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ALLOSTERIC MODULATION OF $GABA_A$ RECEPTORS BY STEROIDS AND RELATED COMPOUNDS: FUNCTIONAL AND BINDING STUDIES

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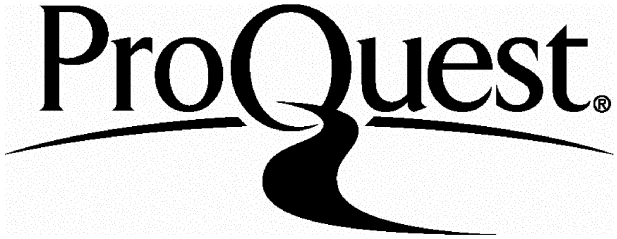

To my parents

谁言寸草心

报得三春晖

ABSTRACT

The allosteric modulation of $GABA_A$ receptors by neuroactive steroids and related compounds was investigated, using ^{3}H-flunitrazepam binding to synaptic membranes prepared from rat whole brain and electrophysiological recording from rat cuneate nucleus in a grease-gap apparatus. The enhancement of ^{3}H-flunitrazepam binding by pregnanolone, a reduced metabolite of progesterone, involves GABA-dependent and GABA-independent components. A low affinity component of pregnanolone's action is selectively antagonised by 11-ketoprogesterone. However, progesterone itself antagonises both high and low affinity components with only a slight selectivity against the low affinity component. Pregnenolone, the precursor of progesterone, has no effect while epipregnanolone, the 3β analogue of pregnanolone, causes a small general depression of the effects of pregnanolone. The properties described above strongly suggest multiple binding sites for pregnanolone on $GABA_A$ receptors. The binding sites for pregnanolone are separate from those for propofol, which enhances ^{3}H-flunitrazepam binding in a largely GABA-independent manner that is not sensitive to 11-ketoprogesterone. The enhancement of ^{3}H-flunitrazepam binding by Δ^{16}-alphaxalone is quite different from the enhancement by pregnanolone and propofol. It is GABA-dependent, with similar potency and efficacy to alphaxalone, but less potent and much less efficacious than pregnanolone. However, the interactions between pairs of steroids when used in combination suggest that Δ^{16}-alphaxalone may share the same range of binding sites as pregnanolone and alphaxalone. Compared with chlormethiazole and pentobarbitone, loreclezole is the most efficacious enhancer of ^{3}H-flunitrazepam binding. The enhancement by loreclezole is due to an increase in binding affinity, is largely GABA-independent and can be partially blocked by chlormethiazole. However, chlormethiazole and pentobarbitone are more efficacious than loreclezole in potentiating responses to muscimol in cuneate nucleus slices. The interactions among loreclezole, chlormethiazole and pentobarbitone suggest separate sites of action for them on the $GABA_A$ receptor complex.

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CHAPTER 1

GENERAL INTRODUCTION

1.1. WHEN WAS GABA ESTABLISHED AS A NEUROTRANSMITTER?

To prove that a substance is a transmitter at a particular junction, the substance has to fulfil certain criteria. In practice, any substance that is a normal constituent of nervous tissue and has a strong excitatory or inhibitory action on nerve or muscle cells is potentially a transmitter: the probability that it is a transmitter increases with the amount of supporting information about the characteristics of its action, its metabolism and turnover in the tissue, its liberation during activity, and the possibility of blocking synaptic transmission by inactivating postsynaptic receptors with either an excess of the supposed transmitter or some more or less specific antagonist (Krnjević, 1974).

γ-Aminobutyric acid (GABA) was first discovered as a brain constituent in 1950 (Roberts & Frankel, 1950). This initiated numerous studies on its distribution, metabolism, action *etc* (eg. Weinstein *et al.*, 1963; Roberts & Frankel, 1951; Krnjević, & Schwartz, 1966). Subsequently, the release of GABA was demonstrated from surface of the cerebral cortex (Jasper *et al.*, 1965), and in response to inhibitory nerve stimulation from lobster inhibitory neurons (Iversen *et al.*, 1967). All this evidence accumulated to suggest that GABA may serve a neurotransmitter function. Now, GABA is recognized as the major inhibitory transmitter in the CNS. It is estimated that, depending on the brain region, 20 to 50 % of all central synapses use GABA as their transmitter (Sieghart, 1995).

Receptors for GABA are divided into three pharmacologically distinct receptor classes, which are denoted $GABA_A$, $GABA_B$ and $GABA_C$. $GABA_A$ receptors, the Cl^- ion channel associated proteins formed by 5 out of 14 subunit subtypes: α (α1-α6), β (β1-β4), γ (γ1-γ3) and δ (see below), are activated by GABA and muscimol, inhibited by bicuculline and picrotoxin (Sieghart, 1995). $GABA_B$ receptors are G-protein associated receptors that can be stimulated by GABA and (-)-baclofen and inhibited by phaclofen (Bowery, 1993). $GABA_C$ receptors, found predominantly in vertebrate retina, are also associated with the Cl^- ion channel. They are defined by their insensitivity to the $GABA_A$ receptor antagonist bicuculline and the $GABA_B$ receptor agonist (-)-baclofen (Bormann & Feigenspan, 1995). The GABA-receptor ρ subunits are likely to be part of the $GABA_C$ receptors as their presence in rat retinal bipolar cells coincides with the $GABA_C$-receptor pharmacology of those cells. Although both the ρ1 and ρ2 polypeptides are often referred to as $GABA_A$-receptor subunits, this designation may be incorrect. In fact, the ρ subunits do not assemble with either $GABA_A$-receptor α or β subunits, but form homo-oligomeric Cl^- ion channels (Bormann & Feigenspan, 1995). This novel class of $GABA_C$ receptors are activated by GABA, muscimol as well as the partially folded GABA analogs *cis*- or *trans*- 4-aminocrotonic acid (CACA and TACA) (Kusama *et al.*, 1993). However, recent evidence suggests that in contrast to the receptors formed by the human ρ1 or ρ2 subunits when expressed in *Xenopus* oocytes (Shimada *et al.*, 1992; Takahashi *et al.*, 1995), $GABA_C$ receptors in the rat are almost insensitive to picrotoxin as an antagonist (Feigenspan *et al.*, 1993; Pan & Lipton, 1995).

In this thesis, only $GABA_A$ receptors will be discussed.

1.2. WHERE ARE $GABA_A$ RECEPTORS?

Electrophysiological and ligand binding studies have revealed that GABA-responsive sites are present in virtually all areas of the CNS, from retina to spinal cord. However, not all neurons respond to GABA with the same sensitivity (Enna, 1983). $GABA_A$ receptors are also known to exist outside the CNS, e.g. the pituitary, the melanotrophs, the α cells of the pancreas, the adrenal medulla *etc.* (McKernan & Whiting, 1996).

GABA can produce hyperpolarization, depolarization or no change in the membrane potential depending on the transmembrane chloride concentration gradient and the instantaneous membrane potential. Most local, short-circuit interneurons and some projection neurons (e.g. cerebellar Purkinje cells) in the CNS are GABAergic. The activation of these neurons can prevent overexcitation of neurons. However, the inhibitory action of GABA at single synapses and neurons is not necessarily translated into overall reduced neuronal activity in a neuronal network, because GABAergic neurons can be themselves the targets of GABAergic inputs; this arrangement of GABAergic neurons in series can produce disinhibition of downstream neurons. Therefore, synaptic inhibition does not necessarily result in depression of complex CNS functions, e.g. in behavioral inhibition (Haefely, 1994).

1.3. WHAT ARE $GABA_A$ RECEPTORS?

1.3.1. The structures of $GABA_A$ receptors

$GABA_A$ receptors are GABA-gated, channel forming proteins located in the cell membrane bilayers. They belong to a gene superfamily of neurotransmitter-gated ion channels, which also includes receptors for acetylcholine (ACh), Glycine, 5-hydroxytryptamine (5-HT), as well as an invertebrate glutamate-gated chloride channel (Karlin & Akabas, 1995). The subunits of these receptors have similar sequences and distributions of hydrophobic, membrane-spanning sequences and are homologous. The receptors in this family are also called Cys-loop receptors, owing to their unique, invariant feature - a 15-residue loop closed by 2 disulfide linked cysteine (Cys) residues in the N-terminal extracellular part of each subunit.

Similar to other members of this superfamily, $GABA_A$ receptors are also composed of several distinct polypeptide subtypes. The cDNAs encoding $GABA_A$ receptor subunits were first cloned nine years ago (Schofield *et al.*, 1987). In the intervening period, the number of known subunits of the $GABA_A$ receptors has grown to 14: α (α1-α6), β (β1-β4), γ (γ1-γ3) and δ (Sieghart, 1995). They exhibit about 70 % amino acid sequence homology within a subfamily, but only about 30 % homology among different subunit families. All subunit subtypes display a marked similarity in their primary structure, their putative secondary structure, and in their transmembrane topology (Schofield *et al.*, 1987).

Most progress toward understanding function in terms of structure of these ionotropic receptors has been made with the ACh receptors. The ACh receptors are pentamers. Their three-dimentional structure in the membrane has been solved to 9Å resolution (Unwin, 1993 & 1995). The subunits are arranged around a central channel that is about 25Å wide in the extracellular domain but narrows to about 7Å close to the cytoplasmic end of the membrane-spanning domain. The N-terminal parts of the subunits, which are extracellular, form the ACh binding sites. The binding of ACh in the interfaces between subunits promotes local conformational changes propagated possibly as small rotations of the subunits.

Each subunit has four membrane-spanning segments (M1-M4), among which M2 plays the major role in lining the channel lumen and in determining its conductance and selectivity. Residues toward the extracellular end of M1 are also exposed in the channel. M2 forms an α helix, interrupted in the middle by a short nonhelical section. The exposed half of M1 does not have a regular secondary structure; M3 and M4, by contrast, are at least partly α helical. The exposures of many residues in M1 and in M2 are different in the presence and absence of ACh. Thus, M1 and M2 undergo conformational changes that may be part of the gating process. The gate itself is close to the cytoplasmic end of the channel and could be opened and closed by the relative movements of the M1 and M2 segments.

In the light of the similarities between the $GABA_A$ receptors and the AChRs, a schematic model for the topology of a $GABA_A$ receptor subunit was proposed

(Schofield *et al.*, 1987) (Fig. 1.1). According to this model, the subunit has four transmembrane domains (MI to MIV), with both N- and C- termini extracellular. The MII domain from each subunit may contribute to the formation of a hydrophilic lining of the chloride channel; the long extracelluar N-terminus may contain binding sites for both $GABA_A$ agonists and some other modulatory drugs; and on some subunits, e.g. β, γ2L and γ2S, the large intracellular loop between MIII and MIV may contain sites for regulation, e.g. phosphorylation (Olsen & Tobin, 1990).

Although the subunit composition of any native $GABA_A$ receptor is not available yet, the quaternary structure of the native $GABA_A$ receptors has been determined by electron microscopic image analysis, which shows that the majority of $GABA_A$ receptors are pentameric rings with a channel in the centre (Nayeem *et al.*, 1994). The diameter of the narrowest portion of the channel is about 6Å (Bormann *et al.*, 1987). Since five subunits are necessary for the formation of $GABA_A$ receptors, and there exist 16 different subunits, a large number of heteropentameric isoforms might occur. Available evidence suggests that 12-24 isoforms are sufficiently abundant to play significant physiological roles (McKernan & Whiting, 1996) (Table 1.1). This diversity presumably serves a number of biological roles, including tissue- and age-dependent transcription control and tissue-dependent functional control at the protein level. The existence of receptor subtypes raises the possibility, not yet realised, of selective drugs of improved clinical profile, e.g. in the treatment of anxiety, epilepsy and insomnia (Smith & Olsen, 1995).

1.3.2. The modulators of $GABA_A$ receptors

$GABA_A$ receptors are not only the recognition sites of GABA, but also the targets of a variety of pharmacologically and clinically important drugs, such as benzodiazepines, barbiturates, neuroactive steroids, cage convulsants, propofol, chlormethiazole and loreclezole (Sieghart, 1995) (Table 1.2). These compounds can bind to the $GABA_A$ receptors at sites distinct from each other, and allosterically modulate the function of the receptor channel positively or negatively.

The interaction of GABA with $GABA_A$ receptors

GABA, by binding to $GABA_A$ receptors, increases the neuronal membrane conductance for chloride ions. These sites are shared by structural analogues of GABA, such as muscimol, and the competitive antagonist, bicuculline. Recently, single point mutation experiments suggest that, in the rat, Phe64 is probably positioned near a GABA binding site, two regions in the β2 subunit and some other residues can affect functional activation by GABA; and these sites may occur at an interface between two dissimilar subunits (Smith & Olsen, 1995).

The interaction of benzodiazepines with $GABA_A$ receptors

Electrophysiological experiments have indicated that classical benzodiazepines, such as diazepam or flunitrazepam, enhance the actions of GABA at the $GABA_A$ receptors

by increasing the frequency of channel opening. Biochemical studies have shown that diazepam or flunitrazepam increase the binding of GABA to $GABA_A$ receptors; reciprocally, GABA and other positive modulators of $GABA_A$ receptors increase the binding of these benzodiazepines to their sites (Martin, 1987). However, the full picture of the allosteric modulation of the $GABA_A$ receptors by benzodiazepines is quite complicated. The modulation could be either positive (facilitation of GABA action) by benzodiazepine agonists / partial agonists; or negative (depression of GABA action) by benzodiazepine inverse agonists / partial inverse agonists; and the benzodiazepine antagonists could block both effects (Wood *et al.*, 1984; Haefely, 1994). Data from some earlier studies suggest that there exist at least two types of benzodiazepine receptors in the brain:. type I benzodiazepine receptors enriched in the cerebellum that exhibit high affinity for compounds such as the triazolopyridazine Cl 218872, 2-oxoquazepam and methyl β-carboline-3-carboxylate (β-CCM); and type II benzodiazepine receptors enriched in the hippocampus and cerebral cortex that exhibit low affinity for these compounds (Sieghart & Schlerka, 1991).

Biochemical evidence suggests that the inclusion of a γ2 subunit is necessary for typical benzodiazepine potentiation to occur, and the type of α subunit present influences the benzodiazepine pharmacology of these receptors greatly. Thus, recombinant $GABA_A$ receptors composed of α1β1γ2 subunit subtypes exhibit properties of the type I benzodiazepine receptors. In contrast, receptors containing α2β1γ2 or α3β1γ2 subunits exhibit properties of the type II benzodiazepine receptors (Pritchett *et al.*, 1989). Recently, it has become clear that this is because

some residues, such as His101, Gly200 in the α1 and Thr142 in the γ2 subunit are playing very important roles here. The benzodiazepine sites may also occur at subunit interfaces, like GABA sites (Smith & Olsen, 1995).

The interaction of barbiturates with $GABA_A$ receptors

Sedative hypnotic barbiturates, such as pentobarbitone, have been shown to have three actions on $GABA_A$ receptors: a potentiation of GABA responses by increasing the mean channel open time; a direct activation of $GABA_A$ receptors at higher concentrations; and at even higher concentrations, a block of the GABA chloride channel (Thompson *et al.*, 1996). Barbiturates can also enhance benzodiazepine receptor affinities and inhibit ^{35}S-t-butylbicyclophosphorothionate (^{35}S-TBPS) (a cage convulsant) binding competitively. The GABA potentiation and direct effects of pentobarbitone are influenced by the type of α subunit present (Thompson *et al.*, 1996).

The interaction of steroids with $GABA_A$ receptors

This is going to be discussed in detail later since this thesis is mainly about the allosteric modulation of $GABA_A$ receptors by steroids.

The interaction of propofol with $GABA_A$ receptors

Propofol is an intravenous anaesthetic which is chemically unrelated to other anaesthetics. At low concentrations (2 - 100 μM), propofol potentiates GABA-activated currents, by increasing the frequency of channel openings; at intermediate concentrations (10 - 1000 μM), propofol directly activates $GABA_A$ channels and induces desensitization; at high concentrations (600 - 10,000 μM), propofol inhibits receptor / channel function presumably by a noncompetitive blocking action (Hales & Lambert, 1991; Orser *et al.*, 1994). The observed modulatory actions of propofol may be due to separate potentiation, activation and inhibitory sites for this anaesthetic on $GABA_A$ receptors (Adodra & Hales, 1995). Propofol also allosterically enhances ^{3}H-muscimol binding, ^{3}H-flunitrazepam binding (Prince & Simmonds, 1992b) and inhibits ^{35}S-TBPS binding (Concas *et al.*, 1991). The sites of action seem to be distinct from those for benzodiazepines, barbiturates, GABA agonists and steroids (Sieghart, 1995), and independent of the γ subunit (Jones *et al.*, 1995). Recently, homomeric β1 $GABA_A$ receptors were reported to be directly activated by propofol but not alphaxalone (Sanna *et al.*, 1995).

The interaction of chlormethiazole with $GABA_A$ receptors

Chlormethiazole is used clinically as a sedative / hypnotic and anticonvulsant agent (Moody & Skolnick, 1989). Electrophysiological studies indicate that chlormethiazole interacts with $GABA_A$ receptors in a manner similar to that of

barbiturates (Harrison & Simmonds, 1983). At lower concentrations, chlormethiazole potentiates GABA-activated currents by prolonging the GABA channel burst duration; and at higher concentrations, it directly activates the $GABA_A$ receptors (Hales & Lambert, 1992). By contrast, biochemical studies suggest that the interactions of chlormethiazole and pentobarbitone with the $GABA_A$ receptors may not be identical. Chlormethiazole can inhibit ^{35}S-TBPS binding and enhance ^{3}H-muscimol binding. But in contrast to pentobarbitone, it cannot enhance ^{3}H-diazepam binding (Leeb-Lundberg *et al.*, 1981), or ^{3}H-flunitrazepam binding (Cross *et al.*, 1989).

The interaction of loreclezole with $GABA_A$ receptors

Loreclezole is a broad spectrum anticonvulsant that is suggested to act via the $GABA_A$ receptor. It inhibits ^{3}H-t-butylbicycloorthobenzoate (^{3}H-TBOB) binding (Van Rijn & Willems-van Bree, 1993), behaves more like a barbiturate than a benzodiazepine in two *in vivo* models (Ashton *et al.*, 1992), and it is not sensitive to the benzodiazepine receptor antagonist, flumazenil (Dawson *et al.*, 1994). Although the site of action for loreclezole has not been identified, recently, it was demonstrated to be highly selective for receptors containing the β2 or β3 subunit over those containing the β1 subunit (Wafford *et al.*, 1994), and this unique subunit dependence is determined by a single amino acid, β2 Asn-289 (β3 Asn-290) (Wingrove *et al.*, 1994).

The interaction of cage convulsants with $GABA_A$ receptors

The convulsant picrotoxin is a noncompetitive antagonist of GABA at $GABA_A$ receptors, in contrast to the competitive antagonist bicuculline. Its binding sites, labelled by ^{3}H-dihydropicrotoxinin and cage convulsants ^{35}S-TBPS and ^{3}H-TBOB, were proposed to be located within the channel (Haefely, 1994). By interacting with these sites, convulsants like picrotoxin and some bicyclic cage compounds can antagonise GABA-induced chloride currents. GABA and compounds which mimic or facilitate the effects of the $GABA_A$ receptors allosterically inhibit ^{35}S-TBPS binding, while compounds reducing the efficacy of GABA, such as some convulsant β-carbolines, enhance ^{35}S-TBPS binding. In some studies, however, biphasic effects were observed (Sieghart, 1995).

1.4. STEROID MODULATION OF $GABA_A$ RECEPTORS

1.4.1. What are neuroactive steroids?

Neuroactive steroids are natural or synthetic steroids that rapidly alter the excitability of neurons by binding to membrane-bound receptors such as those for inhibitory and / or excitatory neurotransmitters (Paul & Purdy, 1992). These acute effects on CNS functions are unrelated to their genomic actions mediated by activation of their intracellular receptors which then act as ligand-activated transcription factors in the

nucleus.

The best-studied neuroactive steroids are a series of sedative / hypnotic 3α-hydroxy ring A-reduced pregnane steroids that include the major metabolites of progesterone and deoxycorticosterone. They do not act at classical intracellular steroid receptors but interact stereoselectively and with high affinity at $GABA_A$ receptors, to modulate the receptors and, thereby, the chloride ion currents (Paul & Purdy, 1992).

Some of these steroids are full agonists on $GABA_A$ receptors, e.g. alphaxalone (5α-pregnan-3α-ol-11,20-dione), pregnanolone (5β-pregnan-3α-ol-20-one), allopregnano-lone (5α-pregnan-3α-ol-20-one), allotetrahydroDOC (5α-pregnan-3α,21-diol-20-one) and androsterone (5α-androstan-3α-ol-17-one). They can allosterically enhance ^{3}H-muscimol and ^{3}H-flunitrazepam binding and displace ^{35}S-TBPS binding. Electrophysiological studies suggest that these steroids not only prolong individual channel openings (barbiturate-like) by promoting the channel to enter a long open-state; but also increase the frequency of single channel openings (benzodiazepine-like) (Macdonald & Olsen, 1994). At higher concentrations, they can directly activate $GABA_A$ receptors, and exacerbate desensitization of the responses to GABA (Lambert *et al.*, 1995).

Pregnenolone sulphate (5-pregnen-3β-ol-20-one 3-O-sulphate) appears to have rather different properties. It exhibits mixed GABA-agonist / antagonist features. It bimodally alters ^{3}H-muscimol binding, weakly enhances ^{3}H-flunitrazepam binding,

but inhibits barbiturate-stimulated ^{3}H-flunitrazepam binding and inhibits $^{36}Cl^{-}$ uptake to $GABA_A$ receptors (Majewska & Schwartz, 1987). Another sulphate derivative of neurosteroids, dehydro-epiandrosterone sulphate (5-androsten-3β-ol-17-one 3-O-sulphate), acts as an allosteric antagonist of the $GABA_A$ receptors. It inhibits barbiturate-mediated enhancement of ^{3}H-flunitrazepam binding and blocks GABA-induced currents (Majewska *et al.*, 1990).

Some other neuroactive steroids in this series have proved to be less active or inactive as agonists on $GABA_A$ receptors. These include progesterone (4-pregnen-3,20-dione) and some of its metabolites, e.g., epipregnanolone (5β-pregnan-3β-ol-20-one), isopregnanolone (5α-pregnan-3β-ol-20-one) and pregnenolone (5-pregnen-3β-ol-20-one). The question arises whether some of the less active steroids might be partial agonists or specific antagonists at the site of steroid action and, thereby, might be able to reduce the potentiation of GABA achieved by more active steroids (Simmonds, 1991). There is evidence for 5α-pregnan-3α,20α-diol being a partial agonist in $^{36}Cl^{-}$ uptake studies where allopregnanolone was used as the more active steroid (Belelli & Gee, 1989). In the studies in our own lab, epipregnanolone was also found to be able to antagonise the potentiating effect of pregnanolone and alphaxalone (Prince & Simmonds, 1993).

1.4.2. Structure-activity relationship for steroids on $GABA_A$ receptors

Inspection of the structures of these most active and less active steroids suggests that

certain features are required for activity (Simmonds, 1991, Lambert *et al.*, 1995) (Fig. 1.2):

i). a hydroxy substituent in the α configuration at position 3 is required, a 3β-hydroxy or 3-keto substituent resulting in little or no activity.

ii). a keto group in the 20 position of the pregnane steroids or in the 17 position of the androstane steroids is a common feature of the active steroids, the reduction of the 20-keto group may result in partial agonists.

iii). the configuration at the 5 position, which markedly affects the shape of the steroid, has little effect on the potency of those pregnane steroids that have only 3α-hydroxy and 20-keto substituents but, 5β is less favorable than 5α configuration when there is a 11-keto or unesterified 21-hydroxy substituent present.

iv). the side chain at position 17 in the pregnane steroids must be in the β configuration for activity.

v). it is possible to confer water solubility upon pregnane steroids, by the introduction of a 2β-morpholinyl moiety, without substantial loss of activity.

vi). a saturated ring system is not an absolute requirement for activity, 4-pregnen-3α-ol-20-one exhibits similar activity to that of 5α-pregnan-3α-ol-20-one.

1.4.3. Sites of action for steroids

1.4.3.1. Binding to lipids or proteins?

Neurosteroids may interact with $GABA_A$ receptors by binding to different kinds of sites: i) specific sites on the receptor proteins, ii) membrane phospholipids, with the

functional groups of the steroids interacting with the receptors and iii) the interface of the receptor proteins and membrane phospholipids (Majewska, 1992).

Both the interaction with specific protein sites and the perturbation of the lipid bilayers have clear structural requirements. However, the first one is likely to be the primary mechanism because intracellularly applied steroids are inactive (Lambert *et al.*, 1990) and, steroids can still enhance ^{3}H-muscimol binding to a solubilized and purified preparation of the $GABA_A$ receptor protein, which was only associated with a minimal amount of lipid (Olsen, 1990). Therefore, it is generally accepted that steroids act through binding sites on the $GABA_A$ receptor protein itself (Simmonds, 1991).

1.4.3.2. Distinct binding sites for steroids

Furthermore, pharmacological analyses utilizing steroid-barbiturate and steroid-benzodiazepine drug combinations strongly support the sites of action for steroids to be distinct from those of the other well-established allosteric modulators (Lambert *et al.*, 1995).

Firstly, steroids do not interact with the allosteric benzodiazepine recognition sites of the $GABA_A$ receptor complex. The potentiation observed with alphaxalone and pregnanolone is unaffected by the benzodiazepine receptor antagonist flumazenil (Ro 15-1788); and the currents elicited by high concentrations (10-100 μM) of these

steroids are greatly potentiated by the benzodiazepine agonist diazepam (Cottrell *et al.*, 1987; Lambert *et al.*, 1990).

Secondly, steroids do not interact with the barbiturate recognition sites associated with the $GABA_A$ receptors. Electrophysiological studies have demonstrated that membrane currents evoked by high concentrations of steroids, e.g., alphaxalone (100 μM) and pregnanolone (30 μM) are enhanced by phenobarbitone. Reciprocally, the membrane currents elicited by pentobarbitone (1mM) can be greatly enhanced by pregnanolone (Cottrell *et al.*, 1987; Peters *et al.*, 1988). Radioligand binding assays provide further evidence by showing that the combination of barbiturates and steroids gave additive synergistic effects when modulating ^{35}S-TBPS binding, ^{3}H-muscimol binding and ^{3}H-flunitrazepam binding (Peters *et al.*, 1988; Gee *et al.*, 1988; Turner *et al.*, 1989).

Recent evidence also suggests that steroids do not share common sites with propofol (Prince & Simmonds, 1992b). Therefore, steroids interact with novel regulatory sites on the $GABA_A$ receptor protein.

1.4.3.3. Heterogeneity of steroid binding sites

Evidence for multiple classes of steroid recognition sites have accumulated during the past several years. For example:

i). 5 sec applications of 5α-pregnan-3α-ol-20-one yielded shallow and biphasic concentration-response curves for the potentiation of muscimol-stimulated Cl^- uptake (Morrow *et al.*, 1990), and GABA-evoked Cl^- currents (Puia *et al.*, 1990). With such short applications of steroids in these studies, it is possible that the component seen at low concentrations resulted from an action on the extracellular portion of the $GABA_A$ protein; the other component might have resulted from an action within the membrane, requiring higher concentrations of steroid in the aqueous medium to achieve effective levels in the lipids within 5 sec. If this is the case, longer exposures to steroid might make the two components appear as one.

ii). Certain binary combinations of steroids upon the binding of ^{35}S-TBPS (Hawkinson *et al.*, 1994) and ^{3}H-flunitrazepam (Prince & Simmonds, 1993) showed complex interactions between two steroids that could not be satisfactorily explained by the competition for a single steroid binding site.

iii). Regional- and species- dependent features were reported for the steroidal modulation of $GABA_A$ receptors (e.g. Nguyen *et al.*, 1995; Jussofie, 1993; Sapp *et al.*, 1992 and Gee *et al.*, 1988 & 1991). In these studies, $GABA_A$ receptor subunit compositions are likely to be different among different brain regions, and the activities of steroids may vary with different receptor subpopulations.

However, in contrast to the clear influence of subunit composition upon the effects of benzodiazepines and other compounds at $GABA_A$ receptor isoforms, similar

investigations with steroids have provided a quite confusing picture:

i). Recombinant $GABA_A$ receptors comprised of $\alpha1\beta1\gamma2L$, $\alpha1\beta1$ or $\beta1$ subunits showed no subunit specificity for steroids (Puia *et al.*, 1990). Thus, the steroid binding site(s) appear to be represented even on homooligomeric $\beta1$ subunit $GABA_A$ receptor. The subtype of β subunit expressed is reported to have little impact upon steroid potentiation of GABA-evoked currents (Hadingham *et al.*, 1993).

ii). So far no clear consensus has emerged from studies investigating the role of the α subunit. In recombinant $GABA_A$ receptors expressing either $\alpha1$, $\alpha2$ or $\alpha3$ subunit together with $\beta1$ or $\beta1\gamma2$ subunits, the $\alpha1$ subunit is preferred by 5α-pregnan-3α-ol-20-one when potentiating GABA-evoked currents (Shingai *et al.*, 1991); however, $\alpha3$ subunit is preferred by the same steroid when enhancing ^{3}H-flunitrazepam binding (Lan *et al.*, 1991). Further discord arises where $\alpha6\beta1\gamma2$ subunit combination exhibited a smaller steroid effect than $\alpha1\beta1\gamma2$, $\alpha3\beta1\gamma2$ and $\alpha5\beta1\gamma2$ subunit combinations in electrophysiological studies (Puia *et al.*, 1993), while $\alpha6\beta2\gamma2$ appeared to be more, not less, sensitive to 5α-pregnan-3α-ol-20-one in radioligand binding experiments (Korpi & Lüddens, 1993).

iii). The γ subunit appears not to be a crucial determinant of steroidal modulation of the receptors (Puia *et al.*, 1990; Shingai *et al.*, 1991). However, the $\gamma1$ subunit seemed to be more sensitive to steroid modulation than $\gamma2$ or $\gamma3$ (Puia *et al.*, 1993, Lambert *et al.*, 1995).

1.5. WHY THIS PROJECT?

This project was started to follow-up some recent findings from our laboratory, at that time. In one paper, epipregnanolone was suggested to be a specific antagonist at the steroid site on the $GABA_A$ receptor complex (Prince & Simmonds, 1992c). Therefore, we thought it to be important to try other pairs of active / inactive steroids which are structurally closely related, e.g., epipregnanolone, pregnenolone against pregnanolone; Δ^{16}-alphaxalone against alphaxalone; progesterone and its derivative, 11-ketoprogesterone against pregnanolone (structures of these steroids can be found in this Chapter as well as Chapter 3, 4 and 5). Dr Prince's work also suggests that there is more than one binding site for steroids on $GABA_A$ receptors, and that steroids and propofol are likely to have different binding sites (Prince & Simmonds, 1992b, 1993). Thus, propofol was also investigated in parallel with pregnanolone. These results are reported in Chapter 3, 4 and 5.

Loreclezole was brought to our attention because it showed interesting subunit dependence (Wafford *et al.*, 1994; Wingrove *et al.*, 1994) and it is structurally related to chlormethiazole, a compound we are quite familiar with (Harrison & Simmonds, 1983). Therefore, loreclezole was compared with chlormethiazole and pentobarbitone, in both functional and binding studies, and reported in Chapters 6 & 7.

The methods used were ^{3}H-flunitrazepam binding and functional studies in a grease-gap apparatus. ^{3}H-flunitrazepam binding is a convenient and well-established technique for monitoring interactions between various modulators of the $GABA_A$ receptors (see Chapter 2). In this study, we also attempted to find out if the binding and functional studies would lead to the same conclusions.

Figure 1.1. Essential structural features of the $GABA_A$ receptor channel (Haefely, 1994).

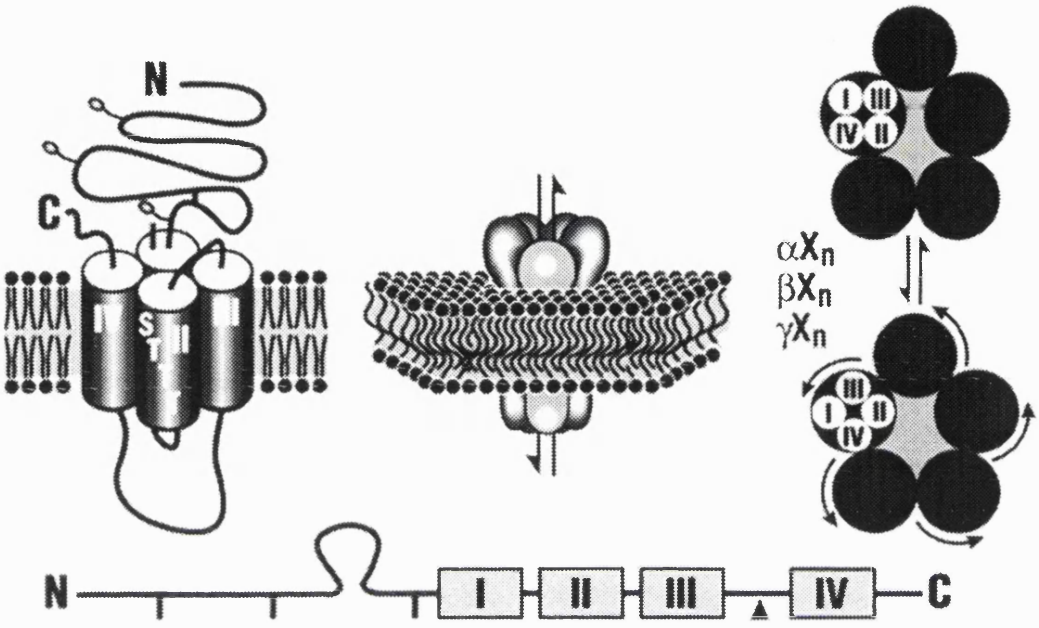
The schematic organization of the linearized subunits is shown at the bottom (not to scale): N terminus on the left, C terminus on the right, disulfide-stabilized β-loop in the ectodomain; strokes and arrowhead indicate potential glycosylation and phosphorylation sites, respectively; transmembrane spanning regions are indicated with Roman numerals.

Top left: speculative topology of subunits; the membrane-spanning stretches (MI to MIV) are indicated by cylinders.

Top centre: speculative side view of the receptor channel complex as suggested by the analogous structure of the AChR.

Top right: cross sectional view through the membrane spanning part with the presumed five tetrahelical subunits in the interconvertible channel-closed (above) and channel-open (below) forms; the helical segments in one subunit are indicated by Roman numerals. The supposed pentamer can consist of any combinatorial assembly of any number (n) of any subunit isoform (X).

$GABA_A$ receptor channel



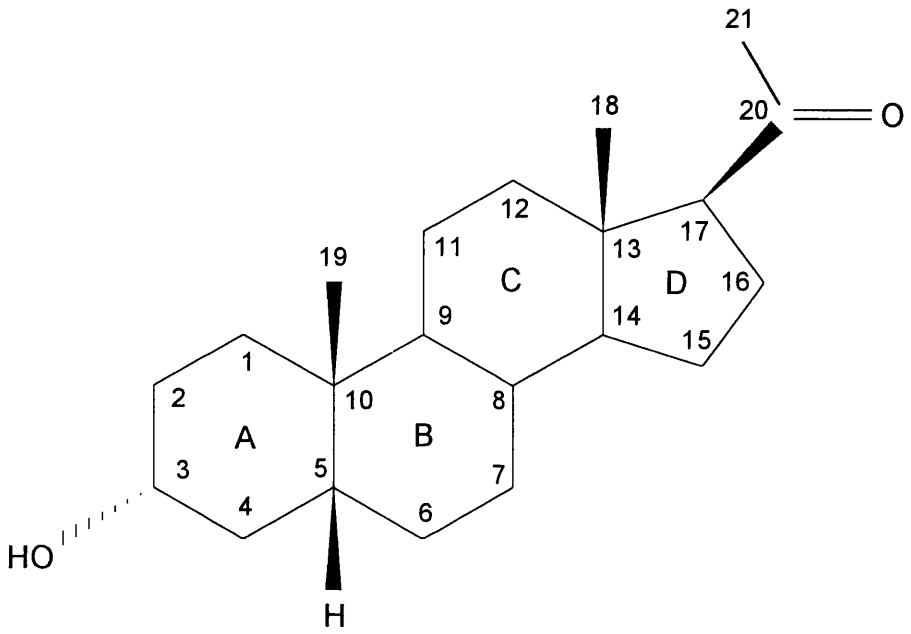


Figure 1.2. The lettering of the steroid rings and numbering of the carbon atoms in the steroid 5β-pregnan-3α-ol-20-one. By convention, the α configuration is that lying below the general plane of the ring system and the β configuration is that projecting above the plane of the ring system. The orientation of the hydrogen at C5 of the reduced pregnane (21 carbon) and androstane (19 carbon) series determines whether the A and B ring fusion is *trans* (5α-series) or *cis* (5β-series) (Lambert *et al.*, 1995).

Table 1.1. Distribution of the major $GABA_A$ Receptor subtypes in the rat brain (McKernan & Whiting, 1996).

Subtype	Relative abundance in rat brain (%)	Location and putative function
α1β2γ2	43	Present in most brain areas. Localized to interneurons in hippocampus, cortex and cerebral Purkinje cells
α2β2/3γ2	18	Present on spinal cord motoneurons and hippocampal pyramidal cells
α3βnγ2/γ3	17	Present on cholinergic and monoaminergic neurons where they regulate ACh and monoamine turnover
α2βnγ1	8	Present on Bergmann glia, nuclei of the limbic systems, and in pancreas
α5β3γ2/γ3	4	Predominantly present on hippocampal pyramidal cells
α6βγ2	2	Present on cerebellar granule cells
α6βδ	2	Present on cerebellar granule cells
α4βδ	3	Present in thalamus and hippocampal dentate gyrus
other minor subtypes	3	Present throughout brain

Location and function are listed where these have been investigated, and are not comprehensive. Other minor subtypes include $\alpha1\alpha6\beta\gamma2$, $\alpha1\alpha3\beta\gamma2$, $\alpha2\alpha3\beta\gamma2$ and $\alpha5\beta\gamma2\delta$ subtypes and are represented together as a small population.

Table 1.2. The modulators of $GABA_A$ receptors.

Modulators	Nature of the modulation
benzodiazepines	potentiation -- antagonism
barbiturates	potentiation
neurosteroids	potentiation
propofol	potentiation
picrotoxin	antagonism
chlormethiazole	potentiation
loreclezole	potentiation

CHAPTER 2

MATERIALS AND METHODS

2.1. RADIOLIGAND BINDING EXPERIMENTS

2.1.1. Background

2.1.1.1. Synaptosomes

In this project, the binding experiments were performed on $GABA_A$ receptors present in the central synapses. Crude rat brain synaptic membranes were prepared as described previously (Prince & Simmonds 1992a), with modification from the traditional synaptic fraction preparation procedure (Zukin *et al.*, 1974). This crude synaptic membrane preparation procedure (detailed below) omitted the "buffy coat" stage. Therefore, the membrane harvested in the end contained mainly synaptosomal and mitochondrial fractions. However, since the ligand ^{3}H-flunitrazepam was a highly selective ligand to central $GABA_A$ receptors (detailed below), the inclusion of some mitochondrial fraction should not interfere with my results. Indeed, at one stage of my experiments, I did compare these two ways of membrane preparations, and did not find any significant difference as far as the results were concerned.

2.1.1.2. The ligand: ^{3}H-flunitrazepam

A variety of ligands are available for probing the function of the $GABA_A$ receptors, e.g. ^{3}H-GABA, ^{3}H-muscimol for the GABA recognition site; ^{3}H-TBOB, ^{3}H-dihydropicrotoxinin and ^{35}S-TBPS for the convulsant site. In my study, I used the binding of ^{3}H-flunitrazepam (^{3}H-FNZ), a ligand widely used, and available with

high specific activity (80-85 Ci/mmol compared with 20-30 Ci/mmol for muscimol) to monitor $GABA_A$ receptor function.

There are both "central" and "peripheral" types of benzodiazepine binding sites in the brain, and only the central type receptors are coupled to the GABA system (Marangos *et al.*, 1982). Flunitrazepam binds with much higher affinity to the central type than to the peripheral type (Quast & Mählmann, 1982; Sieghart, 1992). Therefore, at the concentration of ^{3}H-FNZ used in the studies for this thesis (< 20 nM), it is very unlikely that any binding to peripheral-type receptors would be seen.

Within the central type benzodiazepine binding sites, there are type I and II benzodiazepine receptors, to which ^{3}H-FNZ exhibits a rather similar affinity (Sieghart & Schlerka, 1991). Flunitrazepam binding sites are present at high density in the mammalian brain (Martin, 1987). The binding *in vitro* is rapid, reversible, saturable and with high specificity and affinity (Tallman *et al.*, 1980): Kd approximately 4 nM (Chapter 6), non-specific binding less than 5 % for the conditions used in this thesis. The benzodiazepine receptors are fairly robust and survive solubilization with virtually unchanged affinity and pharmacology, while the pharmacologies of other modulators may disappear (Stephenson & Olsen, 1982; Sigel & Barnard, 1984). Since these sites are allosterically linked to the various modulatory sites of the $GABA_A$ receptors which manifest their potentiating effects via an increase in flunitrazepam affinity (e.g. Tallman *et al.*, 1980; Harrison *et al.*, 1987; Majewska *et al.*, 1986), the modulation of ^{3}H-FNZ binding can be used as a

convenient monitor of the interactions between various modulators of the $GABA_A$ receptors.

One of the most important considerations in any radioligand-binding assay is the determination of specific binding. Specific binding can be defined as binding to the receptor of interest. Nonspecific binding is any other observed binding, in the presence of an appropriate excess of unlabelled drug to block fully the receptors of interest (Bylund & Yamamura, 1990). In choosing a drug to determine nonspecific binding it is best to use a drug that is chemically dissimilar from the radioligand. This is due to the possibility of the drug inhibiting specific but nonreceptor binding sites.

However, in the case of the ^{3}H-FNZ binding, this is not a major concern. The binding I was interested in is the binding to all $GABA_A$-associated benzodiazepine sites (the central type, both type I and II), but not the binding to peripheral type sites. Flunitrazepam has a ten times higher affinity to the central type than the peripheral type, the density of which is only 1/4 of that of the central type (Marangos *et al.*, 1982). Moreover, while the peripheral type benzodiazepine sites are highly localized to nuclear membranes, it is the central type sites that are enriched in the synaptosome fraction that I was using. Therefore although there are ligands which are more selective between central and peripheral type sites, e.g. clonazepam, Ro 15-1788, the use of them to determine specific binding was felt to be unnecessary. Instead, unlabelled flunitrazepam was used as displacer, so that the

specific binding could simply be defined as flunitrazepam binding sites, and the so-called "non-specific" binding is unbound ^{3}H-FNZ trapped in the tissue and filter plus, perhaps, low affinity binding to a high capacity population of binding sites which does not saturate at the 10μM concentration of unlabelled flunitrazepam. Had I needed to investigate the ^{3}H-FNZ binding to type I or type II receptor sites, then type I or type II benzodiazepine receptor specific ligands should be used as the displacer.

2.1.2. Membrane preparation

2.1.2.1. Synaptosome membrane preparation

Crude rat brain synaptic membranes were prepared as described previously (Prince & Simmonds, 1992a). Male Wistar rats (150 - 200 g) were sacrificed by decapitation and their brains, including cerebellum, but excluding pons and medulla, were removed and homogenised in 20 volumes of ice-cold wash buffer, using an Ultra Turrax homogeniser. The homogenate was centrifuged for 10 min at 1000 x g at 4°C in order to pellet any debris and unbroken cells (Beckman J2-21 M/E centrifuge). The supernatant was removed and centrifuged for 20 min at 48,000 x g. The resultant pellet was resuspended in wash buffer and centrifuged for 20 min at 48,000 x g for a further three times. The final pellet was resuspended in wash buffer and then frozen at -20°C until required (14 days maximum). A protein assay was carried out at this stage.

2.1.2.2. Protein assay

Protein concentration was determined by the Biorad assay (Manual from Biorad Laboratories Ltd developed from the method of Bradford, 1976). Briefly, several dilutions of protein standard containing from 0.2 to about 1.4 mg protein.ml^{-1} were prepared. 0.1 ml of sample buffer, standards and appropriately diluted samples were placed in test tubes together with 5.0 ml diluted dye reagent. After a period of 5 minutes to one hour, the OD_{595} versus reagent blank was measured. The standard curve (OD_{595} versus concentration of standards) was plotted and unknowns could be determined.

2.1.2.3. Membrane washing

$GABA_A$ receptors have been assayed traditionally in frozen and thawed, thoroughly disrupted, and multiply washed membranes (Enna & Snyder, 1975; to remove endogenous inhibitors of GABA binding (Greenlee *et al.*, 1978) including GABA (Napias *et al.*, 1980), but possibly other materials (Guidotti *et al.*, 1978)). More recently, multiple washing stages have been used to remove endogenous GABA when preparing membranes (e.g. Concas *et al.*, 1994). I decided to wash the membranes upon thawing rather than before freezing at -20°C in case endogenous GABA and other unknown substances that will affect ^{3}H-FNZ binding may be released by the freeze-thaw process. The membranes received osmotic shock at the two distilled water washing stages (see below), and became quite loose and fluffy.

Therefore, any vesicles formed by the membrane fractions would have been disrupted and GABA leached. With my membrane washing procedure, there was very little endogenous GABA left, as evidenced by the fact that 100 μM bicuculline failed to reduce ^{3}H-FNZ binding significantly (Chapter 3).

On the day of the assay, the tissue was thawed and washed twice with distilled water and twice with the assay buffer by resuspension and centrifugation for 20 min at 48,000 x g. The final pellet was resuspended in assay buffer to give a protein concentration of approximately 2 mg ml^{-1} .

2.1.3. Assays

2.1.3.1. Assay conditions

A concentration of 150-200 mM chloride is often utilised in studies examining benzodiazepine interactions (e.g. Sigel and Barnard, 1984), and a 50 mM Tris, 150 mM NaCl, pH 7.4 at 4°C buffer was adopted in studies on ^{3}H-FNZ binding (Prince & Simmonds, 1993). Therefore, in my studies, this buffer is used as assay buffer.

The affinity of ^{3}H-FNZ binding increases as the incubation temperature decreases (Quast *et al.*, 1982, Prince & Simmonds, 1992a). And it takes about 50 min to reach equilibrium at 4°C (Fig. 2.1). Therefore, all experiments incorporated a 4°C, 60 min incubation to maximize flunitrazepam binding.

2.1.3.2. Assay procedures

All radioligand binding assays were performed in triplicates.

Concentration-effect relationship after 60 min incubation

100 µl aliquots of membranes (0.4 mg protein.ml^{-1}) were pre-incubated with various concentrations of drugs for 10 min at 37°C (Prince & Simmonds, 1992). This was to allow lipophilic modulators to equilibrate with membranes without temperature dependent restrictions on lipid mobility and fluidity. Then 50 µl of ^{3}H-FNZ was added to each tube to give a final concentration of 1 nM and a total volume of 500 µl assay buffer. The samples were incubated for 60 min at 4°C.

Saturation curve

100 µl aliquots of membranes (0.4 mg protein.ml^{-1}) were incubated with 0.1 - 20 nM ^{3}H-FNZ for 60 min at 4°C in the presence or absence of the drug investigated in a total sample volume of 500 µl. Non-specific binding, which was less than 5 % of the total binding, was determined for each concentration of ^{3}H-FNZ by addition of 10 µM unlabelled FNZ.

2.1.3.3. Termination of binding

The binding reaction was terminated by addition of 2 ml ice-cold wash buffer, followed by rapid filtration through Whatman GF-C filters using a Brandel Cell

Harvester. The filters were washed 4 times with 2 ml ice-cold wash buffer. Non-specific binding, which was less than 5 % of the total binding, was determined by addition of 10 μM unlabelled FNZ.

2.1.4. Variations in the solvents used

Drugs that are not water-soluble were initially dissolved either in dimethyl sulphoxide (DMSO), which was present in all samples at a final concentration of 0.45 %, or in acetone with a final concentration of 1 % in all sample tubes. Drugs that are water-soluble were made up into stocks directly with assay buffer.

The effect of DMSO on ^{3}H-FNZ binding at different incubation period was examined (Fig. 2.2). DMSO (0.45 %) did inhibit ^{3}H-FNZ binding, giving 70.9 % of control binding at 60 min (n = 2). Therefore, DMSO was present in all samples at a final concentration of 0.45 % for suitable incubation periods as required, and the effect of DMSO was taken off from the raw results to give the final results.

In all early studies, DMSO - a common solvent used to dissolve steroids in both *in vitro* / *in vivo* studies was adopted. At that time, I got the enhancement of 50-60 % of the control from 60 min exposure experiments, therefore I was not too worried about the reduction in total binding caused by DMSO 0.45 %. But in the subsequent experiments, where I had to deal with much smaller potentiation by alphaxalone *etc.*, then the effect of DMSO seemed to be quite remarkable. However, in order to

compare the results from different experiments, we carried on using DMSO as the solvent. And we also carried out parallel experiments using acetone as the solvent, and did not find any artifactual effect caused by DMSO (data not shown).

But in all subsequent experiments, acetone was used as the solvent. 2-methoxyethanol ethylene glycol (MOE) was recommended to us by some other people recently. I would recommend people to try MOE first if they are going to carry out similar binding studies using ^{3}H-FNZ as the ligand. Please refer to the method section in each experimental chapter for the solvents used for that series of experiments.

2.1.5. Quantification of bound radioactivity

Radioactivity was quantified by conventional liquid scintillation spectrometry. Filter paper discs with membranes sticking to them were placed in 6 ml mini vials and 4 ml scintillant (Emulsifier safe, Canberra Packard) was added. The samples were shaken and left overnight to equilibrate before counting, 2 minutes for each sample. Counting efficiency was about 50 % with a background of 20 - 40 cpm.

2.1.6. Data analysis

All data were analyzed using Prism (Version 2.0, GraphPad, San Diego, CA).

Concentration-effect relationships were analyzed using non-linear regression, and fit to one-site and two-site models where applicable (Hawkinson *et al.*, 1994):

one-site model $Y = A+\{(B-A)/[1+(10^{X}/10^{C})^{D}]\}$,

two-site model $Y = \{A+[(B-A)/100]\}*[\{E/[1+(10^{X}/10^{F})]\}+\{(100-E)/[1+(10^{X}/10^{G})]\}]$

where: Y is the percent specific bound, A is the bottom plateau, B is the top plateau, X is log concentration, C is the log of EC_{50}, D is the Hill coefficient, E is the percentage of high-affinity sites, F is the EC_{50} of high-affinity sites, and G is the EC_{50} of low-affinity sites. The two-site model was chosen if the sum of squares for this fit was significantly lower than for the one-site fit as determined by F-test. The F value was calculated by: $F = [(SS_1-SS_2)/(df_1-df_2)]/(SS_2/df_2)$ where: SS_1 and SS_2 are the sum of squares and df_1 and df_2 are the degrees of freedom for the simpler and more complex models, respectively.

Saturation data were fitted using non-linear regression, hyperbola curve fitting:

$Y=A*X/(B+X)$

where: Y ranges between zero and the maximum plateau value A (B_{max}), B is the dissociation constant (K_D), X has units of concentration.

Statistical comparisons were made by Student's t-test, one-way ANOVA or two-way ANOVA where applicable using Instat.

2.2 ELECTROPHYSIOLOGICAL STUDIES

2.2.1. Background

Many different isolated neuronal preparations have been utilised to investigate some facet of CNS pharmacology. Cultured neurones provide systems in which single cells can be studied independently; slices of brain tissue can be used in several ways to study events at the single cell or higher levels. Another type of preparation is the multineuronal preparation in which responses of a very large population of cells summate to give a gross tissue response. The cuneate nucleus preparation used in this study falls into this category. The "population responses" recorded represent the sum total of a large number of individual neuronal potential changes, and are in principle analogous to the summated contractions of smooth muscle cells which make up the gross contraction of a segment of ileum.

In recent years, the grease-gap arrangement has proved to be quite useful for recording population responses of neurons to drugs that alter membrane potential. It has been used with the roots of hemissected spinal cord from the immature rat (Evans, 1978), the dorsal funiculus in slices of rat cuneate nucleus (Simmonds, 1978), segments of rat optic nerve (Simmonds, 1983), pyramidal cell projections in slices of cerebral cortex from the rat (Harrison & Simmonds, 1985) and the mouse (Burton, 1988), Punkinje cell and granule cell parallel fibre projections in rat cerebellar slices (Garthwaite *et al.*, 1986) and the alvear projection of CA1

neurons in slices of rat hippocampus (Blake *et al.*, 1988). The principal requirement is for a reasonably dense projection of axons within the slice which is arranged in the perfusion bath to pass through a grease-filled gap in a barrier separating the two compartments of the bath.

2.2.2. Preparation of rat cuneate nucleus slices

Rat cuneate nucleus slices were prepared as previously described (Simmonds, 1978; Harrison & Simmonds, 1983). Male Wistar rats were stunned and decapitated, and the medulla oblongata rapidly exposed by removal of the overlying cerebellum, and sectioned *in situ* at the level of the obex. The medulla was then isolated and placed in ice-cold Krebs medium. The pial membranes were teased away from the tissue, and the medulla was placed, dorsal surface uppermost, in a cutting guide. The tissue was sectioned on either side of each dorsal funiculus using a razor blade, producing two slices of tissue of approximately 500-600μm thickness. These slices contained the dorsal funiculus at the dorsal margin, with the underlying cuneate nucleus lying ventrally at the rostral end of the slice. The tissue adjacent to the dorsal funiculus at the caudal end was then trimmed away and the resulting slices were incubated in oxygenated Krebs for 2-3 hours before being placed in the tissue bath.

2.2.3. Tissue bath, perfusion and recording system

A simple two-compartment Perspex bath, divided by a two-piece perspex barrier was

used. The tissue slices were placed in such a position that the dorsal funiculus projected through a grease-filled slot in the lower half of the barrier, with cuneate nucleus only in one compartment; the second part of the barrier was then placed on top of the lower half without damaging the tissue unduly, but tightly enough to ensure that the "grease gap" thus produced formed a high resistance seal between the two compartments.

The tissue slices were perfused with Krebs medium at a rate of 2 ml/min at room temperature (20 ± 1°C). The two compartments were supplied independently via a Watson-Marlow roller pump, and only the compartment containing the terminals of the dorsal funiculus within the cuneate nucleus was superfused with drugs incorporated into the Krebs medium.

Drug-induced negativity in one compartment with respect to the other was recorded via Ag/AgCl electrodes embedded in 3 % agar in saline, which were inserted at one side of the bath, and a high input impedance DC amplifier. Responses were displayed on a chart recorder, and measured at peak amplitude (see Fig. 2.3 for details).

2.2.4. Quantification of drug effects

For quantitative studies of drug effects on the $GABA_A$ receptor system, muscimol was used as an agonist in place of GABA (Harrison & Simmonds, 1983), since it

is both more potent and more slowly accumulated by CNS tissue, than GABA itself (Johnston *et al.*, 1978) and is thus more suitable for this type of investigation.

Muscimol was applied by 2 min superfusion, initially at 1.25 and 2.5μM, to form a control muscimol dose-response line. These concentrations were selected since they were in the lower part of the log dose-response curve for muscimol. Responses to these doses of muscimol were highly reproducible when doses were repeated at 20 min intervals, indicating that cumulative desensitisation of GABA receptors in the preparation did not occur during prolonged experiments.

In each experiment, a muscimol control curve was first obtained, then one drug was superfused for 30 min (sufficient to allow equilibrium for most drugs) before and during the redetermination of responses to muscimol. The same procedure was then carried out with a combination of two drugs. The muscimol dose-response lines were always displaced in an approximately parallel fashion, either to the right (antagonist) or to the left (potentiator). By bridging the same response level as the control line, equi-potent dose ratios could be calculated at an arbitrarily selected response level by taking the ratio of muscimol dose necessary to produce that response during and before perfusion of the drug under test. So the effects of the drugs were measured as equi-effective muscimol dose-ratio.

Muscimol was prepared as a 10 mM solution in distilled water and diluted into the Krebs medium just before use. Other drugs were either dissolved directly in the

Krebs medium or first dissolved in a little acetone and then diluted into the Krebs medium. The final concentration of acetone never exceeded 0.1 % which had no effect on muscimol responses in this preparation.

2.3. BUFFERS AND CHEMICALS USED

2.3.1. Buffers used for binding experiments

Wash buffer: 5 mM Tris-HCl, 1mM EDTA, pH 7.4 at 4°C

Assay buffer: 50 mM Tris, 150 mM NaCl, pH 7.4 at 4°C

2.3.2. Buffers used for electrophysiological studies

Krebs medium contained (mM): NaCl 118, KCl 2.1, KH_2PO_4 1.2, $CaCl_2$ 2.5, $MgSO_4$ 2.2, $NaHCO_3$ 25 and glucose 11, and was continuously gassed with 95% O_2 : 5% CO_2 to give pH 7.4.

2.3.3. Sources for drugs and chemicals

Drugs and chemicals were obtained from various sources as follows:

Glaxo Group Research (Greenford, U.K.): Δ^{16}-alphaxalone, alphaxalone, 11-ketoprogesterone

NEN-Du Pont (Stevenge, U.K.): ^{3}H-FNZ (82.5 Ci mmol^{-1}).

Astra Arcus (Södertälje, Sweden): Chlormethiazole

Janssen Pharmaceutica (Beerse, Belgium): Loreclezole

Sigma: pregnanolone, epipregnanolone, pregnenolone, pentobarbitone, bicuculline, muscimol, flunitrazepam

British Drug House (BDH): most bulk chemicals except Tris and Sucrose which were form Sigma

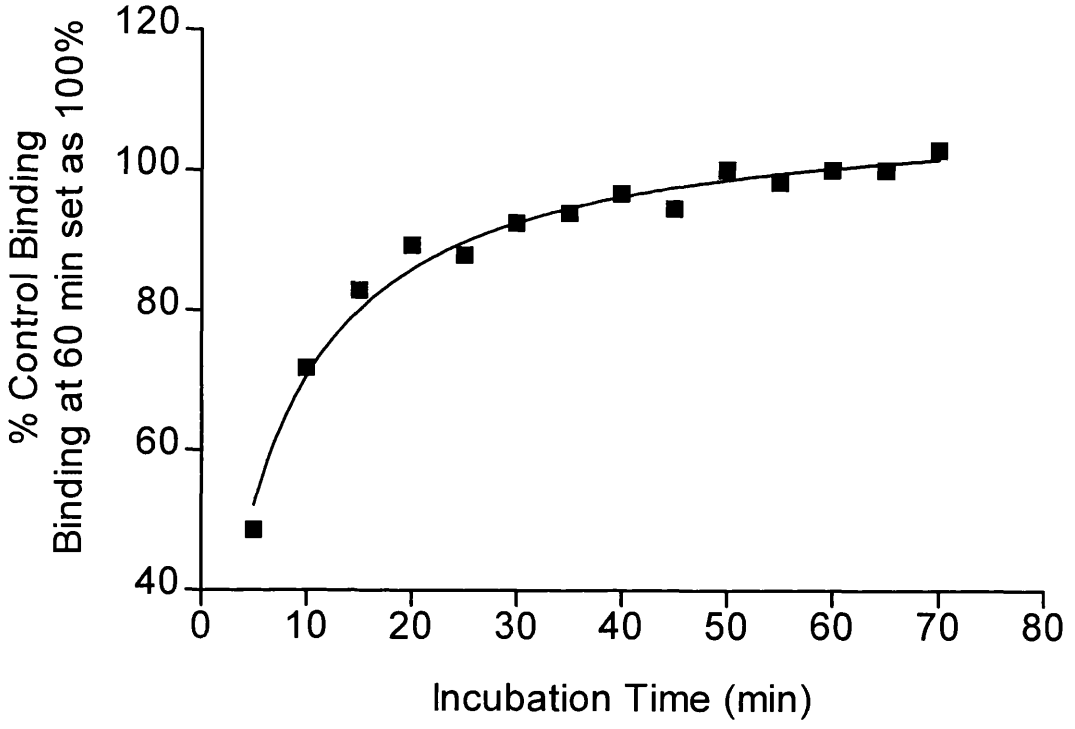


Figure 2.1. Time profile of 1 nM ^{3}H-FNZ binding to 0.4 mg/ml protein at 4°C. Data were obtained from 1 experiment. All data points are the means of triplicates.

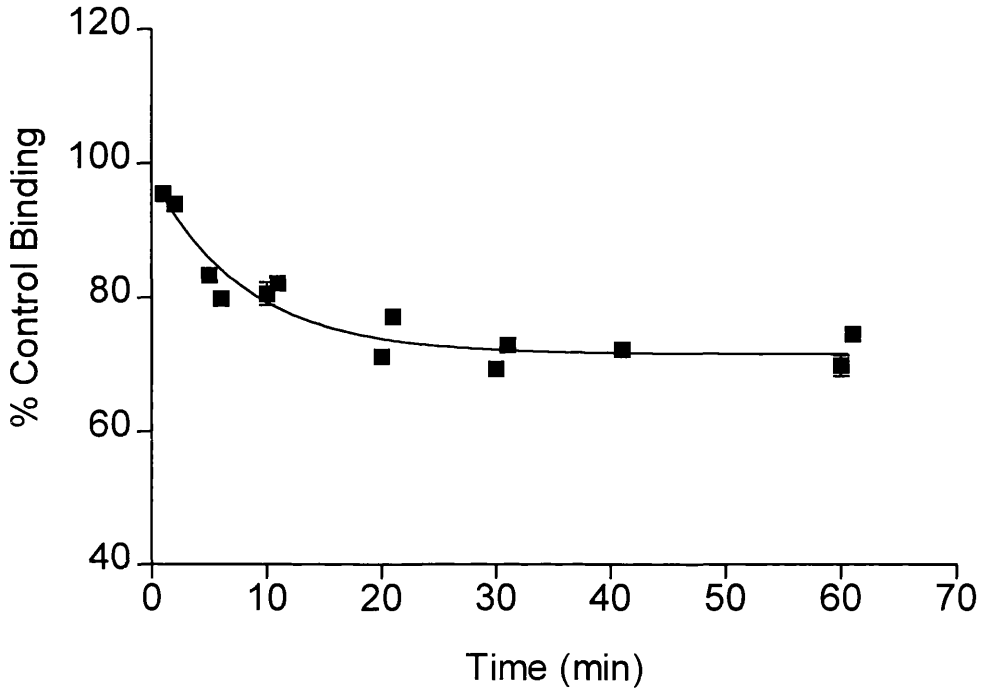
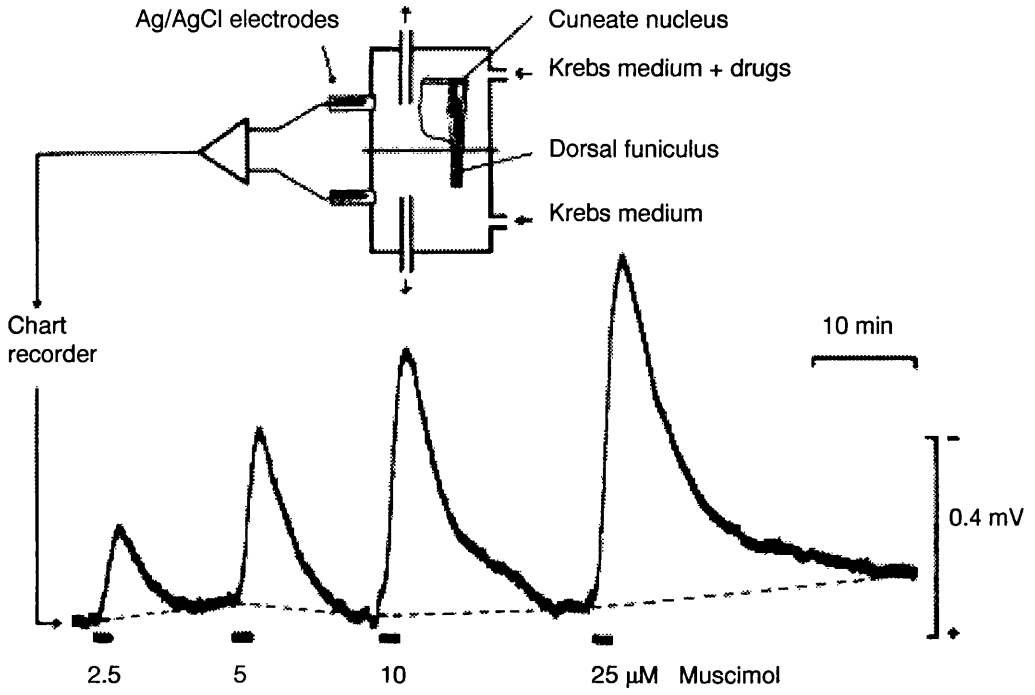


Figure 2.2. Time profile of DMSO (0.45 %) on ^{3}H-FNZ binding. Data were obtained from two experiments. All data points are the means of triplicates.

Figure 2.3. Diagram (not to scale) of a two-compartment bath system used for grease-gap recordings (Simmonds, 1990).

The compartments are separated by a removable barrier which is made in two pieces; the bottom piece contains a 1-mm square slot cut into the centre of its upper edge and the top piece has a straight lower edge. The 1 mm thickness of the barrier is machined down to 0.5 mm in the region of the slot in both the top and bottom pieces. The tissue is placed through the barrier as follows: the bottom piece of the barrier is put in place; the slot having been packed with silicone grease and its upper edge lightly greased; the meniscus of the medium is raised just above the barrier so that the slice can be floated into position over the slot; the top piece of the barrier is then brought down to ease the tissue into the grease in the slot and make a watertight contact with the bottom piece of the barrier. The meniscus is then raised on both sides to submerge the tissue. Each compartment can be perfused by pumping medium into the bottom and sucking off at the meniscus. The fluctuating charge on the medium induced by the roller pump can be avoided with the incorporation of sealed air-breaks into the perfusion lines to isolate electrically the medium passing through the pump from the medium to the bath. A convenient size of bath will contain 1.5-2.0 ml of medium and a flow rate of about 2 ml/min has been found adequate for most purposes. The potential difference between the two compartments is recorded continuously with Ag-AgCl electrodes embedded in agar/saline via a high-impedance, low-gain amplifier onto a chart recorder.

The typical trace shown is from a rat cuneate nucleus slice exposed to 2 min superfusions of muscimol. This can be obtained immediately on setting up the preparation in the bath, after allowing the slice to recover for 2-3 hr in gassed Krebs after cutting. The preparation can usually last for 7-10 hr.


Ag/AgCl electrodes
Cuneate nucleus
Krebs medium + drugs
Dorsal funiculus
Krebs medium
Chart recorder
10 min
0.4 mV
2.5
5
10
25 µM Muscimol

CHAPTER 3

CHARACTERIZATION OF MULTIPLE COMPONENTS IN THE ENHANCEMENT BY PREGNANOLONE AND PROPOFOL OF ^{3}H-FLUNITRAZEPAM BINDING TO $GABA_A$ RECEPTORS[1]

[1] modified from the paper: Zhong, Y. & Simmonds, M. A. (1996a). Pharmacological characterization of multiple components in the enhancement by pregnanolone and propofol of ^{3}H-flunitrazepam binding to $GABA_A$ receptors. *Neuropharmacol.*, **35:** 1193-1198.

3.1. INTRODUCTION

3.1.1. Steroids and $GABA_A$ receptors

With a site of action distinct from those of benzodiazepines and barbiturates, steroids can allosterically enhance ^{3}H-FNZ binding (see Chapter 1 for details). Neurosteroid modulation of the $GABA_A$ receptors shows a distinct structure-activity relationship, with a 3α-OH moiety being a requirement for activity and a 20 keto group being favoured over a 20-hydroxy (Harrison *et al.*, 1987). Certain metabolites of progesterone (4-pregnen-3,20-dione), such as pregnanolone (5β-pregnan-3α-ol-20-one) and allopregnanolone (5α-pregnan-3α-ol-20-one) are among the active potentiators of $GABA_A$ receptors. Close analogues of pregnanolone with a 3-keto group, such as progesterone are much less active as $GABA_A$ potentiators (Harrison *et al.*, 1987).

3.1.2. Propofol and $GABA_A$ receptors

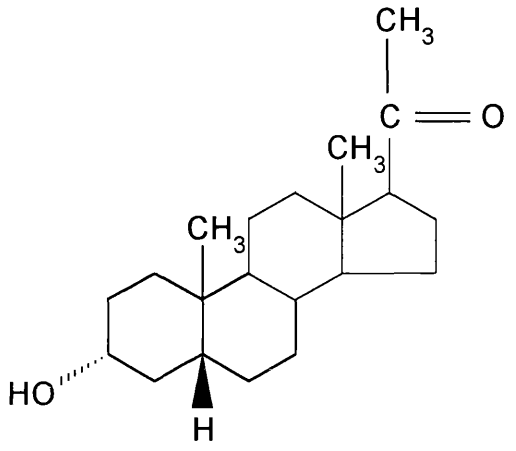
The general anaesthetic, propofol (2,6-diisopropylphenol) has also been shown to enhance ^{3}H-FNZ binding (Prince & Simmonds, 1992b). Recently, however, it was reported that homomeric β_1 $GABA_A$ receptors could be directly activated by propofol but not alphaxalone (Sanna *et al.*, 1995). This provides further support for the suggestion that propofol acts at a different site on the $GABA_A$ receptor complex from the neuroactive steroids.

3.1.3. Biphasic steroid concentration dependencies

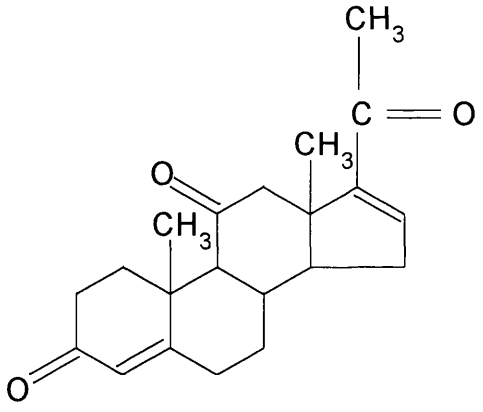
Early studies showed steroids to potentiate GABA responses in a monophasic concentration-dependent manner (e.g. Harrison & Simmonds, 1984). In two more recent studies (Puia *et al.*, 1990 and Morrow *et al.*, 1990), however, distinct biphasic steroid concentration dependencies have been reported and, in both cases, the duration of tissue exposure to the steroid was very short (only 5 seconds).

3.1.4. Aim of this study

In the present investigation, we have sought to determine whether these complex steroid concentration dependencies of GABA potentiation are reflected in the modulation of ^{3}H-FNZ binding. We have determined the bicuculline-sensitivity of the action of pregnanolone and propofol as an indication of their dependence on endogenous GABA. We have also characterised a steroid antagonist effect of 11-ketoprogesterone (4-pregnen-3,11,20-trione) (Fig. 3.1 for structures).



Pregnanolone



11-ketoprogesterone

Figure 3.1. Structures of pregnanolone and 11-ketoprogesterone.

3.2. METHODS

Crude synaptic membranes were prepared from rat whole brain as described in Chapter 2. On the day of the assay, the tissue was thawed and washed 4 times, and the final pellet was resuspended in assay buffer. 100 µl aliquots of membranes (0.4 mg protein.ml^{-1}) were pre-incubated with steroids or propofol for 10 min at 37°C. Then 50 µl of ^{3}H-FNZ was added to each tube to give a final concentration of 1 nM and a total volume of 500 µl assay buffer. The samples were incubated for 60 min at 4°C. The binding reaction was terminated by rapid filtration. Non-specific binding, which was less than 5 % of the total binding, was determined by addition of 10 µM unlabelled FNZ. Radioactivity was quantified by conventional liquid scintillation spectrometry. The steroids were initially dissolved in DMSO, which was present in all samples at a final concentration of 0.45 %. Propofol was initially dissolved in acetone, which was present in all samples at a final concentration of 1 %.

Data were analyzed using Prism (Version 2.0, GraphPad, San Diego, CA). The data were fit to one-site and two-site models (Hawkinson *et al.*, 1994) (see Chapter 2 for details). Statistical comparisons were made by Student's t-test and one-way or two-way ANOVA where applicable.

3.3. RESULTS

3.3.1. Effects of 11-ketoprogesterone on the enhancement by pregnanolone and propofol

on the enhancement by pregnanolone

With a 10 min, 37°C pre-incubation followed by a 60 min 4°C incubation of the membranes with pregnanolone, the log. concentration-effect relationship was approximately linear (Fig. 3.2) having a threshold at 0.1 μM and a 52.2 ± 2.37 % increase in ^{3}H-FNZ binding (mean ± s.e.mean, n = 8) at 30 μM pregnanolone, the highest concentration used. A similar incubation with 60 μM 11-ketoprogesterone instead of pregnanolone showed no enhancement of ^{3}H-FNZ binding (-4.6 ± 0.56 % change from control, mean ± s.e.mean, n = 17). Inclusion of 60 μM 11-ketoprogesterone throughout the incubation with pregnanolone did not affect the lower part of the pregnanolone concentration-effect relationship (up to 1 μM pregnanolone) ($p = 0.55$, not significant, two-way ANOVA), but shifted the upper part to the right in a parallel manner by a factor of 1.9 (0.282 ± 0.013 log unit, mean ± s.e.mean, n = 8) ($p < 0.01$, two-way ANOVA). When this curve was analyzed using the GraphPad software, the two-site model fitted the data significantly better than the one-site model ($p < 0.0001$, F-test). The high-affinity (EC_{50} 358 nM) and low-affinity (EC_{50} 30.0 μM) components constituted 45 % and 55 %, respectively, of the maximal enhancement, which was a 66.7 % increase in binding.

This clear discrimination by 11-ketoprogesterone between the lower and upper parts of the pregnanolone concentration-effect relationship suggested that the enhancement of ^{3}H-FNZ binding by pregnanolone could be separated into a higher affinity component and a lower affinity component, with 11-ketoprogesterone being able to antagonise the lower affinity component. Although the concentration-effect relationship of pregnanolone in the absence of 11-ketoprogesterone appeared to be monophasic, it must be assumed in the light of the foregoing results that there were actually two components in it. Therefore, although the one-site model fitted the control curve significantly better than the two-site model ($p < 0.0001$, F-test), yielding an EC_{50} of 2.59 μM (-5.59 ± 0.10 log units), it is presumed that the two components substantially overlap in the control curve and are difficult to distinguish. In addition, recent experiments in our laboratory have demonstrated that the concentration - effect relationship of pregnanolone on ^{3}H-FNZ binding using rat cortical membranes is clearly biphasic, and significantly better fitted with the two-site model (Goetluck, Schlepper and Simmonds, unpublished results).

on the enhancement by propofol

After a 10 min 37°C pre-incubation followed by a 60 min 4°C incubation with the membranes, propofol yielded a bell-shaped concentration-effect relationship with a maximal 74.5 ± 2.14 % increase in binding at 300 μM (mean ± s.e.mean, n = 4) (Fig. 3.3). 11-ketoprogesterone (60 μM) did not affect the concentration-effect relationship for propofol ($p = 0.57$, not significant, two-way ANOVA). A similar

bell-shaped concentration-effect relationship has been reported from our laboratory before (Prince and Simmonds, 1992b).

3.3.2. Effects of bicuculline and muscimol on the enhancement by pregnanolone, propofol and the interaction of 11-ketoprogesterone and pregnanolone

on the enhancement by pregnanolone

On well washed membranes, there was very little endogenous GABA left, as evidenced by the fact that 100 μM bicuculline failed to reduce ^{3}H-FNZ binding significantly (-2.98 ± 2.46 % from control, mean ± s.e.mean, n = 6). Nevertheless, it was possible that part of the potentiating action of pregnanolone on ^{3}H-FNZ binding was due to potentiation of a subthreshold GABA tone. Therefore, in this series of experiments, 100 μM bicuculline was routinely used. The blank tubes received the same amount of bicuculline as the test tubes, so the baselines of all curves were reset to 100 %.

The influence of 100 μM bicuculline on the concentration-effect relationship for 60 min exposures to pregnanolone is shown in Fig. 3.4A. In the absence of bicuculline, the concentration-effect relationship had a threshold at 0.1 μM and approached a maximum at 10 and 30 μM, with 30 μM giving 63.7 ± 3.25 % increase in binding (mean ± s.e.mean, n = 5). In the presence of bicuculline, however, part of the effect of pregnanolone was blocked, and the remaining concentration-effect relationship

appeared to be monophasic and extended over only the lower affinity end of the concentration range. Sigmoidal fits of the latter data yielded a threshold of 1 μM, an EC_{50} of 3.73 μM (-5.43 ± 0.05 log units), and an E_{max} of 34.2 ± 1.53 % increase in binding (mean ± s.e.mean, n = 4).

Parallel experiments were performed with 0.1 μM muscimol added to the medium to accentuate the GABA-dependent phenomena (Fig. 3.4B). Muscimol 0.1 μM, which enhanced ^{3}H-FNZ binding by 20.6 ± 2.80 % (mean ± s.e.mean, n = 6), was present in all assay tubes and the baselines for all curves were reset to 100 %. Muscimol increased the enhancement of ^{3}H-FNZ binding by pregnanolone over the entire pregnanolone concentration range such that the threshold was reduced to below 0.1 μM and the increase in binding at 30 μM was 79.2 ± 3.18 % (mean ± s.e.mean, n = 3). When 100 μM bicuculline was added, the remaining concentration-effect relationship was very similar to that obtained with bicuculline in the absence of muscimol. Sigmoidal fits of the curve in the presence of both bicuculline and muscimol yielded a threshold of 0.3 μM, an EC_{50} of 3.24 μM (-5.49 ± 0.17 log unit) and an E_{max} of 36.2 ± 5.70 % increase in binding (mean ± s.e.mean, n = 4).

on the interaction of 11-ketoprogesterone and pregnanolone

Since 11-ketoprogesterone could weakly antagonise a low affinity component of the enhancement of ^{3}H-FNZ binding by pregnanolone with no effect on higher affinity components, and there appeared to be both bicuculline-sensitive and bicuculline-

resistant low affinity components, we were interested to see if the action of 11-ketoprogesterone still existed in the presence of bicuculline. As shown in Table 3.1, after a 10 min pre-incubation at 37°C followed by a 60 min incubation at 4°C, pregnanolone 30 μM enhanced ^{3}H-FNZ binding by 63.2 ± 2.29 %, which was significantly reduced to 55.2 ± 2.15 % by 60 μM 11-ketoprogesterone. This is consistent with the results from Fig. 3.2. In the presence of 100 μM bicuculline, the enhancement by pregnanolone 30 μM was 31.5 ± 1.27 %, and 60 μM 11-ketoprogesterone was still able to reduce this enhancement significantly to a 23.8 ± 1.49 % increase in binding. The results were also expressed in specific binding, and the conclusion was the same. This result indicated that 11-ketoprogesterone reduced only the bicuculline-resistant component of pregnanolone's action.

on the enhancement by propofol

In another series of experiments (Fig. 3.5A), in which the membranes were incubated for 60 min at 4°C with propofol, bicuculline 100 μM significantly ($p < 0.05$, Student's t-test) shifted the concentration-effect relationship for propofol to the right without changing the maximum. Sigmoidal fits of the data yielded an EC_{50} of 137 μM (-3.86 ± 0.10 log unit) and an E_{max} of 76.0 ± 10.54 % increase in binding (mean ± s.e.mean, n = 3) in the presence of bicuculline compared with the control EC_{50} of 64.5 μM (-4.19 ± 0.06 log unit) and E_{max} of 64.9 ± 4.87 % increase in binding (mean ± s.e.mean, n = 3). Muscimol 0.1 μM had less effect on the concentration-effect relationship for propofol (Fig. 3.5B) than on that for

pregnanolone, yielding a curve with an EC_{50} of 47.8 μM (-4.32 ± 0.09 log unit) and an E_{max} of 71.6 ± 6.11 % increase in binding, neither of which were significantly different from control values (mean ± s.e.mean, n = 3). However, the effect of muscimol 0.1 μM on the concentration - effect relationship for propofol was still significant ($p < 0.0001$, two-way ANOVA). Bicuculline 100 μM completely inhibited the effect of 0.1 μM muscimol and yielded a curve with an EC_{50} of 87.1 μM (-4.06 ± 0.04 log unit), and an E_{max} of 60.5 ± 3.26 % increase in binding, (mean ± s.e.mean, n = 3) (Fig. 3.5B). Although neither the EC_{50} nor the E_{max} values were significantly different from those with 0.1 μM muscimol present, the effect of bicuculline on the concentration - effect relationship for propofol in the presence of 0.1 μM muscimol was significant ($p < 0.0001$, two-way ANOVA).

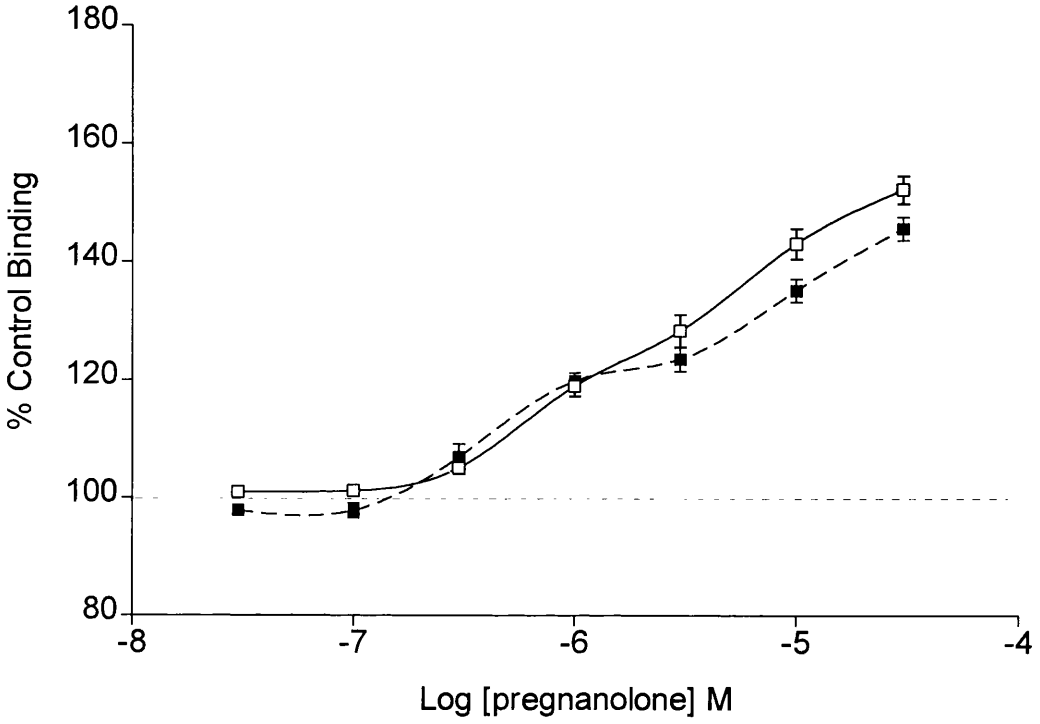


Figure 3.2. Concentration-effect relationships for the enhancement of ^{3}H-FNZ binding by pregnanolone after 10 min preincubation at 37°C + 60 min incubation at 4°C in the absence (□) and presence (■) of 11-ketoprogesterone (60 μM). Data are the means ± s.e.means of 8 experiments. All data points are the means of triplicates.

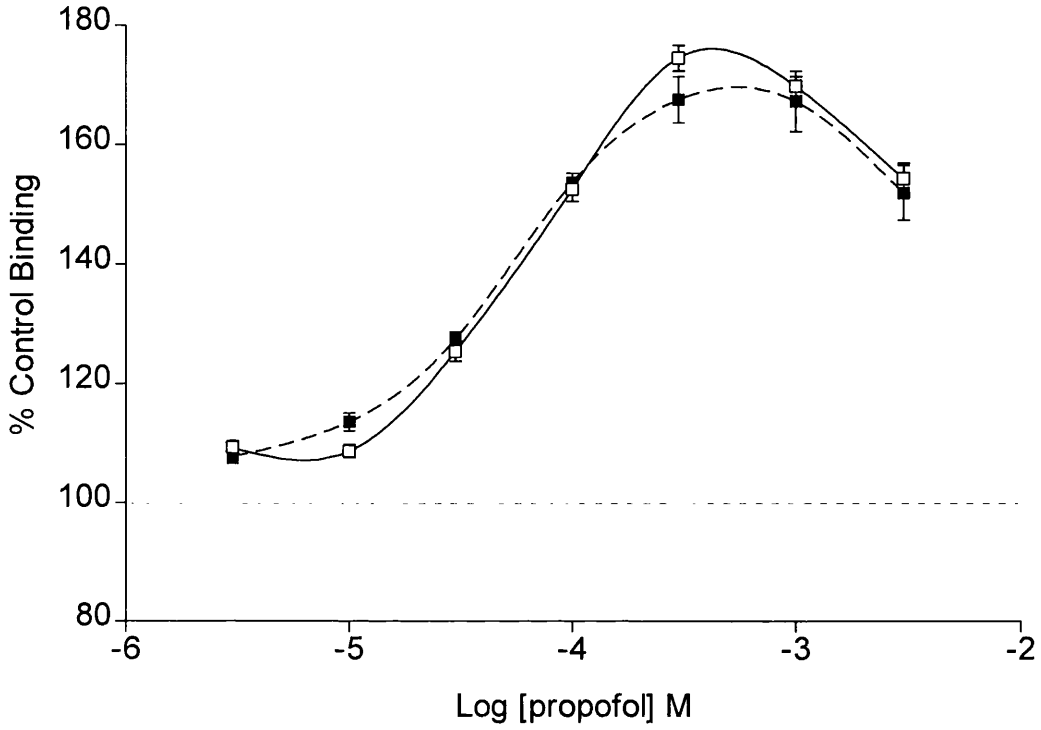


Figure 3.3. Concentration-effect relationships for the enhancement of ^{3}H-FNZ binding by propofol in the absence (□) and presence (■) of 11-ketoprogesterone (60 μM) after 10 min preincubation at 37°C + 60 min incubation at 4°C. Data are the means ± s.e.means of 3 to 4 experiments. All data points are the means of triplicates.

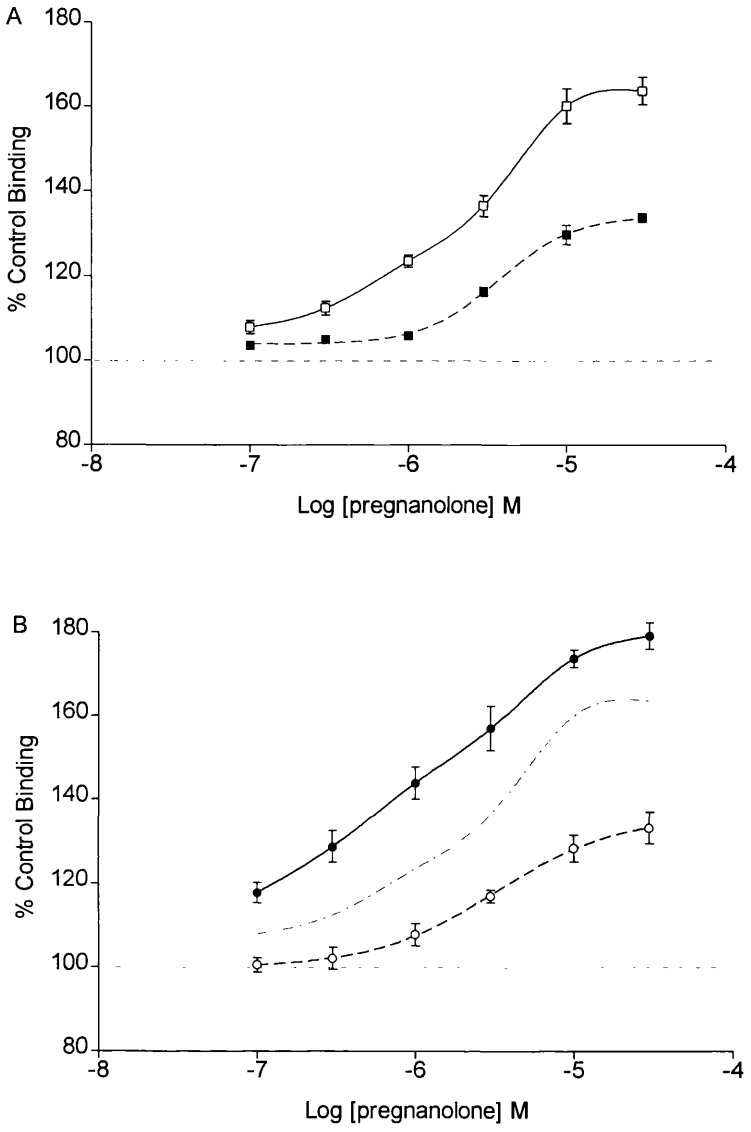


Figure 3.4. Concentration-effect relationships for the enhancement of ^{3}H-FNZ binding by pregnanolone (A) in the absence (□) and presence (■) of bicuculline 100 μM; (B) in the presence of muscimol 0.1 μM (●) and both bicuculline 100 μM and muscimol 0.1 μM (○) after 60 min incubation at 4°C (no pre-incubation). The control curve of pregnanolone in (A) (□) was superimposed as the dashed line in (B) for comparison. Data are the means ± s.e.means of 3 to 5 experiments. All data points are the means of triplicates.

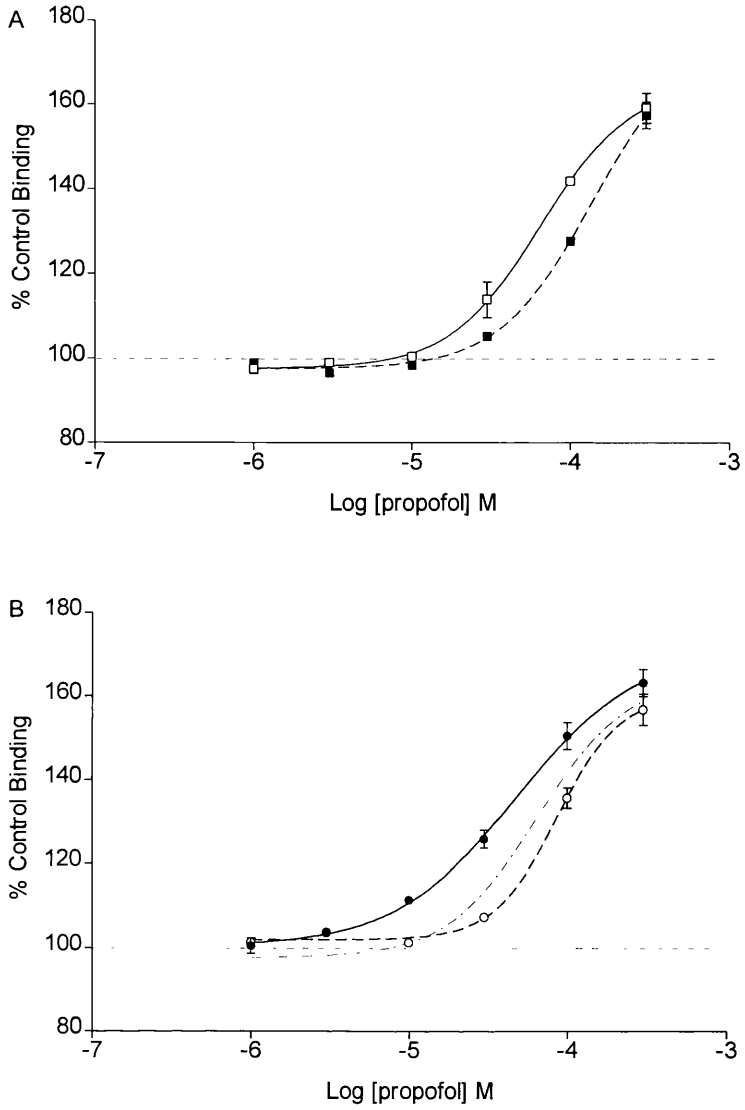


Figure 3.5. Concentration-effect relationships for the enhancement of ^{3}H-FNZ binding by propofol (A) in the absence (□) and presence (■) of bicuculline 100 μM; (B) in the presence of muscimol 0.1 μM (●) and both bicuculline 100 μM and muscimol 0.1 μM (○) after 60 min incubation at 4°C (no pre-incubation). The control curve for propofol in (A) (□) was superimposed as the dashed line in (B) for comparison. Data are the means ± s.e.means of 3 experiments. All data points are the means of triplicates.

Table 3.1. Effect of 11-ketoprogesterone (60 μM) on the enhancement of ^{3}H-FNZ binding by pregnanolone (30 μM) in the presence and absence of bicuculline (100 μM) after 10 min pre-incubation at 37°C and 60 min incubation at 4°C.

Drugs	% enhancement of binding	specific binding (fmol/mg)
Pregnanolone	63.2 ± 2.29	373.4 ± 5.24
Pregnanolone + 11-ketoprogesterone	55.2 ± 2.15 *	355.1 ± 4.92 *
Pregnanolone + bicuculline	31.5 ± 1.27	300.9 ± 2.91
Pregnanolone + bicuculline + 11-ketoprogesterone	23.8 ± 1.49 **	283.3 ± 3.41 **

Data are the means ± s.e.means of 4 experiments. Difference due to 11-ketoprogesterone was significant: * $p < 0.05$, ** $p < 0.01$, Student's t-test.

3.4. DISCUSSION

The major findings of this study are the characterisation of two components in the enhancement of ^{3}H-FNZ binding by pregnanolone and the confirmation of different mechanisms of action between pregnanolone and propofol reported earlier by other groups (see Chapter 1).

In a recent paper (Hawkinson *et al.,* 1994), pregnanolone was demonstrated to enhance ^{3}H-FNZ binding to $GABA_A$ receptors in a biphasic manner. Our present results confirm these observations and show different pharmacologies for two components of the enhancement of ^{3}H-FNZ binding by pregnanolone. By applying 100 μM bicuculline or 0.1 μM muscimol, we differentiated what we presume to be GABA-dependent and GABA-independent components in the action of pregnanolone with respect to its enhancement of ^{3}H-FNZ binding (Fig. 3.4). The GABA-dependent component embraced the whole range of pregnanolone concentrations studied and such a dependence suggests that this action of pregnanolone was due either to potentiation of GABA, which enhanced the ^{3}H-FNZ binding, or to an allosteric action of pregnanolone at a site deep within the channel, access to which was dependent upon GABA opening the channel. The GABA-independent component was apparent only at concentrations of 1 μM pregnanolone and above, which suggests that pregnanolone can enhance ^{3}H-FNZ binding directly at higher concentrations.

The interaction of 11-ketoprogesterone with the concentration-effect relationship for pregnanolone showed a weak antagonism of a low affinity component of pregnanolone's action (Fig. 3.2). Further experiments showed that it was the GABA-independent component that could be antagonised by 11-ketoprogesterone (Table 3.1). These data suggest that the direct, GABA-independent action of pregnanolone involves a separate recognition site from the GABA-dependent action. The relative contributions of these two actions to the total effect of pregnanolone changed with pregnanolone concentration. At concentrations below 1 μM, the GABA-dependent action was dominant but, at higher concentrations, the direct action contributed increasingly. At any particular concentration of pregnanolone, the ratio of the contributions from the two actions will depend on the concentration of GABA present. In the well-washed membranes used in the present study, where the remaining GABA concentration was low, the direct action contributed about half of the potentiation of ^{3}H-FNZ binding by pregnanolone, at best. Fig. 3.4 suggests that higher concentrations of GABA would increase the contribution of the GABA-dependent component without altering the contribution from the direct action of pregnanolone. Thus, the latter action would contribute a smaller proportion to the increased total effect of pregnanolone in the presence of increased GABA concentrations.

Since pregnanolone appears to have two independent actions on ^{3}H-FNZ binding, other steroids may exert their effects on ^{3}H-FNZ binding through similar mechanisms. It would be interesting, therefore, to determine for other neurosteroids,

the relative contributions of GABA-dependent and GABA-independent components to their actions, which could well differ from that found with pregnanolone.

This profile clearly differs from that for propofol which, although enhancing ^{3}H-FNZ binding to a similar maximal extent as pregnanolone, did so in a monophasic manner that was only weakly GABA-dependent (Fig. 3.5). Also, the action of propofol was insensitive to 11-ketoprogesterone (Fig. 3.3). These results indicate that propofol does not bind to the same range of sites as pregnanolone and, thus, confirm and extend previous observations from our laboratory (Prince & Simmonds, 1992b). They are at variance, however, with the report by Concas *et al.* (1990), who failed to obtain any effect of propofol on ^{3}H-FNZ binding to $GABA_A$ receptors, but the lack of NaCl in their buffer and the use of unwashed membranes may be material factors.

3.5. CONCLUSIONS

Altogether, our results indicate that the enhancement of ^{3}H-FNZ binding by pregnanolone involves at least two substantial components whereas the enhancement by propofol is satisfactorily represented by a single component. The actions of both potentiators will be influenced by the amount of GABA agonist present. The action of pregnanolone will be enhanced by GABA to a greater extent than will the action

of propofol but this enhancement occurs with only one of the two components of pregnanolone's action.

CHAPTER 4

EFFECTS OF PROGESTERONE, EPIPREGNANOLONE, PREGNENOLONE ON THE ENHANCEMENT BY PREGNANOLONE OF ^{3}H-FLUNITRAZEPAM BINDING

4.1. INTRODUCTION

4.1.1. Structure-activity relationship of neuroactive steroids

As mentioned in Section 3.1, and detailed in Chapter 1, neurosteroids such as pregnanolone and allopregnanolone, which can be synthesized locally from their precursor, cholesterol (Fig. 4.1 for the synthetic pathway, Simmonds & Prince, 1993), can allosterically enhance ^{3}H-muscimol binding and ^{3}H-FNZ binding and decrease ^{35}S-TBPS binding. Steroid modulation of the $GABA_A$ receptors shows a distinct structure-activity relationship, with a 3α-OH moiety being a requirement for activity and a 20 keto group being favoured over a 20-hydroxy (Harrison *et al.*, 1987). Certain metabolites of progesterone (4-pregnen-3,20-dione), such as pregnanolone(5β-pregnan-3α-ol-20-one),allopregnanolone(5α-pregnan-3α-ol-20-one), as well as synthetic analogues, such as alphaxalone (5α-pregnan-3α-ol-11,20-dione) are among the active potentiators of $GABA_A$ receptors. Close analogues of pregnanolone with a 3-keto group, such as progesterone, or a 3β-OH group, such as pregnenolone (5-pregnen-3β-ol-20-one) and epipregnanolone (5β-pregnan-3β-ol-20-one) are much less active as $GABA_A$ potentiators (Harrison *et al.*, 1987) (see Fig. 4.1 also for structures).

4.1.2. Evidences for multiple components in steroids action

Much evidence suggests that neurosteroids modulate the $GABA_A$ receptors through

a site of action distinct from those of benzodiazepines, barbiturates and propofol (Sieghart, 1992; Prince & Simmonds, 1992b; Chapter 3). Recently the presence of multiple steroid binding sites has been proposed on the basis of the complex (biphasic) concentration-response relationships for some active neurosteroids (Morrow *et al.*, 1990; Puia *et al.*, 1990; Hawkinson *et al.*, 1994 and McCauley *et al.*, 1995). We reported earlier that the enhancement of ^{3}H-FNZ binding by pregnanolone was comprised of two components, of which the low-affinity component was found, serendipitously, to be antagonised by 11-ketoprogesterone (4-pregnen-3,11,20-trione) (Chapter 3). The antagonism was weak but selective.

4.1.3. Aim of this study

In the present investigation, we have sought to determine whether progesterone itself, the precursor of pregnanolone, has similar effects to its analogue, 11-ketoprogesterone; and whether other inactive steroids, such as pregnenolone, has similar effect as epipregnanolone, which showed specific antagonistic effect to pregnanolone (Prince & Simmonds, 1992c).

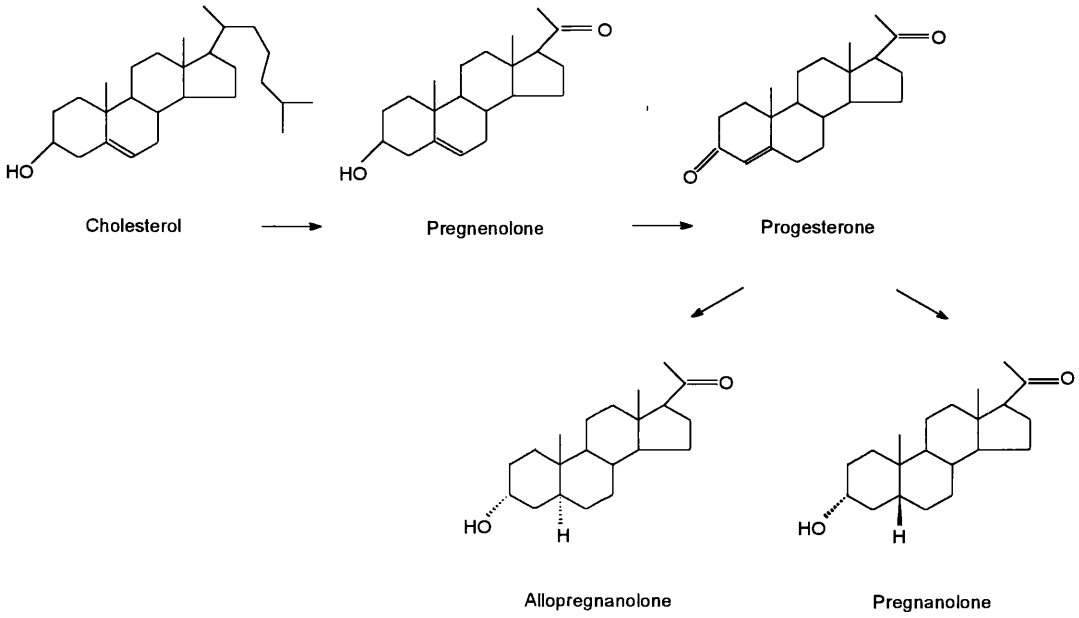


Figure 4.1. The structures and synthetic pathway of some relevant steroids.

4.2. METHODS

Crude synaptic membranes were prepared from rat whole brain as described in Chapter 2. On day of the assay, the tissue were thawed and washed 4 times, and the final pellet was resuspended in assay buffer. Progesterone, epipregnanolone and pregnenolone were investigated as possible antagonists of pregnanolone. In these experiments, 100 µl aliquots of membranes (0.4 mg protein.ml^{-1}) were pre-incubated with less active steroids and pregnanolone for 10 min at 37°C prior to the addition of ^{3}H-FNZ (50 µl) to give a final concentration of 1 nM and a total volume of 500 µl assay buffer., then a 60 min incubation was carried out at 4°C. The binding reaction was terminated by rapid filtration. Non-specific binding, which was less than 5 % of the total binding, was determined by addition of 10 µM unlabelled FNZ. Radioactivity was quantified by conventional liquid scintillation spectrometry. The steroids were initially dissolved in DMSO, which was present in all samples at a final concentration of 0.45 %.

Graphs were generated using Prism (Version 2.0, GraphPad, San Diego, CA). Statistical comparisons were made by Student's t-test.

4.3. RESULTS

4.3.1. Effects of progesterone on the enhancement of ^{3}H-FNZ binding by pregnanolone

With a 10 min, 37°C pre-incubation followed by a 60 min 4°C incubation of the membranes with pregnanolone, the log. concentration-effect relationship was approximately linear (Fig. 4.2), having a threshold at 0.1 μM and a 65.4 ± 0.89 % increase in ^{3}H-FNZ binding (mean ± s.e.mean, n = 4, calculated from the control curve of Fig. 4.2A) at 30 μM pregnanolone, the highest concentration used. From the previous study (Chapter 3), we suggest that there are actually two components in the concentration-effect relationship of pregnanolone, although it appeared to be monophasic. Therefore, we did not feel it appropriate to fit the control curve to a one-site sigmoidal model. A similar incubation with 60 μM progesterone instead of pregnanolone showed no enhancement of ^{3}H-FNZ binding (0.2 ± 1.72 % change from control, mean ± s.e.mean, n = 4).

Inclusion of various concentrations of progesterone affected the concentration-effect relationships of pregnanolone dose-dependently. Progesterone 1 μM (Fig. 4.2A) had no effect on most of the curve, but significantly decreased the enhancement by pregnanolone at 30 μM, the highest concentration used ($p < 0.05$, Student's t-test). Progesterone 10 μM (Fig. 4.2B) gave a similar result to progesterone 1 μM. As the concentration of progesterone was increased to 30 μM (Fig. 4.2C) and 60 μM

(Fig. 4.2D), the antagonism extended over a wider range of the pregnanolone concentration-effect relationship.

4.3.2. Interactions of other less active steroids with pregnanolone

Effect of pregnenolone (60 μM)

Pregnenolone itself at 60 μM showed no enhancement of ^{3}H-FNZ binding (3.0 ± 2.81 % change from control, n = 8). Inclusion of 60 μM pregnenolone did not alter the concentration-effect relationship of pregnanolone significantly (Fig. 4.3).

Effect of epipregnanolone (30 μM)

With a 10 min, 37°C pre-incubation followed by a 60 min 4°C incubation of the membranes with epipregnanolone (30 μM) instead of pregnanolone, the binding was reduced by 4.9 ± 0.75 % from the control ($p < 0.05$, Student's t-test, mean ± s.e.mean, n = 5). Inclusion of epipregnanolone 30 μM significantly decreased the enhancement by pregnanolone at 0.1 μM and 1 μM (Fig. 4.4, $p < 0.05$, Student's t-test). This reduction may be due to the decrease in the basic binding.

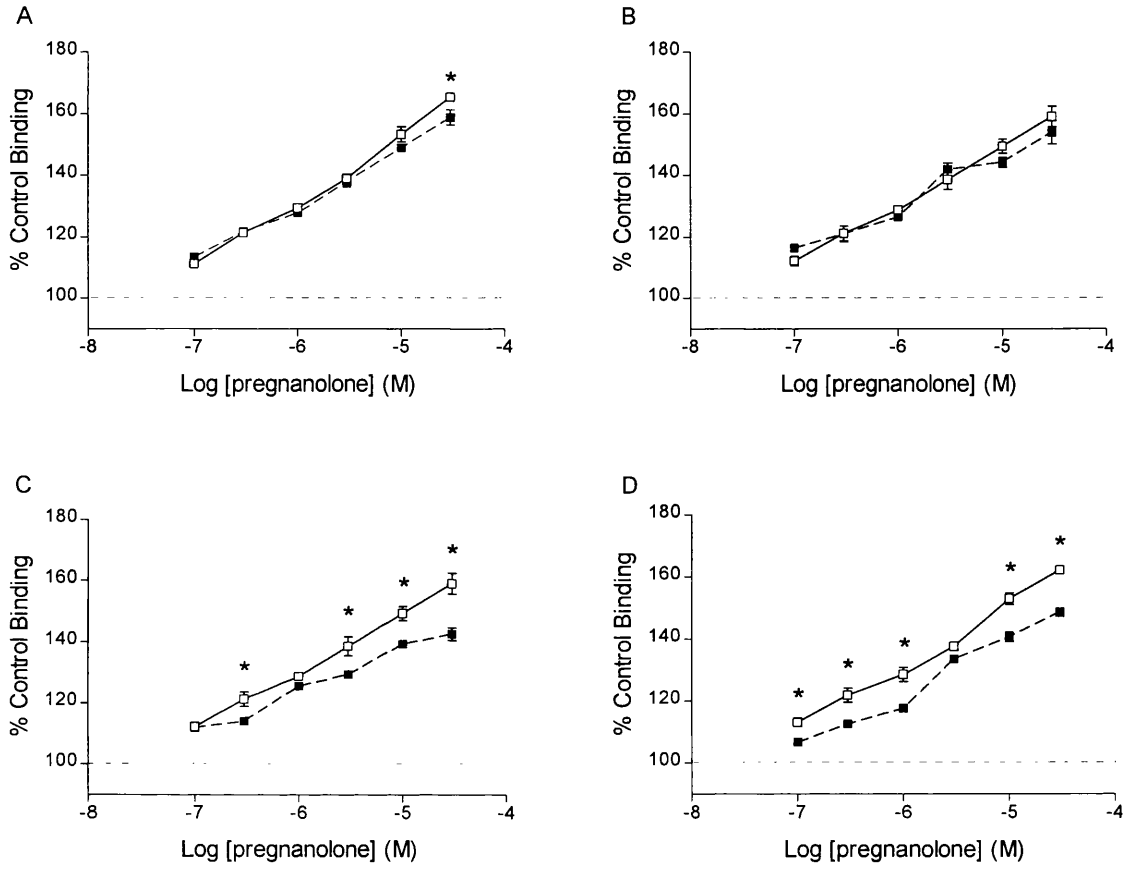


Figure 4.2. The concentration-effect relationships of pregnanolone in the absence (□) and presence (■) of progesterone 1μM (A), 10 μM (B), 30 μM (C) and 60 μM (D). Data are the means ± s.e.means of 4-5 experiments. Where error bars are not shown, they are smaller than the symbols. All data points are the means of triplicates. Student's t-test was applied to all pairs of data in the presence and absence of progesterone, and only those marked with * were significantly different, $p < 0.05$, Student's t-test.

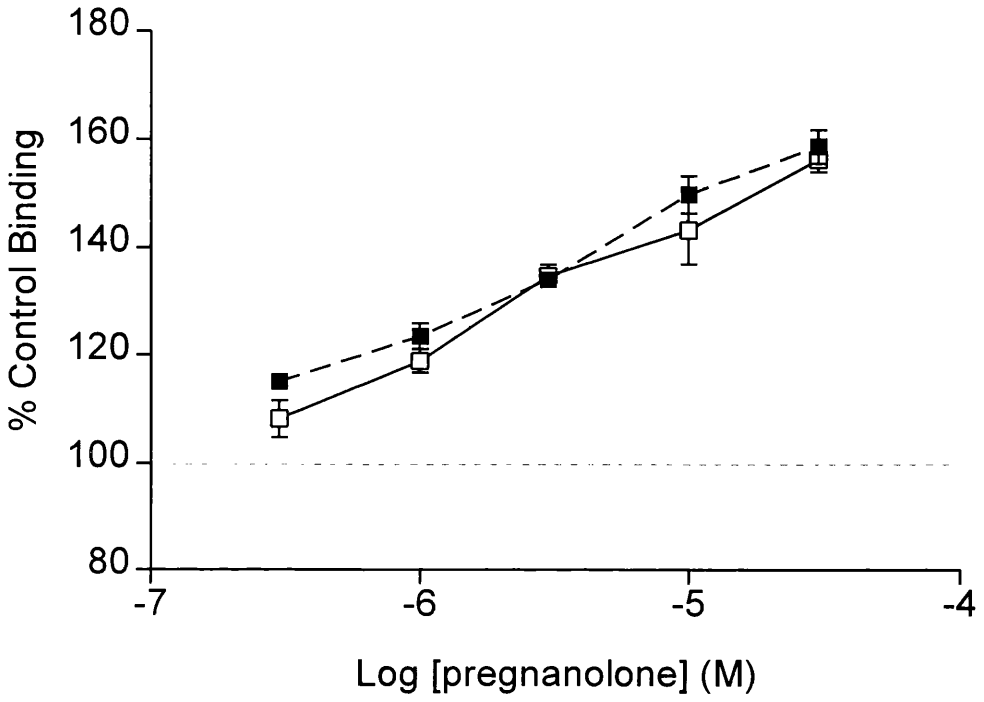


Figure 4.3. The concentration-effect relationships of pregnanolone in the absence (□) and presence (■) of pregnenolone 60 μM. Data are the means ± s.e.means of 4 experiments, where error bars are not shown, they are smaller than the symbols. All data points are the means of triplicates.

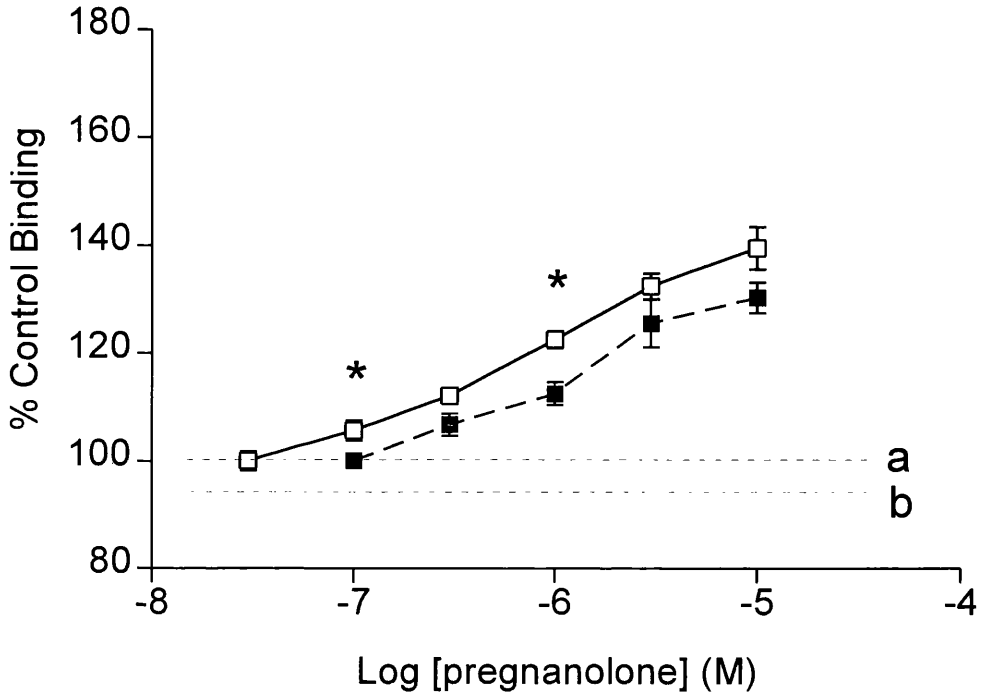


Figure 4.4. The concentration-effect relationships of pregnanolone in the absence (□) and presence (■) of epipregnanolone 30μM. Data are the means ± s.e.means of 5 experiments, where error bars are not shown, they are smaller than the symbols. All data points are the means of triplicates. a. starting level (100 %) for the curve (□); b. starting level (95 %) for the curve (■). Student's t-test was applied to all pairs of data in the presence and absence of epipregnanolone, and only those marked with * were significantly different, $p < 0.05$, Student's t-test.

4.4. DISCUSSION

Pregnanolone enhanced ^{3}H-FNZ binding dose-dependently (Fig. 4.2), while progesterone showed no enhancement, which was in agreement with the results published before (e.g. McAuley *et al.*, 1993). When progesterone was co-applied with pregnanolone, it showed dose-dependent inhibition on the enhancement by pregnanolone, with a slightly greater potency against the higher concentration range.

We were unable to demonstrate clearly, with the present results, the existence of two components in the action of pregnanolone. However, our previous study with 11-ketoprogesterone (Chapter 3) established that low and high affinity components do exist. In the light of this, it may be suggested that the slightly greater potency of progesterone against the highest concentration of pregnanolone used is compatible with a slight selectivity of progesterone against the lower affinity component of pregnanolone's action (Fig. 4.2). This would accord with the results from 11-ketoprogesterone which showed a clear selectivity against the lower affinity component. Like 11-ketoprogesterone, progesterone was only a weak antagonist of pregnanolone in its action as an enhancer of ^{3}H-FNZ binding.

Progesterone is present in cerebral cortex of rat at about 7 ng/g protein, much higher than its level in the plasma (about 0.5 ng/ml) (Purdy *et al.*, 1991). Previous reports of the interaction of progesterone with the $GABA_A$ system may be interpreted either in terms of the genomic properties of progesterone, e.g. in the modulation of

the levels of mRNA for $GABA_A$ receptor subunits (Weiland, 1995), or in terms of the metabolism of progesterone to pregnanolone (Gee *et al.*, 1995), eg. in the inhibition by progesterone *in vivo* of cerebellar purkinje cells (Smith, 1989; Smith *et al.*, 1987a & 1987b). In contrast, our present results demonstrate a weak direct interaction of progesterone with the $GABA_A$ receptor whereby it reduced the potency of its metabolite pregnanolone as an enhancer of ^{3}H-FNZ binding. Since most of this action of pregnanolone, under the conditions of these experiments, does not involve an intermediating modulation of GABA itself (Chapter 6), it remains to be seen to what extent progesterone will antagonise its metabolites in their actions as GABA potentiators. The answer will be of potential physiological as well as pharmacological interest.

Neither the basic ^{3}H-FNZ binding nor the concentration-effect relationship of pregnanolone were affected by pregnenolone (60 μM) (Fig. 4.3). Therefore, pregnenolone was proved to be ineffective either as an agonist or as a competitive antagonist to pregnanolone on $GABA_A$ receptors.

Epipregnanolone was indicated to be a competitive antagonist of pregnanolone on $GABA_A$ receptor, with a derived K_i of 10.5 μM (Prince & Simmonds, 1992c). But in the present study, the shift caused by epipregnanolone 30 μM on the concentration-effect relationship of pregnanolone seemed better described as "downwards" rather than "rightwards", with some contribution from the downwards shift of the baseline (Fig. 4.4). This result is at variance with the work by Dr R.

Prince (Prince & Simmonds, 1992c), but in agreement with (Kokate *et al.*, 1994), who also failed to establish epipregnanolone as the specific antagonist at the neurosteroid sites on $GABA_A$ receptors. In their hands, epipregnanolone neither blocked the potentiation of GABA responses produced by 3α-hydroxy steroids nor prevented the anticonvulsant activity of these compounds. Nevertheless, my results suggested that unlike 11-ketoprogesterone and progesterone, epipregnanolone did not distinguish between the two components of action of pregnanolone in enhancing ^{3}H-FNZ binding to $GABA_A$ receptors, rather, it shifted the whole curve downwards.

4.5. CONCLUSIONS

Altogether, these results showed that similar to 11-ketoprogesterone, progesterone also showed a weak but slight selective antagonistic effect to the lower affinity component of pregnanolone; while pregnenolone was not active either as an agonist, or as a steroid antagonist and epipregnanolone did not antagonise pregnanolone competitively either.

CHAPTER 5

EFFECTS OF Δ^{16}-ALPHAXALONE ON THE ENHANCEMENT BY ALPHAXALONE AND PREGNANOLONE OF ^{3}H-FLUNITRAZEPAM BINDING

5.1. INTRODUCTION

5.1.1. Alphaxalone and $GABA_A$ receptors

Alphaxalone (5α-pregnan-3α-ol-11,20-dione), a steroid developed by Glaxo some 20 years earlier, is a powerful anaesthetic and the main active component in the clinically used anaesthetic Althesin. In 1984, alphaxalone was reported to enhance depolarization responses to GABA and muscimol in slice preparations of the rat cuneate nucleus (Harrison & Simmonds, 1984). Since this pioneer work, considerable data have accumulated demonstrating the interactions between neuroactive steroids and $GABA_A$ receptors. As detailed in Chapter 1, alphaxalone, pregnanolone and allopregnanolone are among the active potentiators of $GABA_A$ receptors.

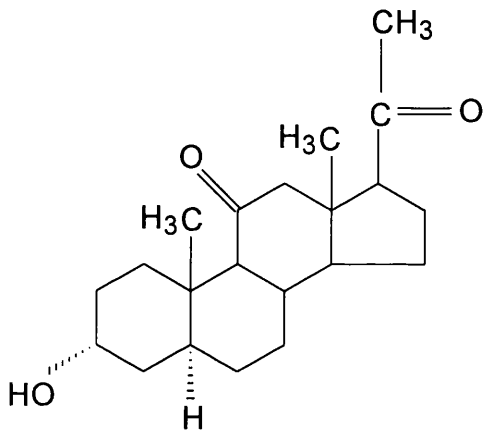
5.1.2. Δ^{16}-alphaxalone and alphaxalone

Δ^{16}-alphaxalone (5α-pregnan-3α-ol-16-ene-11,20-dione) is a non-anaesthetic which differs from alphaxalone only by having a double bond in the C-16 position (see Fig. 5.1 for structures). This double bound favoured a planar configuration for the substituent at position 17 rather than the β configuration as in alphaxalone. This structure change resulted in a loss in membrane perturbation ability, and probably hence the anaesthetic property (Makriyannis *et al.*, 1990). In one study, Δ^{16}-alphaxalone was reported to antagonise the depressant action of alphaxalone on

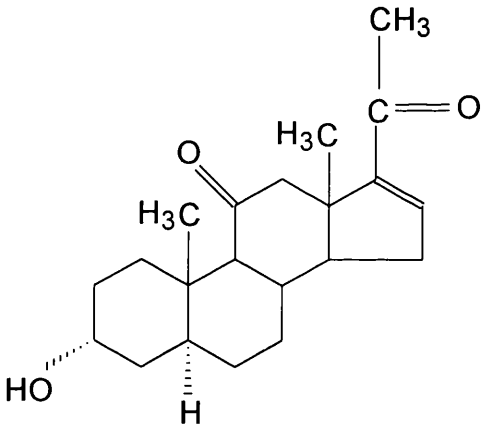
synaptic transmission (Richards & Hesketh, 1975).

5.1.3. Aim of this study

In the present study, we have sought to determine whether Δ^{16}-alphaxalone has any effect on ^{3}H-FNZ binding where alphaxalone was found to be a full agonist, similar to pregnanolone (Prince & Simmonds, 1993); and whether there is any interaction between Δ^{16}-alphaxalone and alphaxalone when enhancing ^{3}H-FNZ binding. As a comparison, the interactions between Δ^{16}-alphaxalone and pregnanolone was also investigated.



Alphaxalone



Δ^{16}- alphaxalone

Figure 5.1. Structures of alphaxalone and Δ^{16}-alphaxalone.

5.2. METHODS

Crude synaptic membranes were prepared from rat whole brain as described in Chapter 2. On day of the assay, the tissue were thawed and washed 4 times, and the final pellet was resuspended in assay buffer. 100 µl aliquots of membranes (0.4 mg protein.ml^{-1}) were pre-incubated with steroids for 10 min at 37°C. Then 50 µl of ^{3}H-FNZ was added to each tube to give a final concentration of 1 nM and a total volume of 500 µl assay buffer. The samples were incubated for 60 min at 4°C. In experiments where the interactions between Δ^{16}-alphaxalone and other full GABA-agonists were investigated, a fixed concentration of Δ^{16}-alphaxalone was included throughout the whole incubation period, while the concentrations of the full agonists varied. The binding reaction was terminated by rapid filtration. Non-specific binding, which was less than 5 % of the total binding, was determined by addition of 10 µM unlabelled FNZ. Radioactivity was quantified by conventional liquid scintillation spectrometry. The steroids were initially dissolved in acetone, which was present in all samples at a final concentration of 1 %.

5.3. RESULTS

5.3.1. Enhancement by Δ^{16}-alphaxalone

To our surprise, Δ^{16}-alphaxalone had a small yet significant potentiating effect on ^{3}H-

FNZ binding (Fig. 5.2, $p < 0.001$, two-way ANOVA). The concentration-effect relationship had a threshold of 0.3 μM, reached a maximal 16.1 % increase in binding at 10 μM (mean of two experiments). Inclusion of bicuculline 100 μM significantly decreased the enhancement by Δ^{16}-alphaxalone ($p < 0.001$, two-way ANOVA). These results indicate that Δ^{16}-alphaxalone could enhance ^{3}H-FNZ binding modestly, in a GABA-dependent manner.

5.3.2. Effects of Δ^{16}-alphaxalone on the concentration-effect relationship of alphaxalone

In my hands, alphaxalone, instead of producing a marked enhancement of ^{3}H-FNZ binding as reported before (Prince & Simmonds, 1993), only showed a slightly bigger enhancement than Δ^{16}-alphaxalone (Fig. 5.3). The concentration-effect relationship had a threshold of 0.3 μM, a maximal 21.9 ± 1.71 % increase in binding at 30 μM, the highest concentration tried (mean ± s.e.mean, n = 4).

Since Δ^{16}-alphaxalone was structurally so close to alphaxalone, yet had antagonistic effect upon alphaxalone in synaptic transmission experiments, we attempted to investigate the interactions between them in the binding studies. In these experiments, Δ^{16}-alphaxalone 60 μM was included in the incubation with varied concentrations of alphaxalone. Δ^{16}-alphaxalone 60 μM on its own gave a 14.5 ± 3.88 % increase in binding (mean ± s.e.mean, n = 4). On top of this, increasing concentrations of alphaxalone did not produce a dose-dependent enhancement.

Instead, in the presence of Δ^{16}-alphaxalone 60 μM, the concentration-effect relationship of alphaxalone was a horizontal line, with a 19.4 ± 0.41 % increase in binding at 30 μM (mean ± s.e.mean, n = 4). These results suggest that in my hand, alphaxalone was quite similar to Δ^{16}-alphaxalone, only enhanced the binding to a modest extent; and on top of the enhancement by Δ^{16}-alphaxalone 60 μM, alphaxalone could not enhance it any further.

5.3.3. Effects of Δ^{16}-alphaxalone on the concentration-effect relationship of pregnanolone

Pregnanolone dose-dependently enhanced ^{3}H-FNZ binding (Fig 5.4), as reported in Chapter 3 and 4. The concentration-effect relationship was approximately linear, having a threshold of less than 0.1 μM, a 58.3 ± 3.29 % increase in binding at 30 μM (highest concentration tried) (mean ± s.e.mean, n = 4). As discussed in Section 3.3.1, we did not feel it appropriate to fit this curve to a one site model, although the software rejected a two-site model.

As a comparison, the effect of Δ^{16}-alphaxalone on the concentration-effect relationship of pregnanolone was also investigated. Δ^{16}-alphaxalone 60 μM on its own gave a 17.4 ± 3.36 % increase in binding (mean ± s.e.mean, n = 4). On top of this, increasing concentrations of pregnanolone enhanced the binding further, reaching a 62.4 ± 1.08 % increase in binding at 30 μM (mean ± s.e.mean, n = 4). These results suggest that pregnanolone is more potent and much more efficacious

than Δ^{16}-alphaxalone and alphaxalone when enhancing ^{3}H-FNZ binding; and on top of the enhancement by Δ^{16}-alphaxalone 60 μM, pregnanolone could enhance the binding dose-dependently, reaching similar maximum as in the absence of Δ^{16}-alphaxalone.

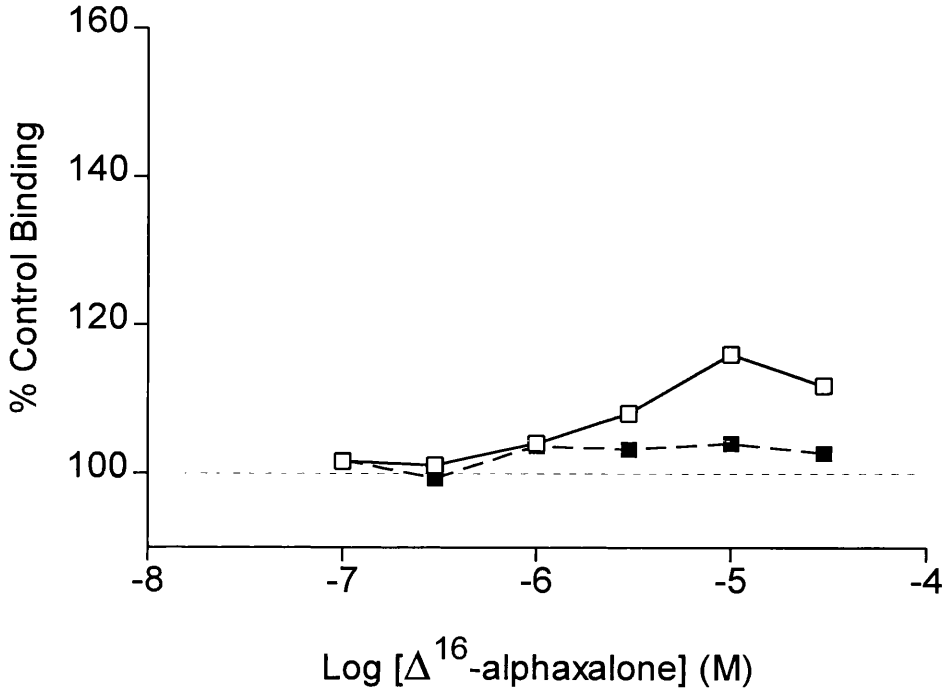


Figure 5.2. Concentration-effect relationships for the enhancement of ^{3}H-FNZ binding by Δ^{16}-alphaxalone after 10 min preincubation at 37°C + 60 min incubation at 4°C in the absence (□) and presence (■) of bicuculline (100 μM). Data are the means of 2 experiments.

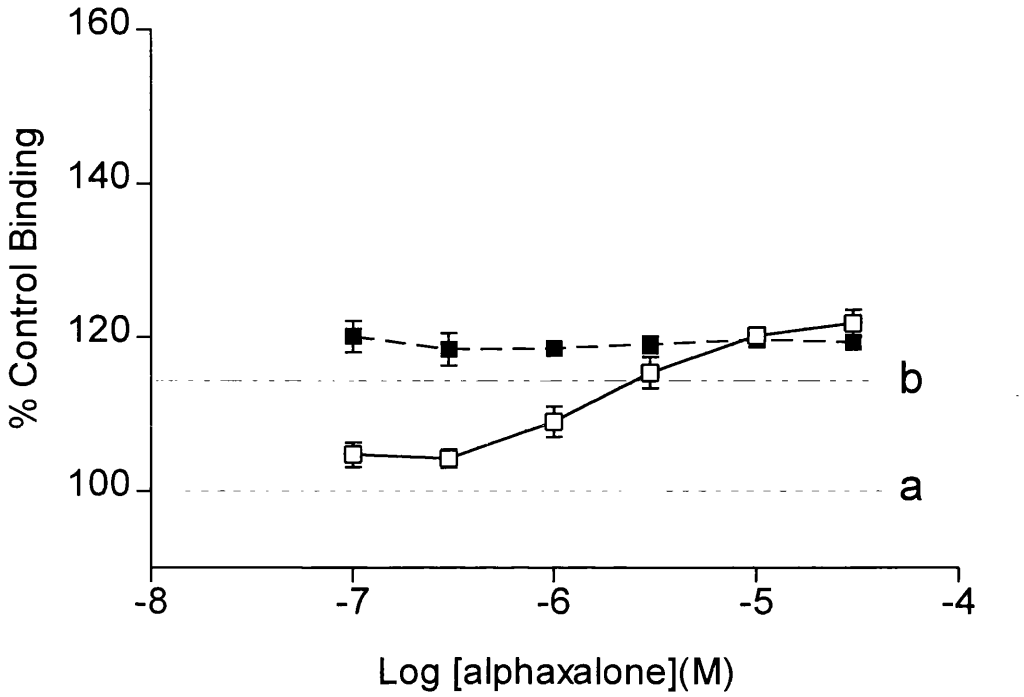


Figure 5.3. Concentration-effect relationships for the enhancement of ^{3}H-FNZ binding by alphaxalone after 10 min preincubation at 37°C + 60 min incubation at 4°C in the absence (□) and presence (■) of Δ^{16}-alphaxalone (60 μM). Data are the means ± s.e.means of 4 experiments and are expressed relative to the binding level a (100 %) in the absence of steroids. The binding level b (114.5 %) is shown as the effect of Δ^{16}-alphaxalone (60 μM) on its own, which could be treated as the baseline for the concentration-effect relationship of alphaxalone in the presence of Δ^{16}-alphaxalone. All data points are the means of triplicates.

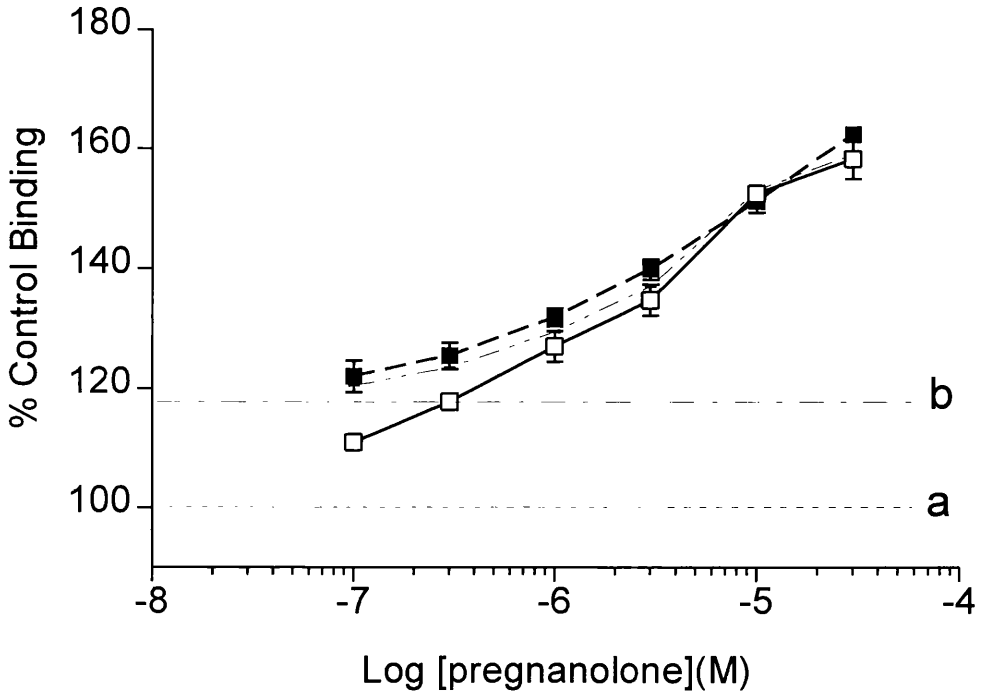


Figure 5.4. Concentration-effect relationships for the enhancement of ^{3}H-FNZ binding by pregnanolone after 10 min preincubation at 37°C + 60 min incubation at 4°C in the absence (□) and presence (■) of Δ^{16}-alphaxalone (60 μM). Data are the means ± s.e.means of 4 experiments and are expressed relative to the binding level a (100 %) in the absence of steroids. The binding level b (117.4 %) is shown as the effect of Δ^{16}-alphaxalone (60 μM) on its own, which could be treated as the baseline for the concentration-effect relationship of pregnanolone in the presence of Δ^{16}-alphaxalone. The predicted concentration-effect relationship of pregnanolone in the presence of Δ^{16}-alphaxalone was also shown (thin dotted curve), calculated from the control curve by assuming Δ^{16}-alphaxalone (60 μM) = pregnanolone (0.3 μM) (see Section 5.4). All data points are the means of triplicates.

5.4. DISCUSSION

Alphaxalone was 10 times more potent than pregnanolone when potentiating depolarizing responses to muscimol in slices of rat cuneate nucleus (Turner & Simmonds, 1989). In other studies, however, pregnanolone and allopregnanolone were reported to be either more potent than alphaxalone, e.g., when potentiating GABA-evoked currents recorded from bovine adrenomedullary chromaffin cells as shown by Lambert *et al.*, 1987 & 1991 and cited by Paul & Purdy, 1992; or equally effective as alphaxalone, e.g., when displacing ^{35}S-TBPS binding (Harrison *et al.*, 1987).

In the present series of experiments, alphaxalone appeared to be less potent and much less efficacious than pregnanolone (Fig. 5.3 & 5.4). This conclusion is in agreement with the conclusions from the Lambert's group, but at variance with the results published by Prince & Simmonds, 1993, who suggested that alphaxalone, pregnanolone and allopregnanolone were nearly equally potent in enhancing ^{3}H-FNZ binding. The reason for the difference is not clear. There were some small differences in the experimental protocols, and there was probably more endogenous GABA in their system. However, this is unlikely to be the cause, since I have tried to mimic their protocol, either by reducing the number of times of the membrane washing, or by adding muscimol into the incubation mixture after washing the membrane extensively, but failed to find any significant change (data not shown).

Δ^{16}-alphaxalone was about 20 - 30 fold less potent than alphaxalone as a potentiator of muscimol on the cuneate nucleus of the rat (Harrison, 1985). In this study, however, Δ^{16}-alphaxalone appeared to be equally potent as alphaxalone when enhancing ^{3}H-FNZ binding (Fig. 5.2 & 5.3). Part of the reason may be that, in the present experiments, alphaxalone demonstrated quite low potency. The enhancement by Δ^{16}-alphaxalone was small yet distinct, and GABA-dependent.

When Δ^{16}-alphaxalone (60 μM) was co-applied with alphaxalone, the concentration-effect relationship yielded a horizontal line, giving an enhancement of nearly 20 %, similar to the maximal effect given by alphaxalone (30 μM) on its own (Fig. 5.3). These results suggest that on top of the enhancement by Δ^{16}-alphaxalone (60 μM), increasing concentrations of alphaxalone failed to give any further enhancement. The explanation for this could be that, either Δ^{16}-alphaxalone binds to the same range of sites as alphaxalone, therefore prevents alphaxalone from producing any effect; or there is a limit for enhancement, therefore one can not expect additive effects from two agonists across the whole concentration range, especially at the higher concentration range, even if they do not compete for the same binding sites. However, the experiments with pregnanolone indicate that greater degrees of enhancement are possible.

When Δ^{16}-alphaxalone (60 μM) was co-applied with pregnanolone, the concentration-effect relationship started from a higher level, due to the enhanced baseline binding by Δ^{16}-alphaxalone (Fig. 5.4). As the concentrations of

pregnanolone increased, the concentration-effect relationships in the absence and presence of Δ^{16}-alphaxalone converged, reaching the same maximal binding at pregnanolone 30 μM. These results suggest that there were some degree of interaction between these two compounds. The best model to explain this interaction is to assume Δ^{16}-alphaxalone 60 μM equals pregnanolone 0.3 μM, since they gave the same enhancements. By doing this, we could predict the concentration-effect relationship in the presence of Δ^{16}-alphaxalone by calculating it from the control curve using the equation: the effective concentration of steroids = varied concentration of pregnanolone + 0.3 μM pregnanolone. The resulting curve correlates very well with the curve we obtained (Fig. 5.4). Therefore, Δ^{16}-alphaxalone acts more like an agonist binding to the same range of binding sites with pregnanolone, but with much lower affinity, than a partial agonist binding to the same sites, whose limited efficacy should have prevented pregnanolone reaching the same maximum as in the control condition.

However, this leaves one question unanswered. If Δ^{16}-alphaxalone is better described as a full agonist with low potency, rather than a partial agonist with low efficacy, why did the steroid itself demonstrate a concentration-effect relationship with a rather modest maximum (Fig. 5.2)? There seemed to be some unknown factors preventing Δ^{16}-alphaxalone from exerting its full effect. One of the factors could be the oil-water partition coefficient of these steroids. Suppose Δ^{16}-alphaxalone has a smaller coefficient than pregnanolone, the real biophasic concentration achieved by Δ^{16}-alphaxalone in the membrane lipids may be less than that by pregnanolone, although

their aqueous concentrations were similar. Hence within the aqueous concentrations we could achieve, Δ^{16}-alphaxalone might not be able to achieve high enough biophasic concentration to exert the same enhancements as pregnanolone. This explanation also applies well to alphaxalone, which showed a modest maximum in the present experiments, while the previous reports suggest it to be a full agonist, even though we do not have strong evidence to support this explanation.

5.5. CONCLUSIONS

Altogether, our results suggest that Δ^{16}-alphaxalone is a full agonist on $GABA_A$ receptors. It enhances ^{3}H-FNZ binding GABA-dependently, by binding to the same sites as pregnanolone and alphaxalone, but with quite low affinity on well-washed membranes.

CHAPTER 6

INTERACTIONS BETWEEN LORECLEZOLE, CHLORMETHIAZOLE AND PENTOBARBITONE IN MODULATION OF ^{3}H-FLUNITRAZEPAM BINDING TO RAT SYNAPTIC MEMBRANES[2]

[2] This chapter and the following chapter were modified from the paper: Zhong, Y. & Simmonds, M.A. (1997). Interactions between loreclezole, chlormethiazole and pentobarbitone at $GABA_A$ receptors: functional and binding studies. *Br. J. Pharmacol.*, **121:** 1392-1396.

6.1. INTRODUCTION

6.1.1. Interactions of loreclezole and chlormethiazole with $GABA_A$ receptors

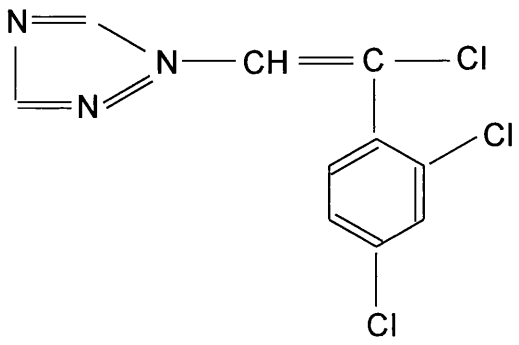
As detailed in Chapter 1, loreclezole (LOR) behaves in a barbiturate-like way on $GABA_A$ receptors and its action is highly dependent on the β subunits present (see Chapter 1 for references). Chlormethiazole (CMZ) was shown to be a potent enhancer in functional studies (Harrison & Simmonds, 1983), but not in ^{3}H-diazepam (Leeb-Lundberg *et al.*, 1981) or ^{3}H-FNZ (Cross *et al.*, 1989) binding studies.

6.1.2. Aim of this study

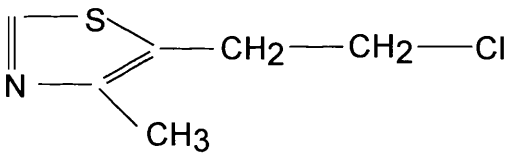
My results in Chapter 3 indicate that the enhancement of ^{3}H-FNZ binding by pregnanolone involves at least two substantial components (the GABA-dependent and GABA-independent components), whereas the enhancement by propofol is largely GABA-independent (Chapter 3). Recently, a bicuculline-resistant enhancement by LOR of the binding of the picrotoxin site ligand ^{35}S-TBPS was reported, and attributed to a direct effect of LOR on the $GABA_A$ receptors (Sanna *et al.*, 1996). It would be very interesting, therefore, to determine for LOR, the relative contributions of GABA-dependent and GABA-independent components in its modulation of ^{3}H-FNZ binding.

Furthermore, since there is some structural resemblance between LOR and CMZ (Fig. 6.1. for structures), we undertook a comparison of these two compounds on modulation of the ^{3}H-FNZ binding where, unusually for a $GABA_A$ potentiator, CMZ did not enhance the binding but, at high concentrations, inhibited it (Moody & Skolnick, 1989). Some further comparisons were also made with pentobarbitone (PB). A preliminary account of a part of this work has been presented (Zhong & Simmonds, 1996b).

In parallel with this work, comparisons between LOR, CMZ and PB were made on a seizure model and additional ligand-binding sites on the $GABA_A$ receptors (Green *et al.*, 1996).



Loreclezole



Chlormethiazole

Figure 6.1. Structures of loreclezole (LOR) and chlormethiazole (CMZ).

6.2. METHODS

Crude synaptic membranes were prepared from rat whole brain as described in Chapter 2. On day of the assay, the tissue were thawed and washed 4 times, and the final pellet was resuspended in assay buffer.

The assays were carried out as the following:

Concentration-effect relationship

100 µl aliquots of membranes (0.4 mg protein.ml^{-1}) were pre-incubated with various concentrations of drugs for 10 min at 37°C (Prince & Simmonds 1992). Then 50 µl of ^{3}H-FNZ was added to each tube to give a final concentration of 1 nM and a total volume of 500 µl assay buffer. The samples were incubated for 60 min at 4°C.

Saturation curve

100 µl aliquots of membranes (0.4 mg protein.ml^{-1}) were incubated with 0.1 - 20 nM ^{3}H-FNZ for 60 min at 4°C in the presence or absence of LOR (100 µM) in a total sample volume of 500 µl.

The binding reaction was terminated by the addition of ice-cold wash buffer, followed by rapid filtration and washing. Non-specific binding, which was less than 5 % of the total binding, was determined for each concentration of ^{3}H-FNZ by addition of 10 µM unlabelled FNZ. LOR was initially dissolved in acetone, which

was present in all samples at a final concentration of 1 %. CMZ and PB were dissolved directly into assay buffer.

6.3. RESULTS

6.3.1. Effects of LOR and CMZ on ^{3}H-FNZ binding

LOR considerably enhanced ^{3}H-FNZ binding to the crude synaptic membranes made from rat whole brain homogenate (Fig. 6.2). The concentration-effect relationship had a threshold at 3 μM, and a maximal 47.3 ± 2.83 % increase in binding at 300 μM (mean ± s.e.mean, n = 3).

In saturation curve analysis, LOR 100 μM significantly decreased the K_d ($p < 0.01$, Student's t-test)but not B_{max} of ^{3}H-FNZ binding (Fig. 6.3). Therefore, the enhancement by LOR of ^{3}H-FNZ binding was due to an increase in binding affinity with no change in the numbers of binding sites.

At 100 μM, CMZ did not significantly change ^{3}H-FNZ binding (1.5 ± 2.35 % change from control, mean ± s.e.mean, n = 8) (Fig. 6.2). This is in agreement with the results published before by other groups (see Chapter 1).

6.3.2. Effects of CMZ and/or bicuculline on the enhancement by LOR of ^{3}H-FNZ binding

Effect of CMZ on the enhancement by LOR of ^{3}H-FNZ binding

At 100 μM, CMZ did not significantly change ^{3}H-FNZ binding (*vide supra*). But in the presence of CMZ 100 μM, LOR gave significantly smaller enhancements (Fig. 6.4), the decreases being significant ($p < 0.05$, Student's t-test) at LOR 100 μM, 300 μM and 1 mM. These results suggested that CMZ could partially block the potentiating effect of LOR on ^{3}H-FNZ binding.

Effect of bicuculline on the enhancement by LOR of ^{3}H-FNZ binding

On well washed membranes, there was very little endogenous GABA left, as evidenced by the fact that 100 μM bicuculline failed to reduce ^{3}H-FNZ binding significantly (-3.9 ± 1.96 % from control, mean ± s.e.mean, n = 3). Nevertheless, it was possible that part of the potentiating action of LOR on ^{3}H-FNZ binding was due to potentiation of a subthreshold GABA tone. Therefore, in this series of experiments, 100 μM bicuculline was routinely used. The blank tubes received the same amount of bicuculline as the test tubes, so the baselines of all curves were reset to 100 %.

The influence of 100 μM bicuculline on the concentration-effect relationship for 60

min exposure to LOR is shown in Fig 6.4. In the presence of bicuculline 100 μM, LOR gave a smaller enhancement of ^{3}H-FNZ binding, the decreases at 30 μM, 300 μM and 1 mM LOR being significant ($p < 0.05$, Student's t-test). These results suggested that there were bicuculline sensitive and bicuculline insensitive components in the enhancement by LOR of ^{3}H-FNZ binding.

Effect of bicuculline and CMZ on the enhancement by LOR of ^{3}H-FNZ binding

Since the concentration-effect relationships of LOR in the presence of CMZ or bicuculline were superimposed on each other, we were interested to find out if the two components identified by CMZ and bicuculline were the same. A combination of 100 μM CMZ and 100 μM bicuculline did not depress the enhancement by LOR of ^{3}H-FNZ binding any further than either CMZ or bicuculline on its own (data not shown).

6.3.3. Enhancement by PB of ^{3}H-FNZ binding

PB did not enhance ^{3}H-FNZ binding until fairly high concentrations (> 300 μM) were reached. Neither CMZ nor bicuculline had any significant influence on the concentration-effect relationship of PB (data not shown).

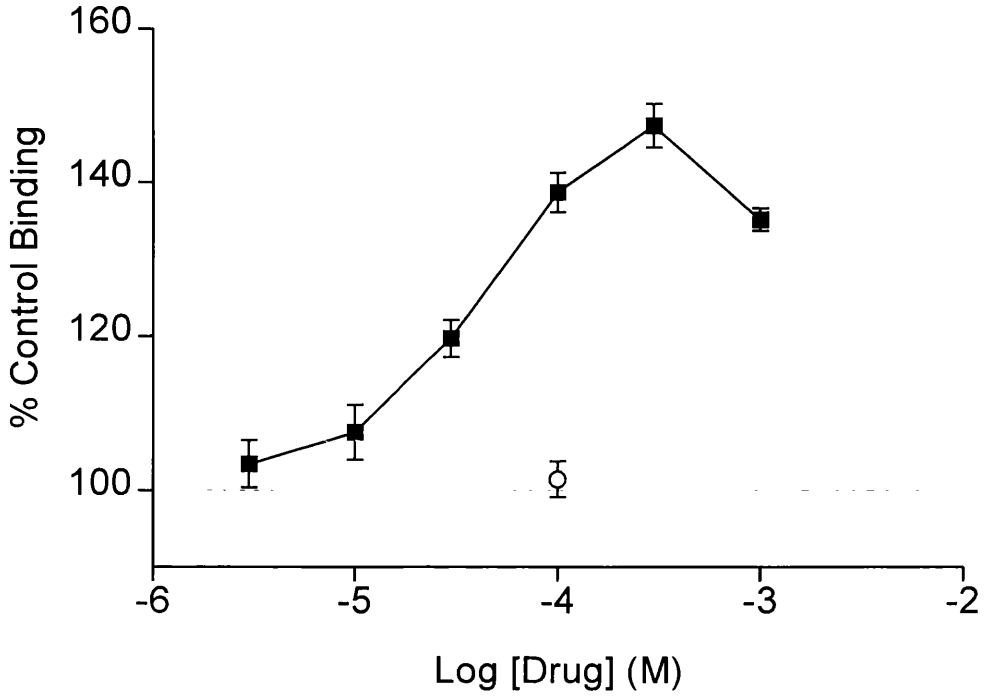


Figure 6.2. Concentration-effect relationship for the enhancement by LOR of ^{3}H-FNZ binding (■). The effect of 100 μM CMZ is also shown (○). Data points are the means ± s.e.means of 3 experiments. All data points are the means of triplicates.

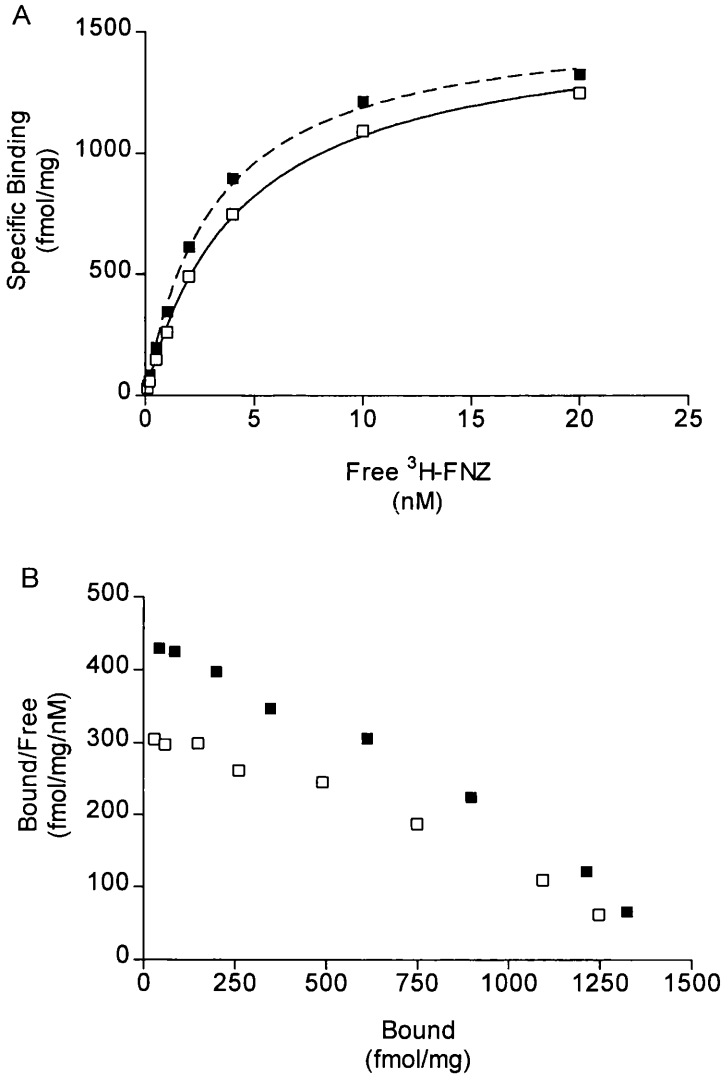


Figure 6.3. Saturation curve (A) and Scatchard plot (B)for ^{3}H-FNZ binding in the presence (■) and absence (□) of LOR (100 μM) from a typical experiment. All data points are the means of triplicates. The results from 4 repeated experiments (means ± s.e.means) showed that, in the absence of LOR, ^{3}H-FNZ binding had a K_d of 3.9 ± 0.29 nM, and a B_{max} of 1149 ± 30 fmol/mg; in the presence of LOR (100 μM), ^{3}H-FNZ binding yielded a K_d of 2.7 ± 0.10 nM *, and a B_{max} of 1204 ± 15 fmol/mg (* significantly different from control value ($p < 0.05$)).

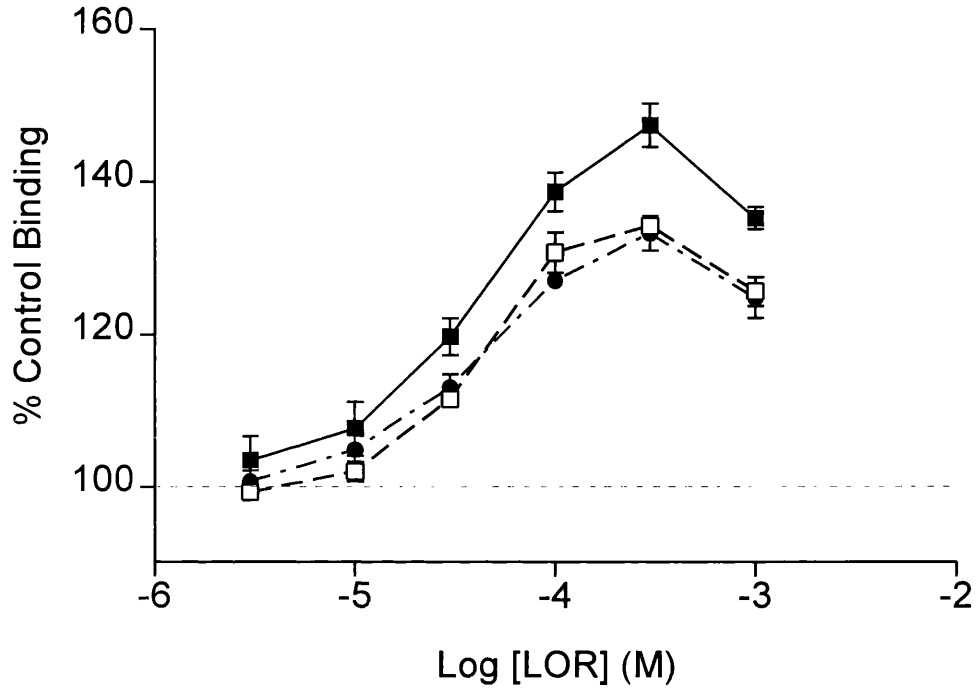


Figure 6.4. Concentration-effect relationships for the enhancement of ^{3}H-FNZ binding by LOR alone (■), and in the presence of bicuculline 100 μM (□) or CMZ 100 μM (●). Data points are the means ± s.e.means of 3 to 4 experiments. All data points are the means of triplicates.

6.4. DISCUSSION

LOR considerably enhanced ^{3}H-FNZ binding to the crude synaptic membranes made from rat whole brain homogenate (Fig. 6.2), and this enhancement was due to an increase in binding affinity rather than a change in the number of binding sites (Fig. 6.3). Both CMZ and bicuculline could block the enhancement by LOR of ^{3}H-FNZ binding to a small extent, and the combination of them failed to depress the enhancement by LOR any further than either CMZ or bicuculline on its own (Fig. 6.4). This suggested that the component that was sensitive to CMZ was also sensitive to bicuculline, therefore GABA dependent, and the remaining component was a direct effect of LOR which was neither dependent on GABA nor influenced by CMZ.

LOR shares some similarity with propofol in enhancing ^{3}H-FNZ binding (Fig. 3.3 & Fig. 6.4). Both yielded bell-shaped concentration-effect relationships, and were only weakly sensitive to bicuculline (Fig. 3.5 & Fig. 6.4). This is quite different from the profile of preganolone (Fig. 3.4). Recently, Sanna *et al.* reported biphasic modulation of ^{35}S-TBPS binding by LOR and propofol (Sanna *et al.*, 1996). These results indicate that LOR and propofol share some similarity in their action on $GABA_A$ receptors. However, their different efficacies in inducing inward Cl^- currents and their different β subunit selectivity may underlie the difference in their pharmacological profiles (Sanna *et al.*, 1995 & 1996).

CMZ, on the other hand, failed to enhance ^{3}H-FNZ binding (Fig. 6.2), while was able to dose-dependently potentiate GABA responses in the cuneate nucleus (Harrison & Simmonds, 1983). PB, also a good potentiator in functional study, did not enhance ^{3}H-FNZ binding until fairly high concentrations (> 300 μM) were reached. Neither CMZ nor bicuculline had any significant influence on the concentration-effect relationship of PB. These results were in agreement with the finding of Moody & Skolnick (1989), but not with the results from Cross *et al.* (1989).

Unusual for a $GABA_A$ potentiator, CMZ did not enhance ^{3}H-diazepam and ^{3}H-FNZ binding. Further more, CMZ could reverse the pentobarbitone's enhancement of ^{3}H-diazepam binding by 50 % (Leeb-Lundberg *et al.*, 1981). In the present study, we demonstrated that CMZ could partially antagonise the enhancement by LOR of ^{3}H-FNZ binding, indicating that CMZ has some unique interactions with $GABA_A$ receptors. These results also suggest that ^{3}H-FNZ binding does not always correlates well with functional studies. In fact, under the conditions of our ^{3}H-FNZ binding experiments on well-washed whole brain membranes, with only low levels of endogenous GABA likely to be present, it is clear that potentiation of GABA contributes little to the enhancement of ^{3}H-FNZ binding.

6.5. CONCLUSIONS

We conclude that LOR, being different from CMZ and PB, is a good enhancer of ^{3}H-FNZ binding. This enhancement is due to an increase in binding affinity, is largely GABA-independent, and can be partially blocked by CMZ.

CHAPTER 7

INTERACTIONS BETWEEN LORECLEZOLE, CHLORMETHIAZOLE AND PENTOBARBITONE AT $GABA_A$ RECEPTORS IN RAT CUNEATE NUCLEUS *IN VITRO*

7.1. INTRODUCTION

Aim of this study

In Chapter 6, the comparisons of the modulations of ^{3}H-FNZ binding by LOR, CMZ and PB were reported. In parallel with that work, the effects of LOR, CMZ and PB on rat cuneate nucleus slices were also compared, where CMZ and PB have been shown to potentiate responses to muscimol (Harrison & Simmonds, 1983).

7.2. METHODS

As detailed in Chapter 2, experiments were performed on slices of medulla oblongata containing the dorsal funiculus and cuneate nucleus, prepared from male Wistar rats (100 - 150 g) as previously described (Simmonds 1978; Harrison & Simmonds 1983). Each slice was placed in a two-compartment bath so that the dorsal funiculus projected through a grease-filled gap in a barrier separating the two compartments. The slices were superfused with Krebs medium at room temperature and only the compartment containing the terminals of the dorsal funiculus within the cuneate nucleus was superfused with drugs incorporated into the Krebs medium. The potential difference between the two compartments was recorded continuously and the negativity induced in the drug-perfused compartment was interpreted as a depolarization of the terminals of dorsal funiculus fibres projecting through the

barrier. Responses were measured at their peak amplitudes.

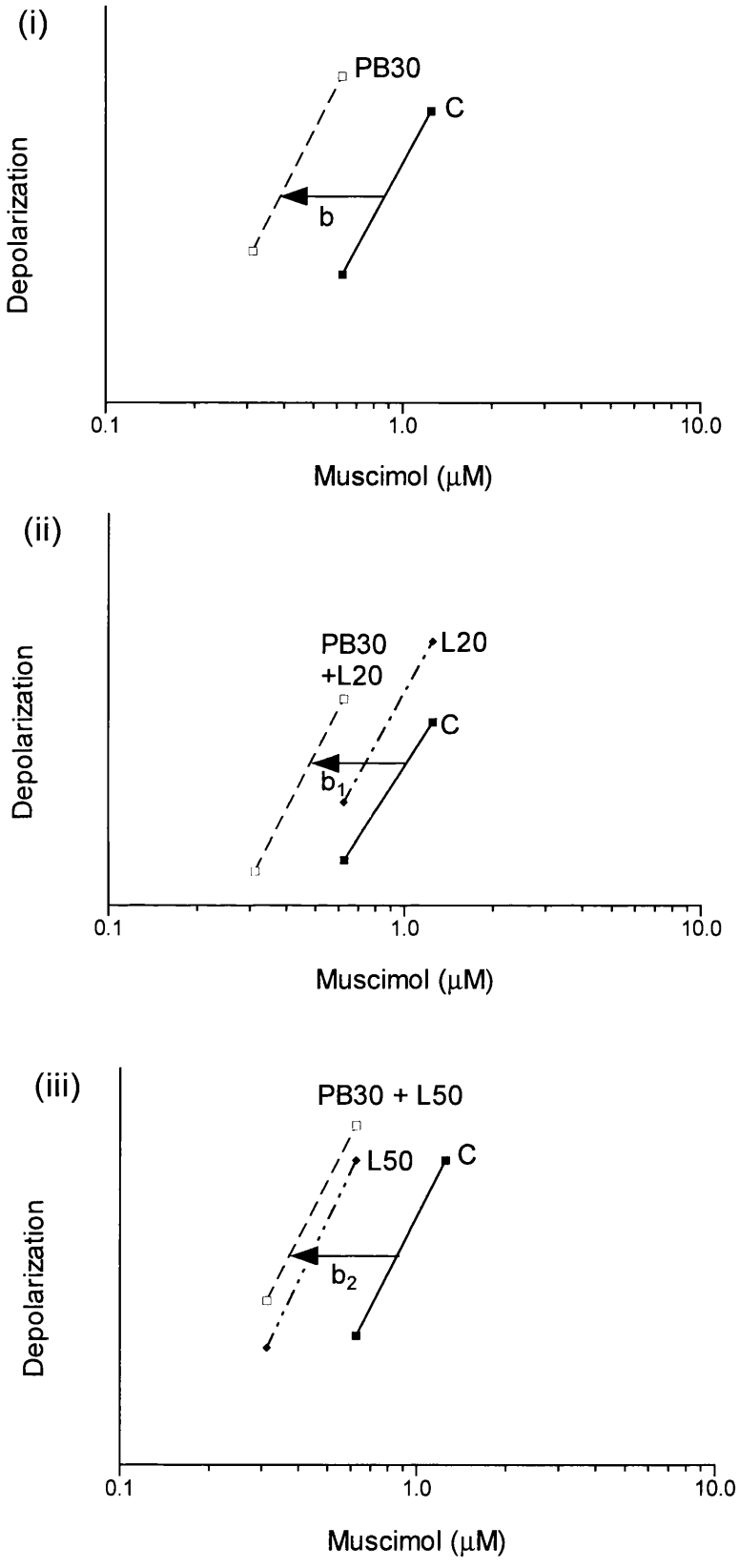
Muscimol was used routinely as the $GABA_A$ receptor agonist. To minimize the problem of desensitization, control responses in the lower part of the log dose-response curve were used and these were routinely obtained from 2 min superfusions of 1.25 and 2.5 μM muscimol. In each experiment, a muscimol control curve was first obtained, then one drug was superfused for 30 min before and during the redetermination of responses to muscimol. The same procedure was then carried out with a combination of two drugs. The muscimol dose-response lines were always displaced in an approximately parallel fashion so the effects of the drugs were measured as equi-effective muscimol dose-ratio. The experimental design is illustrated in Fig. 7.1.

The Krebs medium was continuously gassed with 95% O_2 : 5% CO_2 to give pH 7.4. Muscimol was prepared as a 10 mM solution in distilled water and diluted into the Krebs medium just before use. CMZ and PB were dissolved directly in the Krebs medium. LOR was first dissolved in a little acetone and then diluted into the Krebs medium. The final concentration of acetone never exceeded 0.1% which had no effect on muscimol responses in this preparation.

Figure. 7.1. The experimental design: experiments measuring the effects of PB in the absence and presence of LOR were performed in the following way:

(i). Two-point muscimol log dose-response lines were obtained under control (C) conditions, then in the presence of PB 30 μM (PB30). (ii). In related experiments, two-point muscimol log dose-response lines were obtained under control (C) conditions, then in the presence of LOR 20 μM (L20) and then in the presence of a combination of PB 30 μM and LOR 20 μM (PB30+L20). (iii). Experiments were performed as in (ii) except that the LOR dose was 50 μM (L50). The effects of PB or the combinations of PB and LOR were expressed as left shifts of muscimol log dose / response curve (log unit) (b, b_1 and b_2).

Experiments measuring the effects of CMZ in the absence and presence of LOR, or the effects of PB in the absence and presence of CMZ were performed in similar ways.


(i)
PB30
C
b
Depolarization
0.1
1.0
10.0
Muscimol (μM)
(ii)
PB30
+L20
L20
C
b1
Depolarization
0.1
1.0
10.0
Muscimol (μM)
(iii)
PB30 + L50
L50
C
b2
Depolarization
0.1
1.0
10.0
Muscimol (μM)

7.3. RESULTS

7.3.1. Effects of CMZ, PB and LOR individually

In agreement with the results published before from our laboratory (Harrison & Simmonds, 1983), CMZ dose-dependently potentiated responses to muscimol on the cuneate nucleus (Fig. 7.2, $p < 0.0001$, one-way ANOVA). A submaximal concentration of PB (30 μM) gave a potentiation effect similar to 50 μM CMZ, and in agreement with the results published by Simmonds & Turner (1987).

LOR, however, showed a rather different profile (Fig. 7.3). At 5, 10 and 20 μM, LOR gave quite small leftward shifts. At 10 and 20 μM, the shifts were significant ($p < 0.05$, $p < 0.01$, respectively, Student's t-test). At 50 μM, the responses were very variable, ranging from a clear potentiating effect to an antagonizing effect and the mean shift was not significant.

These results suggested that compared to CMZ and PB, LOR was a much weaker potentiator of GABA responses. At higher concentrations, LOR could induce very variable responses.

7.3.2. Effects of CMZ, PB and LOR applied in pairs

When PB 30 μM was co-applied with CMZ 50 μM, the leftward shifts induced by

these two drugs were very similar to the sum of their effects when applied separately (Fig. 7.4). However, when LOR 20 μM and 50 μM were co-applied with PB 30 μM, the total shifts were less than the sums of their individual effects. As the concentration of LOR increased, the total shift decreased and, at LOR 50 μM, was significantly different from the predicted sum of the individual effects ($p < 0.05$, Student's t-test). Similarly, when LOR 10 μM and 50 μM were co-applied with CMZ 50 μM, the total shifts were less than the sums of their individual effects and, at LOR 50 μM, this difference was significant ($p < 0.05$, Student's t-test).

These results suggested that CMZ and PB had no interaction between them when potentiating responses to GABA. However, LOR could dose-dependently attenuate the effects of CMZ and PB.

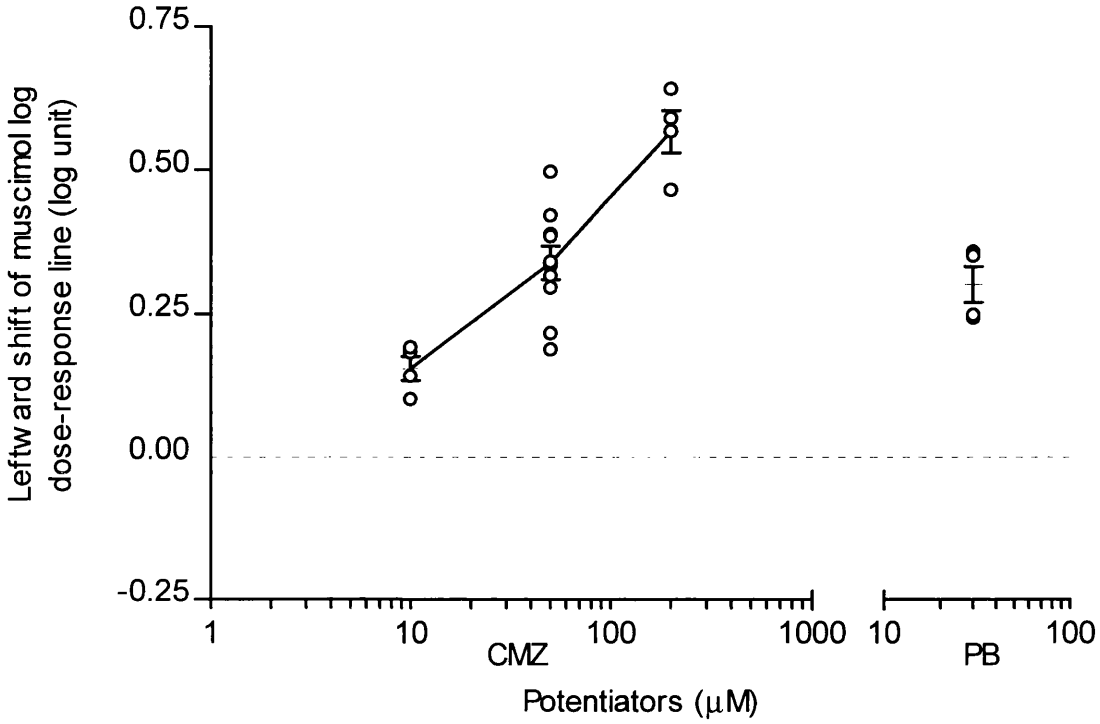


Figure 7.2. Potentiation by CMZ and PB of responses to muscimol, expressed as the leftward shift (log unit) of the muscimol log dose-response line. All data points as well as means ± s.e.means of 4 to 10 experiments are shown to display the distribution of the data.

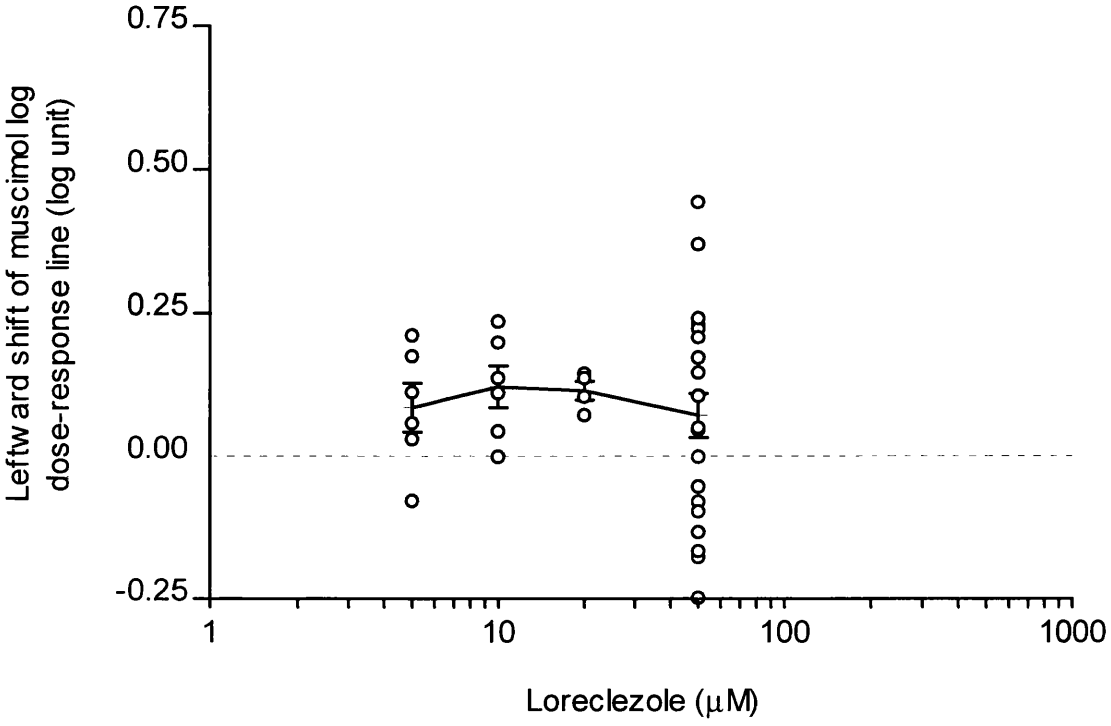


Figure 7.3. Effects of LOR on responses to muscimol, expressed as the leftward / rightward shift (log unit) of the muscimol log dose-response line. All data points as well as means ± s.e.means of 4 to 10 experiments are shown to display the distribution of the data.

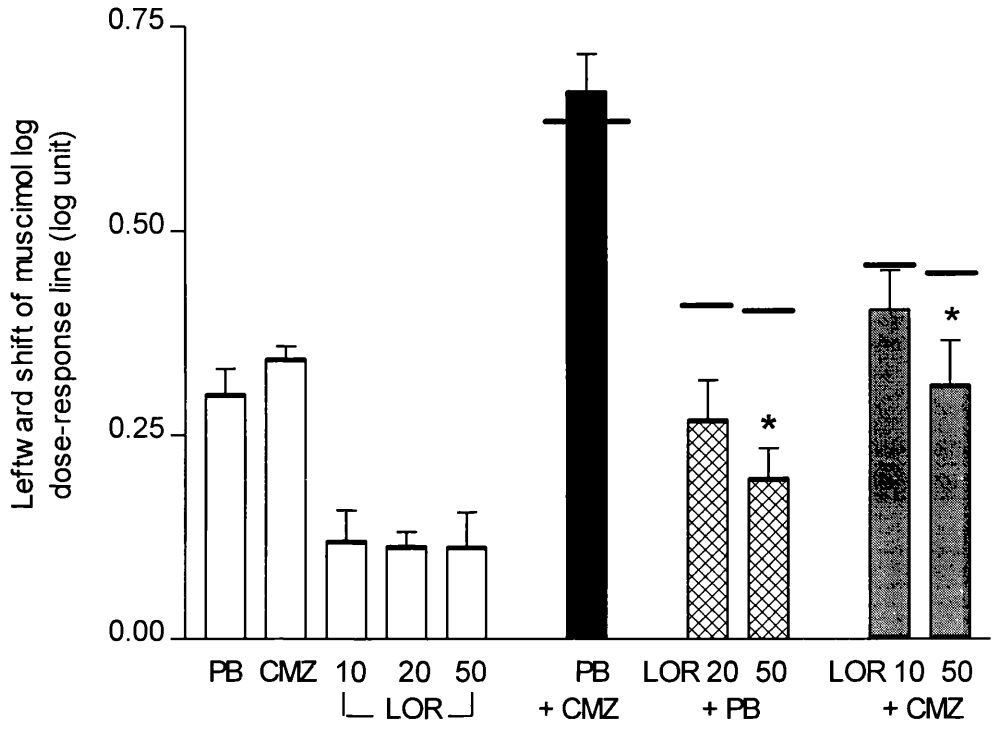


Figure 7.4. Effects of combinations of drugs on responses to muscimol, expressed as the leftward shift (log unit) of the muscimol log dose-response line. The open bars show the effects of (from left to right) PB 30 μM, CMZ 50 μM, LOR 10, 20, 50 μM. The filled bars show the effects of combinations of drugs (from left to right): PB 30 μM + CMZ 50 μM, PB 30 μM + LOR 20 μM, PB 30 μM + LOR 50 μM, CMZ 50 μM + LOR 10 μM, CMZ 50 μM + LOR 50 μM. For the combinations of drugs, the calculated dose-ratios assuming simple additive effects with no interactions are shown as short solid lines. Comparisons with the actual shifts obtained were made by Student's t-test (* $p < 0.05$). The results are the means ± s.e.means of 4 to 10 experiments.

7.4. DISCUSSION

CMZ could dose-dependently potentiate GABA responses in the cuneate nucleus, and PB was also a good potentiator in this functional study (Fig. 7.2).

Compared to CMZ and PB, LOR was a much weaker potentiator of GABA responses on the cuneate nucleus (Fig. 7.3). At high concentrations (50 μM), LOR could induce very variable responses, ranging from a clear potentiating effect to an antagonizing effect. When LOR was co-applied with PB or CMZ, it dose-dependently attenuated the effects of CMZ and PB (Fig. 7.4). One explanation could be that LOR acted on the same site as CMZ and PB and acted as a partial agonist to antagonize their potentiating effects. However, the fact that CMZ and PB had no interaction between themselves when potentiating responses to GABA suggested that CMZ and PB did not share common binding sites. The most plausible explanation of the attenuations by LOR of the actions of CMZ and PB, and the variable responses induced by LOR on its own, is that LOR not only potentiates the muscimol responses, but also induces desensitization of $GABA_A$ receptors. Direct evidence in support of this explanation has come in a recent paper (Donnelly & Macdonald, 1996), in which, it is clearly shown that LOR increases desensitization of $GABA_A$ receptor-mediated responses to GABA recorded intracellularly. Unlike the GABA-potentiating property of LOR, its action to increase desensitization is not $GABA_A$ subunit specific.

In this study (Chapter 6 & 7), where we compared LOR, CMZ and PB in functional and binding studies, the data on GABA potentiation were obtained on tissue from a lower level of the neuraxis than the tissue on which ^{3}H-FNZ binding was studied. It is conceivable, therefore, that the spectrum of subunit compositions of the $GABA_A$ receptors differed between these two preparations. We do not know to what extent, if at all, this may have contributed to the different patterns of modulation of muscimol and ^{3}H-FNZ binding. Nevertheless, the different profiles of LOR, CMZ and PB in the functional and binding studies do indicate that a drug-induced change in ^{3}H-FNZ binding in well-washed membranes is not a reliable predictor of how that drug will affect functional responses to GABA.

7.5. CONCLUSIONS

Overall, LOR showed a distinctly different profile of action from CMZ and PB at the $GABA_A$ receptors. Of the drugs, LOR was the most efficacious positive modulator of ^{3}H-FNZ binding but its ability to act as a GABA potentiator was obscured by a probable exacerbation of GABA receptor desensitization. CMZ, in contrast, did not enhance ^{3}H-FNZ binding and even antagonized any GABA-dependent component of enhancement by another drug, e.g. LOR, but it was a more efficacious GABA potentiator than LOR. PB was similar to CMZ in potentiating responses to GABA, but only enhanced ^{3}H-FNZ binding at high concentrations.

There can be little doubt that LOR, CMZ and PB have separate sites of action on the $GABA_A$ receptor complex.

CHAPTER 8

General Discussion

8.1. MULTIPLE BINDING SITES FOR STEROIDS ON $GABA_A$ RECEPTORS

There has been quite strong evidence suggesting multiple binding sites for steroids on $GABA_A$ receptors (Chapter 1). As my work went on, more evidence came out. Pregnanolone was demonstrated to enhance ^{3}H-FNZ and inhibit ^{35}S-TBPS binding to the $GABA_A$ receptors in a biphasic manner, apart from in thalamus. The percentages of the high and low affinity components vary among different brain regions. However, this biphasic modulatory ability is not shared by allopregnanolone (Hawkinson *et al.*, 1994). In another paper (McCauley *et al.*, 1995), certain pregnanediols(5α-pregnan-3α,20α-diol and 5β-pregnan-3α,20β-diol) were observed to have limited efficacy as allosteric modulators of ^{35}S-TBPS binding, which may be explained in part by their selectivity for the high affinity site recognized by pregnanolone, and could be due to different subunit compositions of the $GABA_A$ receptors.

My results confirm the observations by Hawkinson *et al.*, 1994 and show different pharmacologies for the two components of the enhancement of ^{3}H-FNZ binding by pregnanolone (Chapter 3). The GABA-dependent component embraces the whole range of pregnanolone concentrations studied; while the direct effect of pregnanolone is apparent only at concentrations of 1 μM and above, and can be weakly antagonised by 11-ketoprogesterone. These data can be best explained by assuming that there is more than one site for the action of pregnanolone. Thus, the direct action of pregnanolone may involve a separate site from the GABA-dependent

action, and this site is shared by 11-ketoprogesterone.

As discussed in Chapter 1, although there is no clear subunit dependence of the effects of steroids on $GABA_A$ receptors established so far, the steroidal modulation of $GABA_A$ receptors still demonstrate regional- and species- dependent features. Thus, steroids may still show different sensitivities to receptors composed of different subunits, even if the differences are not large.

Then, where are these multiple sites? How do different combinations of subunits influence steroids' activity?

Studies on the AChR have shown that there are strong interactions between the receptor and phospholipids and sterols, which result in two classes of lipid binding sites on the receptor: *annular sites* at the lipid-protein interfaces and *nonannular sites* in the "axial clefts" between the receptor subunits (Jones & McNamee, 1988). Phospholipids bind strongly to the annular sites, while cholesterol binds preferentially to the nonannular sites and fatty acids bind to both (Fig. 8.1). Since the nonannular sites are formed at the interstices of the receptor subunits, in receptors which are hetero-oligomers, these nonannular sites will not be equivalent. A range of 5 - 10 nonannular sites per 250,000 - dalton monomer has been proposed by Jones & McNamee, 1988.

The natural steroid cholesterol is an abundant component of the postsynaptic

membrane. It can interact with high affinity with the AChR (Ellena *et al.*, 1983), stabilize its structure in reconstituted vesicles (Artigues *et al.*, 1989), and augment AChR-mediated ion influx (Criado *et al.*, 1982). Short-term incubation of cells or membrane patches excised therefrom with cholesterol gives rise to a fourfold increase in the frequency of AChR channel opening, with no changes occurring in conductance or mean open time (Barrantes, 1993).

The general idea emerging from the work on lipid / AChR protein interactions is that most if not all lipophilic compounds may exert their action by altering the protein / lipid interfaces (annular sites), or by reaching specific sites on the protein (e.g. nonannular sites) via a membrane pathway. It is interesting to speculate whether steroids could bind to analogous sites at the $GABA_A$ receptors.

As discussed in Chapter 1, the sites for steroids are probably on the receptor protein rather than in the lipid bilayer and these sites are unlikely to be intracellular. Therefore, it is possible for steroids to bind to the transmembrane domain of the receptor protein via a membrane pathway, ie., the nonannular sites. If this suggestion is true, then the possibility for further binding sites for steroids will be greatly raised. For example, in a receptor containing 2 α1 subunits, 2 β2 and 1 γ2, in the arrangement of αβγβα, there would exist five distinct binding domains: α-β; β-γ; γ-β; β-α and α-α (with - representing the axial cleft). In the arrangement of βαγαβ, there would also be 5 distinct domains but these would then be: β-α; α-γ; γ-α; α-β and β-β. As discussed in Chapter 1, 12 - 24 isoforms of $GABA_A$ receptors

may occur in the brain. Then, a large number of binding sites may exist for steroids.

Recent findings in our laboratory suggest that there is little evidence for a selective competition between cholesterol and pregnanolone at its binding sites. Nevertheless, cholesterol does influence the functional couplings between various sites on the $GABA_A$ receptors (Bennett & Simmonds, 1996). These include the functional couplings between flunitrazepam sites and pregnanolone sites, flunitrazepam sites and propofol sites, flunitrazepam sites and muscimol sites *etc.* The binding sites for benzodiazepines and GABA have been proposed to occur at the subunit interfaces (Smith & Olsen, 1995) (Fig. 8.2). If the sites for steroids are also located at the interfaces of two dissimilar subunits, as illustrated in Fig. 8.2, it is plausible that these steroid sites may have different couplings with benzodiazepine sites and GABA sites depending on their relative proximities. This may explain in part the different GABA dependencies of pregnanolone's action I observed.

9.2. PHYSIOLOGICAL IMPLICATIONS OF STEROIDS INTERACTION WITH $GABA_A$ RECEPTORS

Of all the pharmacologically defined recognition sites for $GABA_A$ potentiators such as benzodiazepines, barbiturates, steroids, those for the steroids are currently the strongest candidates for a physiological role (Simmonds & Prince, 1993). In animals, adrenal cortex, gonads and liver are predominant sources of steroids

(Majewska, 1987). Progesterone and deoxycorticosterone, the precursors of inhibitory steroids, allopregnanolone (5α-pregnan-3α-ol-20-one) and THDOC (5α-pregnan-3α,21-diol-20-one), are the major steroid hormones released by ovaries and adrenals. High lipophilicity of steroids ensures their easy penetration of biological membranes, enabling access to all cells and organs, including the CNS. Furthermore, neural tissues and pituitary contain the enzymes responsible for converting the inactive precursors, progesterone and deoxycorticosterone, to the anaesthetic steroids allopregnanolone and THDOC. This suggests that steroids can also be synthesized *de novo* in the brain (Majewska, 1987). Indeed, some steroids have higher concentrations in the brain than in the plasma. For example, allopregnanolone is detectable in the cerebral cortex (2-5 ng/g protein) in adult male rats, while the level in plasma is undetectable. Progesterone also has higher concentration (about 7 ng/g protein) in the brain than in the plasma (about 0.5 ng/ml) (Purdy *et al.*, 1991).

In female rats, brain levels of ring A-reduced pregnanes change with oestrous cycle and pregnancy. For example, in pregnant rats, the concentration of allopregnanolone can be substantially raised to more than 30 nM, a level known to enhance $GABA_A$ receptor function *in vitro* (Paul & Purdy, 1992). Thus, some cycle dependent changes in mood and behaviour may be related to the amounts of ring A-reduced pregnanes entering the CNS (Holzbauer, 1976). In male rats, the levels of allopregnanolone and THDOC are relatively low, but a brief swim-stress can produce a rapid increase of their concentration in both brain and plasma, into the range

known to enhance $GABA_A$ receptor function *in vitro* (Purdy *et al.*, 1991). The source of these steroids was partly central and partly peripheral since adrenalectomy reduced, but failed to prevent, the stress-induced rise in brain allopregnanolone, even though it prevented the concomitant rise in plasma progesterone and allopregnanolone. Recently, Barbaccia *et al.* demonstrated an elevation of brain THDOC concentration with foot shock (from 3-4 nM in control male rats, to about 15 nM in rats subjected to foot shock) (Barbaccia *et al.*, 1996). This increase in brain THDOC concentration may be sufficient to potentiate the effect of GABA at $GABA_A$ receptors (Puia *et al.*, 1990), and hence may be related to the recovery of the $GABA_A$ receptor function after stress. Therefore, the concentration of steroids in the CNS may be influenced by fluctuations in their release from peripheral endocrine glands and putatively by their local synthesis from cholesterol (Fig. 8.3).

Progesterone itself is, at best, only a weak potentiator of GABA *in vitro* although it appears to be more active when administered into the rat brain *in vivo*, possibly by forming endogenously more active metabolites (Smith *et al.*, 1987b). In rodents, ovarian release of allopregnanolone closely follows the phasic release of progesterone during the estrous cycles and is highest at met-oestrus (Majewska, 1987). In the human, progesterone can be reduced by either 5α-reductase or a 5β-reductase, at least in the periphery. Circulating pregnanolone, but not allopregnanolone, could be detected at the luteal phase of the menstrual cycle (Paul & Purdy, 1992). Thus, the modulation of $GABA_A$ channels by 5β-reduced steroids might have physiological significance in the human. Furthermore, my results demonstrate a weak direct

interaction of progesterone with the $GABA_A$ receptor whereby it reduced the potency of its metabolite pregnanolone as an enhancer of ^{3}H-FNZ binding (Chapter 4). Since most of this action of pregnanolone, under the conditions of those experiments, does not involve an intermediating modulation of GABA itself (Chapter 6), it remains to be seen to what extent progesterone will antagonise its metabolites in their actions as GABA potentiators. Would there be any suppression by progesterone on pregnanolone's GABA potentiation effect as their concentrations increase? Would that then be followed by an increase of pregnanolone's effect because of the removal of the suppression as the concentration of progesterone starts to drop? The answer will be of potential physiological as well as pharmacological interest.

An interaction of pregnenolone sulphate with the $GABA_A$ channel might also occur physiologically. Pregnenolone is an intermediate in the biosynthesis of progesterone from cholesterol and its formation has been shown to occur in the rat brain, predominantly in the white matter (Baulieu *et al.*, 1987). At micromolar concentrations, pregnenolone sulphate has mixed agonist / antagonist effects (Chapter 1). Since these steroids are lipophilic, the aqueous concentrations required for pharmacological effects may be physiologically relevant (Simmonds & Prince, 1993).

8.3. "MULTIPLE BINDING SITES", A COMMON FEATURE FOR MODULATORS ON $GABA_A$ RECEPTORS?

Various drugs with anticonvulsant, hypnotic / sedative, and / or anaesthetic activities are known to interact with $GABA_A$ receptors. The most extensively studied of these drugs are the benzodiazepines and hypnotic barbiturates. More recently, further drugs have been added to this list. These include chlormethiazole, propofol and steroids. However, their actions on $GABA_A$ receptors are far from simple.

The modulation by benzodiazepines could be either positive or negative, with the antagonists blocking both. Barbiturates and propofol could potentiate, activate and block the $GABA_A$ receptor channel depending on the different concentration ranges, possibly through different binding sites (see Chapter 1 for references). Steroids could also act as full agonists, partial agonists or antagonists, and there is more than one site involved in one steroid's action (see Chapter 1 for references and Chapter 8 for discussion).

Furthermore, in the study of this thesis, loreclezole was also demonstrated to have mixed effects on the $GABA_A$ channels (Chapter 7). Loreclezole can enhance ^3H-FNZ binding quite efficaciously, but its ability to act as a GABA potentiator is obscured by a probable exacerbation of $GABA_A$ receptor desensitization. Unlike the GABA-potentiating property of loreclezole, its action to increase desensitization is not $GABA_A$ subunit specific (Donnelly & Macdonald, 1996). Hence, more than one

binding site may be involved in the action of loreclezole as well. Therefore, there exist on $GABA_A$ receptors, multiple binding sites not only for steroids, but also for GABA, barbiturates, propofol, loreclezole and possibly many other modulators. It would be very interesting to find out the physiological and pharmacological significance of this phenomenon.

8.4. DIFFERENT PICTURES FROM BINDING AND FUNCTIONAL STUDIES

^{3}H-FNZ binding is a well-established, reliable and convenient technique for monitoring interactions between various modulators of $GABA_A$ receptors. GABA and non-benzodiazepine potentiators of electrophysiological responses to GABA can enhance ^{3}H-FNZ binding; while negative modulators can inhibit ^{3}H-FNZ binding (Chapter 2). However, the findings in this study suggest that a drug-induced change in ^{3}H-FNZ binding in well-washed membranes is not always a reliable predictor of how that drug will affect functional responses to GABA (Chapter 6 & 7). Chlormethiazole potentiates muscimol responses in cuneate nucleus slices but fails to enhance ^{3}H-FNZ binding. On the other hand, loreclezole shows rather complex effects in the functional model while being a good enhancer of ^{3}H-FNZ binding.

One reason might be the different couplings between the sites for chlormethiazole, loreclezole and those for benzodiazepine and GABA. If, for example, the sites for chlormethiazole are quite close to those for GABA but not those for

benzodiazepines, then chlormethiazole may allosterically modulate the GABA effects but not the benzodiazepine binding.

Alternatively, the spectrum of subunit compositions of the $GABA_A$ receptors may contribute to the different patterns of modulation of GABA and ^{3}H-FNZ binding observed. In my study, the data on GABA potentiation were obtained from cuneate nucleus, a lower level of neuraxis than the brain, where ^{3}H-FNZ binding was studied (Chapter 6 & 7). The subunit compositions of $GABA_A$ receptors in cuneate nucleus are likely to be similar to those in spinal cord, which are different from those in the brain, and showed different sensitivity to steroid modulation (Chapter 1; Gee & Lan, 1991).

Different levels of endogenous GABA present in the isolated tissues may also affect the responses obtained. This is clearly demonstrated in the enhancement of ^{3}H-FNZ binding by pregnanolone (Chapter 3). Under the conditions of our ^{3}H-FNZ binding experiments on well-washed membranes, only low levels of endogenous GABA were likely to be present. Therefore, the potentiation of GABA contributes little to the enhancement of ^{3}H-FNZ binding (Chapter 6). It will be interesting to investigate the correlation between these two studies under more physiological conditions.

8.5. FUTURE PERSPECTIVES

One of the major findings of this study is the selective and weak antagonism by 11-ketoprogesterone on a low affinity component of the enhancement by pregnanolone of ^{3}H-FNZ binding (Chapter 3). However, the effect is quite small. Is this due to 11-ketoprogesterone being a weak antagonist? Or could the shift observed in whole brain be the net effect of some bigger and smaller shifts in different parts of the brain?

Different brain regions demonstrate different sensitivities to the steroid modulation (Nguyen *et al.*, 1995). It is plausible that different subunit subtypes of $GABA_A$ receptors from different brain regions may form different nonannular binding sites, to which 11-ketoprogesterone and pregnanolone may show different affinities. Furthermore, recent findings from our laboratory also demonstrate different concentration-effect relationships for the enhancement by pregnanolone of ^{3}H-FNZ binding to membranes from cerebral cortex, cerebellum and spinal cord (Bennett & Simmonds, 1996). The rank order for the maximal enhancements by pregnanolone in the cholesterol unenriched membranes is cerebellum > cerebral cortex > spinal cord. This correlates well with the rank order for the levels of cholesterol in these cholesterol unenriched membranes, which is cerebellum > cerebral cortex > spinal cord. Although the authors made no comments about this in that paper, it appears to me that the different maximal enhancements by pregnanolone in different regions may be due, in part, to the different cholesterol levels in those regions. Thus, the

binding of steroids, the competition for the same binding sites by steroid agonists and antagonists may be differentially influenced by different levels of cholesterol in those regions. Therefore, it would be very interesting to investigate the selective antagonism by 11-ketoprogesterone further using different brain regions.

Alternatively, studies using recombinant $GABA_A$ receptors may answer this question more directly. Although so far, no clear subunit dependence for steroids has been established (Chapter 1), there still exists the possibility that the competition for the same nonannular binding sites by two steroids are subunit sensitive.

Since pregnanolone appears to have more than one component in its action on ^{3}H-FNZ binding, other steroids may exert their effects on ^{3}H-FNZ binding through similar mechanisms. It would be interesting, therefore, to determine for other neuroactive steroids, the relative contributions of GABA-dependent and GABA-independent components to their actions, and the selective and weak antagonism by 11-ketoprogesterone, which could well differ from those found with pregnanolone. These further studies may provide us invaluable information for understanding the physiological role, as well as the pharmacological effects, of this remarkable class of neuroactive steroids; and may eventually help us to design drugs that have selective behavioral activity.

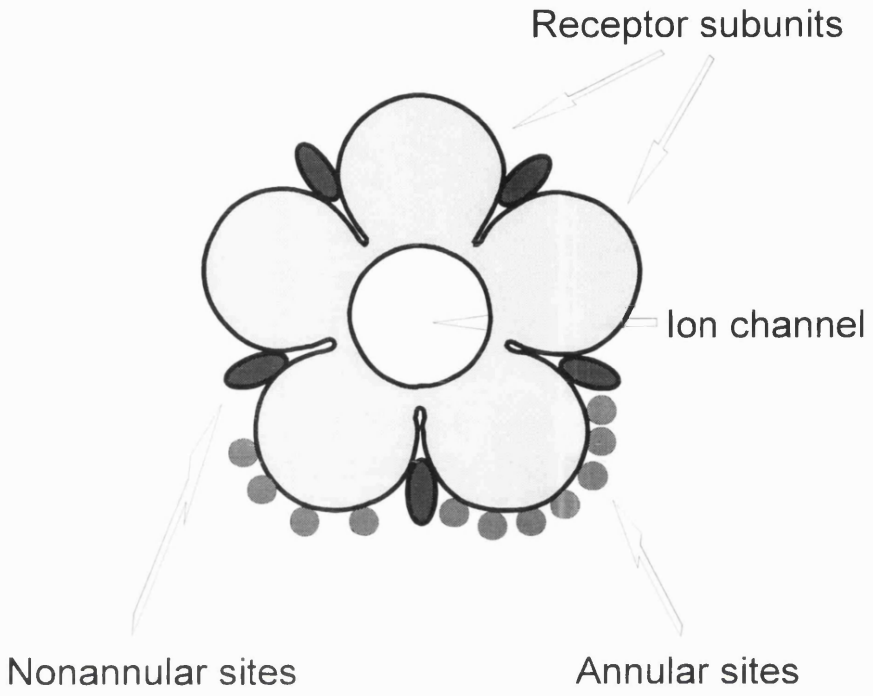


Figure 8.1. A model (not to scale) for the interaction of lipids with ligand gated ion channels (adapted from Jones & McNamee, 1988). The lipids may interact with the subunits of the receptors at two classes of site: i) the nonannular sites, to which cholesterol binds preferentially and ii) the annular sites, which attract phospholipids predominantly. The annular sites are around the outer surface of the protein, while the nonannular sites may be located at the protein / protein interfaces of the subunits. In receptors which are hetero-oligomers, these nonannular sites will not be equivalent.

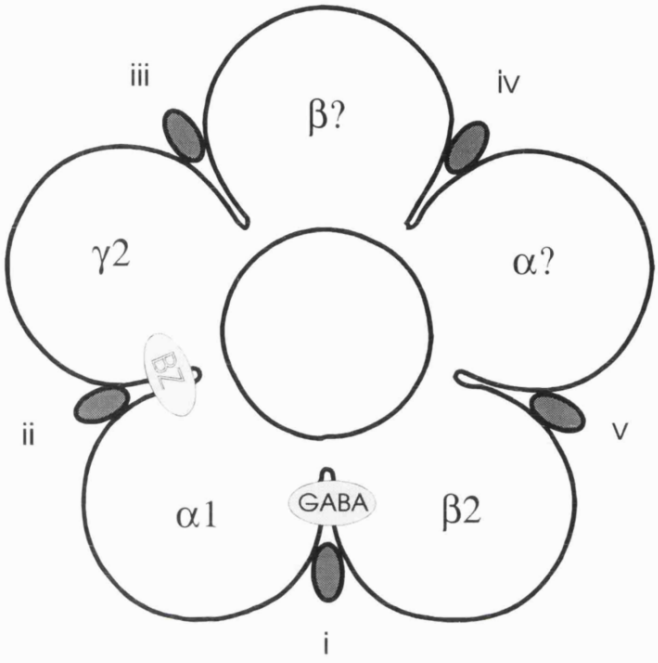


Figure 8.2. A model (not to scale) of $GABA_A$ receptor protein with five subunits (adapted from Smith & Olsen, 1995). The tentative binding sites of GABA and benzodiazepines (BZ) and the nonannular sites (i, ii, iii *etc*) to which steroids may bind have been indicated. Pregnanolone may bind to extracellular sites as well as nonannular sites such as (i)(ii)(iii) *etc*. The action on ^{3}H-FNZ binding by pregnanolone at site (ii) may be direct, while that at site (i) may be influenced by GABA (see main text). Steroids binding to nonannular sites such as (iii)(iv)(v) *etc* may also have allosteric interactions with BZ sites and GABA sites.

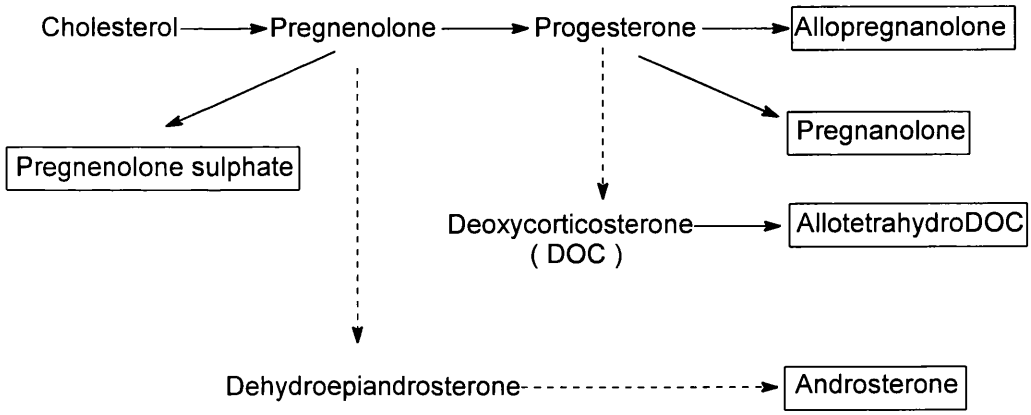


Figure 8.3. Outline of the metabolic pathways by which neuroactive steroids may be synthesized (Simmonds & Prince, 1993). Solid arrows indicate pathways that may operate in both the brain and the periphery and broken arrows indicate pathways thought to operate in the periphery only. The steroids shown in boxes have modulatory activity at the $GABA_A$ receptor protein.

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