1123.

45. Leidenheimer, N.J., Whiting, P.J., & Harris R.A. (1993), Activation of calcium-phospholipid-dependent protein kinase enhances benzodiazepine and barbiturate potentiation of the $GABA_A$ receptor, *J. Neurochem.*, **60,** 1972-1975. [42]

46. Levitan, E.S., Schofield. P.R., Burt, D.R., Rhee, L.M., Wisden, W., Kohler, M., Fujita, N., Rodriguez, H.F., Stephenson, A , Darlison, M.G., Barnard, E.A., & Seeburg, P.H. (1988), Structural and functional basis of $GABA_A$ receptor heterogeneity, *Nature*, **335,** 76-79.

47. Lin, L., Whiting, P., & Harris, R.A. (1993), Molecular determinants of general anesthetic action: Role of $GABA_A$ receptor structure, *J. Neurochem.*, **60,** 1548-1553.

48. Lin, L., Chen, L.L., Zirrolli, J.A., & Harris, R.A. (1992), General anesthetics potentiate γ-aminobutyric acid actions on γ-Aminobutyric $Acid_A$ receptors expressed by *xenopus* oocytes: lack of involvement of intracellular calcium, *The Journal of Pharmacology and Experimental Therapeutics*, **263 (2),** 569-578.

49. Luddens, H., Prichett, D.B., Kohler, M., Killisch, I., Keinanen, K., Monyer, H., Sprengel, R., & Seeburg P.H. (1990), Cerebellar $GABA_A$ receptor selective for a behavioural alcohol antagonist, *Nature*, **346,** 648-651.

50. Luddens, H., Killisch, I., & Seeburg, P.H. (1991), More than one α variant may exist in a $GABA_A$/benzodiazepine receptor complex, *J. Recept. Res.*, **11 (1-4),** 535-551.

51. Macdonald, R.L., & Olsen, R.W. (1994), $GABA_A$ receptor channels, *Annu. Rev. Neurosci.*, **17,** 569-602.

52. Machu, T.K., Olsen, R.W., & Browning, M.D. (1993), Immunochemical characterization of the β2 subunit of the $GABA_A$ receptor, *J. Neurochem.*, **61 (6),** 2034-2040.

53. Mihic, S.J., Whiting, P.J., Klein, R.L., Wafford, K.A., & Harris, R.A. (1994), A single amino acid of the human γ-aminobutyric acid type A receptor γ2 subunit determines benzodiazepine efficacy, *J. Biol. Chem.*, **269 (52),** 32768-32773.

54. Miralles, C.P., Gutierrez, A., Khan, Z.U., Victoria, J., & De-Blas, A.L. (1994), Differential expression of the short and long forms of the γ2 subunit of the $GABA_A$/benzodiazepine receptors, *Brain Res. Mol. Brain Res.*, **24 (1-4),** 129-139.

55. Moffett, J.R., Namboodiri, A., & Neale, J.H. (1989), Two forms of the $GABA_A$ receptor distinguished by anion-exchange chromatography, *FEBS Lett.*, **247**, 81-85.

56. Mohler, H., Malherbe, P., & Draguhn, A. (1989), $GABA_A$-receptors expressed from rat α- and β-subunits in *xenopus* oocytes are modulated by benzodiazepine receptor ligands, *Soc. Neurosci. Abstr.*, **15**, 997.

57. Moss, S.J., Smart, T.G., Blackstone, C.D., Huganir, R.L. (1992), Functional modulation of $GABA_A$ receptors by cAMP-dependent protein phosphorylation, *Science*, **257**, 661-665.

58. Olson, T.S., & Lane, M.D. (1989), A common mechanism for post-translational activation of plasma membrane receptors, *FASEB J.*, **3**, 1618-1624.

59. Nayeem, N., Green, T.P., Martin, I.L., & Barnard, E.A. (1994), Quarternary structure of the native $GABA_A$ receptor determined by electron microscopic image analysis, *J. Neurochem.*, **62 (2)**, 815-818.

60. Orser, B.A., Wang, L., Pennefather, P.S., & MacDonald, J.F. (1994), Propofol modulates activation and desensitization of $GABA_A$ receptors in cultured murine hippocampal neurons, *The Journal of Neuroscience*, **14 (12)**, 7747-7760.

61. Ortells, M.O., & Lunt, G.G. (1995), Evolutionary history of the ligand-gated ion-channel superfamily of receptors, *Trends Neurosci.*, **18**, 121-127.

62. Peduto, V.A., Concas, A., Santoro, G., Biggio, G., Gessa, G.L. (1991), Biochemical and electrophysiologic evidence that propofol enhances GABAergic transmission in the rat brain, *Anesthesiology*, **75**, 1000-1009.

63. Prince, R.J., & Simmonds, M.A. (1992), Propofol potentiates the binding of [^{3}H]flunitrazepam to the $GABA_A$ receptor complex, *Brain Research*, **596**, 238- 242.

64. Prince, R.J., & Simmonds, M.A. (1992), Temperature and anion dependence of allosteric interactions at the γ-aminobutyric acid-benzodiazepine receptor, *Biochemical Pharmacology*, **44 (7)**, 1297-1302.

65. Pritchett, D.B., & Seeburg, P.H. (1990), γ-Aminobutyric $Acid_A$ receptor α5-subunit creates novel type II benzodiazepine receptor pharmacology, *J. Neurochem.*, **54**, 1802-01804.

66. Pritchett, D.B., Luddens, H., & Seeburg, P. H. (1989), Type I and type II $GABA_A$-Benzodiazepine receptor produced in transfected cells, *Science*, **245**, 1389-1392.

67. Pritchett, D.B., Sontheimer, H., Shivers, B.D., Ymer, S., Kettenmann, H., Schofield,

P.R., & Seeburg, P.H. (1989), Importance of a novel $GABA_A$ receptor subunit for benzodiazepine pharmacology, *Nature*, **338,** 582-585.

68. Quirk, K., Gillard, N.P., ragan. C.I., Whiting, P.J., & McKernan, R.M. (1994), Model of subunit composition of γ-aminobutyric acid A receptor subtypes expressed in rat cerebellum with respect to their α and γ/δ subunits, *The Journal of Biological Chemistry*, **269 (23),** 16020-16028.

69. Roth, T., & Roehrs, T.A. (1991), A review of the safety profiles of benzodiazepine hypnotics, *J. Clin. Psychiatry*, **52 (Suppl 9),** 38-41.

70. Sanna, E., Garau, F., & Harris, R.A. (1995), Novel properties of homomeric $\beta 1$ γ-aminobutyric acid type A receptors: actions of the anesthetics propofol and pentobarbital, *Molecular Pharmacology*, **47,** 213-217

71. Sequier, J.M., Richards, J.G., Malherbe, P., Price. G.W., Mathews, S., & Mohler, H. (1988), Mapping the brain areas containing RNA homologous to cDNAs encoding the α and β subunits of the rat $GABA_A$ γ-aminobutyrate receptor, *Proc. Natl. Acad. Sci. USA*, **85,** 7815-7819.

72. Short, T.G., & Chui, P.T. (1991), Propofol and midazolam act synergistically in combination, *Br. J. Anaesthesia*, **67,** 539-545.

73. Shyr, M., Tsai, T., Tan, P.P.C., Chen, C., & Chan, S.H.H. (1995), Concentration and regional distribution of propofol in brain and spinal cord during propofol anesthesia in the rat, *Neuroscience Letters*, **184,** 212-215.

74. Sieghart, W. (1992), $GABA_A$ receptors: ligand-gated Cl^- ion channels modulated by multiple drug-binding sites, *TiPS*, **13,** 446-450.

75. Sigel, E., Baur, R., Trube, G., Mohler, H., & Malherbe, P. (1990), The effect of subunit composition of rat brain $GABA_A$ receptors on channel function, *Neuron*, **5,** 703-711.

76. Simmonds, M.A., & Turner, J.P. (1987), Potentiators of responses to activation of γ-aminobutyric acid ($GABA_A$) receptors, *Neuropharmacology*, **26,** 923-930.

77. Sivilotti, L., & Nistri, A. (1991), Gaba receptor mechanisms in the central nervous system, *Progress in Neurobiology*, **36,** 35-92.

78. Smart, T.G., Houamed, K.M., Van Renterghem, C., & Constanti, A. (1987), mRNA-directed synthesis and insertion of functional amino acid receptors in *xenopus laevis* oocytes, *Biochem. Soc. Trans.*, **15,** 117-122.

79. Smith, I., White, P.F., Nathanson, M., & Gouldson, R. (1994), Propofol, an update on its clinical use (Biebuyck, J.F., ed.), *Anesthesiology*, **81,** 1005-1043.

80. Smith, L.D., Xu, W., & Varnold, R.L. (1991), Oogenesis and oocyte isolation, Chapter 4, *Methods in Cell Biology*, **Vol. 36,** Academic Press, Inc., 45-60.

81. Snutch, T.P. (1988), The use of *xenopus* oocytes to probe synaptic communication, *TINS*, **11 (6)**, 250-256.

82. Soreq, H. (1985), *CRC Crit. Rev. Biochem.*, **18,** 199-238.

83. Stephenson, F.A., Duggan, M.J., & Pollard, S. (1990), The $\gamma 2$ subunit is an integral component of the γ-Aminobutyric $Acid_A$ receptor but the $\alpha 1$ polypeptide is the principal site of the agonist benzodiazepine photoaffinity labelling reaction, *The Journal of Biological Chemistry*, **265 (34),** 21160-21165.

84. Sumikawa, K., Parker, I., & Miledi, R. (1988), Effects of tunicamycin on the expression of functional brain neurotransmitter receptors and vpltage operated channels in *xenopus* oocytes, *Mol. Brain Res.*, **4,** 191-199.

85. Swope, S.L., Moss, S.J., Blackstone, C.D., & Huganir, R.L. (1992), Phosphorylation of ligand-gated ion channels: a possible mode of synaptic plasticity, *FASEB J.*, **6,** 2514-2523.

86. Thompson, C.L., Bodewitz, G., Stephenson, F.A., & Turner, J.D. (1992), Mapping of $GABA_A$ receptor $\alpha 5$ and $\alpha 6$ subunit-like immunoreactivity in rat brain, *Neurosci-Lett.*, **144 (1-2),** 53-56.

87. Trifiletti, R.R., & Snyder, S.H. (1984), Anxiolytic cyclopyrrolones zopiclone and suriclone bind to a novel site linked allosterically to benzodiazepine receptors, *Life Science*, **26,** 458-469.

88. Ueda, I., & Kamaya, H. (1984), Molecular mechanisms of anesthesia, *Anesth. Analg.*, **63,** 929-945.

89. Uhl, G.R., O'Hara, B., Shimada, S., Zaczek, R., DiGiorgianni, J., & Nishimori, T. (1991), Dopamine transporter: expression in *xenopus* oocytes, *Molecular Brain Research*, **9**, 23-29.

90. Unwin, N. (1993), Neurotransmitter action: opening of ligand-gated ion channels, *Neuron*, **10 (Suppl.),** 31-41.

91. Wafford, K.A., Whiting, P., & Kemp, J.A. (1992), Functional Modulation of cloned $GABA_A$ receptors expressed in xenopus oocytes, *GABAergic Synaptic Transmission*,

edited by G. Biggio, A. Concas & E. Costa, Raven Press, New York, 75-79.

92. Wafford, K.A., Bain, C.J., Whiting, P.J., & Kemp, J.A. (1993), Functional comparison of the role of γ subunits in recombinant human γ-Aminobutyric $Acid_A$/Benzodiazepine receptors, *Molecular Pharmacology*, **44,** 437-442.

93. Wahl, P., Ragsdale, D., Schousboe, A., & Miledi, R. (1993), Expression of neurotransmitter receptors by mRNAs from neurons developing in vitro: A *xenopus* oocyte expression study, *J. Neurochem.* **60**, 57-65.

94. Whiting, P., McKernan, R.M., & Iversen, L.L. (1990), Another mechanism for creating diversity in γ-aminobutyrate type A receptors: RNA splicing directs expression of two forms of γ2 subunit, one of which contains a protein kinase C phosphorylation site, *Proc. Natl. Acad. Sci. USA*, **87,** 9966-9970.

95. Wieland, H.A., Luddens, H., and Seeburg, P.H. (1992), A single histidine in $GABA_A$ receptors is essential for benzodiazepine agonist binding, *J. Biol. Chem.*, **267,** 1426.

96. Wisden, W., Herb, A., Wieland, H., Keinanen, K., Luddens, H., & Seeburg, P.H. (1991), Cloning, pharmacological characteristics and expression pattern of the rat $GABA_A$ receptor α4 subunit, *FEBS*, **289 (2),** 227-230.

97. Wisden, W., Laurie, D.J., Monyer, H., & Seeburg, P.H. (1992), The distribution of 13 $GABA_A$ receptor subunit mRNAs in the rat brain. I. Telencephalon, diencephalon, mesencephalon, *The Journal of Neuroscience*, **12 (3),** 1040-1062.

98. Zimmerman, S.A., Jones, M.V., & Harrison, N.L. (1994), Potentiation of γ-Aminobutyric $Acid_A$ raceptor Cl^- current correlates with *in vivo* anesthetic potency, *The Journal of Pharmacology and Experimental Therapeutics*, **270 (3),** 987-991.

APPENDIX I

Solutions

Transfection of micro-organism with cDNA

Preparation of liquid broth (LB):

1% w/w Bacto Tryptone

0.5% w/w Bacto Yeast Extract

1% w/w sodium chloride

distilled water to make up the volume and adjusted pH to 7.0 with NaOH

Preparation of Tris-EDTA (TE) Buffer:

50 mM Tris[hydroxymethyl]aminomethane pH 8.0

1 mM ethylenediamine tetraacetic acid (EDTA)

Maxi plasmid preparation

Solution 1	Solution 2
50 mM glucose	0.2 M NaOH
25 mM Tris pH 8.0	1% Sodium dodecyl sulphate (SDS)
10 mM EDTA pH 8.0	(Made up fresh before each plasmid preparation)

Solution 3

For 100 mL:

29.4 g KOAc

6.26 mL glacial acetic acid

distilled water to make up volume

Solution 4

TE buffer:

50 mM Tris pH 8.0

1 mM EDTA

Electrophoresis Gel

1. 10X Tris borate EDTA buffer (TBE):

   108 g Tris base

   55 g boric acid

   40 mL 0.5 mM EDTA pH 8.0

   dH_2O to make up volume to 1 L

   autoclaved.

2. 10X loading buffer:

   0.42% bromophenol blue

   0.42% xylene cyanol FF

   25% ficoll (Type 400; Pharmacia) in water

3. 1.0% agarose gel

***In vitro* transcription**

1. 5X transcription buffer (5X TB)

   200 mM Tris-HCl pH 7.5

   30 mM $MgCl_2$

   10 mM spermidine

   10 mM NaCl

   Made up in DEPC treated water, filtered, sterilized and stored at -20°C.

2. Bovine serum albumin 2 mg/mL (Grade V)

3. 100 mM dithiothreitol

4. rNTP mix

   For 8 μL:

   10 mM rATP - 2.5 μL

   10 mM rUTP - 2.5 μL

   10 mM rCTP - 2.5 μL

   DEPC water - 0.5 μL

5. 0.5 mM rGTP

6. DNAase buffer

   10 mM Tris pH 7.5

   5 mM $MgCl_2$

   10 mM NaCl

   DEPC Water

7. Restriction enzymes

Bam HI for human α_1 cDNA

Pst I for human α_2 cDNA

Sca I for human β_2 cDNA

Xho I for human γ_{2L} cDNA

Oocyte storage solution

NaCl - 88 mM

KCl - 2 mM

$CaCl_2$ - 1 mM

$MgCl_2$ - 1 mM

$NaHCO_3$ - 2.4mM

HEPES - 5 mM

pyruvic Acid - 2 mM

caffeine or theophylline - 0.5 mM

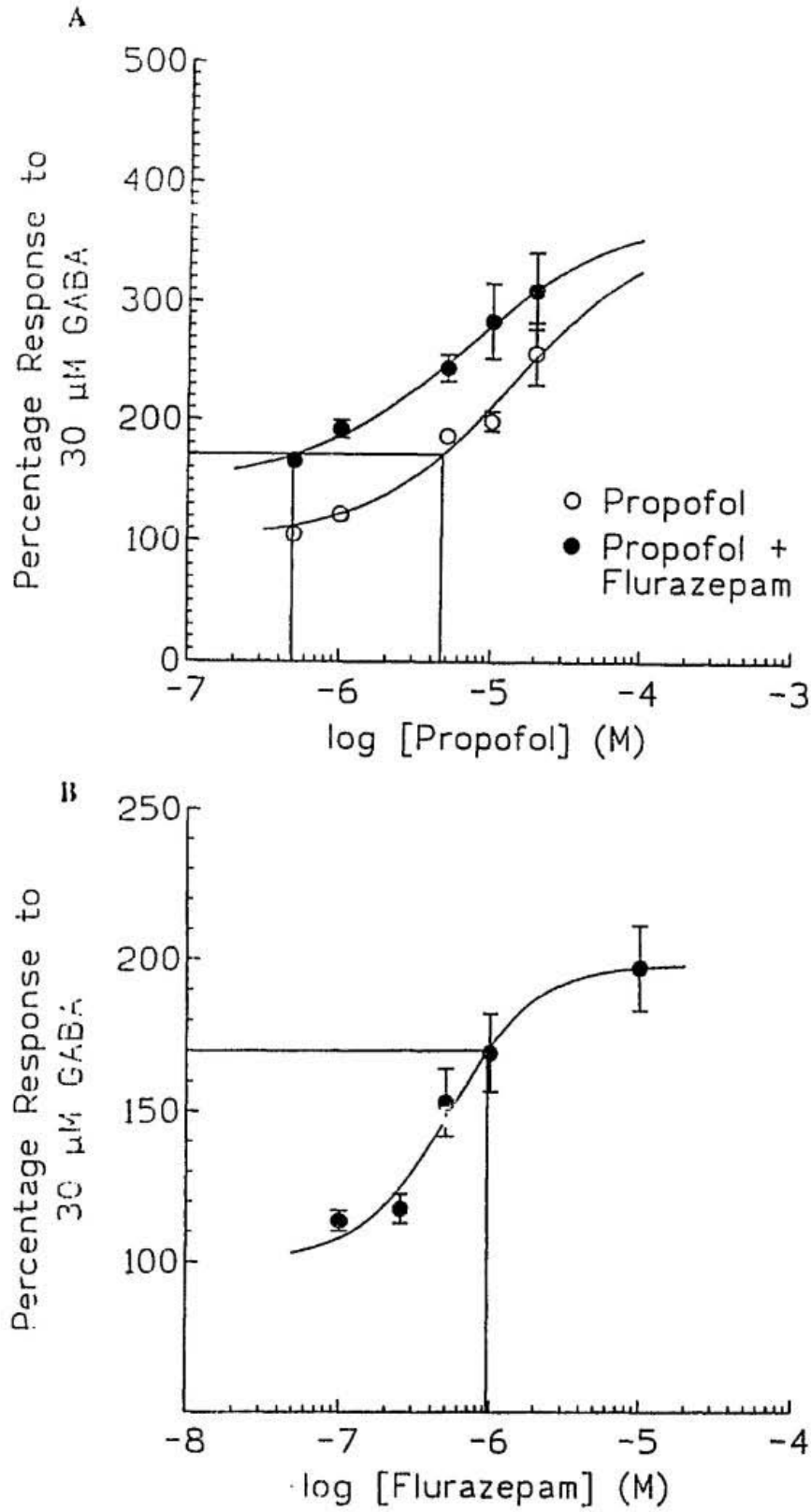
penicillin - 100 U/mL

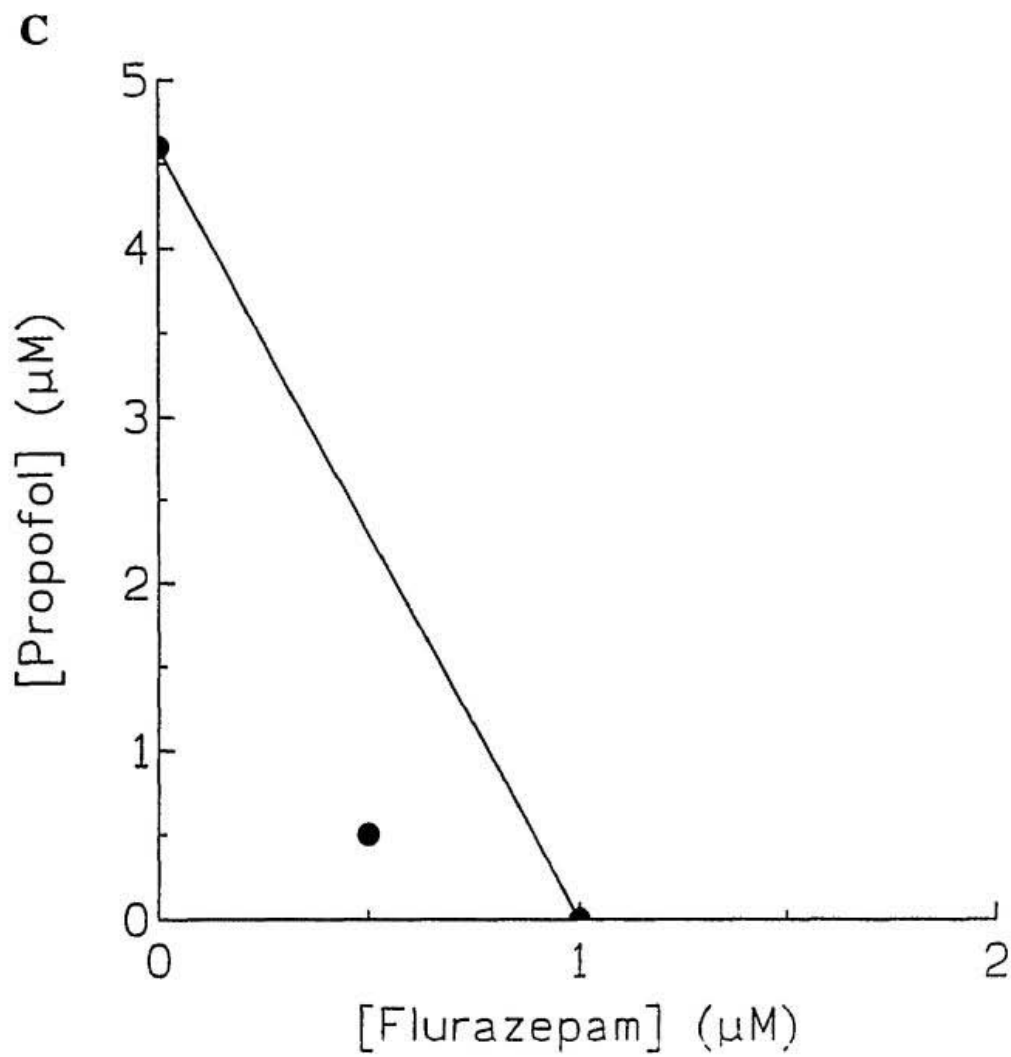
streptomycin - 100 μg/mL

APPENDIX II

Isobole analysis of interaction between propofol and flurazepam

GABA-activated inward current was potentiated both by propofol and flurazepam at the $\alpha_2\beta_2\gamma_{2L}$ $GABA_A$ receptor. An effect level was arbitrarily choosen from the concentration-response relationships of propofol (Fig.A) and flurazepam (Fig.B) in presence of 30 μM GABA. A 170% potentiation of I_{GABA} was produced by 4.6 μM and 1 μM of propofol and flurazepam respectively. The analysis was carried out according to the method described by Berenbaum, M.C. (What is synergy? *Pharmacol. Rev.*, **41** (1989) 93-141). Combined application of 0.5 μM flurazepam with propofol (0.5 - 20 μM) shows a concentration dependent response (Fig.A). Concentration of propofol in presence of flurazepam producing the desired effect was calculated from the sigmoidal relationship. Points joining the concentration of flurazepam and propofol producing 170% augmentation gives a straight line (Fig.C) and any data point for the combined effect of the drugs falling on that line is indicative of zero interaction. Combination of propofol and flurazepam which produces 170% enhancement of I_{GABA} falls far to the left of the straight line on the graph, thus showing that the drugs act in synergy (Fig.C).


A
Percentage Response to 30 µM GABA
500
400
300
200
100
0
-7
-6
-5
-4
-3
log [Propofol] (M)
o Propofol
• Propofol + Flurazepam
B
Percentage Response to 30 µM GABA
250
200
150
100
-8
-7
-6
-5
-4
log [Flurazepam] (M)


C
5
4
3
2
1
0
[Propofol] (μM)
0
1
2
[Flurazepam] (μM)