Effects of The Intravenous General Anaesthetic Propofol
on Human Recombinant GABA<sub>A</sub> Receptors
Expressed in Xenopus Oocytes

by

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A thesis submitted to the
Department of Pharmacology and Toxicology in conformity with the
requirements for the degree of Master of Science

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Abstract


Propofol (2,6-diisopropylphenol) is an intravenous general anaesthetic which can directly activate and positively modulate the GABA\(_\alpha\) receptor. The effect of propofol on human recombinant GABA\(_\alpha\) receptors was studied in Xenopus oocytes expressing either \(\alpha_1\beta_2\), \(\alpha_1\beta_2\gamma_{2L}\), or \(\alpha_2\beta_2\gamma_{2L}\) receptor constructs. In all receptor constructs, propofol was able to potentiate the GABA-activated currents in a concentration dependent manner. Although propofol potentiated both \(\alpha_1\beta_2\) and \(\alpha_1\beta_2\gamma_{2L}\) receptors with equal affinity, the efficacy of propofol potentiation was markedly greater in \(\alpha_1\beta_2\) receptors. In contrast, potentiation of \(\alpha_2\beta_2\gamma_{2L}\) receptors by propofol occurred with higher affinity and lower efficacy than in \(\alpha_1\beta_2\gamma_{2L}\) receptors. Propofol directly activated all three receptor constructs in a concentration dependent manner. Addition of a \(\gamma_{2L}\) subunit to the \(\alpha_1\beta_2\) receptor decreased receptor sensitivity to direct activation by propofol. Replacement of the \(\alpha_1\) subunit with the \(\alpha_2\) subunit increased receptor sensitivity to propofol's direct effects. A low concentration of GABA enhanced the response to propofol in \(\alpha_1\beta_2\) receptors but had no effect on either \(\alpha_1\beta_2\gamma_{2L}\) or \(\alpha_2\beta_2\gamma_{2L}\) receptors. Thus, both the \(\alpha\) and the \(\gamma_{2L}\) subunits influence the direct and modulatory actions of propofol on GABA\(_\alpha\) receptor function.

Various general anaesthetics can act synergistically with benzodiazepines to potentiate
GABA$_A$ receptor activation. Anaesthetics and benzodiazepines occupy distinct binding sites on the GABA$_A$ receptor and modulate receptor function in different ways. Such differences are thought to contribute to the observed synergy. Anaesthetics such as propofol and alfaxalone also occupy distinct sites on the GABA$_A$ receptor. However, the potential for synergistic interaction between anaesthetics from different chemical classes had not previously been investigated. This study also attempted to determine possible interaction between propofol and alfaxalone at the GABA$_A$ receptor. Combining increasing concentrations of one anaesthetic with a fixed concentration of the other in the presence of GABA revealed that these two anaesthetics acted in a simple additive manner to potentiate GABA$_A$ receptor function.
Acknowledgements

First and foremost, I would like to thank my supervisor, Dr. James Reynolds, for his continual guidance and support. Over the past two years, Dr. Reynolds has allowed me to independently grow as a student and researcher, all the while providing instruction and insight whenever necessary. He gave me the freedom to make my own decisions and the encouragement to carry them out. He is a friend and mentor without whom I would not be where I am now.

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Finally, I would like to thank my family and friends who continue to provide a seemingly endless source of support and encouragement.
Dedication

This thesis is dedicated to my loving parents
and to Margaret, my favourite girl in the world.
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<th>Definition</th>
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<tr>
<td>5HT&lt;sub&gt;3&lt;/sub&gt;R</td>
<td>5-hydroxytryptamine (serotonin) type-3 receptor</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>cDNA</td>
<td>cloned deoxyribonucleic acid</td>
</tr>
<tr>
<td>cRNA</td>
<td>cloned ribonucleic acid</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>DNA</td>
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</tr>
<tr>
<td>dNTP</td>
<td>deoxynucleotide triphosphate</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>EC&lt;sub&gt;20&lt;/sub&gt;</td>
<td>drug concentration producing the 20% effect level</td>
</tr>
<tr>
<td>EC&lt;sub&gt;50&lt;/sub&gt;</td>
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<tr>
<td>HEPES</td>
<td>4-(2-hydroxymethyl)-1-piperazineethanesulfonic acid</td>
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<td>GABA</td>
<td>γ-aminobutyric acid</td>
</tr>
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<td>GABA&lt;sub&gt;A&lt;/sub&gt;</td>
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<td>GlyR</td>
<td>glycine receptor</td>
</tr>
<tr>
<td>GTP</td>
<td>guanine nucleotide triphosphate</td>
</tr>
<tr>
<td>LP</td>
<td>lysophospholipid</td>
</tr>
<tr>
<td>MOPS</td>
<td>[N-morpholino]propanesulfonic acid</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>nAChR</td>
<td>nicotinic acetylcholine receptor</td>
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<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
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<td>uridine ribonucleotide triphosphate</td>
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<td>TBPS</td>
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</tr>
<tr>
<td>TE</td>
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<tr>
<td>THIP</td>
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INTRODUCTION

1.1 Anesthesia and The Central Nervous System

1.1.1 Non-Specific Mechanisms of Anesthesia

Since the advent of drug-induced anaesthesia in the mid 1800s, many new and improved anaesthetic agents have been developed and introduced into clinical practice. An anaesthetic was historically defined as an agent which could reversibly block neural conduction without significantly affecting the resting membrane potential [Seeman, 1972]. However, new knowledge of the central nervous system (CNS), especially receptor mechanisms, has resulted in modifications of the theories concerning the cellular mechanisms of anaesthesia. The clinical symptoms associated with the anaesthetic state, however, have not changed. The state of general anaesthesia usually includes clinical symptoms such as analgesia, loss of consciousness, inhibition of sensory and autonomic reflexes, skeletal muscle relaxation, and amnesia upon recovery.

Alongside drug development came attempts to explain the mechanism(s) of general anaesthesia. The structural diversity present among general anaesthetics led to the suggestion that anaesthetics exert their action in a non-specific manner rather than interacting at specific target proteins. Furthermore, several lines of evidence suggested that the site of drug interaction causing anesthesia was within the plasma membrane [Larrabee and Posternak, 1971; Mueller and Rudin, 1967; Narahashi et al., 1971]

Entering the twentieth century, two investigators independently reported a correlation between the potency and lipid solubility of anaesthetics [Meyer, 1899; Overton, 1901]. Their conclusion, now known as the Meyer-Overton Solubility Rule states that anaesthetic potency of a
drug is directly proportional to its lipid solubility. This rule was extended to suggest that the hydrophobic nature of anaesthetics allowed for their incorporation into cell membranes, and that anaesthetic potency was proportional to the volume within the membrane occupied by the drug [Mullins, 1954]. When anaesthetics were shown to expand artificial and biological membranes [Clements et al., 1963; Selye, 1942; Seeman and Roth, 1972; Roth et al., 1974], a model known as the Membrane Expansion Theory arose. According to this theory, anaesthetic molecules that become lodged within the neuronal membrane distort and expand the membrane. As a result, integral membrane proteins such as sodium channels are also distorted and cease to function properly. To further support the possibility that membrane expansion causes anaesthesia, studies revealed that pressure can reverse anaesthesia in living organisms, presumably by compressing the membrane back to its original, "functional" state [Johnson and Flagler, 1950; Miller et al., 1973; Halsey and Eger, 1971]. Work from several other investigators resulted in yet another theory of anaesthetic action, the Membrane Fluidization Model [Metcalfe et al., 1968; Lee, 1976; Trudell et al., 1973]. This model predicts that incorporation of anaesthetics into the lipid bilayer increases the disorder of the membrane thereby making it more fluid, which then causes alterations in the function of membrane proteins such as ion channels.

1.1.2 Receptor-Mediated Mechanisms of Anesthesia

Despite extensive effort spent to explain the anaesthetic phenomenon in terms of non-specific mechanisms of action, much criticism has arisen. For example, in order for anaesthetics to produce detectable changes in membrane fluidity, toxic concentrations of some anaesthetic are required [Boggs et al., 1976b]. Other studies have shown that increasing the temperature by a few degrees can induce equivalent membrane fluidization without causing anaesthesia [Allott et
Some anaesthetics, when incorporated into artificial or biological membranes, did not cause subsequent membrane expansion [Franks and Lieb, 1981]. In addition, pressure does not antagonize all aspects of anaesthesia, and may even enhance the anaesthetic effect [MacDonald, 1978; Boggs et al., 1976a].

Also compelling, however, is the body of information now supporting a specific mechanism of anaesthetic action. Various volatile anaesthetics were shown to interact directly with proteins [Ueda, 1965, Ueda and Kamaya, 1973]. At clinically relevant concentrations, these anaesthetics could inhibit the luciferase-induced reaction which causes bioluminescence in fireflies. More recently, further support was lent to a specific mechanism of anaesthetic action when the luciferin-luciferase inhibition by isoflurane was shown to be stereoselective [Franks and Lieb, 1993]. Evers and colleagues [1987] demonstrated that the anaesthetic effect of halothane correlated with binding of the anaesthetic to saturable sites in the brain. Recent technological advances in molecular biology have allowed us to determine that such saturable binding sites are likely receptor proteins, in particular, ion channels. Stereoselectivity of anaesthetic action has been shown with such ion channels as well [Puia et al., 1990]. In fact, potentiation of the GABA\textsubscript{A} receptor-ion channel complex by anaesthetics most closely correlates with their ability to produce general anaesthesia [Lin et al., 1993; Mihic et al., 1994; Zimmerman et al., 1994]. These studies not only demonstrated that clinically relevant concentrations of anaesthetics potentiate GABA\textsubscript{A} receptor function, but also that nonanaesthetic structural analogues fail to potentiate the GABA\textsubscript{A} receptor.

1.1.3 The Ligand-Gated Ion Channels

Within the CNS are a group of functional protein complexes known as ligand-gated ion
channels. The γ-aminobutyric acid type A receptor (GABA<sub>A</sub> receptor), nicotinic acetylcholine receptor (nAChR), glycine receptor (GlyR), and serotonin type-3 receptor (5-HT<sub>3</sub>R) are members of this ion channel superfamily. Many properties characterize these ion channels. First of all, each receptor is a complex of several protein subunits, each subunit possessing four transmembrane domains [Lindstrom <i>et al.</i>, 1979; Raftery <i>et al.</i>, 1980]. Five protein subunits assemble in a pseudopentameric fashion to form the functional ion channel [Langosch <i>et al.</i>, 1988; Nayeem <i>et al.</i>, 1994; Unwin, 1993]. The pentamer forms a pore which is permeable to monovalent cations (nAChR, 5-HT<sub>3</sub>R) or anions (GABA<sub>A</sub> receptor, GlyR). These protein complexes are referred to as receptors, attributable to the fact that various chemical compounds, or ligands, can bind to one or more sites on the protein complex. In addition, opening of the ion channel, also known as gating or activation, requires binding of one or more such ligands.

1.2 The GABA<sub>A</sub> Receptor

1.2.1 Molecular Biology of The GABA<sub>A</sub> Receptor

The GABA<sub>A</sub> receptor (Fig. 1) is a member of the superfamily of ligand-gated ion channels [Schofield <i>et al.</i>, 1987]. Although five protein subunits are required for assembly of a functional receptor, each subunit of the receptor may not be identical to the others. Indeed, assembly of the nAChR involves four different subunit subtypes [for review, see Karlin, 1993]. With respect to the GABA<sub>A</sub> receptor, 15 distinct subunits have been identified [Luddens and Wisden, 1991; Seeburg <i>et al.</i>, 1990] and classified into five general subunit families (α, β, γ, δ, ε) based on similarities in amino acid sequence [Schofield <i>et al.</i>, 1987; Olsen and Tobin 1990; Davies <i>et al.</i>, 1997a]. Within most subunit families are multiple subunit subtypes (α<sub>1</sub>-6, β<sub>1</sub>-4, γ<sub>1</sub>-3, δ, ε) which
have also been determined based on amino acid sequence homology. Additional diversity arises from RNA splice variants. Such alternative splicing has been identified for genes encoding α-, β-, and γ-subunits [Korpi et al., 1994; Kirkness and Fraser 1993; Whiting et al., 1990; Kofugi et al., 1991]. Often, these splice variants result in the addition or removal of a putative phosphorylation site on the translated protein subunit. For example, the γ2L-subunit contains eight amino acids which are absent from the γ2S-subunit and which represent a site of phosphorylation by Ca2+/phospholipid-dependent protein kinase [Kofugi et al., 1994].

1.2.2 Structure and Assembly of The GABA\textsubscript{A} Receptor

The muscle and neuronal nicotinic acetylcholine receptors, as well as the glycine receptor, are known to possess a quasisymmetrical pentameric quaternary structure [Brisson and Unwin, 1985; Cooper et al., 1991; Langosch et al., 1988]. To date, the crystal structure of the GABA\textsubscript{A} receptor cannot be used to directly determine whether it, too, exists as a pentamer. However, electron image analysis revealed that GABA\textsubscript{A} receptors isolated from porcine brain have a rotational symmetry of 5 which is consistent with a pentameric structure [Nayeem et al., 1994]. In addition, hydrodynamic analysis of bovine GABA\textsubscript{A} receptors estimates the molecular weight of the receptor complex to be approximately 240 kDa [Mamalaki et al., 1989]. Since the molecular weight of individual GABA\textsubscript{A} receptor subunits ranges from 48-62 kDa, this estimation is also consistent with a pentameric assembly.

If GABA\textsubscript{A} receptor subunits could assemble in any combination of five, the identified subunits would allow over 100000 possible constructs for the GABA\textsubscript{A} receptor. The cloning of genes encoding specific GABA\textsubscript{A} receptor subunits has led to the use of in vitro expression systems to study recombinant GABA\textsubscript{A} receptors. Homomeric receptors consisting of but one
receptor subunit, as well as heteromeric receptors assembled from two to four different subunit subtypes can form in vitro [Angelotti et al., 1993; Mathews et al., 1994; Pritchett et al., 1989; Shivers et al., 1989]. Not surprising, however, is the discovery that relatively few subunit combinations may actually exist in vivo. One study has demonstrated that when α-subunits are co-expressed with β- and γ-subunits, only receptors with αβ and αβγ combinations produce functional receptors on the cell surface [Connolly et al., 1996]. In addition, co-expression of α-, β-, and γ-subunits leads to highly preferential, if not exclusive, assembly of αβγ receptors over αβ receptors [Angelotti et al., 1993].

Despite the wealth of knowledge regarding GABA\textsubscript{A} receptor structure and molecular biology, there remains a relative paucity of data addressing stoichiometry of the GABA\textsubscript{A} receptor. From what is known, the α\textsubscript{3}β\textsubscript{2}γ\textsubscript{2} GABA\textsubscript{A} receptor subtype appears to assemble in α:β:γ subunit ratios of 2:1:2, 2:2:1, or 1:2:2 with the first being the most favoured stoichiometry [Backus et al., 1993]. The α\textsubscript{4}β\textsubscript{3}γ\textsubscript{2} receptor subtype appears to assemble from two α-subunits, two β-subunits, and a single γ-subunit [Tretter et al., 1997]. Expression of only α\textsubscript{1} and β\textsubscript{3} subunits yields an α:β ratio of 1:1:1, suggesting either a mixed population of receptors containing three subunits of one type and two of the other or formation of a tetrameric GABA\textsubscript{A} receptor. However, the use of fusion proteins of α-β subunits tends to exclude the possibility of tetrameric receptors [Kellenberger et al., 1996]. Whether the varied results are due to the expression system, the analytical technique, or true variation in expression in vivo remains to be seen.
Figure 1.

The GABA<sub>A</sub> receptor is a complex of protein subunits, each of which consists of four membrane-spanning domains, a large extracellular N-terminal domain, and a large intracellular loop between transmembrane domains III and IV (left). Five such subunits are believed to assemble in a pseudopentameric ring surrounding a central pore through which anions can flow (middle). Binding of an agonist to the GABA<sub>A</sub> receptor is thought to induce a conformational change in the pentameric ring which, in turn, opens the channel and allows anions to pass (right). [Modified from Haefely, 1994]
GABA<sub>A</sub> receptor channel
1.2.3 Distribution of GABA$_A$ Receptor Subunits in The CNS

Alongside non-random assembly of GABA$_A$ receptor subunits into functional receptors, it is well established that GABA$_A$ receptor subunits are not uniformly distributed throughout the mammalian CNS [for review see McKernan and Whiting, 1996]. The most striking example of regional distribution of a subunit subtype within the CNS is the $\alpha_6$-subunit which is found only in cerebellar granule cells [Kato, 1990; Luddens et al., 1990]. The many approaches used to study the distribution of GABA$_A$ receptor subunits within the brain demonstrate that: i) mRNA for certain subunit subtypes are found only in certain regions/cell populations of the brain [Kato, 1990; Laurie et al., 1992; Wisden et al., 1992], ii) the abundance of mRNA for a given subunit does not necessarily remain the same from one brain region/cell population to the next [Esclapez et al., 1996; Laurie et al., 1992; Wisden et al., 1992], iii) the actual subunit proteins expressed appear to co-assemble in different combinations throughout the brain [Khan et al., 1996; Quirk et al., 1994], and iv) many factors can influence the expression level of GABA$_A$ receptor subunits [Devaud et al., 1995; Fenelon and Herbison, 1996; Gutierrez et al., 1996].

By far the most abundant GABA$_A$ receptor subtype in the brain is $\alpha_1\beta_2\gamma_2$. Almost half of all GABA$_A$ receptors in the brain, hippocampal and cortical interneurons in particular, contain this subunit combination [Fritschy et al., 1992; Gao and Fritschy, 1994]. Receptors containing $\alpha_3\beta_{23}\gamma_2$ are the next most abundant subtype in the brain, making up 18% of all GABA$_A$ receptors. These are localized mainly in spinal cord motoneurons and hippocampal pyramidal cells [Laurie et al., 1992; Persohn et al., 1991; Wisden et al., 1992].

1.2.4 Modulation of GABA$_A$ Receptor Function

GABA is the major inhibitory neurotransmitter within the CNS. GABA has been found to
activate the receptor in a concentration-dependent fashion, and the slope of the logarithmic concentration-response curve in native GABA_α receptors is 2, indicating that two molecules of GABA bind to the GABA_α receptor in order to cause full activation of the channel [Sakmann et al., 1983].

At the single channel level, GABA_α receptor activation results in rapid opening and closing of the channel which can be described in terms of conductance, duration, and burst properties [Bormann et al., 1987; Hamill, et al., 1983; MacDonald et al., 1989]. Detailed analysis of single-channel properties has allowed for development of a kinetic model of GABA_α receptor channel gating [reviewed by MacDonald and Twyman, 1992]. First of all, the GABA_α receptor can exist in an unbound, singly bound, or doubly bound state of receptor occupancy. Singly and doubly bound receptors are thought to open into multiple open states. In addition, the receptor can exist in multiple closed states and at least one desensitized state. The measurable properties such as channel conductance and channel opening are influenced by the subunit composition of a given GABA_α receptor. For example, the ion channel associated with the GABA_α receptor opens to a main conductance level as well as one or more subconductance levels. α_1β_1 receptors open to a main conductance level of 15 pS [Angelotti and Macdonald, 1993], whereas α_1β_2 receptors open to a main conductance level of 11 pS [Verdoon et al., 1990]. The same studies found that addition of a γ_2-subunit produced main conductance levels of 29 pS and 32 pS for α_1β_1γ_2 and α_1β_2γ_2 receptor subtypes, respectively. Open duration is another channel property that depends upon receptor subunit composition. While the mean open duration for α_1β_1γ_2 receptors is 6.0 ms, the open duration for α_1β_1 receptors was only 2.3 ms [Angelotti and Macdonald, 1993]. Angelotti and Macdonald [1993] further demonstrated that burst properties of the channel are
influenced by subunit composition. Bursts are defined as repeated openings of an ion channel separated by brief closures of the channel. In their study, \( \alpha_1\beta_1\gamma_2 \) receptors displayed reduced burst frequency, but greater burst duration, than \( \alpha_1\beta_1 \) receptors.

There exist many natural and synthetic compounds which possess GABA-mimetic actions. These include muscimol, 4,5,6,7-tetrahydroisoxazolopyridin-3-ol (THIP), piperidine-4-sulphonate (P4S), and isoguvacine [Falch and Krogsgaard-Larsen, 1982]. Bicuculline acts as a competitive antagonist acting at the GABA binding site, whereas t-butylibicyclophosphorothionate (TBPS) and picrotoxin are non-competitive antagonists acting on the ion channel itself [Ticku and Maksay, 1983; Concas et al., 1990].

In addition, many anaesthetic, anxiolytic, and sedative/hypnotic compounds act as allosteric modulators of the GABA\(_A\) receptor. That is, they are capable of enhancing, or potentiating, GABA-mediated activation of the GABA\(_A\) receptor. This large class of compounds includes the benzodiazepines, volatile anaesthetics (e.g. halothane, isoflurane), and non-volatile anaesthetics. Included in the latter anaesthetic class are barbiturates, neuroactive steroids, alcohols, as well as structurally distinct anaesthetics such as etomidate and propofol [Concas et al., 1990; Ghiani et al., 1996; Harris et al., 1995; Hill-Venning et al., 1997; Reynolds and Prasad, 1991; von Blankenfeld, et al., 1990]. While the non-anaesthetic benzodiazepines can alter GABA\(_A\) receptor function only in the presence of GABA [Hattori et al., 1986], anaesthetics such as the barbiturates, steroids, and propofol can also directly activate the receptor in the absence of GABA [Puia et al., 1990; Sanna et al., 1995b].

Single channel analysis reveals that anaesthetic modulation of GABA\(_A\) receptor function may take several forms. Barbiturate potentiation of the GABA\(_A\) receptor involves prolonging
burst duration with no change in burst frequency [MacDonald et al., 1989; Twyman et al., 1989]. Potentiation by anaesthetic steroids, on the other hand, increases both mean open duration and mean open frequency [Twyman and MacDonald, 1992]. Direct activation by both barbiturates and anaesthetic steroids produces similar main conductance, mean open time and mean burst duration compared with activation by GABA [Barker et al., 1987; Rho et al., 1996]. Although direct activation by propofol is similar to barbiturate or steroid activation, propofol potentiation involves increasing the channel open frequency, but not open duration or conductance levels [Orser et al., 1994].

Biochemical studies demonstrate that many of these compounds do not share the same binding sites on the GABA\textsubscript{A} receptor. In particular, the benzodiazepines, barbiturates and neurosteroids each bind to distinct sites on the GABA\textsubscript{A} receptor [Concas et al., 1990, Concas et al. 1991]. Multiple binding sites on the GABA\textsubscript{A} receptor allow for possible drug interactions, not only between an allosteric modulator and GABA, but also between different allosteric modulators. This has been shown to be the case for anaesthetic steroids and barbiturates, each of which can enhance benzodiazepine binding affinity at the GABA\textsubscript{A} receptor [Harris et al., 1995]. Other studies have shown that propofol and benzodiazepines act synergistically to potentiate GABA\textsubscript{A} receptor activation [Prince and Simmonds, 1992; Reynolds and Maitra, 1996]. Although this type of drug-drug interaction adds complexity to the GABA\textsubscript{A} receptor pharmacology, the data agree with clinical observations that combination of such agents leads to synergistic enhancement of anaesthesia [Short and Chui, 1991].

1.2.5 Anaesthetic Effects at The GABA\textsubscript{A} Receptor: Subunit Dependence

Subunit subtype diversity leads to receptor heterogeneity. Numerous investigators have
studied recombinant $\text{GABA}_A$ receptors in an attempt to investigate differences in pharmacological profiles. The wealth of data obtained demonstrates that, indeed, the subunit composition of a given $\text{GABA}_A$ receptor subtype determines its sensitivity to different ligands. Receptors containing $\alpha$-, $\beta$-, and $\gamma$-subunits are generally less sensitive to GABA than those containing only $\alpha$- and $\beta$-subunits [Angelotti et al., 1993]. In addition, $\alpha\beta$ receptor constructs are exquisitely sensitive to inhibition by $\text{Zn}^{2+}$, whereas, $\alpha\beta\gamma$ receptor constructs are completely insensitive to the effects of $\text{Zn}^{2+}$ [Draguhn et al., 1990]. Among $\alpha\beta\gamma$ receptor constructs, changing any of the subunits can alter receptor sensitivity to GABA [Ducic et al., 1995].

Effects of many anaesthetic and anxiolytic agents on $\text{GABA}_A$ receptors are dependent upon subunit composition. A classical example is the subunit dependence associated with the anxiolytic/anticonvulsant agents known as benzodiazepines. Benzodiazepine action at the $\text{GABA}_A$ receptor requires the presence of a $\gamma_2$-subunit [Pritchett et al., 1989] and can be influenced by the type of $\alpha$-subunit present [Hadingham et al., 1993a]. The $\beta$-subunit, in contrast, has no effect on the affinity or efficacy of benzodiazepine action [Hadingham et al., 1993b].

The actions of general anaesthetics are also dependent upon subunit composition of the receptor. However, their ability to directly activate as well as allosterically modulate the $\text{GABA}_A$ receptor complicates the roles of various subunits. The type of $\alpha$-subunit present, but not the $\beta$-subunit, influences modulation of the GABA response by barbiturates [Hadingham et al., 1993b; Thompson et al., 1996]. Direct activation of the $\text{GABA}_A$ receptor by barbiturates can be influenced by both $\alpha$- and $\beta$-subunits [Thompson et al., 1996]. In contrast, neither modulatory nor direct neurosteroid actions on $\text{GABA}_A$ receptors are influenced by $\alpha$- or $\beta$-subunits [Sanna et
al., 1997]. In fact, while barbiturates can directly activate homomeric $\beta_1$ or $\beta_3$ receptors, the neurosteroids could not [Davies et al., 1997b; Sanna et al., 1995a]. These studies not only suggest a putative role for the $\beta$-subunit in the direct effects of barbiturates, they also confirm biochemical studies demonstrating that barbiturates and steroids have distinct binding sites. Together with single channel experiments [Puia et al., 1990], the evidence supports the notion that barbiturates and neurosteroids interact differently at the $\text{GABA}_A$ receptor.

1.3 Propofol

1.3.1 Propofol: A New Anaesthetic Agent

2,6-Diisopropylphenol, also known as propofol (or by its trade name, Diprivan®) is an intravenous general anaesthetic first developed in the late 1970s [Kay and Rolly, 1977] and has, in the past decade, gained great popularity in the clinical setting. Propofol is favoured because it exhibits a rapid onset and short duration of anaesthetic action [Langley and Heel, 1988; Sebel and Lowdon, 1989, Kortilla et al., 1992]. Since nausea has often been associated with recovery from general anaesthesia, a further advantage is that propofol has potent anti-emetic properties at subanaesthetic concentrations [Borgeat et al., 1992; Gunwardene and White, 1988]. In addition, propofol has been shown to act in synergy with benzodiazepines which are commonly co-administered with anaesthetics as anxiolytics [Reynolds and Maitra, 1996; Short and Chui, 1991]. While the most common side effect of propofol administration is pain at the site of injection due to drug infusion, cardiovascular depression during propofol anaesthesia is likely the most potentially harmful side effect [Merin, 1990]. Nonetheless, the incidence of adverse reactions associated with propofol anaesthesia is low [Green and Jonsson, 1993]. One study also proposed
that propofol be used as the alternative to volatile anaesthetic administration for individuals with compromised pulmonary function [Ludbrook et al., 1997]. For these reasons, propofol has become one of the anaesthetics of choice for out-patient surgical procedures [Smith et al., 1994]

1.3.2 Multiple Target Sites for Propofol Within The CNS

Determining a molecular basis for the clinical effects of a drug has always been a challenge, and the study of propofol is no exception. Propofol has been shown to act at many target sites within and outside the CNS, though the general consensus is that its primary anaesthetic and anti-emetic actions occur within the CNS. Among its many actions, propofol can enhance GABA$_\alpha$ receptor activation [Hales and Lambert, 1991], inhibit N-methyl-D-aspartate (NMDA) receptor activation [Orser et al., 1995], inhibit nAChR function [Dilger et al., 1994], inhibit voltage-gated Na$^+$ and K$^+$ channel function [Frenkel and Urban, 1991; Mozrzymas et al., 1996], and suppress lysophosphatidate (LP) receptor function [Rossi et al., 1996]. From these same studies, however, difficulties arise when one attempts to link these proteins to the mechanism of anaesthesia. For instance, the effects on NMDA receptors require concentrations of propofol beyond the anaesthetic range. Functional alteration of voltage-gated ion channels and LP receptors are small relative to that observed in GABA$_\alpha$ receptors. K$^+$ channels susceptible to propofol are found on T lymphocytes which are probably not directly associated with propofol-induced anaesthesia. Moreover, with the exception of the GABA$_\alpha$ receptor, functional alterations of these targets by propofol are actions which do not appear to be consistently shared among the other general anaesthetics. Therefore, the great majority of clinical and experimental evidence indicates that the GABA$_\alpha$ receptor represents the most likely site of action for general anaesthetics such as propofol.
1.3.3 Actions of Propofol on The GABA\textsubscript{A} Receptor

As with virtually every known anaesthetic, sedative/hypnotic, and anxiolytic agent to date, clinically relevant concentrations of propofol dramatically enhance activation of the GABA\textsubscript{A} receptor. Similar to other general anaesthetics, propofol enhances the function of the GABA\textsubscript{A} receptor in different areas of the rat brain (Concas et al., 1990; Concas et al., 1991). The common action is, however, not supported by a common binding site for the various anaesthetics. In fact, biochemical studies have shown that benzodiazepines, barbiturates, and neuroactive steroids all have unique binding sites on the GABA\textsubscript{A} receptor [Concas et al., 1990, Concas et al. 1991]. Furthermore, the same studies show that propofol does not compete with any of these drugs for a common binding site.

In accordance with other anaesthetics, low concentrations (1-10 \(\mu\text{M}\)) of propofol can potentiate GABA-mediated activation of the GABA\textsubscript{A} receptor [Jones et al., 1995; Lin et al., 1992]. Patch clamp studies [Orser et al., 1994] determined that the potentiating effect of propofol was due to increases in the frequency but not the duration of channel openings, with no change in the single channel conductance. In addition to potentiation of GABA\textsubscript{A} receptor activation, general anaesthetics such as the barbiturates, neurosteroids, and propofol also possess the ability to directly activate the GABA\textsubscript{A} receptor in the absence of GABA. Higher concentrations (10-500 \(\mu\text{M}\)) of propofol can directly activate the GABA\textsubscript{A} receptor in this fashion [Sanna et al., 1995a; Sanna et al. 1995b].

Similar to other general anaesthetics, subunit composition may also influence the effects of propofol at the GABA\textsubscript{A} receptor. As with barbiturates, propofol can directly activate \(\beta_{1/3}\)
homomers [Davies et al., 1997b; Sanna et al., 1995a]. Also similar to barbiturate action, propofol potentiation of GABA_\alpha receptor function is not influenced by the type of \beta-subunit present within the receptor [Hill Venning et al., 1997]. Less is known about the influence of the \alpha- and \gamma-subunits. One study reported that the \gamma-subunit was not required for direct activation of the receptor by propofol [Jones et al., 1995]. Another study systematically tested propofol action on several different \alpha\beta and \alpha\beta\gamma receptor constructs [Sanna et al., 1995b]. The results varied extensively, and the investigators concluded from their data that propofol did not require any one particular subunit for its modulatory effects, but that individual subunits might contribute to the degree of potentiation of receptor function by propofol. In agreement with this suggestion, previous work from this laboratory has demonstrated that the \gamma_2-subunit significantly influences the ability of propofol to modulate the GABA response [Reynolds and Maitra, 1996].

Enhancement by propofol of benzodiazepine binding to the GABA_\alpha receptor has been observed [Prince and Simmonds, 1992]. Functional studies on recombinant GABA_\alpha receptors have also found that propofol and benzodiazepines act synergistically to potentiate GABA-induced activation of the receptor [Reynolds and Maitra, 1996]. Furthermore, barbiturates and neuroactive steroids can enhance benzodiazepine binding to the GABA_\alpha receptor [Harris et al., 1995]. These studies demonstrate that anaesthetics and benzodiazepines facilitate the actions of one another on the GABA_\alpha receptor. Little is known, however, about the possible interactions between different anaesthetics.
Rationale, Objectives and Hypotheses

The GABA_A receptor is an important target involved in inhibitory neurotransmission within the CNS. Many anaesthetic agents dramatically alter GABA_A receptor function either by enhancing GABA-mediated activation of the receptor or by directly activating the receptor in the absence of GABA. These actions are thought to play a major role in the mechanism of general anaesthesia. However, different anaesthetics often possess unique pharmacological properties, both at the molecular and behavioural levels.

Within the GABA_A receptor class, there exists a great deal of receptor heterogeneity due to the fact that protein subunits assemble in various combinations to form different receptor subtypes. The actions of traditional anaesthetic agents such as barbiturates and neurosteroids are influenced by the subunit composition of the GABA_A receptor. Furthermore, GABA_A receptor subunits are unevenly distributed in brain regions and cell types throughout the CNS. Although somewhat presumptuous to assume that abundance and selective distribution is associated with functional relevance, receptor heterogeneity has been shown to exist in vivo as well as in vitro and may therefore contribute to the unique pharmacological properties of different anaesthetics. Furthermore, the abundance of \( \alpha_1\beta_2\gamma_2 \) and \( \alpha_2\beta_2\gamma_2 \) receptor subtypes in vivo suggests that such receptor subtypes may be involved in regulation of neuronal signalling, which in turn may contribute to the mechanism of anesthesia.

Propofol is a relatively new general anaesthetic which is structurally unrelated to other known anaesthetics. Due to its favourable clinical properties, propofol has become one of the most popular anaesthetics in use today. Although the ability of propofol to act at the GABA_A
receptor has been established, the influence of subunit composition on propofol action is not very well understood. The β-subunit appears to contribute to the direct actions of propofol. Recently, work from this laboratory demonstrated that the presence or absence of the γ2-subunit significantly influences both potency and efficacy of propofol to potentiate GABA<sub>A</sub> receptor activation. The purpose of this study, therefore, was to quantify the effects of α- and/or γ-subunits on the modulatory and direct actions of propofol on the GABA<sub>A</sub> receptor.

Many anaesthetics including propofol have been shown to facilitate the actions of benzodiazepines, indicating that allosteric modulators of the GABA<sub>A</sub> receptor can act in synergy. Whether different anaesthetics in combination have a similar effect remains to be seen. Thus, the nature of the interaction between propofol and alfaxalone, when administered in combination, to potentiate GABA<sub>A</sub> receptor activation, was also investigated.

The objectives of this thesis were as follows:

1) To express human recombinant GABA<sub>A</sub> receptor subtypes (α<sub>1</sub>β<sub>2</sub>, α<sub>1</sub>β<sub>2</sub>γ<sub>2L</sub>, or α<sub>2</sub>β<sub>2</sub>γ<sub>2L</sub>) in Xenopus oocytes.

2) To measure changes in membrane current due to activation of recombinant GABA<sub>A</sub> receptors by using the two-electrode voltage clamp recording technique.

3) To compare the efficacy and potency for potentiation of GABA-activated current from α<sub>1</sub>β<sub>2</sub>, α<sub>1</sub>β<sub>2</sub>γ<sub>2L</sub>, and α<sub>2</sub>β<sub>2</sub>γ<sub>2L</sub> receptor subtypes by propofol.

4) To compare the efficacy and potency for direct activation of α<sub>1</sub>β<sub>2</sub>, α<sub>1</sub>β<sub>2</sub>γ<sub>2L</sub>, and α<sub>2</sub>β<sub>2</sub>γ<sub>2L</sub> receptor subtypes by propofol.
5) To determine whether co-application of propofol and alfaxalone produces synergistic enhancement of GABA-activated current in different receptor subtypes.

The hypotheses to be tested were as follows:

1) The presence of the $\gamma_{2L}$-subunit significantly alters the ability of propofol to potentiate $\text{GABA}_A$ receptor activation, but does not alter the ability of propofol to directly activate the $\text{GABA}_A$ receptor.

2) The type of $\alpha$-subunit present significantly alters the ability of propofol to potentiate $\text{GABA}_A$ receptor activation, but does not alter the ability of propofol to directly activate the $\text{GABA}_A$ receptor.

3) Propofol and alfaxalone act in synergy to potentiate activation of recombinant $\text{GABA}_A$ receptors.
METHODS

2.1 Purification of $\text{GABA}_A$ Receptor Subunit cDNAs

Cloned DNA (cDNA) encoding individual human $\text{GABA}_A$ receptor subunits, cloned into the pCDM8 vector, were received as a gift from Dr. P. Whiting, Merck Sharpe & Dohme (Terlings Park, Essex). Prior to the following procedures, the cDNAs were transfected into the MC1601/P3 strain of *Escherichia coli* (*E. Coli*) bacterial cells (Invitrogen Corp.). Following successful transfection, the cDNA plasmids produced were isolated and purified by alkali lysis of the transfected cells.

2.2 Preparation of cRNA From Plasmid cDNA

2.2.1 Linearization of Plasmid cDNA

An aliquot of each plasmid cDNA (2 µg/µL) was placed in a separate microcentrifuge tube. The appropriate restriction enzyme (50 units) and 5 µL of the corresponding buffer stock was then added to each tube. Nuclease-free water was added to bring the reaction mixture to a final volume of 50 µL. The restriction enzymes used were as follows: BamH I (Life Technologies Inc.) for $\alpha_1$ cDNA, Pst I (Life Technologies Inc.) for $\alpha_2$ cDNA, Sca I (Pharmacia Biotech) for $\beta_2$ cDNA, and Xho I (Life Technologies Inc.) for $\gamma_2$ cDNA. The reaction mixture was gently mixed and subsequently incubated at 37°C for 4 hours. The linearized cDNA was then isolated and purified using a standard phenol/chloroform extraction followed by ethanol precipitation as described for the RiboMAX Large Scale RNA Production System from Promega Corp.
(Madison, WI). Finally, the cDNAs were resuspended in 50 µL of Tris-EDTA (TE) buffer and stored at 4°C.

2.2.2 Filling Up The 3' Overhangs

Cleaving of plasmid cDNAs with certain enzymes such as Pst I yields linearized fragments which possess 3' overhangs. These overhangs allow complementary cDNA ends to anneal. The linked cDNA will cause inefficient and improper transcription to cRNA. Therefore, the 3' overhangs must be end-capped prior to in vitro transcription. Since only α₂ plasmid cDNA was linearized with Pst I which yields a 3' overhang, the following procedure pertains to this cDNA alone.

10 µL (approx. 10 µg) of linear cDNA was combined with 10 µL of T4 DNA polymerase buffer, and 1µL of T4 DNA polymerase. Nuclease-free water was added to bring the mixture to a final volume of 100 µL. The reaction mixture was left at room temperature for 2 minutes in order for the enzyme to associate with the cDNA template. The four dNTPs (2 mM, final concentration) were then added, and the reaction mixture was incubated at 37°C for 30 minutes. Following incubation, the converted cDNA fragments were again purified using phenol/chloroform extraction and ethanol precipitation. The purified linear cDNA was resuspended in 10 µL of TE for a final concentration of approximately 1 µg/µL.

2.2.3 DNA Agarose Gel Electrophoresis

To confirm successful linearization of plasmid cDNA, samples of the cDNA product were run on an agarose gel. Agarose powder was combined with 1X Tris-boric acid-EDTA (TBE) buffer and heated until dissolved to make a 1% agarose solution. 3.3 µL of ethidium bromide (10
μg/μL) was mixed into 50 mL of agarose solution. The solution was then poured into a gel casting platform, containing a comb for well formation, and allowed to set (approx 20 minutes). The set gel was placed in an electrophoresis bath containing 1X TBE buffer. Within the bath, the comb was carefully removed, and the DNA (marker and samples) loaded into individual wells. The composition of DNA marker and cDNA samples were as follows:

\[
\begin{align*}
1 \text{Kb DNA ladder or cDNA sample} & \quad 1 \mu\text{L} \\
10 \times \text{loading buffer (bromophenol blue)} & \quad 1 \mu\text{L} \\
\text{DNase-free water} & \quad 8 \mu\text{L}
\end{align*}
\]

The gel was run at 100V for 30 minutes. The gel was analyzed for single bands (specific cleavage product) with the predicted molecular weight, as measured by the 1Kb ladder.

2.2.4 \textit{In vitro} Transcription

The \textit{in vitro} transcription procedure was performed according to the RiboMAX Large Scale RNA Production System (Promega Corp.). The following description highlights the salient aspects of the protocol. Following linearization of plasmid cDNAs, separate 50 μL reaction mixtures were created by adding reagents in the listed order:

\[
\begin{align*}
\text{T7 Polymerase transcription 5X buffer} & \quad 10 \mu\text{L} \\
\text{rNTPs (25mM rATP, rCTP, rUTP)} & \quad 15 \mu\text{L} \\
\text{rGTP (25mM)} & \quad 2.5 \mu\text{L} \\
7\text{-methyl capped-GTP (25 mM)} & \quad 7.5 \mu\text{L} \\
\text{Linear DNA template} & \quad 5 \mu\text{L}
\end{align*}
\]
Nuclease-free water

T7 Polymerase Enzyme Mix

Each reaction mixture was mixed by gentle pipetting and incubated at 37°C for 4 hours. Upon completion of the transcription reaction, 5 units of RQ1 RNase-free DNase was added, and the reaction mixture was incubated at 37°C for 15 minutes.

2.2.5 Isolation and Purification of cRNA Transcript

In order to isolate and purify the cloned ribonucleic acid (cRNA) transcript, phenol/chloroform extraction was performed as before. It is important to note, however, that the phenol/chloroform/isoamyl alcohol reagent used here was buffered at pH 4.5. The difference in pH reflects the different pH values at which DNA and RNA are ionized and thus can be separated from each other as well as from protein. Following extraction from the reaction mixture, the cRNA was precipitated with isopropanol and resuspended in 50 μL of TE buffer.

2.2.6 RNA Denaturing Gel Electrophoresis

To confirm production of cRNA from cDNA, samples of the cRNA were run on a RNA denaturing gel. Aseptic lab technique for treating and handling all materials was practiced in order to minimize RNase contamination. 18.6 mL of (diethylpyrocarbonte) DEPC-treated water was added to 0.54 g of agarose and heated until dissolved. Once dissolved, 6 mL of 5X 3-[N-Morpholino]propanesulfonic acid (MOPS) buffer and 5.4 mL of formaldehyde (37%) were added. The solution was poured into a gel casting platform and allowed to set (40 minutes). A sample buffer was prepared as follows (for 240 μL stock):

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>5X MOPS buffer</td>
<td>30 μL</td>
</tr>
</tbody>
</table>
10-30 μg of cRNA or marker were mixed with 20 μL of sample buffer. 2 μL of bromophenol blue was added to the marker to aid visualization of relative migration during gel electrophoresis. RNA samples were incubated at 65°C for 10 minutes and then chilled on ice to ensure no RNA has annealed together. The set gel was placed in an electrophoresis bath containing 1X MOPS buffer. Samples were loaded into the wells, and the gel was run at 80V of 75-90 minutes. Following electrophoresis, the gel was analyzed for single bands (specific RNA transcript) with the predicted molecular weights.

The concentration of each cRNA stock was determined using a spectrophotometer. With the concentration determined, the cRNAs were diluted to a final concentration of 3 μg/μL. All cRNA solutions were stored at -70°C until used.

2.3 Preparation of Oocytes
2.3.1 Isolation of Oocytes From Xenopus laevis

Female *Xenopus laevis* frogs (Xenopus, Ann Arbor, MI) were housed in pairs within 2' x 2' x 3' containers filled with approximately 15 cm of water. The storage environment was maintained at room temperature (20-22°C) with a 12 hour alternate light and dark cycle. Frogs were fed two times a week with commercially available frog pellets. Water was also changed twice a week.
In order to isolate oocytes for experimentation, individual frogs were anaesthetized by immersion in 0.2% 3-aminobenzoic acid ethyl ester (Sigma Chemical Co., St. Louis, MO). The anaesthetic was dissolved in ice cold water which further promoted anaesthesia by reducing the metabolic rate of the frog. Failure to respond to noxious stimuli was used as the endpoint of anaesthesia. This was determined by pinching of lower limbs.

The anaesthetized frog was placed on a bed of crushed ice to maintain a low core body temperature during surgery. A small (1.5 cm) incision was made with a surgical scalpel through the epidermal layer of the lower abdomen to the right or left of the midline. An incision of similar size was then made through the muscle layer using fine surgical scissors and 1-2 lobes of ovary were removed. From these ovarian pieces, stage V and VI oocytes were isolated. The thecal, epithelial, and follicular layers were manually dissected away from each oocyte using fine forceps. Isolated oocytes were then stored in agar-coated petri dishes containing a buffered storage solution (in mM): 88 NaCl, 2 KCl, 1 CaCl₂, 1 MgCl₂, 2.4 NaHCO₃, 5 HEPES, 2 sodium pyruvate, 0.5 theophylline, pH 7.4. The storage solution was further supplemented with penicillin (100 U/mL) and streptomycin (100 mg/mL).

Upon removal of oocytes, the muscle layer of the abdomen was sutured with surgical gut suture while the epidermal layer was sutured with a surgical silk suture. The frog was then transferred to a small plastic box containing 500 mL of water supplemented with penicillin (100 units) and streptomycin (100 µg) Upon regaining consciousness, a further 500 mL of water was added. The antibiotic-supplemented water was replaced daily and the frog monitored for three days following surgery.
2.3.2 Injection of cRNA Combinations Into Oocytes

Frozen cRNA (3 μg/μL) solutions were incubated at 65°C for 5 minutes and placed on ice prior to injection. Individual oocytes were injected with 20-50 nL of a mixture containing either α1 and β2 cRNA, α1, β2, and γ2L cRNA, or α2, β2, and γ2L cRNA (1 ng/nL of each subunit) using a Drummond Nanoject Automatic Injector (Drummond Scientific, Broomall, PA). Following injection, the oocytes were incubated in agar-coated petri dishes containing storage solution at room temperature for 2-3 days prior to electrophysiological recording. The incubation time allows for optimal expression of protein from the cRNA.

2.3.3 Electrophysiological Recording

Experiments with oocytes were performed using the two electrode voltage clamp technique. Individual oocytes were placed in a perfusion apparatus and constantly bathed with a buffered salt solution composed of (in mM): 88 NaCl, 2 KCl, 1 CaCl2, 1 MgCl2, 2.4 NaHCO3, 5 HEPES, pH 7.4. The perfusion apparatus consisted of a nylon mesh (1.5 x 1.5 mm) adhered to the bottom of a small petri dish. Two glass capillary tubes (1.5 mm diameter), connected to a series of polypropylene tubes, were fastened side-by-side to a three dimensional manipulator and positioned at a 45° angle close (1-2 mm) to the oocyte with one capillary tube directly above the oocyte. The first capillary tube (barrel A) was connected to a series of tubes containing drug/drug combinations while the second (barrel B) was connected to a tube containing control solution (physiological buffer). All solutions perfused the oocytes at a rate of 2-3 mL/min. Drug was applied by positioning barrel A directly over the oocyte while the appropriate drug/drug combination flowed through the tubing. Choice of drug application was controlled manually by a
series of valves which connected the drug-filled tubes to barrel A. This apparatus allowed for rapid application and wash off drugs from each oocyte. Peak current amplitudes in response to GABA applications could be achieved within 5-15 seconds.

Each oocyte was impaled in the animal pole with two glass microelectrodes containing 3 M KCl, and voltage-clamped at -60 mV using an Axoclamp 2A amplifier (Axon Instruments, Inc.). The glass microelectrodes were pulled in two stages using a Narishige PB-7 Micropipette Puller (Setagaya-Ku, Tokyo). Membrane current in response to application of GABA or propofol was recorded on a strip chart recorder for later analysis. GABA and propofol and alfaxalone were applied, either alone, or in combination, to the oocyte until a steady-state peak current response was obtained. A wash period of 4-15 minutes between drug applications was sufficient to obtain complete reversal of drug effects.

2.3.4 Data Analysis

Concentration-response relationships for GABA in the presence and absence of propofol were determined using a nonlinear curve-fitting protocol (Inplot4, GraphPad, Inc.). The data were fitted to the logistic equation \( I = I_{\text{max}} \left[ \frac{C^n}{(C^n+EC_{50}^n)} \right] \), where \( I \) represents the measured current response and \( C \) represents the drug concentration, to obtain estimates of maximal response \( I_{\text{max}} \), \( EC_{50} \) values and Hill coefficient \( n \). Unless otherwise noted, data were expressed as mean ± S.E.M. Where appropriate, statistical significance was tested using the Student’s t-test for paired or unpaired data, repeated measures or ordinary one-way analysis of variance (ANOVA) coupled with Student Newman-Keuls post-hoc test for multiple comparisons (Instat2, Graphpad, Inc.).
RESULTS

3.1 *Xenopus* Oocytes Express Functional GABA<sub>α</sub> Receptors

Oocytes injected with combinations of cRNA encoding GABA<sub>α</sub> receptor subunits were voltage-clamped at -60mV. Perfusion of these oocytes with GABA produced inward currents which increased in amplitude as the GABA concentration increased. Uninjected oocytes did not respond to GABA. Figure 2 shows representative current traces generated from application of increasing concentrations of GABA in the presence or absence of 2.5 μM propofol on α<sub>1</sub>β<sub>2</sub> and α<sub>1</sub>β<sub>2</sub>γ<sub>2L</sub> receptor constructs. The enhanced responses represent facilitation of GABA-mediated activation of the receptor by propofol. Analysis of the concentration-response relationship for GABA in oocytes expressing recombinant α<sub>1</sub>β<sub>2</sub> receptors yielded an EC<sub>50</sub> for GABA of 8 ± 1 μM, and a Hill slope of 0.94 ± 0.06. Oocytes expressing α<sub>1</sub>β<sub>2</sub>γ<sub>2L</sub> receptors produced an EC<sub>50</sub> for GABA of 41 ± 3 μM, and a Hill slope of 1.28 ± 0.04. In order to confirm proper expression of recombinant receptors in *Xenopus* oocytes, GABA was applied to oocytes expressing α<sub>1</sub>β<sub>2</sub> and α<sub>1</sub>β<sub>2</sub>γ<sub>2L</sub> receptors in the presence of Zn<sup>2+</sup>. Application of GABA in the presence of Zn<sup>2+</sup> (10 μM) reduced the GABA response by greater than 80% in oocytes expressing α<sub>1</sub>β<sub>2</sub> receptors. However, this concentration of Zn<sup>2+</sup> did not alter the GABA response in oocytes expressing α<sub>1</sub>β<sub>2</sub>γ<sub>2L</sub> (data not shown). As α<sub>1</sub>β<sub>2</sub> receptors are extremely sensitive to low concentrations of Zn<sup>2+</sup> [Draguhn et al., 1990; Smart et al., 1991], this experiment confirms that there is negligible expression of αβ receptors when the γ<sub>2</sub>-subunit is present.
Current responses in oocytes expressing $\alpha_1\beta_2$ receptors (upper traces) and $\alpha_1\beta_2\gamma_2$ receptors (lower traces). Increasing concentrations of GABA (μM) were applied alone or in combination with 2.5 μM propofol. Propofol enhanced the GABA response in both receptor subtypes.
$\alpha_1\beta_2$

\begin{align*}
1 \text{ G} &+ 2.5 \text{ P} \\
10 \text{ G} &+ 2.5 \text{ P} \\
100 \text{ G} &+ 2.5 \text{ P}
\end{align*}

12 \text{ min}

20 \text{ nA}

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

\begin{align*}
5 \text{ G} &+ 2.5 \text{ P} \\
5 \text{ G} &+ 2.5 \text{ P} \\
30 \text{ G} &+ 2.5 \text{ P} \\
30 \text{ G} &+ 2.5 \text{ P} \\
500 \text{ G} &+ 2.5 \text{ P} \\
500 \text{ G} &+ 2.5 \text{ P}
\end{align*}

12 \text{ min}

200 \text{ nA}
3.2 Propofol Potentiation of GABA<sub>A</sub> Receptor Function

In order to study the ability of propofol to potentiate GABA-activated currents, it was important to use a concentration of propofol that could not directly activate GABA<sub>A</sub> receptors on its own. Below 10 μM, propofol does not directly activate GABA<sub>A</sub> receptors. Therefore, 2.5 μM propofol was co-applied with increasing concentrations of GABA. Co-application of propofol and GABA resulted in a leftward shift of GABA concentration response profiles for both receptor subtypes (Fig. 3A and 2B). The EC<sub>50</sub> values for GABA in α<sub>1</sub>β<sub>2</sub> and α<sub>1</sub>β<sub>2</sub>γ<sub>2L</sub> receptor subtypes were 4 ± 1 μM and 21 ± 4 μM, respectively as compared with control EC<sub>50</sub> values of 8 μM and 41 μM, respectively. Both EC<sub>50</sub> values were significantly less than their respective controls (p < 0.05). Propofol did not alter the Hill slope in either receptor construct. Thus, 2.5 μM propofol approximately doubled the potency of GABA to activate the receptor. In oocytes expressing α<sub>1</sub>β<sub>2</sub>γ<sub>2L</sub> receptors, the presence of propofol did not significantly alter the maximal response to GABA. In contrast, the presence of propofol significantly increased the maximal response to GABA in oocytes expressing α<sub>1</sub>β<sub>2</sub> receptors (p < 0.05). These data are summarized in Table 1. 2.5 μM propofol alone did not elicit detectable current responses from oocytes expressing either receptor construct (not shown).

To further compare propofol modulation of the GABA response across the two receptor subtypes, propofol (0.1 - 50 μM) was co-administered with a constant and equieffective concentration of GABA. From the concentration response profiles for GABA, the EC<sub>20</sub> for α<sub>1</sub>β<sub>2</sub> and α<sub>1</sub>β<sub>2</sub>γ<sub>2L</sub> receptor subtypes were approximately 1 μM and 10 μM, respectively. In oocytes expressing the α<sub>1</sub>β<sub>2</sub> receptor subtype, propofol produced concentration-dependent enhancement
of the response to GABA. The maximal potentiation by propofol was \(3244 \pm 577\%\) of control with an \(EC_{50}\) of \(25 \pm 6\ \mu M\) (Fig. 4A). Propofol also enhanced the response to GABA in oocytes expressing the \(\alpha_1\beta_2\gamma_{2L}\) receptor subtype. The maximal potentiation achieved was \(1437 \pm 165\%\) of control with an \(EC_{50}\) of \(25 \pm 6\ \mu M\) (Fig. 4B). The \(EC_{50}\) values for propofol potentiation did not differ significantly across receptor subtypes. However, the maximal potentiation of the GABA response by propofol was significantly higher with \(\alpha_1\beta_2\) receptors than with \(\alpha_1\beta_2\gamma_{2L}\) receptors (\(p < 0.05\)).

The ability of the \(\alpha\)-subunit to influence propofol potentiation of the GABA response was subsequently tested with the \(\alpha_2\beta_2\gamma_{2L}\) receptor subtype. Propofol (0.1-50 \(\mu M\)) potentiated the response to 10\(\mu M\) GABA (the \(EC_{20}\) concentration in this receptor construct) in a concentration-dependent manner. The maximal potentiation induced by propofol was \(517 \pm 103\%\) of control, with an \(EC_{50}\) of \(9 \pm 1\ \mu M\). The efficacy of propofol potentiation of \(\alpha_2\beta_2\gamma_{2L}\) receptor activation was significantly less than that of the \(\alpha_1\beta_2\gamma_{2L}\) receptor subtype (\(p < 0.0005\)). Moreover, the potency of propofol potentiation was significantly greater with \(\alpha_2\beta_2\gamma_{2L}\) receptors than with \(\alpha_1\beta_2\gamma_{2L}\) receptors (\(p < 0.05\)). These data are summarized in Table 2.
Propofol (2.5 μM) potentiates the GABA response in both α₁β₂ (n=8) and α₁β₂γ₂L (n=5) receptors. (A) For α₁β₂ receptors, propofol decreased the EC₅₀ for GABA from 8 ± 1 μM to 4 ± 1 μM (p < 0.05, paired t-test). In addition, the presence of propofol increased the maximum GABA response to 126 ± 9% of control (p < 0.05, one sample t-test). (B) For α₁β₂γ₂L receptors, propofol decreased the EC₅₀ for GABA from 41 ± 3 μM to 21 ± 4 μM (p < 0.05, paired t-test) without changing the maximum GABA response.
Table 1. Effect of 2.5 μM propofol on the GABA concentration-response profile for α₁β₂ and α₁β₂γ₂L receptor subtypes.

<table>
<thead>
<tr>
<th>Subtype</th>
<th>EC₅₀ (μM)</th>
<th>Maximum Response (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GABA</td>
<td>GABA + Propofol</td>
</tr>
<tr>
<td>α₁β₂</td>
<td>8 ± 1.2</td>
<td>4 ± 1.3*</td>
</tr>
<tr>
<td>α₁β₂γ₂L</td>
<td>41 ± 3.0</td>
<td>21 ± 3.9*</td>
</tr>
</tbody>
</table>

All values represented as mean ± S.E.M.
* p<0.05 as compared with respective GABA EC₅₀
** p<0.05 as compared with GABA maximum response
Figure 4.

Propofol (0.1-50 µM) potentiates the GABA EC$_{20}$ response in oocytes expressing $\alpha_1\beta_2$ (n=7), $\alpha_4\beta_2\gamma_2L$ (n=6), and $\alpha_2\beta_2\gamma_2L$ (n=8) receptors in a concentration-dependent manner. (A) Propofol potentiated the GABA response in $\alpha_1\beta_2$ and $\alpha_4\beta_2\gamma_2L$ receptors with equal potency (EC$_{50}$: 25 ± 6 µM and 25 ± 6 µM, respectively). However, the maximal degree of potentiation was greater in $\alpha_4\beta_2$ receptors than in $\alpha_1\beta_2\gamma_2L$ receptors (3244 ± 577% and 1437 ± 165% respectively, p < 0.05). (B) Replacing the $\alpha_1$ subunit with the $\alpha_2$ subunit decreased the EC$_{50}$ to 9 ± 1 µM (p < 0.05) and reduced the maximal degree of potentiation to 517 ± 103% (p < 0.0005). For above comparisons, statistical analyses were performed using the paired t-test or ANOVA with subsequent post-hoc tests.
Table 2. Propofol potentiation of response to GABA EC$_{20}$ (1 or 10 µM) from $\alpha_1\beta_2$, $\alpha_1\beta_2\gamma_{2L}$ and $\alpha_2\beta_2\gamma_{2L}$ receptor subtypes

<table>
<thead>
<tr>
<th>Subtype</th>
<th>EC$_{50}$ (µM)</th>
<th>Maximum Potentiation (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha_1\beta_2$</td>
<td>25 ± 6.1</td>
<td>3244 ± 577</td>
</tr>
<tr>
<td>$\alpha_1\beta_2\gamma_{2L}$</td>
<td>25 ± 6.1</td>
<td>1437 ± 165**</td>
</tr>
<tr>
<td>$\alpha_2\beta_2\gamma_{2L}$</td>
<td>9 ± 1.1*</td>
<td>517 ± 103§</td>
</tr>
</tbody>
</table>

All values represented as mean ± S.E.M.

* p<0.05 as compared with EC$_{50}$ for $\alpha_1\beta_2\gamma_{2L}$ subtype

** p<0.05 as compared with maximum potentiation of $\alpha_1\beta_2$ subtype

§ p<0.0005 as compared with maximum potentiation of $\alpha_2\beta_2\gamma_{2L}$
3.3 Direct Activation of The GABA<sub>α</sub> Receptor By Propofol

In addition to their ability to potentiate GABA-mediated responses, general anaesthetics including propofol are able to directly activate the receptor in the absence of GABA. It has been suggested that direct activation of the GABA<sub>α</sub> receptor is the property that distinguishes anaesthetic agents from non-anaesthetic modulators such as benzodiazepines. To investigate the contribution of the γ<sub>2</sub>-subunit to the propofol’s direct effects on the GABA<sub>α</sub> receptor, propofol (10-100 μM) was applied in the absence of GABA to oocytes expressing recombinant α<sub>1</sub>β<sub>2</sub> or α<sub>1</sub>β<sub>2</sub>γ<sub>2L</sub> receptor subtypes. Figure 5 shows representative current traces of direct activation by propofol of α<sub>1</sub>β<sub>2</sub> and α<sub>1</sub>β<sub>2</sub>γ<sub>2L</sub> receptors, respectively. Activation of both receptor subtypes by propofol was concentration-dependent. Also notable, however, is the time course of propofol activation. While the peak amplitude of currents generated by GABA were attained within the first 30 seconds of application (Fig. 2), propofol responses did not attain peak amplitude until 2-5 minutes after onset of action. Also shown are current traces generated from co-application of propofol with a very low concentration of GABA (0.1 μM) to oocytes. 0.1 μM GABA alone produced no detectable currents in oocytes expressing either receptor construct (data not shown).

In oocytes expressing α<sub>1</sub>β<sub>2</sub> receptors, 0.1 μM GABA dramatically enhanced direct activation of the receptor by propofol (Fig. 6A). This enhancement was more pronounced at the lower concentrations of propofol and achieved statistical significance (p < 0.001) with 10 μM and 50 μM propofol. In contrast, oocytes expressing α<sub>1</sub>β<sub>2</sub>γ<sub>2L</sub> receptors did not show a significantly enhanced response to propofol in the presence of 0.1 μM GABA (Fig. 6B). Comparison of propofol’s ability to directly activate α<sub>1</sub>β<sub>2</sub> and α<sub>1</sub>β<sub>2</sub>γ<sub>2L</sub> receptor subtypes revealed that, at high
concentrations, $\alpha_1\beta_2$ receptors were much more susceptible to activation by propofol (Fig. 6C).

The role of the $\alpha$-subunit on direct activation of the GABA$_A$ receptor by propofol was then studied. Propofol (10-100 $\mu$M) was applied to oocytes expressing $\alpha_1\beta_2\gamma_2L$ or $\alpha_2\beta_2\gamma_2L$ receptors (Fig. 7A and B). Propofol directly activated both receptor subtypes in a concentration-dependent manner. The presence of 0.1 $\mu$M GABA, however, did not significantly alter propofol's ability to activate either receptor subtype. At high concentrations, $\alpha_2\beta_2\gamma_2L$ receptors appear to be more sensitive to the direct effects of propofol than $\alpha_1\beta_2\gamma_2L$ receptors (Fig. 7C).

The data from Figures 6 and 7 were subsequently expressed as a percent enhancement of the response to propofol alone (Fig. 8). In the $\alpha_1\beta_2$ receptor subtype, 0.1 $\mu$M GABA potentiated the response to 10 $\mu$M propofol by 1615 $\pm$ 296.2%. This degree of potentiation was markedly greater than that which was produced from $\alpha_1\beta_2\gamma_2L$ or $\alpha_2\beta_2\gamma_2L$ receptors ($p < 0.001$).
Figure 5.

Current responses in oocytes containing $\alpha_1\beta_2$ receptors (upper) and $\alpha_1\beta_2\gamma_2$ receptors (lower). Propofol (10-100 μM) was applied in the presence and absence of 0.1 μM GABA. In $\alpha_1\beta_2$ receptors, GABA enhanced the response to propofol.
$\alpha_1\beta_2\gamma_{2L}$

10 P 10 P 50 P 50 P 100 P 100 P
+ + + + +
0.1 G 0.1 G 0.1 G 0.1 G 0.1 G

12 min

20 nA

$\alpha_1\beta_2$

10 P 10 P 50 P 50 P 100 P 100 P
+ + + + +
0.1 G 0.1 G 0.1 G 0.1 G 0.1 G

12 min

50 nA
Propofol (10–100 μM) directly activates both α₁β₂ (A) receptors (n=6) and α₁β₂γ₂L (B) receptors (n=7) in a concentration-dependent manner. Addition of a low concentration of GABA (0.1 μM) enhanced propofol activation of α₁β₂ receptors (*, p < 0.001). However, GABA did not significantly alter the response to propofol in α₁β₂γ₂L receptors. At high concentrations, propofol directly activated α₁β₂γ₂L receptors to a lesser extent than α₁β₂ receptors (C) (#, p < 0.05). Statistical comparisons were performed using ANOVA, with subsequent post tests.
Propofol (10-100 μM) directly activates both $\alpha_1\beta_2\gamma_2$ (A) receptors ($n=7$) and $\alpha_2\beta_2\gamma_2$ (B) receptors ($n=8$) in a concentration-dependent manner. Addition of a low concentration of GABA (0.1 μM) did not significantly alter propofol activation of $\alpha_1\beta_2\gamma_2$ receptors or $\alpha_2\beta_2\gamma_2$ receptors. However, high concentrations of propofol directly activated $\alpha_2\beta_2\gamma_2$ receptors to a greater extent than $\alpha_1\beta_2\gamma_2$ receptors (C) ($\#$, $p < 0.05$). Statistical comparisons were performed using ANOVA with subsequent post tests.
Figure 8.

In the presence of 0.1 µM GABA, propofol activation is enhanced. Bars represent the difference between propofol responses in the presence and absence of GABA normalized as a percent of control (propofol alone). The modulation of 10 µM propofol activation by GABA was significantly greater in α₁β₂ receptors than in α₁β₂γ₂L or α₂β₂γ₂L receptors (**, p < 0.001). Statistical comparisons were made using ANOVA, with subsequent post tests.
3.4 **Propofol and Alfaxalone Do Not Act Synergistically at The GABA<sub>A</sub> Receptor**

In oocytes expressing α<sub>1</sub>β<sub>2</sub> receptors, both propofol (0.1-50 μM) and alfaxalone (0.01-50 μM) potentiated the GABA response in a concentration-dependent manner (Fig. 9A and B). Similarly, α<sub>1</sub>β<sub>2</sub>γ<sub>2</sub> receptor activation was facilitated by propofol and alfaxalone (Fig. 10A and B).

In order to study any possible interaction between the two anaesthetic agents, the control GABA concentration for each receptor subtype was combined with a fixed concentration of one anaesthetic and increasing concentrations of the other. In experiments where increasing concentrations of alfaxalone were applied, the propofol concentration was fixed at 1 μM. Accordingly, experiments which involved application of increasing propofol concentrations used a fixed concentration of 250 nM alfaxalone. Both 1 μM propofol and 250 nM alfaxalone significantly enhanced the control GABA responses in α<sub>1</sub>β<sub>2</sub> and α<sub>1</sub>β<sub>2</sub>γ<sub>2L</sub> receptors. Notable is the potency and efficacy of alfaxalone potentiation in the two receptor subtypes. The EC<sub>50</sub> values for α<sub>1</sub>β<sub>2</sub> and α<sub>1</sub>β<sub>2</sub>γ<sub>2L</sub> receptors (6.0 ± 1.1 and 3.3 ± 1.4, respectively) were not different. Moreover, the maximal potentiation of the GABA response by alfaxalone for α<sub>1</sub>β<sub>2</sub> and α<sub>1</sub>β<sub>2</sub>γ<sub>2L</sub> receptor constructs (1036 ± 157% and 762 ± 150%, respectively) was not different.

Experimental values were compared against the theoretical additive (R<sub>T</sub>) response predicted by the equation: R<sub>T</sub> = [G<sub>p</sub> + G<sub>α</sub>]-100%, where G<sub>p</sub> represents the percent potentiation of the GABA response by propofol alone, and G<sub>α</sub> represents the percent potentiation of the GABA response by alfaxalone alone. For oocytes expressing α<sub>1</sub>β<sub>2</sub> receptors, 250 nM alfaxalone did not enhance potentiation of the control GABA response by propofol beyond the theoretical additive response (Fig. 11A). These results were supported by the finding that 1 μM propofol failed to
enhance potentiation of the GABA response by alfaxalone beyond the theoretical additive response (Fig. 11B). Oocytes expressing $\alpha_1\beta_2\gamma_{1L}$ receptors generated similar results to those obtained with $\alpha_1\beta_2$-expressing oocytes. Neither anaesthetic could enhance potentiation of the GABA response by the other beyond the calculated theoretical additive responses (Fig. 12A and B).
Figure 9.

(A) Alfaxalone (0.01-50 μM) potentiates the response to a low concentration (0.5 μM) of GABA in oocytes (n=7) expressing α₁β₂ GABA₅ receptors. (B) Propofol (0.1-50 μM) also potentiates the response to 0.5 μM GABA in oocytes (n=5) expressing α₁β₂ GABA₅ receptors.
A

% Control Response (0.5 µM GABA)

Log [Alfaxalone] (M)

B

% Control Response (0.5 µM GABA)

Log [Propofol] (M)
Figure 10.

(A) Alfaxalone (0.01-50 μM) potentiates the response to a low concentration (5 μM) of GABA in oocytes (n=5) expressing α₁β₂γ₂L GABAₐ receptors. (B) Propofol (0.1-50 μM) also potentiates the response to 5 μM GABA in oocytes (n=6) expressing α₁β₂γ₂L GABAₐ receptors.
Figure 11.

(A) 250 nM alfaxalone potentiated the response to 0.5 µM GABA in oocytes (n=5) expressing α₄β₂ receptors (open bar). GABA was then combined with either propofol (0.1-50 µM) alone (solid bars), or with propofol and 250 nM alfaxalone (speckled bars). ANOVA revealed that co-application of propofol and alfaxalone did not enhance the GABA response beyond the theoretical additive response (hatched bars). (B) 1 µM propofol potentiated the response to 0.5 µM GABA in oocytes (n=7) expressing α₄β₂ receptors (open bar). GABA was then combined with either alfaxalone (0.01-50 µM) alone (solid bars), or with alfaxalone and 1 µM propofol (speckled bars). ANOVA revealed that co-application of propofol and alfaxalone did not enhance the GABA response beyond the theoretical additive response (hatched bars).
Figure 12.

(A) 250 nM alfaxalone potentiated the response to 0.5 μM GABA in oocytes (n=6) expressing α₁β₂γ₂L receptors (open bar). GABA was then combined with either propofol (0.1-50 μM) alone (solid bars), or together with propofol and 250 nM alfaxalone (speckled bars). ANOVA revealed that co-application of propofol and alfaxalone did not enhance the GABA response beyond the theoretical additive response (hatched bars). (B) 1 μM propofol potentiated the response to 0.5 μM GABA in oocytes (n=5) expressing α₁β₂γ₂L receptors (open bar). GABA was then combined with either alfaxalone (0.01-50 μM) alone (solid bars), or together with alfaxalone and 1 μM propofol (speckled bars). ANOVA revealed that co-application of propofol and alfaxalone did not enhance the GABA response beyond the theoretical additive response (hatched bars).
DISCUSSION

4.1 Anaesthetic Actions on GABA<sub>\alpha</sub> Receptors: The Role of Subunit Composition

Propofol belongs to a group of anaesthetic, anxiolytic, and sedative-hypnotic agents which have a profound effect on GABA<sub>\alpha</sub> receptor function. Similar to benzodiazepines, barbiturates, alcohols, and the anaesthetic class of steroids, propofol can enhance GABA-mediated activation of the GABA<sub>\alpha</sub> receptor [Hara et al., 1994; Peters et al., 1988; Reynolds & Prasad, 1991; Asano & Ogasawara, 1981]. What is not as well known, however, is the relationship between propofol action and GABA<sub>\alpha</sub> receptor subunit composition.

Modulation of GABA<sub>\alpha</sub> receptor function by drugs is often, if not always, influenced by the subunit composition of a given GABA<sub>\alpha</sub> receptor subtype. For example, benzodiazepine action on GABA<sub>\alpha</sub> receptors is dependent upon the presence of a \(\gamma_2\)-subunit within the receptor construct [Pritchett et al., 1989; Wafford et al., 1993]. In addition, the type of \(\alpha\)-subunit present within the GABA<sub>\alpha</sub> receptor can influence the degree to which benzodiazepines modulate receptor function [Pritchett et al., 1989; Luddens et al., 1990]. The pattern of subunit dependence is not necessarily shared identically among other classes of compounds known to modulate GABA<sub>\alpha</sub> receptor function [Wafford et al., 1992; Ducic et al., 1995; Harris et al., 1995; Wafford et al., 1994]. In contrast to benzodiazepines for instance, propofol can potentiate the activity of GABA<sub>\alpha</sub> receptors assembled with or without a \(\gamma\)-subunit [Jones et al., 1995]. Data from another study indicate that different receptor subtypes may be differentially sensitive to the modulatory actions of propofol [Sanna et al., 1995b]. Previous data from our laboratory suggest that the \(\gamma_2\)-
subunit contributes to sensitivity of the GABA<sub>A</sub> receptor to modulation by propofol [Reynolds and Maitra, 1996]. In addition, the study demonstrated that propofol acts synergistically with benzodiazepines to potentiate GABA<sub>A</sub> receptor activation, and that the presence of the γ<sub>2</sub>-subunit significantly altered both the efficacy and potency of propofol to potentiate GABA<sub>A</sub> receptor function.

4.2 Role of The γ<sub>2</sub>-Subunit on The Modulatory and Direct Effects of Propofol

To investigate the role of the γ<sub>2</sub>-subunit on propofol potentiation of GABA<sub>A</sub> receptor function, α<sub>i</sub>β<sub>i</sub> and α<sub>i</sub>β<sub>2γ<sub>2</sub>L</sub> receptor subtypes were studied. Increasing concentrations of GABA activated both receptor subtypes in a concentration-dependent manner. The presence of 2.5 μM propofol, however, significantly increased the potency of GABA to activate both GABA<sub>A</sub> receptor subtypes by approximately two-fold. For the α<sub>i</sub>β<sub>2γ<sub>2</sub>L</sub> receptor subtype, propofol did not alter the maximal response to GABA, and a parallel shift of the GABA concentration-response curve was produced. In contrast, propofol significantly increased the maximal response of the α<sub>i</sub>β<sub>2</sub> receptor subtype to GABA, thereby producing a non-parallel shift in the GABA concentration-response curve. These results provide evidence that the γ<sub>2</sub>-subunit influences potentiation of GABA<sub>A</sub> receptor activation by propofol.

The ability of propofol to produce a non-parallel shift in the GABA concentration-response profile in αβ receptor constructs is common to anaesthetics such as barbiturates and neurosteroids, but not to the non-anaesthetic benzodiazepines [Horne et al., 1993]. It is not clear what causes the increased efficacy of GABA-mediated activation. However, one possible
explanation is that anaesthetics are able to change the conductance properties of the channel. A main conductance level as well as multiple subconductance levels are associated with GABA_\alpha receptor channel opening, with \( \alpha\beta \) receptors opening into a lower main conductance level than \( \alpha\beta\gamma \) receptors [reviewed by Macdonald and Olsen, 1994]. Anaesthetics such as propofol may increase the probability of \( \alpha\beta \) receptors opening to a higher conductance level than the main conductance level gated by GABA alone. Such an event would be able to produce the non-parallel shift observed with anaesthetic modulation. The open and burst properties of \( \alpha\beta \) and \( \alpha\beta\gamma \) receptors are also different [Angelotti and Macdonald, 1993]. On average, \( \alpha\beta\gamma \) receptors opened almost three times longer than \( \alpha\beta \) receptors. Moreover, while three openings constituted a burst from \( \alpha\beta\gamma \) receptors, \( \alpha\beta \) receptors only opened once per burst. Based on these data, one can also speculate that propofol may increase the efficacy of GABA at \( \alpha\beta \) receptors by increasing open duration, number of bursts per opening, or both.

Increasing concentrations of propofol (0.1-50 \( \mu \)M) applied to each receptor subtype in combination with the GABA EC_{20} produced a concentration-dependent potentiation of the GABA response. Although propofol potentiated the GABA response in both \( \alpha_1\beta_2 \) and \( \alpha_1\beta_2\gamma_{2L} \) receptor subtypes with equal potency, the maximal potentiation found with \( \alpha_1\beta_2 \) receptors was double that found with \( \alpha_1\beta_2\gamma_{2L} \) receptors. The ability of propofol to differentially potentiate equieffective GABA responses in the two receptor subtypes supports our hypothesis that the \( \gamma_{2} \)-subunit indeed influences the modulatory actions of propofol on the GABA_\alpha receptor. More specifically, the absence of the \( \gamma_{2} \)-subunit confers upon GABA_\alpha receptors increased sensitivity to positive modulation by propofol. This greater efficacy for propofol in \( \alpha_1\beta_2 \) receptors might also be
explained by an increase in the single channel conductance, open properties, or burst properties of GABA-gated currents in $\alpha_1\beta_2$, but not in $\alpha_1\beta_2\gamma_{2L}$ receptors by propofol.

In contrast, by testing the ability of alfaxalone to potentiate GABA-mediated activation of $\alpha_1\beta_2$ and $\alpha_1\beta_2\gamma_{2L}$ receptors we have demonstrated that neither the potency nor the efficacy of alfaxalone potentiation were different between the two receptor subtypes. These results, together with other biochemical and single channel data, demonstrate that propofol and alfaxalone modulate GABA$_\alpha$ receptor function via distinct mechanisms [Concas et al., 1991; Twyman and Macdonald, 1992, Orser et al., 1994] and have different subunit dependence. Indeed, other work currently being done in this laboratory suggests that the $\beta$-subunit is most critical in determining the efficacy for alfaxalone potentiation of the GABA response.

The ability of propofol to directly activate $\alpha_1\beta_2$ and $\alpha_1\beta_2\gamma_{2L}$ receptors was then tested. Application of propofol (10-100 $\mu$M) directly activated both receptor subtypes in a concentration-dependent manner. At higher concentrations, however, propofol produced much larger currents in $\alpha_1\beta_2$ receptors than in $\alpha_1\beta_2\gamma_{2L}$ receptors. This indicates that, contrary to our hypotheses, direct activation of the GABA$_\alpha$ receptor by propofol is, indeed, altered by the $\gamma_2$-subunit. Thus, the $\gamma_2$-subunit contributes to direct activation, as well as positive modulation, of the GABA$_\alpha$ receptor by propofol. Furthermore, 0.1 $\mu$M GABA, which on its own produced no detectable current, markedly enhanced the response to propofol in $\alpha_1\beta_2$ receptors. However, this concentration of GABA failed to alter the degree of activation of $\alpha_1\beta_2\gamma_{2L}$ and $\alpha_2\beta_2\gamma_{2L}$ receptors by propofol. These data indicate that, just as propofol can modulate GABA-mediated activation of the GABA$_\alpha$ receptor, GABA can likewise modulate propofol-mediated activation. In addition, the ability of
GABA to potentiate propofol-mediated activation of the GABA_\textsubscript{A} receptor is dependent upon subunit composition. In this case, the presence of a \gamma_2-subunit decreases GABA modulation of the propofol response.

Since \alpha_1\beta_2 receptors appear to be more sensitive than \alpha_1\beta_2\gamma_2\alphaL receptors to propofol potentiation and direct activation, the two effects of propofol may possess similar subunit dependence. If so, it follows that propofol potentiation and direct activation may represent a mechanism similar to that of GABA activation. For example, two binding sites for propofol may exist. While binding of one molecule of propofol is sufficient to potentiate activation of the GABA_\textsubscript{A} receptor by another agonist, full activation of the receptor requires binding of two molecules of propofol.

One other notable feature is that while activation of \alpha_1\beta_2 or \alpha_1\beta_2\gamma_2\alphaL receptors by 10 \textmu M propofol was not significantly different, maximal potentiation of the propofol response by GABA occurred at this concentration in the \alpha_1\beta_2 receptor subtype (Fig. 8). During induction of anaesthesia, the blood concentration of propofol in humans is approximately 50 \textmu M, whereas maintenance of anaesthesia is associated with a blood concentration of approximately 9 \textmu M [Schuttler et al., 1988; Jensen et al., 1994]. Therefore, 10 \textmu M propofol is well within the clinical range of concentrations required to induce and maintain anaesthesia. Since GABA is found endogenously throughout the central nervous system, it is possible that if neuronal populations containing the \alpha_1\beta_2 receptor subtype exist in the CNS, these cell populations would be sensitive to both direct and modulatory effects of propofol during anaesthesia.
4.3 Role of The α-Subunit on The Modulatory and Direct Effects of Propofol

To investigate the role of the α-subunit on propofol potentiation of GABA<sub>A</sub> receptor function, α<sub>1</sub>β<sub>2</sub>γ<sub>2L</sub> and α<sub>2</sub>β<sub>2</sub>γ<sub>2L</sub> receptor subtypes were studied. Interestingly, maximal potentiation of the GABA EC<sub>50</sub> concentration by propofol in the α<sub>2</sub>-containing receptors was only one-third of that found with the α<sub>1</sub>-containing receptors. Furthermore, propofol potentiated responses in α<sub>2</sub>β<sub>2</sub>γ<sub>2L</sub> receptors with over twice the potency observed with α<sub>1</sub>β<sub>2</sub>γ<sub>2L</sub> receptors. These results clearly demonstrate that the type of α-subunit present in the GABA<sub>A</sub> receptor influences the ability of propofol to positively modulate the receptor. The difference in apparent affinity also implicates the α-subunit as a major contributor to the binding site of propofol on the GABA<sub>A</sub> receptor.

Application of 10-100 μM propofol directly activated both α<sub>1</sub>β<sub>2</sub>γ<sub>2L</sub> and α<sub>2</sub>β<sub>2</sub>γ<sub>2L</sub> receptors. Once again, however, the degree of propofol activation differed between receptor subtypes. Here, α<sub>2</sub>-containing receptors were more sensitive to direct activation by propofol than α<sub>1</sub>-containing receptors. Interestingly, neither receptor subtype was sensitive to potentiation of the propofol response by 0.1 μM GABA. First of all, the data reveal that, contrary to our hypothesis, the α-subunit has a significant influence on the ability of propofol to directly activate GABA<sub>A</sub> receptors. Secondly, the results indicate that the γ-subunit (or rather the lack thereof), but not the α-subunit, determines whether or not low concentrations of GABA can positively modulate receptor activation by propofol. An intriguing difference arises when the role of the α-subunit on propofol potentiation and direct activation are examined together. While α<sub>1</sub>-containing receptors displayed greater efficacy for propofol potentiation of the GABA response than α<sub>2</sub>-containing...
receptors, propofol was less efficacious at directly activating $\alpha_1$-containing receptors than $\alpha_2$-containing receptors. The results therefore suggest that although both the modulatory and direct effects of propofol are influenced by receptor subunit composition, they do not share identical subunit dependence.

These subunit combinations are co-localized in different areas of the mammalian brain. While $\alpha_1$-, $\beta_2$-, and $\gamma_2$-subunits are co-localized in hippocampal and cortical GABAergic interneurons as well as cerebellar Purkinje cells [Fritschy et al., 1992; Gao and Fritschy, 1994], $\alpha_2$-, $\beta_2$-, and $\gamma_2$-subunits are present on spinal cord motoneurons and hippocampal pyramidal cells [Laurie et al., 1992; Wisden et al., 1992; Persohn et al., 1991]. Taken together, one can speculate that an explanation behind the broad spectrum of behavioural effects observed from anaesthetic administration may be based, in part, upon GABA$_A$ receptor sensitivity. For example, spinal cord motoneurons are thought to be involved in mediation of motor co-ordination. Therefore propofol modulation of GABA$_A$ receptors in this cell type may explain the loss of motor co-ordination in anaesthesia. Similarly, propofol action on hippocampal and cortical pyramidal neurons may contribute to propofol’s sedative/hypnotic effects.

### 4.4 Interaction Between General Anaesthetics at The GABA$_A$ Receptor

Using $\alpha_1\beta_2$ and $\alpha_1\beta_2\gamma_2L$ receptor subtypes, we attempted to determine if a combination of propofol and alfaxalone produced a supra-additive potentiation of a constant GABA response. Application of either propofol or alfaxalone to a constant GABA concentration produced a concentration-dependent potentiation of the GABA response in both $\alpha_1\beta_2$ or $\alpha_1\beta_2\gamma_2L$ receptor
subtypes. Propofol (1 μM) did not augment the degree of potentiation of the GABA response by alfaxalone in either receptor subtypes. Similarly, alfaxalone (250 nM) did not facilitate potentiation of the GABA response by propofol. Thus, it appears that although propofol and alfaxalone occupy distinct sites on the GABA\textsubscript{A} receptor and produce distinct changes in single channel activity, their combined presence does not allosterically alter each other's modulatory abilities. These results, however, do not preclude the possibility that supra-additive interaction may occur between other classes of anaesthetics. Just as potentiation of propofol-mediated activation of the receptor by GABA seems to depend upon the presence or absence of a γ-subunit, one should not disregard the possibility that lack of interaction between propofol and alfaxalone may be due to the presence or absence of a particular subunit(s).

4.5 Future Studies

This study has demonstrated that the presence or absence of the γ\textsubscript{2}-subunit influences the effects of propofol. However, most neurons within the central nervous system appear to co-assemble with either γ- or δ- subunits, rather than existing simply as αβ oligomers. Therefore, an interesting study would be to determine whether replacing, rather than omitting the γ-subunit confers onto the receptor differential sensitivity to propofol action. Single channel studies of anaesthetic action on GABA\textsubscript{A} receptors only exist for GABA\textsubscript{A} receptors characteristic of the αβγ subtypes. In order to more clearly understand the differential effects of propofol seen on populations of αβ and αβγ receptors, it would be prudent to study propofol's ability to modulate the single channel properties of α₁β₂ receptors. The experiments involving direct activation of
GABA\textsubscript{A} receptors by propofol were not based on equieffective concentrations of propofol. Propofol may not exhibit the same potency when activating $\alpha_1\beta_2$, $\alpha_1\beta_2\gamma_2\lambda$, or $\alpha_2\beta_2\gamma_2\lambda$ receptors. Therefore, plotting complete dose-response relationships for propofol activation of these receptor subtypes would help clarify this issue. Interaction between propofol and alfaxalone does not appear to exist. However, barbiturates and volatile anaesthetics also possess distinct binding sites on the GABA\textsubscript{A} receptor. Therefore, it would be interesting to study whether propofol interacts with other classes of anaesthetic compounds at the GABA\textsubscript{A} receptor.
SUMMARY AND CONCLUSIONS

5.1 Summary

1) The presence of the $\gamma_{2L}$-subunit significantly alters the ability of propofol to both potentiate $\text{GABA}_A$ receptor activation and directly activate the $\text{GABA}_A$ receptor.

2) The type of $\alpha$-subunit present significantly alters the ability of propofol to both potentiate $\text{GABA}_A$ receptor activation and directly activate the $\text{GABA}_A$ receptor.

3) The modulatory and direct effects of propofol do not share the same form of subunit dependence.

4) The $\gamma_{2L}$-subunit significantly influences the ability of GABA to potentiate $\text{GABA}_A$ receptor activation by propofol.

5) The abilities of propofol and alfaxalone to potentiate $\text{GABA}_A$ receptor activation do not share the same type of subunit dependence.

6) Propofol and alfaxalone interact in a simple additive nature at the $\text{GABA}_A$ receptor.

5.2 Conclusions

The intravenous general anaesthetic, propofol, shares with other classes of general anaesthetics, such as barbiturates and anaesthetic steroids, the ability to modulate $\text{GABA}_A$ receptor function. These anaesthetics can potentiate $\text{GABA}_A$ receptor activation as well as directly activate the receptor in the absence of another agonist. The $\beta$-subunit likely contributes...
to the direct effects of propofol on GABA receptors. We have now demonstrated that the α- and γ-subunits also contribute to both modulatory and direct effects of propofol on GABA receptors. While there is now clear evidence that subunit dependence exists for propofol action, the nature of the subunit dependence is different from at least one other class of anaesthetic, the neurosteroids. Furthermore, subunit dependence is different for the modulatory and direct effects of propofol, suggesting the possibility that multiple sites/mechanisms govern propofol’s dual actions on the GABA receptors.

Another novel finding from this research is the observation that with one receptor construct (α1β2), low concentrations of GABA can dramatically enhance activation of GABA receptors by propofol. In addition, the modulatory action of GABA is also dependent upon receptor subunit composition. The reciprocal facilitation of receptor activation between propofol and GABA leads to the possibility that neurons expressing this receptor construct would be sensitive to the direct and modulatory effects of propofol during anaesthesia.

Finally, this research has determined that combination of propofol and the anaesthetic steroid alfaxalone produces a simple additive potentiation of GABA receptor function. In contrast to these results, previous findings by our lab demonstrated that propofol and the anxiolytic benzodiazepines act synergistically to potentiate GABA receptor activation. Taken together, this study has established a new set of determinants for propofol action at the GABA receptor.
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