TITLE

'The influence of membrane cholesterol on the rat $GABA_A$ receptor'

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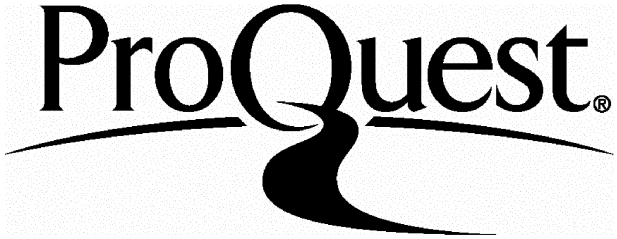

Abstract

The $GABA_A$ receptor is an integral membrane protein surrounded by synaptic membrane lipids that may exert a biological control upon the receptor. An important component of the lipid milieu is cholesterol that constitutes over 20% of the membrane lipids which may interact with the neurosteroid binding sites of the $GABA_AR$. To investigate this possibility synaptic membranes were enriched with cholesterol by incubating with liposomes comprising 50 phosphatidylcholine : 50 cholesterol in the presence of 1% BSA. The effects of cholesterol and modulatory drugs on the $GABA_A$ channel were assessed via changes induced in [^{3}H]-FNZ, [^{3}H]-muscimol or [^{3}H]-TBOB binding. Cholesterol enrichment did not affect the affinity of FNZ, muscimol or TBOB for their binding sites; however, the enhancement of [^{3}H]-FNZ binding by pregnanolone, muscimol or propofol; the modulation of [^{3}H]-muscimol by propofol and the modulation of [^{3}H]-TBOB binding by FNZ, pregnanolone, muscimol or propofol were affected.

In enriched cerebral cortex membranes, the potency of pregnanolone to enhance [^{3}H]-FNZ binding was reduced. By contrast, in membranes from spinal cord, the potency of pregnanolone was increased following cholesterol enrichment. In membranes from cerebellum, there was little overall change in pregnanolone potency although the effects of threshold concentrations were increased.

The enhancement of [^{3}H]-FNZ binding by propofol was reduced and the potency of muscimol to potentiate [^{3}H]-FNZ binding was increased upon enrichment of whole brain, cerebral cortex and spinal cord membranes.

Enhancement of [^{3}H]-muscimol binding by propofol in whole brain membranes was reduced upon cholesterol enrichment.

In whole brain membranes modulation of [^{3}H]-TBOB binding by FNZ, pregnanolone, muscimol or propofol was reduced upon cholesterol enrichment.

These results provide little evidence for a selective competition between cholesterol and pregnanolone at its binding site. Rather, they suggest an influence of membrane cholesterol on the functional coupling between the modulatory sites on the $GABA_A$ channel. The detailed pattern of influence on the enhancement of [^{3}H]-FNZ binding by pregnanolone depended on the region of CNS and may be related to the subunit composition of the $GABA_A$ channels present.

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Preliminary reports of some sections of this thesis have appeared in the following publications:

BENNETT, P.J. & SIMMONDS M.A. (1996). 'The influence of membrane cholesterol on the $GABA_A$ receptor'. *Br. J. Pharmacol.*, **117** (1), 87-92.

BENNETT, P.J. & SIMMONDS M.A. (1995). 'Cholesterol enrichment of neuronal membranes alters the enhancement of [^{3}H]-flunitrazepam binding by pregnanolone.' *Br. J. Pharmacol.*, **114**, 294P.

Abbreviations used: ANOVA, analysis of variants; BNZ, benzodiazepine; BSA, bovine serum albumin; Cl^-, chloride; CNS, central nervous system; DMSO, dimethylsulfoxide; FNZ, flunitrazepam; GABA, γ-aminobutyric acid; $GABA_AR$, γ-aminobutyric acid type A receptor; nAChR, nicotinic acetylcholine receptor; nsL-TP, non-specific lipid transfer protein; PC, phosphatidylcholine; pre-nsL-TP, precursor form of the non-specific lipid transfer protein; pregnanolone, 3α-hydroxy-5β-pregnan-20-one; TBOB, t-butylbicycloorthobenzoate; TBPS, t-butylbicyclophosphorothionate.

Buffers Used: Assay Buffer, (50 mM Tris-base, 150 mM $NaCl^-$, pH 7.4 at 4°C); SET Buffer, (0.25 M sucrose, 1 mM EDTA, 50 mM Tris-base, 0.02 % sodium azide, pH 6.9 at 25°C); Wash Buffer, (5 mM Tris-base, 1 mM EDTA, pH 7.4 at 4°C).

Chapter 1

General Introduction

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1.1 Plasma membrane structure

The conventional view of biological membrane organization is the fluid mosaic model proposed by Singer & Nicolson (1972). This model proposes that proteins are embedded within a lipid environment containing static and dynamic regions of the membrane. This heterogeneity of the eukaryote plasma membrane is controlled by couplings between the lipid membrane and the cytoskeleton, lipid-lipid and lipid-protein interactions (Mouritsen & Bloom, 1993). The lipids surrounding an integral membrane protein are selected by hydrophobic matching with the protein leading to a local lipid environment. The lipids consequently interact with the protein by electrostatic and hydrogen bonding, and therefore are immobilized and ordered while the remaining lipids in the membrane form a fluid bilayer (Benga, 1985). Lipids in the boundary layer still may exchange with the bulk lipids in the fluid bilayer but due to their interaction with the protein the time constant (10^{-4} sec) for such an exchange is greater than it otherwise would be. Cholesterol does not significantly interact with intrinsic membrane proteins at the lipid-protein interface because the planar ring of the sterol molecule is unable to adapt to the surface of the protein. This makes cholesterol a poor agent for sealing proteins into the bilayer. Lipids may also interact with hydrophobic pockets within the intrinsic protein leading to their retention.

1.2 Cholesterol's interaction with the nicotinic Acetylcholine Receptor (nAChR)

Cholesterol and negatively charged phospholipids are required to maintain ion-gating activity of the nAChR (Fong & McNamee, 1986). A minimum of 45 molecules of lipid are necessary to prevent irreversible loss of both the allosteric transitions and the ion gating functions (Jones & McNamee, 1988). The agonist dissociation constants are not affected by change of the lipid environment since the agonist binding sites are located at the extracellular domain of the protein above the membrane surface. Meanwhile, the conformational state interconversion of the receptor is dependent upon the nature of the lipid environment, which affects the ability of the nAChR to undergo low to high affinity state transition. This state transition allows desensitization of the receptor (Lena & Changeux, 1993) causing a difference in the protein structure between the resting and the agonist induced desensitized conformations of the nAChR, both in AChR rich native membranes from *Torpedo* and in reconstituted lipid vesicles containing purified nAChR as the sole protein component (Fernandez-Ballester *et al.*, 1994). Cholesterol though is not required for the agonist to produce structural changes leading to nAChR desensitization (Fernandez-Ballester *et al.*, 1994). Apart from affecting the transition states, the secondary structure of the protein is also altered by cholesterol. The α helix content of the receptor is increased when reconstituted into membranes containing cholesterol (Butler & McNamee, 1993).

There are high cholesterol concentrations present within electroplax membranes that interact strongly with the AChR. The lipid environment around the AChR is controlled by the protein as mentioned above allowing optimum conditions for its

functioning. The interactions between cholesterol and negative phospholipids thus allow dynamic changes in the conformation of the receptor which control the opening and closing of the channel.

1.3 Nature of cholesterol's binding site at the nAChR

Neural membranes comprise of up to 50 % cholesterol on a molar basis (Jones & McNamee, 1988) making the interaction between the cholesterol molecule and nAChR particularly important. There seems to be two pools of cholesterol within the membranes: a fraction influencing the bulk lipid fluidity and a more tightly bound fraction thought to be associated with the receptor. Fluorescence quenching studies suggests that two lipid binding sites exist at the nAChR; annular sites at the lipid-protein interface and non-annular binding sites which occlude lipids. Negatively charged phospholipids preferentially interact with the receptor at the annular sites while cholesterol appears to interact strongly with the non-annular sites but less well with the annular sites due to its rigid planar structure. The non-annular binding sites may be at the interstices between the subunits which form hydrophobic pockets (Sunshine & McNamee, 1992) with 5-10 per nAChR (Jones & McNamee, 1988). The receptor can easily be labelled by a photoactivatable cholesterol analogue and such labelling can be displaced by unlabeled cholesterol and is sensitive to the protein structural rearrangement induced by desensitization (Fernandez-Ballester *et al.*, 1994). These observations show that there are specific interactions between cholesterol and sites on the nAChR. Cholesterol may stabilize the amphipathic helical structure in the nAChR by fitting into the grooves of tilted α helices or β sheets of

the M1, M3, and M4 transmembrane domains of the receptor, in the vicinity of a cysteine residue (Narayanaswami & McNamee, 1993; Fong & McNamee, 1987; Fernandez-Ballester *et al.*, 1994; Butler & McNamee, 1993). This stabilization of the nAChR structure is responsible for supporting the ion-gating activity. Additionally the non-annular sites may be pharmacologically important in respect to local and general anaesthetics. Local anaesthetics, such as procaine and tetracaine, may compete for the cholesterol binding sites (Sunshine & McNamee, 1992).

1.4 Superfamily of ligand-gated ion channels

The nAChR belongs to a superfamily of receptors to which five families of ligand-gated receptors belong. Notably this group includes the inhibitory neuronal γ-amino butyric acid Type A ($GABA_A$) channel which conducts Cl^- ions. This superfamily of receptors share common features of four putative spanning regions denoted M1-M4 for each subunit (Figure 1.1) (Schofield *et al.*, 1987); strong homology sequence in these hydrophobic transmembrane sequences especially in M2 the hydrophobic stretch of 20 amino acids that lines the ion conducting channel; and a quasi-symmetric pentameric arrangement of similar or identical subunits (Bertazzon *et al.*, 1992; Nayeem *et al.*, 1994; Stroud *et al.*, 1990; Unwin, 1993).

Comparing the nAChR and the $GABA_AR$ reveals a similar homologous cysteine-bridged loop found on the extracellular side of the subunits in both receptors. This loop contains an N-linked glycosylation site which is conserved between the $GABA_A$ β subunit

and the nAChR subunits. The α and β subunits of the $GABA_A$ receptor are homologous to the nAChR subunits throughout their length (Schofield *et al.*, 1987). In the AChR serines and threonines found in the M2 region of the anion conducting channel are found in analogous positions of the $GABA_A$ receptor. There is however, a higher number of serine, threonine or polar residues in the $GABA_A$ receptor, about 2 per turn compared to the 1.5 per turn in the nAChR. The $GABA_A$ receptor M2 domains are also richer in threonine than serine compared to the nAChR. The nAChR vestibule that is above the membrane is negatively charged to gain channel selectivity by repelling anions (Stroud *et al.*, 1990). Meanwhile, the $GABA_A$ receptors vestibule displays a positive charge to repel cations (Schofield *et al.*, 1987). Both receptors undergo desensitization if an agonist is present continuously and require two agonist molecules to bind for maximal ion flux (Macdonald & Olsen, 1994; Stroud *et al.*, 1990).

The common features shared by the $GABA_AR$ and the nAChR are not surprising since they both evolved from a common ancestor (Ortells & Lunt, 1995). Therefore, it might be likely that binding sites for cholesterol at 'non-annular' sites within the $GABA_A$ receptor exist.

1.5 Molecular biology of the $GABA_A$ receptor

The main subunits are split into the groups α, β, γ, ρ and δ. The α, β and γ subunits are the most commonly expressed, while the ρ and δ subunits are found only in bicuculline and benzodiazepine insensitive receptors, respectively (Sieghart, 1995).

Bicuculline insensitive receptors display neither $GABA_A$ or $GABA_B$ receptor pharmacology and the ρ subunit also displays the greatest divergence among the subunit subtypes. For these reasons it has been suggested that the ρ subunit may constitute a homo-oligomeric receptor that belongs in the $GABA_C$ category (Rabow *et al.*, 1995). With the advent of molecular biology evidence has accumulated for the existence of six α, four β, three γ, two ρ and one δ subunit. This means that hundreds to thousands of different combinations are possible but only a small number are presumably used. The exact composition of even one native $GABA_A$ receptor is as yet unknown.

The amino acid sequence of the subunits show 20-40% homology to each other but subunits of the same group such as the α subunit show 70% homology (Olsen & Tobin, 1990). Subunits can be combined in a multitude of ways giving rise to different pharmacological profiles. The function of the subunits and the way that they alter the pharmacology of the receptor is outlined below:

i) The β subunit is required for neurosteroid activity, but the type expressed has little impact on steroid potentiation of GABA evoked currents. This is not true however for BNZ modulation of [^{35}S]-TBPS binding in the construct $\alpha_1\beta_x\gamma_2$. Here the rank order of modulation is $\beta_1>\beta_2>\beta_3$. When an α_5 subunit is expressed this must be coupled with a β_3 subunit to gain [^{35}S]-TBPS binding. The action of the anticonvulsant loreclezole is also dependent on the type of β subunit present. This compound potentiated the action of GABA with a higher affinity in receptors containing β_2 or β_3 variants compared to β_1 subunits.

ii) The type of α subunit present gives no differential interaction of neurosteroids between $\alpha_1\beta_1\gamma_2$, $\alpha_3\beta_1\gamma_2$ and $\alpha_5\beta_1\gamma_2$ subunit combinations in electrophysiology experiments. However, the potentiation of GABA evoked currents by allopregnanolone (5α-pregnan-3α-ol-20-one) was greater in recombinants with the α_1 subunit and the enhancement of [^{3}H]-FNZ binding by pregnanolone preferred α_3 rather than α_1. The α subunit variants are also responsible for differential BNZ pharmacology. The type of α subunit present effects BNZ agonist affinities but not the affinities of the antogonists and inverse agonists.

iii) The γ subunit greatly influences the BNZ pharmacology of the $GABA_AR$ and is obligatory for the formation of the BNZ binding site. Receptors with an γ_1 or γ_3 variant instead of the γ_2 subtype show a reduced affinity for BNZs. Although the γ subunit is not a crucial determinant of steroidal modulation, the type may still exert some influence. This has been shown by steroidal modulation of GABA evoked currents being higher in cells expressing $\alpha_1\beta_1\gamma_1$ compared to that with a corresponding γ_2 or γ_3. Two copies of the γ_2 subunit exist, a long and short variant (γ_{2L} and γ_{2S}, respectively). The γ_{2L} variant has an eight amino acid insert which has a consesus sequence for a protein kinase C binding site. This site is thought to be important for ethanol modulation of $GABA_ARs$.

iv) The δ subunit can replace the γ subunit in a receptor. Preliminary reports suggest that replacing γ_2 with δ forms a BNZ insensitive receptor.

v) When either ρ subunit is expressed this produces a receptor that is not modulated by pentobarbital or BNZs and is insensitive to bicuculline and baclofen. Due to this pharmacology the ρ subunit maybe part of the BNZ insensitive retinal specific $GABA_AR$ that resembles a $GABA_CR$.

(Lambert *et al.*, 1995; Luddens *et al.*, 1994; Sieghart, 1995)

With expression studies forming homooligomeric ion channels it was seen that some of them can be activated by GABA and inhibited by bicuculline (Burt & Kamatchi, 1991). Such channels display multiple conductance states and desensitization. Channels containing two subunits are formed more easily and can be activated by a lower GABA concentration, and the induced ion fluxes were greater (Sigel *et al.*, 1990; Knoflach *et al.*, 1992). The cooperativity though of the $GABA_A$ receptor was absent in most of these dual subunit combinations. Coexpression of α, β, and γ_2 subunits resulted in large GABA gated Cl^- flux which could be inhibited by bicuculline. Cooperativity was now present, but as in previous studies the conductance properties were dependent upon the subunit combination used (Sigel *et al.*, 1990; Knoflach *et al.*, 1992).

These studies show that the binding sites for picrotoxinin, barbiturates and possibly steroids seem to be formed by the assembly of most $GABA_A$ receptor subunits. Modulation of the channel by BNZs was observed when α, β and γ2 subunits were expressed together in a single cell (Burt & Kamatchi, 1991; Sigel *et al.*, 1990; Knoflach *et al.*, 1992). From these studies it was concluded that receptors comprised of α, β, and γ subunits most closely resemble the native $GABA_A$ receptors found in the brain. The subunit composition alters the BNZ binding properties and is especially influenced by the type of α or γ subunits present which alter the affinities of various selective and non-selective BNZ ligands for the receptor (Burt & Kamatchi, 1991; Lüddens & Wisden, 1991; Doble & Martin, 1992) and also the efficacy of BNZ ligands to enhance GABA-induced Cl^- ion flux (Puia *et al.*, 1991; Puia *et al.*, 1992).

Regions of the subunits contain conserved and variable amino acid sequences suggesting the presence of structural and functional domains. All the subunits have four transmembrane domains M1-M4 that are α helices or β-sheet configurations with 22-23 amino acids as shown by hydrophobicity studies, occurring at residues 225-246, 251-272, 284-306 and 394-415 in the carboxyl terminal half of the protein which are conserved (Figure 1.1) (Olsen & Tobin, 1990; Unwin, 1993).

The hydrophilic amino terminal half of the subunit has potential aparagine-glycosylation sites at 10 and 110 amino acids of the α_1 subunit as well as a conserved cystine bridge between two cysteine residues (Figure 1.1) that may help in ligand binding. In other subunits it is assumed there are other glycosylation sites (two in the α subunit and three in the β subunit), since the peptide sizes determined by SDS-PAGE are larger than the deduced sequences (Mamalaki *et al.*, 1987; Sweetnam & Tallman, 1986). The C-terminus ends shortly after the M4 hydrophobic transmembrane domain as a hydrophilic chain on the extracellular side (Figure 1.1). Between M3 and M4 a large hydrophilic loop responsible for subunit specificity is prone to phosphorylation in the β subunit at the consensus Arg-Arg-Arg-Ala-Ser and the chain length differs in size between the α and β subunits (87 and 124 amino acids in the α and β subunits respectively) (Feramisco *et al.*, 1980).

Each receptor contains at least one α, one β and one γ (which can be substituted by δ) subunit to form a fully functional $GABA_AR$ (McKernan & Whiting, 1996). The major subtypes of receptor and their relative abundance in rat brain are: $\alpha_1\beta_2\gamma_2$ 43% (present in most brain areas); $\alpha_2\beta_{2/3}\gamma_2$ 18% (present in spinal cord and hippocampal pyramidal cells); $\alpha_2\beta_x\gamma_{2/3}$ 17% (present on cholinergic and monoaminergic neurones); $\alpha_2\beta_x\gamma_1$ 8% (present on Bergmann glia); $\alpha_5\beta_3\gamma_{2/3}$ 4% (present on hippocampal pyramidal cells) and $\alpha_6\beta\gamma_2$ 2% (present on cerebellar granule cells) (McKernan & Whiting, 1996). When the subunits are clustered together a structure is gained of five α helices and β sheet in a barrel configuration contributing to or stabilizing the 5.6Å ion channel

(Bormann *et al.*, 1987; Unwin, 1993). The M2 domain of the $GABA_A$ receptor subunits by analogy to that of the M2 domain in the AChR is thought to line the ion channel pore. Evidence for the M2 transmembrane domain lining the channel pore comes from the rich content of threonine and serine residues which form a hydrophilic environment within the pore conducive to ion conductance (Schofield *et al.*, 1987; Mamalaki *et al.*, 1987). The M2 transmembrane domain in the α_1 and β_1 subunits of the $GABA_A$ receptor and the α_2 and β_2 subunits of the nAChR have an invariant leucine residue that introduces a bend into the transmembrane helix (Figure 1.2) (Unwin, 1993). The leucine 251 on the α subunit of the AChR and the leucine residues at homologous positions on other subunits project into the centre of the pore and associate by side to side interactions of their branched chains (Figure 1.3). This creates a tight hydrophobic ring preventing the passage of hydrated ions until the $GABA_A$ receptor is bound by GABA (or acetylcholine in the case of the AChR) at its binding sites (Unwin, 1993). When this occurs the stability of this weak association would presumably be affected by nearby hydrophobic and electrostatic interactions causing a conformational change in the helix and opening of the gate (Alber, 1992). The M4 domain interacts with the lipids at the protein-lipid boundary which may also regulate the receptor.

At both ends of the transmembrane domains that form the channel's mouth, positively charged amino acids are found which act as an anion filter to increase the Cl^- flow through the pore when opened (Schofield *et al.*, 1987). The GABA and BNZ agonist and antagonist binding sites are probably located at the N-terminal extracellular

domains that have ten potential N-glycosylation sites per receptor complex. Evidence for the binding sites locations comes from the fact that deglycosylation of rat $GABA_A$ receptors causes modification in the BNZ and GABA agonist and antagonist binding (Sweetnam & Tallman, 1986).

1.6 Pharmacology of the $GABA_A$ receptor

The various binding sites of the receptor are outlined below. These binding sites are all specific and distinct from one another upon the $GABA_A$ receptor. Allosteric interactions between these binding sites occur thus modulating the gating of the $GABA_A$ channel (Sieghart, 1992).

1.6.1 GABA binding sites

Binding of GABA to the $GABA_A$ receptor increases the neuronal conductance of the Cl^- ions resulting in membrane hyperpolarization and a reduction in neurone excitability (Study & Barker, 1981; Bormann, 1988). This can be competitively inhibited by bicuculline (Bormann, 1988).

The sites show high and low affinity for GABA and its agonists (such as muscimol) with K_d values in the nanomolar or micromolar range respectively. The low affinity site appears to be antagonist preferring (Schumacher & McEwen, 1989), being selectively labelled by the antagonists, such as (+)-bicuculline and SR95531. Both low and high affinity forms of the $GABA_A$ binding site show similar drug specificity, and pentobarbital increases the high affinity sites at a cost of the low affinity sites. These different types of

binding may represent a conformational change of the receptor occurring (Schumacher & McEwen, 1989). It is presumed that the low affinity sites activate the Cl^- channel (Schumacher & McEwen, 1989) since micromolar concentrations of GABA or its analogues are required for this (Segal & Barker, 1984) and to modulate the other binding sites at the $GABA_A$ receptor (Olsen 1982; Squires *et al.*, 1983). The high affinity state of binding could be a desensitized form of the receptor.

1.6.2 Benzodiazepine binding site

These compounds are widely used for their anxiolytic, anticonvulsant, muscle relaxant, sedative and antidepressant properties. BNZs enhance the action of GABA at the $GABA_A$ receptor by increasing the frequency of the Cl^- ion channel opening (Study & Barker, 1981). Binding experiments have shown that these sites are found on membranes closely associated with the $GABA_A$ receptors (Olsen, 1982). Binding of [^{3}H]-flunitrazepam (FNZ) to brain membranes was potentiated by GABA (and the vice versa could be seen) (Skerrit *et al.*, 1982), which could be inhibited by (+)-bicuculline (Olsen, 1982).

There is a high correlation between the clinical potency of the benzodiazepines and their affinity for the [^{3}H]-FNZ binding site which brought the conclusion that these are the binding sites by which the benzodiazepines exert their clinically important actions (Haefely *et al.*, 1985). Inverse benzodiazepine receptor agonists reduce the GABA-induced Cl^- flux (Haefely *et al.*, 1985). The antagonists of the benzodiazepines do not influence GABA-

induced Cl^- flux but antagonize benzodiazepine agonists or inverse agonists (Haefley *et al.*, 1985).

1.6.3 Picrotoxinin/TBPS binding site

These compounds are convulsants which antagonize the Cl^- ion influx induced by GABA (Olsen, 1982; Bormann, 1988). Their binding site seems to be closely associated with the Cl^- channel of the $GABA_A$ receptor since GABA and BNZ binding was not inhibited by these compounds (Olsen, 1982). Also they directly reduce the Cl^- conductance by sterically inhibiting the passage of Cl^- ions (Gee, 1988). GABA and compounds that modulate the $GABA_A$ receptor such as the BNZs, barbiturates and neurosteroids allosterically inhibit [^{35}S]-TBPS binding by decreasing its affinity (Gee, 1988). Compounds that reduce the efficacy of GABA at $GABA_A$ receptors like the convulsant β-carbolines, enhance [^{35}S]-TBPS binding affinity through specific interactions with the BNZ binding site. This points to high affinity TBPS binding being associated with a closed conformation of the Cl^- ion channel (Gee, 1988).

1.6.4 Barbiturate binding site

These compounds enhance the effect of GABA by increasing the mean channel open time (Study & Barker, 1981; Bormann, 1988). At higher concentrations they can directly enhance the Cl^- conductance (Bormann, 1988). The barbiturates have low affinity sites so the interactions of barbiturates with the GABA site and the BNZ site were investigated indirectly. Barbiturates increase GABA and BNZ affinities (Olsen, 1982) in

order of their potency as anaesthetics and hypnotics. They also inhibit binding of TBPS in a similar rank order (Olsen, 1982; Squires *et al.*, 1983). The barbiturates seem to interact allosterically with the TBPS binding sites, suggesting that the depressant barbiturates enhance Cl^- ion flux by interacting with a specific binding site.

1.6.5 Propofol binding site

Propofol exerts its actions through the $GABA_A$ receptor. Propofol dose dependently potentiates GABA activated currents, and at higher concentrations directly activates the receptor by increasing the channel conductance to Cl^- ions in a bicuculline sensitive manner (Hales & Lambert, 1991). Propofol inhibits allosterically the binding of [^{35}S]-TBPS and enhances the binding of [^{3}H]-muscimol and [^{3}H]-FNZ (Concas *et al.*, 1990, 1991; Prince & Simmonds, 1992).

1.6.6 Neurosteroid binding site

Steroids intensify GABA Cl^- ion conductance in brain membranes by prolonging the open time of the Cl^- channels (Peters *et al.*, 1988). They also increase the binding affinity of GABA and BNZ agonists, but inhibited binding of TBPS (Gee, 1988; Schumacher & McEwen, 1989).

The location of the neurosteroid site has not, as yet, been determined. Neurosteroid action was thought to be brought about by perturbations of the lipid membrane due to a correlation between the potency of a general anaesthetic increasing in proportion to its solubility in fat like solvents (Franks & Lieb, 1987). Interactions with the

$GABA_A$ channel though, suggest structure-activity relationships: the requirement of a 3-hydroxyl group in the α configuration; a keto group in the 20 position of the pregnanes and in the 17 position of the androstanes; and the position 17 side chain must be in the β configuration to maintain GABA potentiating activity (Franks & Lieb, 1994; Simmonds & Prince, 1992; Harrison *et al.*, 1987). Furthermore, Oliver *et al.* (1991), have shown that sensitivity to steroid anaesthetics at the $GABA_A$ receptor only appeared late in evolution. Pregnanolone only affects organisms belonging to the phylum Chordata. The insensitivity of invertebrates that do possess $GABA_A$ receptors excludes a lipid bilayer site for the actions of neurosteroids. Removal of most of the membrane lipids after solubilization and purification of the $GABA_A$ receptor, maintains pregnanolone potentiation of [^{3}H]-muscimol binding, further supporting an interaction with the channel protein (Olsen, 1990). By analogy to the acetylcholine receptor (Jones & McNamee, 1988), cholesterol may bind at the axial clefts between the $GABA_A$ receptor's subunits which may be the locus of certain drug binding sites (Changeux, 1990), such as the neurosteroids. These sites would be accessible to membrane cholesterol and would suggest a model for neurosteroid binding via diffusion through the membrane to a hydrophobic pocket at the $GABA_A$ receptor subunit interstices.

1.7 Effect of the lipid environment upon the $GABA_A$ receptor

Apart from the lipid milieu influencing the nAChR an influence is also seen upon the $GABA_A$ receptor. To maintain modulatory characteristics after purification and solubilization of the $GABA_A$ receptor, this procedure must be carried out in the presence

of a natural brain lipid extract and the cholesterol analogue cholesteryl hemisuccinate (Bristow & Martin, 1987). Cholesterol also seems to be an essential requirement for reconstitution of the protein into liposomes.

1.8 Aims and objectives

Considering that cholesterol binding sites have been shown in the nAChR and there is a requirement for cholesterol if modulation of the $GABA_A$ receptor by the allosteric sites is to be retained after solubilization and reconstitution of the receptor into liposomes, the aim of this PhD thesis is to investigate ways of enriching synaptic membranes with exogenous cholesterol and to determine the nature of this enrichment. Upon achieving cholesterol enrichment, evidence will be sought to see if cholesterol competes for the neurosteroid binding site or interacts more generally with the $GABA_A$ receptor. Such a competition between cholesterol and neurosteroids or a more general influence of the cholesterol molecule upon the $GABA_A$ receptor will be investigated using radioligand binding studies.

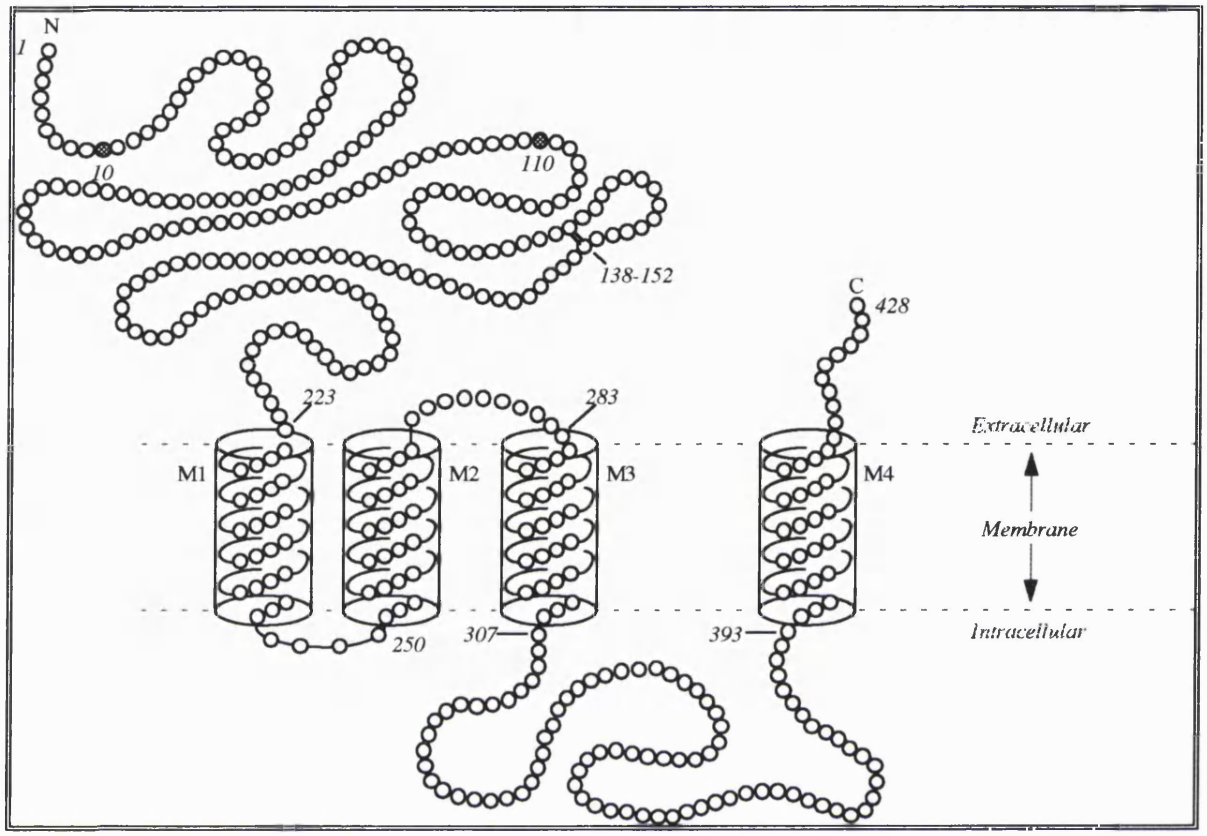


Figure 1.1 Generic $GABA_A$ receptor protein subunit sequence and topological structure. The numbering follows that of the rat α_1 sequence. The extracellular NH_2 terminal has asparagine glycosylation sites at positions 10 and 110 (filled circles) and a conserved cystine bridge (solid line connecting 138 and 152) between all members of the gene superfamily. Four putative membrane spanning α-helical cylinders M1, M2, M3, and M4 are shown. The COOH terminal is again extracellular. A large intracellular cytoplasmic loop between M3 and M4 is present. Modified from Olsen & Tobin, 1990.

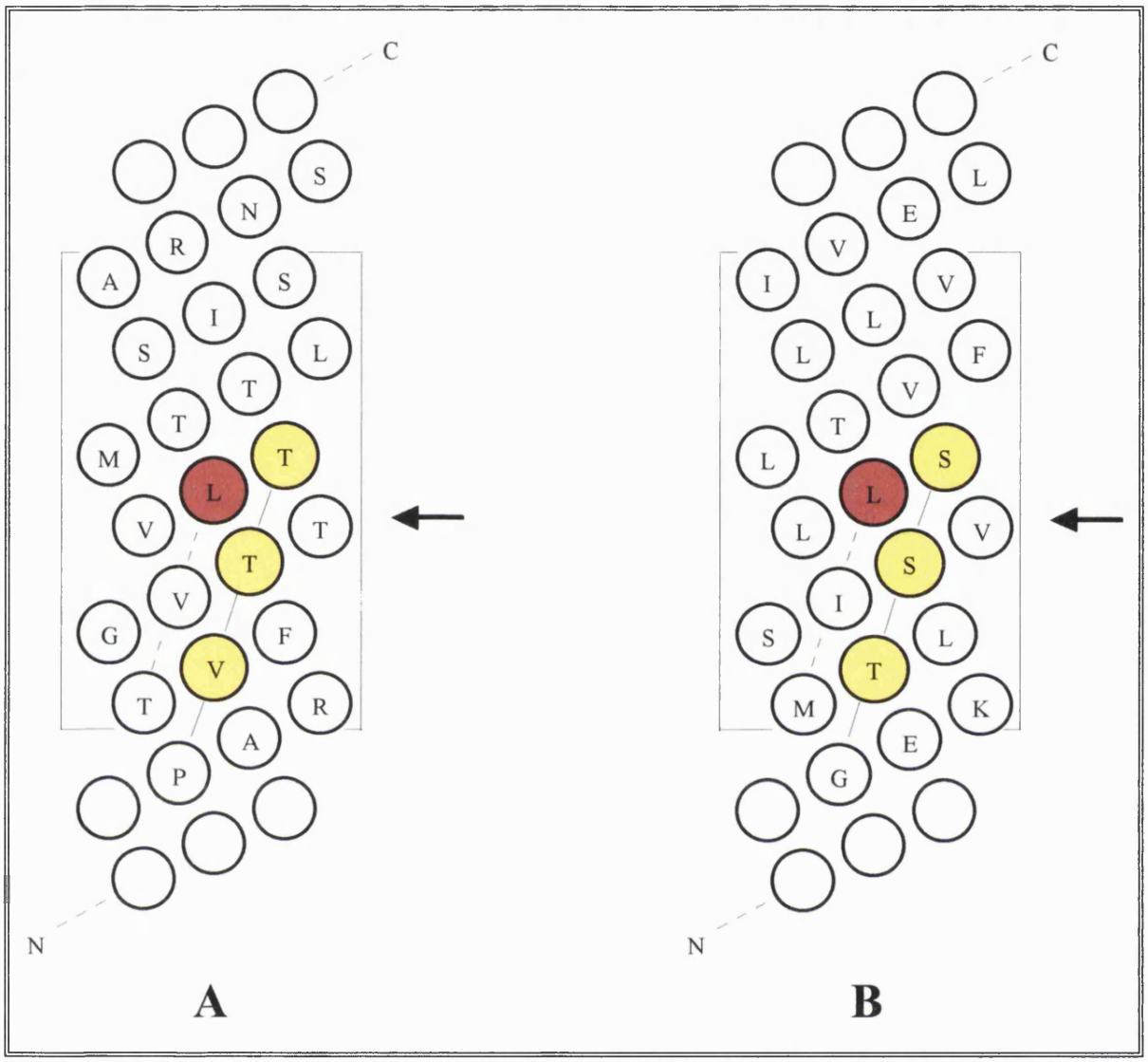


Figure 1.2 Helical net plot of the amino acid sequence around M2 of the $GABA_A$ receptor α_1 subunit (A) and the Torpedo AChR α subunit (B). The solid line indicates a face on the helix composed entirely of small residues. This face is flanked by bulky hydrophobic side chains (dashed line), which confer a kink shaped helix whose position is indicated by the arrow. The rectangle indicates the bilayer spanning segment. The red filled circle indicates the conserved leucine residue and the yellow shaded circles the residues which when mutated affect the ion flow through the channels (Unwin, 1993).

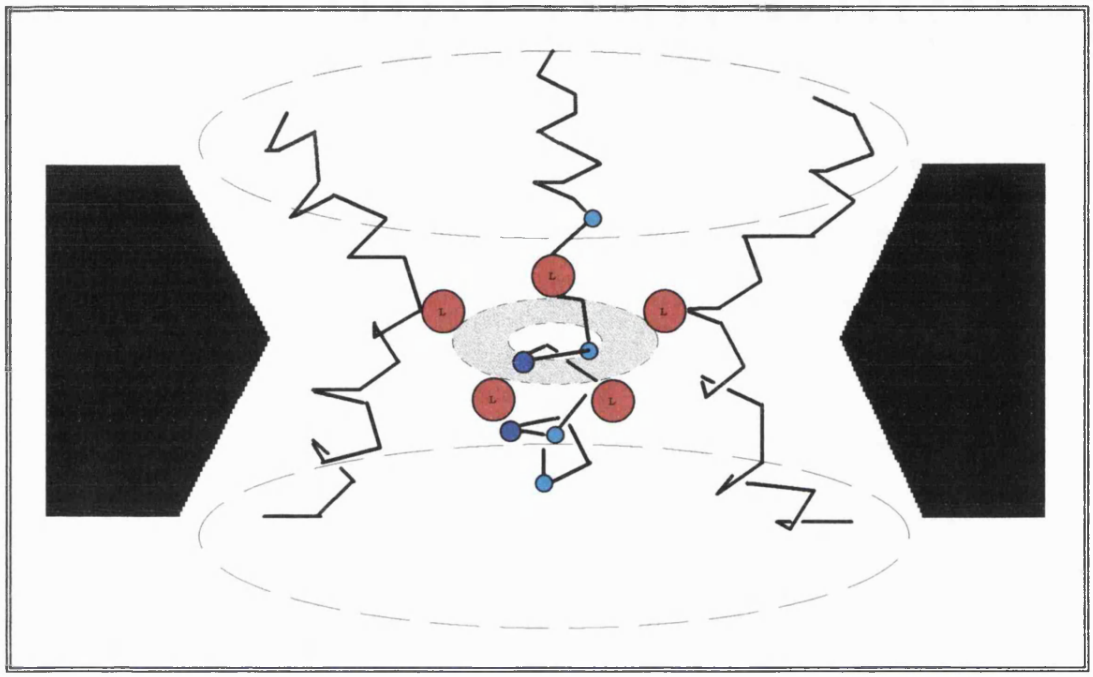


Figure 1.3 A diagram illustrating how the gate of a closed channel might be constructed. The M2 helix, which is kinked about half way across the bilayer, allows leucine side chains (red circles) to project close to the centre of the pore. The leucine residues associate by side to side interactions of their branched chains, forming a tight hydrophobic ring. The ring makes a barrier through which hydrated ions cannot pass. Adjacent to and below the leucine residue is a sloping line of small amino acids (small cyan cirles), flanked by larger hydrophobic side chains (blue circles) (Unwin, 1993).

Chapter 2

Cholesterol Enrichment of Synaptic Membranes

Chapter 2

Cholesterol enrichment of synaptic membranes

2.1 Introduction

The lipid content of the plasma membrane has a considerable influence on the membrane viscosity (Shinitzky & Inbar, 1976) and also the lipid microenvironment surrounding the integral lipid proteins (Benga, 1985). Increasing the cholesterol content increases the membranes microviscosity and the degree of order in the system. With increasing cholesterol content the physical properties of the membrane change abruptly with different superlattices being formed (Virtanen *et al.*, 1995). Changes of this kind may induce or alter various cellular functions which depend on the dynamics of the cell surface membrane such as transport processes (Shinitzy & Inbar, 1976).

In the AChR an optimum fluidity is proposed to account for conformational transition properties between high and low affinity states of the receptor (Fong & McNamee, 1986). Only AChRs in membranes containing both cholesterol and negatively charged phospholipids retain ion gating properties, due to these lipids interacting strongly and stabilizing the amphipathic helical structure of the AChR whilst the ion channel is opened upon agonist binding. The role of cholesterol in stabilization is important since, synaptic membrane lipids are composed of over 20% of cholesterol whose interactions with integral membrane proteins are thought to be mainly of a hydrophobic nature which maybe at hydrophobic crevices within the protein (Benga, 1985). Unsaturated fatty acids, though

inhibit AChR function by perturbing interactions between AChRs and cholesterol and/or negatively charged phospholipids (Andreasen & McNamee, 1980; Pjura *et al.*, 1982). The dissociation constants of agonists for the AChR will not be altered significantly by different lipid environments since the agonist binding sites project into the extracellular domains above the membranes.

Modulation by cholesterol is also seen in other integral membrane proteins. The catalytic activity of Ca^{2+}-ATPase is inhibited by cholesterol (Warren *et al.*, 1975) and a critical concentration of cholesterol is required for maximal catalytic activity of adenylate cyclase (Whetton *et al.*, 1983). Cholesterol depletion from liver plasma membranes results in an increased 5'-nucleotidase activity (Whetton & Hously, 1983).

Cholesterol enrichment of membranes has in the past been achieved by a number of methods including dietary means (Kroes & Ostwald, 1971), incubation of membranes *in vitro* with PC : cholesterol liposomes (Bruckdorfer *et al.*, 1968) and use of lipid transfer proteins such as nsL-TP (North & Fleischer, 1983). Dietary manipulation (*in vitro* or in culture) of cholesterol is not effective for all cell types and more importantly will result in fatty acid changes as well (Ostwald *et al.*, 1970; Baldassare *et al.*, 1979). Previous studies by North & Fleischer (1983) reported that liposome partitioning between PC : cholesterol liposomes and synaptic membranes showed only minor changes of 10-20% of the PC : cholesterol ratio over a few hours. With longer incubations a greater cholesterol exchange is achieved but an increased degradation of the tissue will occur (Shapiro & Barchi, 1981). North & Fleischer (1983) have reported the use of the nsL-TP to facilitate large rapid changes in membrane lipid composition after its extraction from bovine liver, acting as a

non-specific sterol carrier between donor and acceptor membranes eliminating a chemical gradient by mediating both net mass transfer and a random exchange of lipids. This nsL-TP has now been expressed in the E. Coli strain BL21 (DE3) as the pre-nsL-TP and purified by anion exchange chromatography (Ossendorp *et al.*, 1992). Unesterified cholesterol will also undergo spontaneous exchange between membranes in one kinetic pool, even though distributed between two leaflets (Kan & Bittman, 1991; Fugler *et al.*, 1985). The rate of spontaneous transfer is dependent upon the degree of saturation of the fatty acid acyl chains, the sphingomyelin content of the membrane (Phillips *et al.*, 1987; Bittman, 1988), and also the curvature of the surface from which desorption occurs (McLean & Phillips, 1984; Fugler *et al.*, 1985)

Here I report the use of the nsL-TP, pre-nsL-TP and spontaneous lipid transfer to study the transport of cholesterol from PC : cholesterol liposomes to synaptic rat whole brain membranes to evaluate the best system. Also I have determined the nature of this cholesterol enrichment whether the cholesterol enriches as a single pool and mixes with the endogenous cholesterol or enriches to a separate pool.

2.2 Methods

2.2.1 Membrane preparation

Membranes were prepared from the whole brain, cerebral cortex, cerebellum or spinal cord of male Wistar rats (130-200g) obtained in-house or from Harlan U.K. (The source of the CNS regions used are stated in the figures). In-house rats were killed by decapitation with prior stunning and their brains rapidly removed and placed on ice. The

spinal cord was then forced from the spinal column under compressed N_2 and treated as above. The cerebral cortex and cerebellum dissected from the whole brain, spinal cord or whole brain were then either used fresh or the spinal cord and whole brains frozen until required. The whole brains and spinal cords obtained from Harlan U.K. were delivered frozen. To gain cortex membranes from Harlan U.K. whole brains, they were first thawed and then dissected. The 'buffy coat' fraction of the tissue homogenate was obtained by the method of Zukin *et al.* (1974) as follows. The tissue was homogenized in 0.32 M sucrose (at pH 7.4) with an Ultra-Turrax T-25 homogenizer, then with eight strokes of a teflon homogenizer. The homogenate was centrifuged at 1520 g and 4°C for 10 min to form a nucleic pellet of debris (Robertis *et al.*, 1962). The supernatant was collected and centrifuged at 31000 g for 20 min. The resulting mitochondrial pellet contained myelin fragments, nerve endings, synaptic vesicles and free mitochondria (Robertis *et al.*, 1962). This mitochondrial pellet was resuspended in 20 ml distilled H_2O and incubated for 30 min on ice to rupture the nerve endings by osmotic shock, thereby releasing the enclosed components such as mitochondria and synaptic vesicles (Robertis *et al.*, 1963). This suspension was centrifuged at 12900 g for 20 min and the buffy coat layer was resuspended in the supernatant to separate it from the mitochondrial pellet below. The suspended buffy coat was then centrifuged at 48400 g for 20 min to form a pellet of synaptic membranes whilst the supernatant contained synaptic vesicles. The pellet was resuspended in distilled H_2O and this washing step was repeated a further two times, with the final centrifugation being carried out in ice cold wash buffer (5 mM Tris-Base, 1 mM

EDTA, pH 7.4). The final pellet was resuspended in ice cold wash buffer and stored at -20°C until required.

2.2.2 Non specific - lipid transfer protein extraction

The nsL-TP was extracted by the method of Crain & Zilversmit (1980) as follows, but with the omission of the heat treatment and octylagarose column chromatography steps. Porcine liver (2 kg) was diced and the excess fat removed prior to rinsing with 0.25 M sucrose to remove the blood. The tissue was then homogenized in a Waring blender yielding a 35% (v/v) suspension of tissue in SET buffer (0.25 M sucrose, 1 mM EDTA, 50 mM Tris-base, 0.02% (w/v) sodium azide, pH 6.9 at 25°C). The homogenate was centrifuged at 13000 g for 30 min and the supernatant carefully decanted, this was then brought to pH 5.1 with 3 M HCl and stirred for 30 min at room temperature. The solution was then stirred for 30 min on ice and spun at 13000 g for 30 min after which the pellet was discarded. The supernatant was adjusted to pH 7.4 with 3 M NaOH and stored in 600 ml aliquots at -20°C for a maximum of 6 months.

When required the supernatant was thawed overnight and the pH altered to 5.1. Over a period of an hour ammonium sulphate was added to a 40% (w/v) saturation and stirred for a further hour. The protein precipitated by this step was pelleted by centrifugation at 13000 g for 30 min and then the clear supernatant brought to 90% (w/v) ammonium sulphate saturation over a period of an hour. The solution was then stirred for a further hour and the protein precipitated by this step removed by centrifuging at 13000 g for 30 min at 4°C.

The pellet from this step was resuspended in 40 ml resuspension buffer (25 mM sodium sulphate, 10 mM mercaptoethanol, 0.02% (w/v) sodium azide, pH 7.4 at 4°C) and dialyzed extensively against dialysis buffer (5 mM mercaptoethanol, 5 mM sodium sulphate and 0.02% (w/v) sodium azide). This involved three changes of 2 l of dialysis buffer at intervals of 4 or 12 h.

After dialysis the solution was applied to a PD10 sephadex G-25 column pre-equilibriated with equilibrium buffer (5 mM sodium phosphate, 5 mM mercaptoethanol, 0.02% (w/v) sodium azide). The column was washed with 160 ml equilibrium buffer and the bound protein eluted with 160 ml column wash buffer (0.25 mM sodium phosphate, 45 mM sodium chloride, 5 mM mercaptoethanol, 0.02% (w/v) sodium azide, pH 7.4 at 4°C). The elutant was stored at 0-4°C until required. Immediately before use the transferase protein was concentrated 5-10 times using a centrifugal concentrator centrifuged at 5000 g for 45 min. To remove any residual protein the concentrated transferase was then centrifuged at 48000 g for 15 min.

2.2.3 Liposome preparation

Liposomes were prepared by a modified version of North & Fleischer's method (1983a) as detailed below. PC and cholesterol (0.30 mg cholesterol and 0.75 mg PC in preliminary studies and later 0.75 mg of each per ml of buffer added) were dissolved in a small amount of chloroform. The chloroform was evaporated under a stream of nitrogen to leave a thin film of lipids and prevent their oxidation. SET buffer (0.25 M sucrose, 1

mM EDTA, 50 mM Tris-Base, 0.02% sodium azide, pH 6.9 at 25°C) was added and the lipids allowed to hydrate for 1 h at room temperature. The lipids were then dispersed with a Branson Sonifier 250 fitted with a half inch tip on 80% duty cycle under a stream of nitrogen, whilst incubating on an ice bath. Three periods of 4 min sonication were carried out at 90 W allowing the same time in between each sonication for cooling. After sonication the translucent solution was centrifuged at 48400 g for 20 min at room temperature and the pellet containing titanium fragments and undispersed lipids discarded. This procedure generally incorporated about 80% of the cholesterol into liposomes. Pure PC and PC : sphingomyelin liposomes were prepared by the same procedures.

2.2.4 Cholesterol enrichment of synaptic membranes

Previously prepared membranes were washed at 4°C with wash buffer and centrifuged at 8000 g for 25 min at 4°C and resuspended in SET buffer. Cholesterol-enrichment was achieved by incubating the membranes (0.4 mg protein ml^{-1}) in SET buffer containing 1% BSA, and approximately 0.5 mg total liposome lipids ml^{-1} (approximately 0.35 mg total liposome lipids ml^{-1} in preliminary studies) at 37°C. In some preliminary studies, 5 μg ml^{-1} of the lipid transfer protein pre-nsL-TP (Ossendorp *et al.*, 1992) was included to determine whether it enhanced the lipid transfer or 1-2 units of nsL-TP. The cholesterol transfer was terminated after 7 h (in initial experiments) or 4 h (in all subsequent studies) by addition of an equal volume of ice cold assay buffer (50 mM Tris-base, 150 mM $NaCl^-$, pH 7.4 at 4°C) and the mixture centrifuged at 48000 g for 20 min.

The pellets were resuspended in wash buffer. Essentially the same procedures were used to expose membranes to pure PC liposomes and PC : sphingomyelin liposomes. For comparative purposes, a portion of the original membrane preparation was subjected to these same procedures but with the omission of the liposomes and these membranes were designated as 'unenriched'. All membranes were frozen until required.

To monitor the time course of cholesterol enrichment, experiments were performed in which the membranes were sampled at intervals during the incubation, for assay of membrane cholesterol (Sigma diagnostics kit) and protein (BioRad reagent). The stability of enrichment in membranes held at 4°C was assessed by repeated washing of the membranes with the wash buffer.

In experiments to investigate the disposition of the cholesterol enrichment, whole brain membranes were incubated with PC : cholesterol liposomes as detailed above but including [^{3}H]-cholesterol (specific activity 1.86 mCi mmol^{-1}). Following enrichment, the membranes were incubated for a further 4 h with or without PC liposomes (0.25 mg PC ml^{-1}) at 37°C in SET buffer containing 1% (w/v) BSA to achieve substantial reversal of the cholesterol enrichment. Aliquots were removed for assay of [^{3}H]-cholesterol, total cholesterol and protein after 2 min and 4 h.

All chemicals and drugs were obtained from Sigma with the exception of [^{3}H]-cholesterol (51.1 Ci mmol^{-1} specific activity) which was obtained from Amersham International. The pre-nsL-TP was obtained from Prof. K.W.A. Wirtz, Utrecht University. Data were fitted using the Inplot package Prism (Graphpad Software). Statistical comparisons were made by Student's *t* test for independent samples.

2.3 Results

2.3.1 Cholesterol enrichment

Preliminary studies using nsL-TP extracted from bovine liver for the cholesterol enrichment of rat whole brain membranes gave a cholesterol enrichment of 140 ± 18% and 108 ± 8% above basal levels for the nsL-TP compared to spontaneous enrichment respectively (incubated with liposomes comprising 0.30 mg cholesterol and 0.75 mg PC per ml of SET buffer added. Mean and s.e.m. of 6 experiments). Meanwhile, pre-nsL-TP yielded an enhancement no greater than that of spontaneous cholesterol enrichment (196% and 208% increase above basal, respectively) in the presence of the 0.75 mg PC and 0.75 mg cholesterol per ml of SET buffer liposomes. Therefore in subsequent studies, spontaneous cholesterol transfer was routinely used.

A time course study of the spontaneous enrichment of whole brain membranes with cholesterol from liposomes comprised of 50 PC : 50 cholesterol showed that the enrichment reached equilibrium after 3 h (Figure 2.1). On the basis of these results all subsequent cholesterol enrichment studies involved 4 h incubations. Washing of the membranes to remove liposomes and a further 4 washes at 4°C with wash buffer resulted in a loss of about 20% of the cholesterol enrichment.

2.3.2 Site of cholesterol enrichment

Evidence was sought to distinguish between the possibilities that the exogenous cholesterol mixed entirely with the endogenous membrane cholesterol, and the alternative

possibility that the exogenous cholesterol was associated with the membrane as a separate pool. Whole brain membranes were enriched with [^{3}H]-cholesterol and the specific activity (Sp.Act.) of the [^{3}H]-cholesterol in the membranes was measured at intervals during subsequent washing and depletion of cholesterol from the membranes at 37°C with a buffer containing 1% (w/v) BSA. It was predicted that depletion of [^{3}H]-cholesterol should result in no change in Sp.Act. of the [^{3}H]-cholesterol remaining in the membranes if depletion was from a homogenous mixed pool of exogenous and endogenous cholesterol. In contrast, depletion from a separate exogenous pool having the same Sp.Act. as the [^{3}H]-cholesterol in the liposomes, should result in a clear reduction in the whole membrane Sp.Act. of the remaining [^{3}H]-cholesterol. When this was tested experimentally the results obtained were as shown in Table 2.1. In the wash period following enrichment with [^{3}H]-cholesterol, a 28-34% depletion of total cholesterol was associated with no change in Sp.Act. of [^{3}H]-cholesterol remaining in membranes. This contrasts with a predicted reduction in Sp.Act. of 14143 ± 1439 d.p.m. μmol^{-1} cholesterol (68%) or 10757 ± 732 d.p.m. μmol^{-1} cholesterol (52%), depending on the presence or absence of PC liposomes, if the depleted cholesterol had come from a separate exogenous pool.

2.3.3 Cholesterol depletion of synaptic membranes

A study was made to see if the endogenous cholesterol within synaptic membranes could be depleted by use of PC : sphingomyelin liposomes. Rat whole brain synaptic

membranes incubated with these liposomes should show a decrease in the endogenous cholesterol. The results showed no decrease in the cholesterol content after a 4 h incubation in the presence of 1% BSA (w/v) at 37°C (Figure 2.2).

2.3.4 Cholesterol enrichment of discrete areas of the C.N.S.

In many of the experiments involving the binding of [^{3}H]-FNZ and [^{3}H]-muscimol, membranes from discrete areas of the CNS were used. The cholesterol enrichments in membranes from cerebral cortex, cerebellum and spinal cord following a 4 h incubation with 50 PC : 50 cholesterol liposomes are shown in Table 2.2.

A comparison was made between the cholesterol enrichment of synaptic membranes from in-house rats and those purchased from Harlan U.K. following a 4 h incubation with 50 PC : 50 cholesterol liposomes. In whole brain, cerebral cortex and spinal cord synaptic membranes a lower cholesterol enrichment was achieved with Harlan U.K. rat membranes (Figures 2.3-2.5).

2.4 Discussion

The primary reason for carrying out these studies was to investigate the possibility that cholesterol enrichment of synaptic membranes may alter the binding properties and modulatory coupling of the benzodiazepines, neurosteroids, propofol, GABA and cage convulsants acting at the $GABA_A$ receptor in later studies. The nature of this cholesterol enrichment thus had to be determined. Due to the different subunit constructs of the $GABA_A$ receptor in different regions of the CNS and the distinct lipid environment in

these regions a study was also made of the cholesterol enrichment in cerebral cortex, cerebellum and spinal cord synaptic membranes. The synaptic membranes derived from Harlan U.K. and in-house rats may differ in their endogenous cholesterol content. The endogenous cholesterol can be affected by diet and the way that the CNS regions and whole brain were prepared. Therefore a comparison was made between the cholesterol enrichment of Harlan U.K. and in-house whole brain, cerebral cortex and spinal cord synaptic membranes.

Cholesterol enrichment of synaptic membranes was achieved by cholesterol partitioning between liposomes and synaptic membranes. We found that the lipid transfer protein pre-nsL-TP did not enhance cholesterol transfer above the spontaneous enrichment obtained in its absence, in agreement with Nichols & Pagano (1983). However, the nsL-TP did show an enhancement over spontaneous cholesterol transfer with the lower cholesterol concentration in the liposomes over a 7 h period. A draw back of the nsL-TP is that the protein aggregates upon storage thus preventing interactions with the membrane and decreasing the overall transfer activity. The rapid spontaneous transfer required the presence of 1% (w/v) BSA at 37°C, the BSA presumably acting as a carrier for the cholesterol (Bartholow & Geyer, 1982; Lundberg & Suominen, 1985), since it stimulates the rate of exchange (Clejan & Bittman, 1984). Both enrichment and de-enrichment could be achieved under these conditions. Although the enrichment thus achieved was stable at 4°C in the absence of BSA and shown to mix with the endogenous lipids, we were unable to deplete cholesterol below the levels found in the membranes at the end of the membrane isolation procedure (Figure 2.2). This suggests that there is a basal level of cholesterol in

plasma membranes. With the lability of higher levels of cholesterol, it is not known whether the membranes *in vivo* contained cholesterol above the basal level, the extra cholesterol having been lost during the isolation procedure. Meanwhile, Phillips *et al.* (1987) have reported that cholesterol depletion is possible with small unilamellar vesicles of egg PC being present at a concentration of more than 1 mg ml^{-1} of medium to give a maximum flux. The data here may reflect the low concentration of PC : sphingomyelin liposomes in the buffer medium used for cholesterol depletion.

In manipulating the lipid composition of membranes, it cannot be assumed that the various components are homogeneously distributed. Membrane proteins have specific affinities for different lipids, thus creating an intimate shell of lipids that has a different composition from the bulk phase. This may lead to the presence of cholesterol rich domains within the plasma membrane (Lund-Katz, 1995). Nevertheless, the composition of the shell is likely to be influenced by the availability of specific lipids in the bulk phase (Benga, 1985). The nicotinic acetylcholine receptor is reported to interact strongly with sterol molecules but they are absent from the lipid shell. The physical chemical evidence suggests that cholesterol may bind in the axial clefts between the protein subunits (Jones & McNamee, 1988). The axial clefts may also be the locus of the binding sites for certain drugs (Changeux, 1990). Since the $GABA_A$ receptor is structurally related to the nicotinic acetylcholine receptor, an analogous model may be applied to the neurosteroid sites on the $GABA_A$ channel protein. A requirement for the presence of cholesteryl hemisuccinate a cholesterol analogue and natural brain lipids to preserve agonist activity after reconstitution of the $GABA_A$ receptors in liposomes (Bristow & Martin, 1987) has been

shown and may well be due to an interaction between cholesterol and the $GABA_A$ receptor.

The nsL-TP will transfer a variety of phospholipids (Bloj & Zilversmit, 1983; Crain & Zilversmit, 1980) between membranes by way of a carrier mechanism (Wirtz & Gadella, 1990). The model proposed involves nsL-TP being instrumental in juxtaposing two membrane interfaces (Van Amerongen, 1989). NsL-TPs tendency to form dimers (Van Amerongen, 1985), may indicate that dimers are essential for the collisional complex between membranes, enabling lipids to redistribute subsequently at these collision sites. If a particular phospholipid though, is strongly associated with a membrane protein it may not be removed by this transfer protein (Dawidowicz, 1987). The pre-nsL-TP of rat liver supplied by Prof. Wirtz was expressed in E.Coli (strain BL21) as a 15 kDa precursor to the nsL-TP. It has a presequence of 20 amino acids but still retains the lipid transfer activity identical to the mature nsL-TP (Ossendorp *et al.*, 1992).

Spontaneous cholesterol transfer occurs by aqueous diffusion from a donor particle (Pownall *et al.*, 1983; Phillips *et al.*, 1987; Dawidowicz, 1987; Bittman 1988). Cholesterol forms the transitional state when it is attached to the outer surface of the liposome by its hydrophobic tail, this then desorbs from the outer leaflet to the aqueous phase which is the rate limiting step (Phillips *et al.*, 1987). Only a single pool of cholesterol for desorption has been detected (Kan & Bittman, 1991) indicating cholesterol exchanges between the two leaflets rapidly. Exchange will be first order provided an excess of acceptor particles are present at 37°C. Using the soybean polyunsaturated PC gives a rapid half time for cholesterol transfer ($t_{1/2}$ equals 0.3 h) due to shortening of the

acyl chains and bending of them (if cis double bonds are present) which decreases possible Van der Waal associations with cholesterol causing looser packing (Fugler *et al.*, 1985). PC also undergoes exchange between the liposomes and synaptic membranes but to a lesser degree (six times lower than cholesterol), since it is less soluble in the aqueous phase (Pownall *et al.*, 1983; Phillips *et al.*, 1987). Lundberg & Suominen (1985) have reported that inclusion of BSA will further decrease this transfer but will increase cholesterol transfer. The small unilamellar vesicles (liposomes) used produce highly curved membranes leading to looser packing of PC fatty acyl chains due to the assymetry of the phospholipid packing within the bilayer. The polar heads of the PC take up a large area compared to the acyl chains causing the decrease in lateral packing (Fugler *et al.*, 1985). This assymetry thus offers the smallest kinetic barrier to cholesterol redistribution which must be overcome for desorption (Yeagle & Young, 1986). Due to this kinetic barrier the exchange is temperature dependent (Dawidowicz, 1987). PC : sphingomyelin liposomes were used for cholesterol depletion since sphingomyelin forms preferential interactions with cholesterol such as H-bonds (Phillips *et al.*, 1987; Fugler *et al.*, 1985), so should readily accept cholesterol from synaptic membranes. The Van der Waal forces associated between cholesterol and sphingomyelin are greater than the corresponding forces between cholesterol and PC because of PCs extensive branching. Sphingomyelin is essentially saturated in its interaction with cholesterol unlike PC that has a double bond at C-12 causing bending of the acyl chain thus allowing closer packing (Darke *et al.*, 1972). These properties of sphingomyelin will therefore allow unidirectional spontaneous transfer of cholesterol from the synaptic membranes to the liposomes down a simple concentration

gradient where it becomes closely associated with the sphingomyelin resulting in cholesterol depletion.

In summary it has been shown that Harlan U.K. and in-house synaptic membranes from specific CNS regions can be enriched with cholesterol by spontaneous transfer mediated by 1% (w/v) BSA with a routine incubation with PC : cholesterol liposomes for 4 h at 37°C. This cholesterol enrichment though is lower in the Harlan U.K. synaptic membranes which may be explained by the fact that a lower endogenous cholesterol content was seen in in-house whole brain membranes compared to the Harlan U.K. whole brain membranes (Figure 2.3). Upon cholesterol enrichment of Harlan U.K. and in-house membranes the same cholesterol content was gained (Figure 2.3), but the lower endogenous cholesterol content of the in-house membranes enabled a greater enrichment. Thus the difference in the cholesterol enrichment cannot be accounted for in terms of strain since both in-house and Harlan U.K. rats were Wistar males, but rather a difference in the endogenous cholesterol content which is likely to be caused by diffences in diet or handling of the tissue. The exogenous cholesterol has been shown to mix entirely with the endogenous and the enrichment is stable in the absence of BSA. On this basis the effect of cholesterol enrichment on binding studies could be studied as shown in the following chapters.

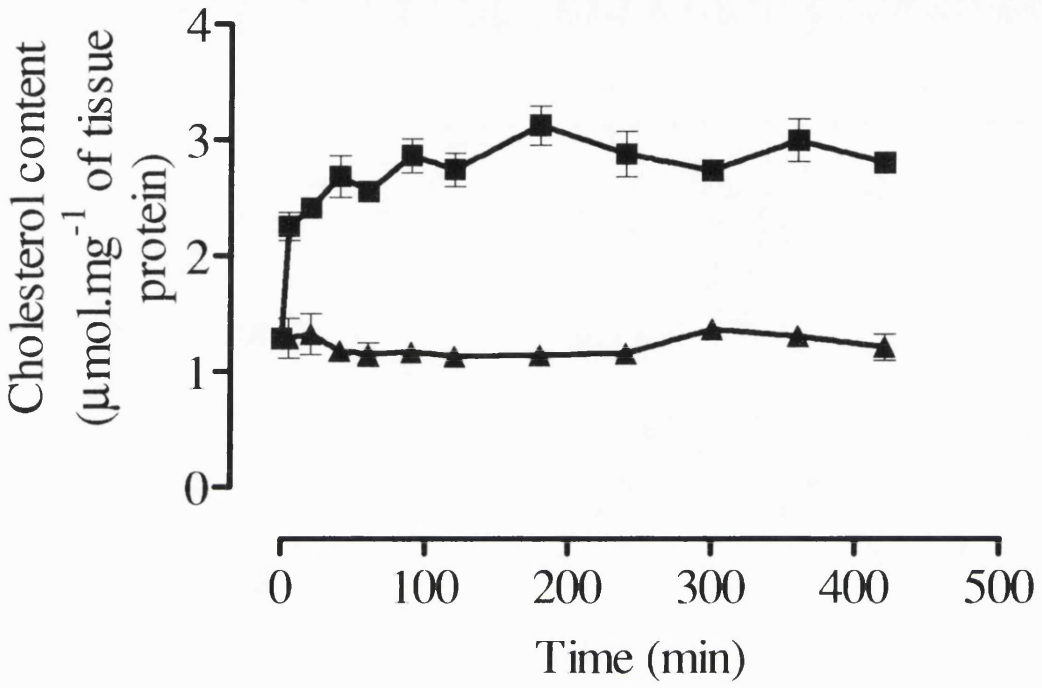
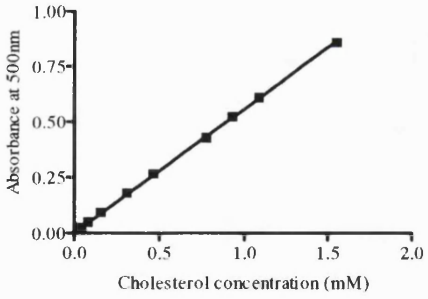


Figure 2.1 The time course of a cholesterol enrichment of rat whole brain membranes from in-house after incubating with 50 PC : 50 cholesterol liposomes (squares) or in their absence (triangles). Cholesterol enrichment was rapid at first but plateaued after 180 minutes. The control incubation showed no change in cholesterol content. The values shown are the means of cholesterol content measured in triplicate ± s.e. and mean. Similar results were obtained in 3 other experiments.



Standard Cholesterol curve number	Slope Mean	95% Confidence interval	Confidence Interval % mean
1	0.558	0.013	2.33
2	0.605	0.054	8.313
3	0.616	0.141	22.30
4	0.595	0.018	3.08
Overall mean	0.593 ± 0.01	0.057 ± 0.06	9.161 ± 4.77

Above is a typical cholesterol standard curve obtained with the Sigma cholesterol assay kit used to determine the cholesterol content of synaptic membranes. The data points show the mean of 3 readings ± s.e. and mean with the error bars lying within the symbols. The cholesterol standards and samples were incubated with the reagent for 5 mins at 37°C which caused a colour change due to the production of a quinoneimine dye that was measured at 500nm.

The above table indicates the 95% confidence intervals determined from the gradient of 4 standard cholesterol curves in order to show the intra-assay coefficient. This is expressed in the final column as a percentage of the slope value. In order to calculate the inter-assay coefficient the overall mean was calculated with the s.e. and mean.

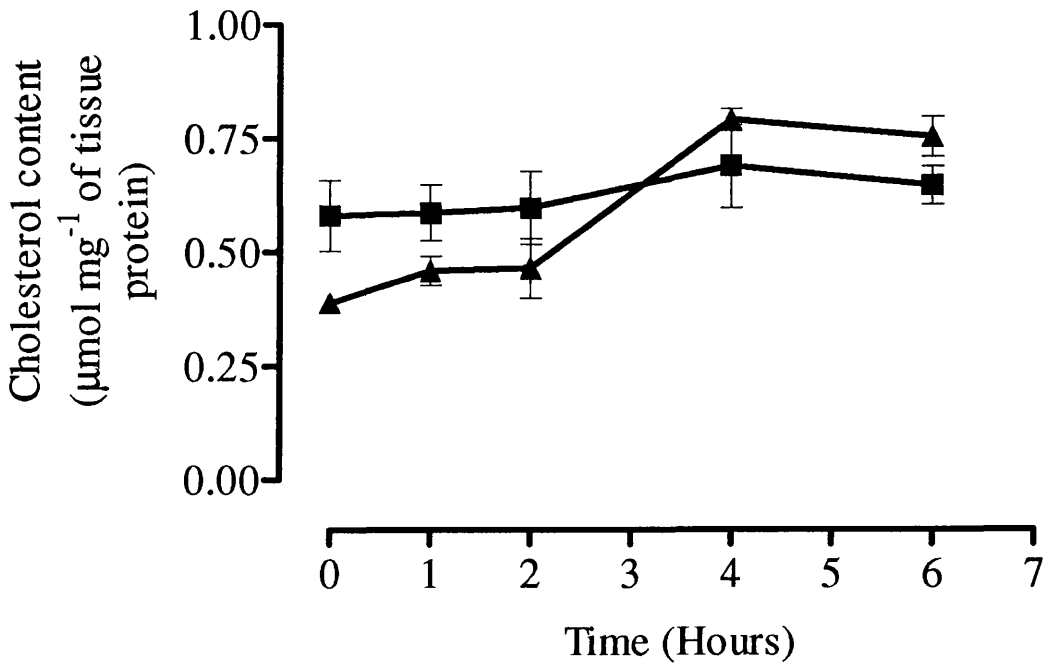


Figure 2.2 The time course of a cholesterol depletion of rat whole brain membranes from in-house after incubating with 50 PC : 50 sphingomyelin liposomes (squares) or in their absence (triangles). The membranes were from a single membrane preparation. No cholesterol depletion was seen to occur under the conditions present throughout the time course studied ($P>0.05$), but it would be desirable to repeat this experiment on other membrane preparations. Data are the means of 3 readings of cholesterol content ± s.e.mean.

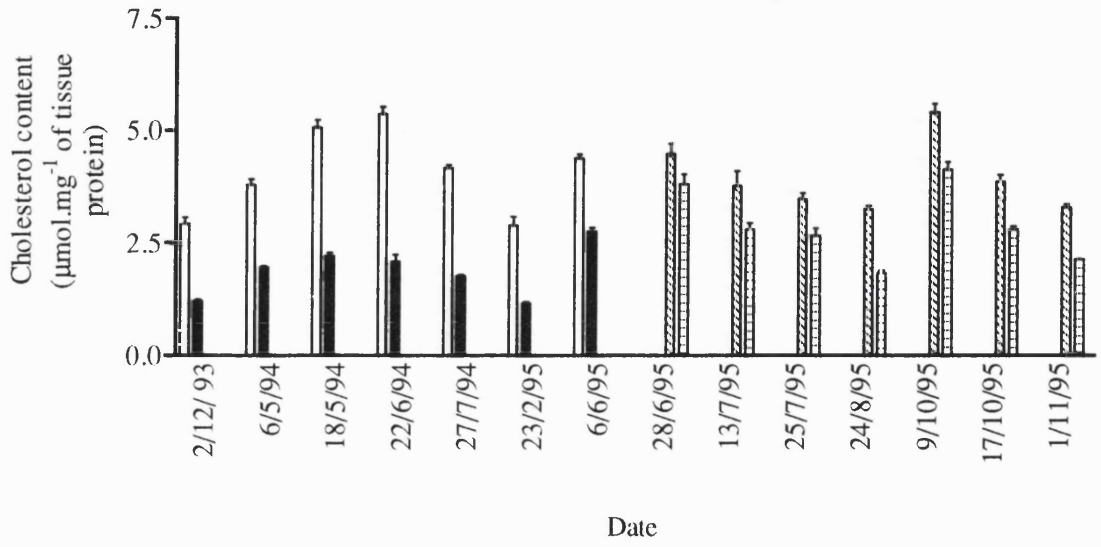


Figure 2.3 The cholesterol content of rat whole brain membranes following incubation with or without 50 PC : 50 cholesterol liposomes for 4 hours in the presence of 1% BSA at 37°C. Clear and filled bars indicate rat whole brain membranes derived from in-house stock of male Wistar rats, incubated with or without liposomes respectively. Diagonally and horizontally hatched bars indicate rat whole brain membranes derived from Harlan U.K. male Wistar rats, incubated with or without liposomes respectively. The mean cholesterol content of the cholesterol enriched in-house membranes (4.068 ± 0.363 $\mu mol.mg^{-1}$ of tissue protein) was not significantly different to that of the enriched Harlan U.K. membranes (3.905 ± 0.293 $\mu mol.mg^{-1}$ of tissue protein). However, the mean cholesterol content of the unenriched in-house membranes (1.872 ± 0.211 $\mu mol.mg^{-1}$ of tissue protein) was significantly different to that of the Harlan U.K. unenriched membranes (2.866 ± 0.309 $\mu mol.mg^{-1}$ of tissue protein) ($P = 0.0209$, $n = 7$). The dates on the graph refer to the days on which membranes were prepared. Data are the means of 3 readings of cholesterol content ± s.e.mean.

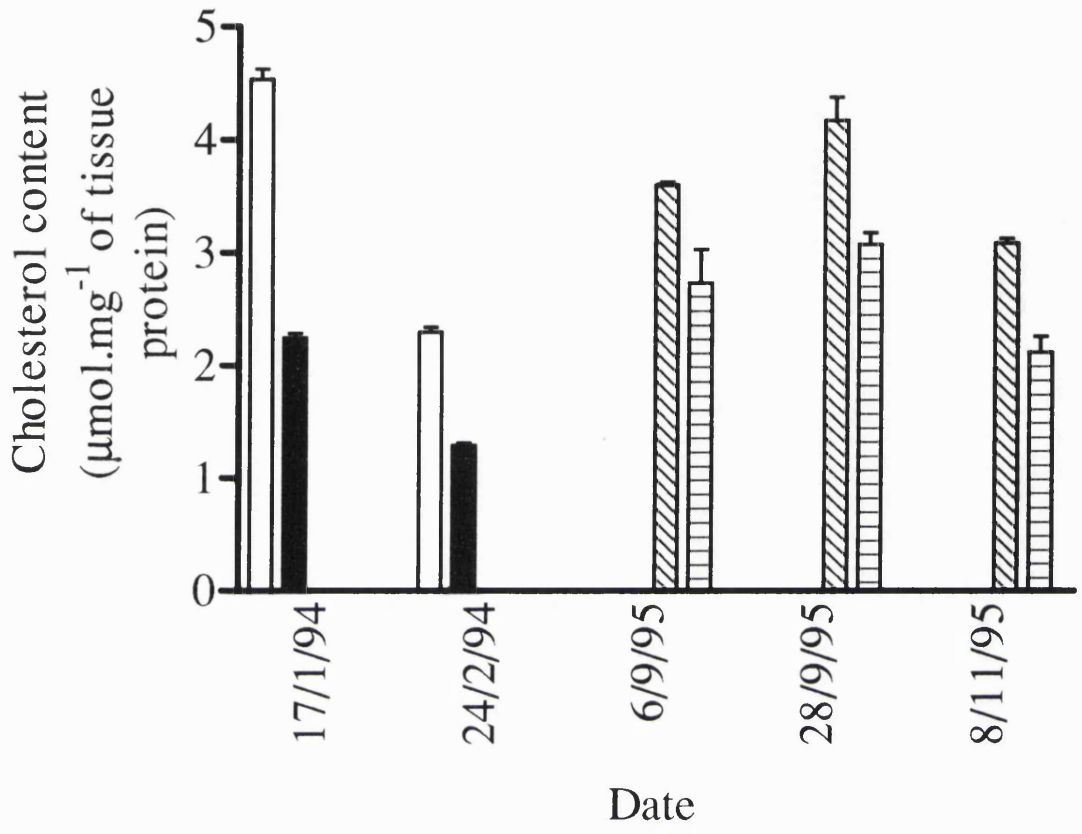


Figure 2.4 The cholesterol content of rat cerebral cortex membranes following incubation with or without 50 PC : 50 cholesterol liposomes for 4 hours in the presence of 1% BSA at 37°C. Clear and filled bars indicate membranes derived from in-house male Wistar rats, incubated with or without liposomes, respectively. Diagonally and horizontally hatched bars indicate membranes derived from Harlan U.K. male Wistar rats, incubated with or without liposomes, respectively. The average cholesterol content of the cholesterol enriched in-house membranes and the enriched Harlan U.K. membranes was 3.411 and 3.611 µmol.mg^{-1} of tissue protein, respectively. The average cholesterol content of the unenriched in-house membranes and the unenriched Harlan U.K. membranes was 1.770 and 2.590 µmol.mg^{-1} of tissue protein, respectively. The dates on the graph refer to the days on which the membranes were prepared. Data are the means of 3 readings of cholesterol content ± s.e.mean.

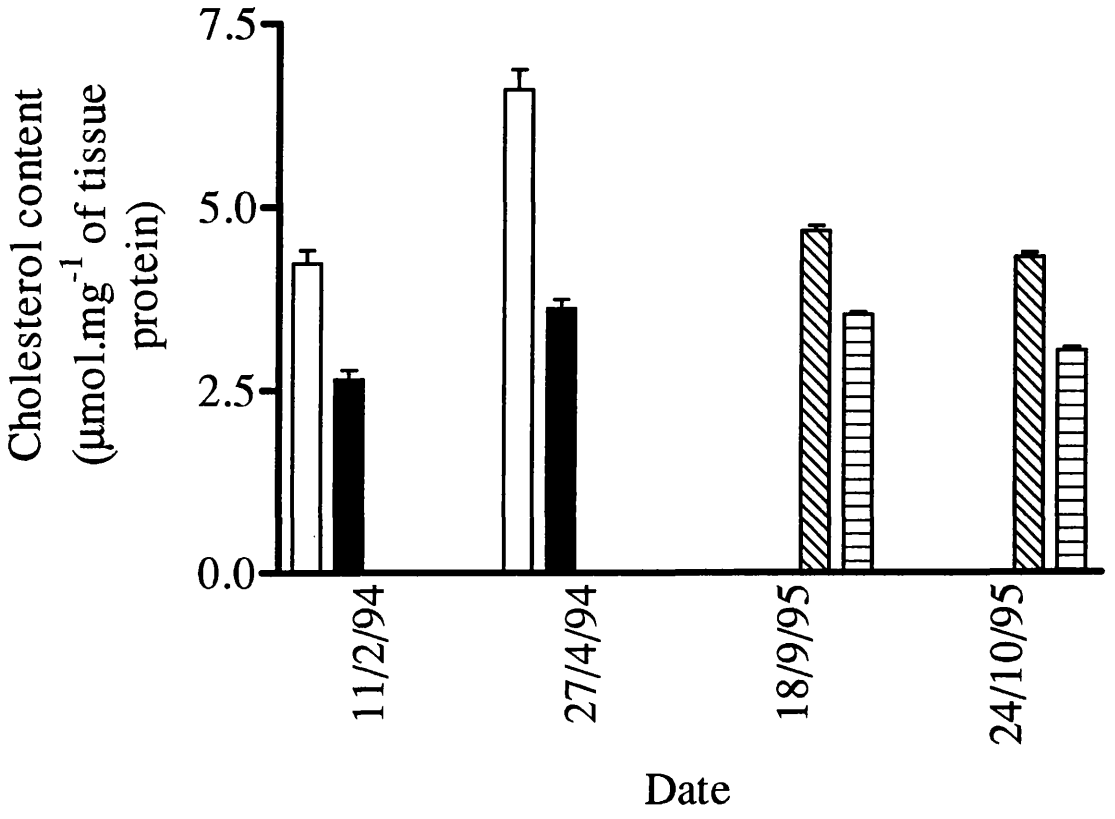


Figure 2.5 The cholesterol content of rat spinal cord membranes following incubation with or without 50 PC : 50 cholesterol liposomes for 4 hours in the presence of 1% BSA at 37°C. Clear and filled bars indicate membranes derived from in-house male Wistar rats, incubated with or without liposomes, respectively. Diagonally and horizontally hatched bars indicate membranes derived from Harlan U.K. male Wistar rats, incubated with or without liposomes, respectively. The average cholesterol content of the cholesterol enriched in-house membranes and the enriched Harlan U.K. membranes was 5.405 and 4.478 $\mu mol.mg^{-1}$ of tissue protein, respectively. The average cholesterol content of the unenriched in-house membranes and the unenriched Harlan U.K. membranes was 3.114 and 3.279 $\mu mol.mg^{-1}$ of tissue protein, respectively. The dates on the graph refer to the days on which the membranes were prepared. Data are the means of 3 readings of cholesterol content ± s.e.mean.

Table 2.1 Changes in cholesterol and in [^{3}H]-cholesterol specific activity after a 4 h [^{3}H]-cholesterol enrichment and a further 4 h incubation with or without PC liposomes both carried out with SET buffer.

	Membrane cholesterol (μmol mg^{-1} protein)	**% change from 2 min wash**	**Specific activity of membrane [^{3}H]-cholesterol** (d.p.m.μmol^{-1} cholesterol)	**% change from 2 min wash**
Before incubation	2.10 ± 0.10		0	
After incubation with [^{3}H]-cholesterol : PC liposomes				
2 min wash	3.22 ± 0.12		20887 ± 1197	
4 h wash with PC liposomes	2.17 ± 0.07	-34%	20914 ± 504	-0.1%
4 h wash without PC liposomes	2.33 ± 0.16	-28%	20852 ± 476	0.2%

After incubation with [^{3}H]-cholesterol : PC liposomes the cholesterol content of the membranes was significantly increased (P = 0.004). This enrichment was then significantly reduced by washing the membranes with or without PC liposomes (P = 0.003 and 0.0043, respectively). There was no significant difference though between the membranes washed for 4 h with PC liposomes and those washed for 4 h in their absence. After incubating the membranes with [^{3}H]-cholesterol there was no significant difference between the specific activities of the membrane [^{3}H]-cholesterol following a 2 min wash, 4 h wash with PC liposomes or 4 h wash without PC liposomes. Values shown are the mean ± s.e.mean of 4 experiments. The membranes were prepared from whole brains of Harlan U.K. male Wistar rats.

Table 2.2 Total membrane cholesterol following a 4 h incubation at 37°C with 50 PC : 50 cholesterol liposomes (enriched) and without liposomes (unenriched).

	Cholesterol content (μmol cholesterol mg^{-1} protein)	
CNS region	**Unenriched**	**Enriched**
Cortex	2.25 ± 0.04	4.54 ± 0.09
Cerebellum	1.96 ± 0.10	3.63 ± 0.13
Spinal cord	2.64 ± 0.13	4.23 ± 0.17

A single batch of membranes was prepared for each area of CNS from in-house rats. The cholesterol enriched cerebral cortex, cerebellum and spinal cord membranes all had significantly higher cholesterol levels compared to their unenriched counterparts ($P < 0.0001$, $P = 0.0005$ and $P = 0.018$, respectively). Values shown are mean ± s.e.mean of 3 readings.

Chapter 3

The Effect of Cholesterol Enrichment of Synaptic Membranes upon Pregnanolone Potentiation of [^{3}H]-FNZ Binding

Chapter 3

The effect of cholesterol enrichment of synaptic membranes upon pregnanolone potentiation of [^{3}H]-FNZ binding

3.1 Introduction

γ-Aminobutyric acid the most abundant inhibitory neurotransmitter in the mammalian brain, acts at its pentameric $GABA_A$ receptor (Nayeem *et al.*, 1994), causing hyperpolarization of the neurone by allowing an influx of Cl^- ions, thus preventing an action potential (Schofield *et al.*, 1987; Sieghart, 1992). This activity is regulated or mimicked by several anxiolytic, hypnotic and convulsant compounds that include benzodiazepines (Sieghart, 1989) and neurosteroids (Turner & Simmonds, 1989; Paul & Purdy, 1992). Agonist benzodiazepines increase GABA invoked Cl^- currents by increasing opening frequency (Rogers *et al.*, 1994). First CNS effects of neurosteroids were demonstrated by Selye (1941) who showed progesterone was capable of inducing sedation when injected intraperitoneally. Figdor *et al.* (1957) found that it was the metabolites of progesterone that brought about the CNS depressant actions. The sedative actions of neurosteroids are due to enhancement of $GABA_A$ channel current via an increase in the mean open duration. They also increase the opening frequency (Twyman & Macdonald, 1992). The potentiation of GABA-evoked Cl^- currents are seen at the nM range and at μM concentrations neurosteroids directly activate the $GABA_A$ receptor mediated Cl^- conductance (Puia *et al.*, 1990). Neurosteroids have also been seen to

potentiate the enhancement of [^{3}H]-Flunitrazepam (FNZ) binding by GABA and at higher concentrations to enhance [^{3}H]-FNZ binding directly (Harrison *et al.*, 1987; Majewska *et al.*, 1986) with a dependence on the presence of Cl^- ions (Prince & Simmonds, 1992a). Clear structural activity requirements have been seen for neurosteroid potentiation of $GABA_A$ receptor mediated responses (Simmonds, 1991). All this is compatible with the idea that there are specific recognition sites for the neurosteroids on the $GABA_A$ channel protein.

An interaction may occur between the high concentration of cholesterol in the lipid membrane and the neurosteroid site due to their similar structures. The alternative scenario is that cholesterol may interact more generally with the $GABA_A$ receptor. Such an association has been seen between cholesterol and the nicotinic acetylcholine receptor (Jones & McNamee, 1988). To investigate these possibilities neuronal membranes have been enriched with cholesterol and the effect this has upon FNZ binding to the $GABA_A$ receptor and the potentiation of [^{3}H]-FNZ binding by pregnanolone studied.

3.2 Methods

3.2.1 Membrane preparation

Membranes were prepared either from the whole brain, cerebral cortex, cerebellum, or spinal cord of male Wistar rats (130-200 g) as in chapter 2 (see section 2.2.1).

3.2.2 Liposome preparation

PC and cholesterol (0.75 mg of each per ml of buffer added) liposomes were prepared as in chapter 2 (see section 2.2.3).

3.2.3 Cholesterol enrichment of synaptic membranes

Cholesterol enrichment of synaptic membranes was achieved by the standard incubation set out in Chapter 2 (see section 2.2.4).

To monitor the cholesterol enrichment, membranes were sampled at 5 min and 4 h during the incubation, for assay of membrane cholesterol (Sigma diagnostics kit) and protein (BioRad reagent).

3.2.4 Binding experiments ✳

Experiments were performed to determine the effects of FNZ and pregnanolone on [^{3}H]-FNZ binding to cholesterol-enriched, PC-incubated and unenriched membranes. On the day of assay the membranes were thawed and centrifuged at 48400 g for 20 min at 4°C and the pellets were resuspended in assay buffer to give a final protein concentration of about 1 mg ml^{-1}. Aliquots of membranes (100 µl) were pre-incubated with FNZ 10 pM-1 µM or pregnanolone 30 nM-10 µM in assay buffer at 37°C for 10 min. [^{3}H]-FNZ (3α-hydroxy-5β-pregnan-20-one) was added to give a 1 nM concentration in a final volume of 0.5 ml and the samples were then incubated on ice for 60 min. The reaction was terminated with the addition of 2 ml ice cold wash buffer, followed by rapid filtration through Whatman GF-C filters using a

✳ (Modified from the method of Prince & Simmonds, 1993).

Brandell Cell Harvester. The filter mat was washed a further four times with 2 ml wash buffer and the radioactivity determined by standard liquid scintillation techniques (Beckman 5801).

In Scatchard experiments to determine the nature of pregnanolone's modulation of [^{3}H]-FNZ binding, essentially the same procedures as above were used, except 1 μM pregnanolone or 0.45% DMSO were pre-incubated with synaptic membranes from cerebral cortex or spinal cord that had been enriched with cholesterol or were unenriched. [^{3}H]-FNZ was added to give a concentration range of 0.2-20 nM in a final volume of 0.5 ml.

Non-specific binding was determined by use of 1 μM FNZ and was in the region of 5-10%. FNZ was initially dissolved in ethanol and pregnanolone was dissolved in dimethylsulphoxide (DMSO). The final concentration of ethanol was less than 0.4% and DMSO was 0.45%. Since DMSO caused a consistent reduction in [^{3}H]-FNZ binding by about 30%, it was present in all assay tubes for experiments involving pregnanolone. [^{3}H]-FNZ (1 nM) was used as a trace concentration which should have no functional effect on the $GABA_A$ receptor. The incubation was carried out on ice, since a decrease in [^{3}H]-FNZ binding affinity occurs with increasing temperature though this does not affect the B_{max} (Quast *et al.*, 1982). EDTA (1 mM) was included in the wash buffer to chelate divalent cations.

All chemicals and drugs were obtained from Sigma with the exception of [^{3}H]-FNZ (82.5 Ci mmol^{-1} specific activity) which was obtained from Amersham International.

Data were plotted using the Prism package (Graphpad Software). Statistical comparisons were made by Student's *t* test for independent samples and ANOVA.

3.3 Results

3.3.1 The effect of cholesterol enrichment on FNZ binding

In initial experiments to determine the effect of cholesterol enrichment on FNZ binding, displacement of [^{3}H]-FNZ by unlabelled FNZ (10 pM-1 μM) was measured in rat whole brain membranes. Although [^{3}H]-FNZ binding was slightly lower in enriched membranes, the displacement by unlabelled FNZ was found to be unaffected by cholesterol enrichment (Figure 3.1).

3.3.2 The effect of cholesterol enrichment upon the modulatory action of pregnanolone at the $GABA_A$ receptor

The foregoing result allowed an investigation into the influence of cholesterol enrichment upon the modulatory action of pregnanolone on the $GABA_A$ receptor by determining its effect on [^{3}H]-FNZ binding. These experiments were performed on membranes from whole brain and discrete areas of the CNS. In unenriched membranes from cerebral cortex, cerebellum and spinal cord, the threshold concentrations of pregnanolone for enhancement of [^{3}H]-FNZ binding were about 0.3 μM (Figure 3.2). In unenriched whole brain membranes, the threshold concentration of pregnanolone for enhancement of [^{3}H]-FNZ binding was about 0.1 μM (Figure 3.3). The effects of

pregnanolone increased with concentrations up to 3 μM. These concentration-effect relationships were compared with analogous data from enriched membranes. Where cholesterol enrichment caused lateral shifts of the concentration-response curves, the log shifts were calculated to determine changes in the potency of pregnanolone. In whole brain and cerebral cortex membranes, enrichment with cholesterol was associated with a 3.1 and a 3.2 fold reduction in pregnanolone potency, respectively (Figures 3.3 and 3.2). In cerebellar membranes, enrichment with cholesterol was associated with no change in pregnanolone potency over most of the concentration range. However, at the threshold of the concentration-effect curve in the enriched cerebellar membranes, concentrations of 0.1 and 0.3 μM pregnanolone gave a significant enhancement of [^{3}H]-FNZ binding to 108.2 ± 1.4% of control compared with no enhancement in unenriched membranes (mean ± s.e.mean, n=4) (Figure 3.2). In spinal cord membranes, enrichment with cholesterol was, by contrast, associated with an 8.4 fold increase in pregnanolone potency (Figure 3.2).

3.3.3 The effect of PC liposome incubation upon the modulatory action of pregnanolone at the $GABA_A$ receptor

To ascertain whether the changes in the pregnanolone potency were due to cholesterol alone or to possible changes in the PC content of the membranes, experiments were performed with membranes pre-incubated with pure PC liposomes (0.25 mg ml^{-1}). In membranes from the cerebral cortex, pregnanolone (30 nM-10 μM) potentiated [^{3}H]-FNZ binding to a similar extent in membranes pre-incubated with PC liposomes and

membranes not exposed to PC (Figure 3.4). Likewise, in cerebellar membranes, potentiation of [^{3}H]-FNZ binding by 1 μM pregnanolone was found to be 116.7 ± 4.28% of control binding for membranes pre-incubated with PC liposomes and 110.1 ± 4.46% of control binding for membranes not exposed to PC (mean ± s.e.mean, n=4). A similar experiment in spinal cord membranes showed 1 μM pregnanolone potentiation of [^{3}H]-FNZ binding to be 140.1 ± 15.92% of control binding and 128.3 ± 12.75% of control binding, respectively, for membranes pre-incubated with PC liposomes and membranes not exposed to PC liposomes (mean ± s.e.mean, n=7). In neither case was the difference statistically significant.

3.3.4 Scatchard analysis of pregnanolone's modulation of [^{3}H]-FNZ binding

An explanation was sought for the different effects of cholesterol enrichment on the modulatory actions of pregnanolone on [^{3}H]-FNZ binding in cerebral cortex and spinal cord membranes. Cerebral cortex or spinal cord membranes enriched or unenriched were subjected to Scatchard experiments in the presence of either 1 μM pregnanolone or 0.45% DMSO to determine if there was a change in the B_{max} or K_d of [^{3}H]-FNZ binding. The experimental results obtained are shown in Table 3.1. Cerebral cortex membranes showed no change in affinity of [^{3}H]-FNZ binding in the presence of 0.45% DMSO between cholesterol enriched or unenriched (Figure 3.5). However, the B_{max} was significantly reduced upon cholesterol enrichment (P=0.008). The effect of pregnanolone, compared with 0.45% DMSO, in both enriched and unenriched membranes was a reduction in K_d

(P=0.014 in enriched, P=0.02 in unenriched membranes) (Figure 3.6). In enriched membranes there was also a reduction in B_{max} due to pregnanolone (P=0.046).

Spinal cord membranes in the presence of 0.45% DMSO showed no differences in either the B_{max} or K_d of [^{3}H]-FNZ in cholesterol enriched and unenriched membranes (Table 3.1) (Figure 3.7). In the presence of 1 μM pregnanolone both the K_d and B_{max} are reduced significantly (P=0.0051 and P=0.0002 respectively) upon cholesterol enrichment. The pregnanolone potentiation of [^{3}H]-FNZ binding in cholesterol enriched and unenriched spinal cord membranes shows opposing influences of pregnanolone upon [^{3}H]-FNZ binding. Pregnanolone decreases the affinity of [^{3}H]-FNZ binding and increases B_{max} in both membrane preparations (Table 3.1) (Figure 3.8). The dominant factor here is likely to be the affinity of [^{3}H]-FNZ binding in cholesterol enriched membranes since the affinity reduction is only half that in unenriched membranes although the increase in B_{max} is also less in the presence of pregnanolone.

3.4 Discussion

The primary purpose of this investigation was to investigate if cholesterol of neuronal membranes competes for the neurosteroid binding site of the $GABA_A$ receptor. Cholesterol constitutes over 20% of the membrane lipids so even a low affinity at the neurosteroid site would result in effective competition.

If cholesterol in the membrane was competing with pregnanolone for its site of action on the $GABA_A$ receptor, enrichment of the membrane with cholesterol would be expected to reduce the potency of pregnanolone as a potentiator of [^{3}H]-FNZ binding.

The results from whole brain and cerebral cortex were in accord with this but the results from cerebellum and spinal cord were not. We cannot, therefore, interpret the observations with pregnanolone on whole brain and cerebral cortex membranes as a competitive displacement of pregnanolone by cholesterol, although such a mechanism has not been excluded. The similar results from whole brain and cerebral cortex were probably due to the large component of the cerebral cortex within the whole brain.

The substantial increase in pregnanolone potency following cholesterol enrichment of spinal cord membranes presumably arose from an allosteric interaction on the $GABA_A$ protein. The opposite effects seen in spinal cord and cerebral cortex membranes with respect to the interactions between cholesterol and pregnanolone are difficult to interpret (Figure 3.2). Conceivably, they were the net of two opposing effects which were operating to different extents in both preparations. Scatchard analysis supports this view with an increased B_{max} and decreased [^{3}H]-FNZ binding affinity due to pregnanolone in cholesterol enriched spinal cord membranes compared with a decreased B_{max} and increased [^{3}H]-FNZ binding affinity due to pregnanolone in cholesterol enriched cortical membranes (Table 3.1). The reduction in B_{max} of [^{3}H]-FNZ binding in the cholesterol enriched cerebral cortex membranes causes a depression in the maximal response of the curve (Figure 3.2). The cerebellar membranes tend to show a more even balance between the two effects. A further complication in the cerebellum is the biphasic nature of the unenriched curve (Figure 3.2), which suggests more than one component of pregnanolone binding.

Biphasic actions of pregnanolone in the cerebellum and cerebral cortex in binding experiments have been reported previously (Hawkinson *et al.*, 1994). Pregnanolone showed a two component modulation of [^{3}H]-FNZ and [^{35}S]-TBPS binding in agreement with the cerebellum results in this study. Electrophysiology studies also show biphasic concentration response curves for steroid facilitation of $GABA_A$ receptor activated Cl^- currents (Morrow *et al.*, 1990). The biphasic modulation may represent high and low affinity sites present on a single receptor complex or they may be present on structurally distinct receptor complexes. One of these binding sites maybe responsible for facilitation of GABA action, whilst the other, for direct channel opening properties of these steroids observed in the absence of GABA. At present we cannot discriminate between these two views since both may occur. Prince & Simmonds (1992b) postulated the presence of multiple binding sites for steroids due to the differential antagonism by epipregnanolone of alphaxalone and pregnanolone potentiation of [^{3}H]-FNZ binding. If the neurosteroids do bind at the interstices of the $GABA_A$ receptor channel protein subunits, there are likely to be more than one binding site per channel which would explain these observations. The range and proportions of subunits that make up the $GABA_A$ channels also differ in the CNS regions studied, leading to heterogeneity of the channels and their pharmacological profiles as has been seen (Gee & Lan, 1991). Furthermore the distribution of $GABA_A$ receptor subunit mRNA has been shown to be: α_1, α_2, α_3, α_4, (β_1), β_2, β_3, γ_2, (γ_3) and (δ) in the cerebral cortex; α_1, α_6, β_2, β_3, γ_1, γ_2 and δ in the cerebellum and (α_1), α_2, α_3, β_2, β_3 and γ_2 in the spinal cord (subunits in brackets indicate they were only weakly detected) (Wisden *et al.*, 1991; 1992; Persohn *et al.*, 1991; 1992; Laurie *et al.*, 1992).

Affinity and maximum binding of [^{3}H]-FNZ binding varied among the CNS regions investigated. A lower number of binding sites for [^{3}H]-FNZ in the spinal cord compared to the cerebral cortex membranes (Table 3.1) were present. However, [^{3}H]- FNZ exhibits a similar order of affinity for benzodiazepine binding sites in all brain areas studied in agreement with Sieghart & Schlerka (1991). Pregnanolone modulation of [^{3}H]-FNZ binding results as a reduction in both the B_{max} and K_d in the cerebral cortex membranes enriched with cholesterol and a reduction of K_d in the unenriched cerebral cortex membranes. Whereas, the spinal cord membranes enriched or unenriched with cholesterol showed an increase in both the K_d and B_{max} values. The modulation by pregnanolone at the two different CNS regions is obviously heterogenous. Majewska *et al.* (1986) showed neurosteroids increased the apparent affinity of [^{3}H]-FNZ binding in rat whole brain membranes without altering the apparent B_{max}. This is in agreement with the data presented here for the unenriched cerebral cortex membranes. Cholesterol thus appears to alter the modulatory actions of pregnanolone.

The modulatory actions of pregnanolone seem to be altered both by cholesterol content and the heterogeneity of steroid binding sites. Further work to address these influences will require a study with recombinant receptors and also an investigation of whether fluctuations in neuronal cholesterol occur. If neuronal membrane cholesterol content does fluctuate, this may alter the responsiveness of the $GABA_A$ channels to neurosteroids, which are present physiologically.

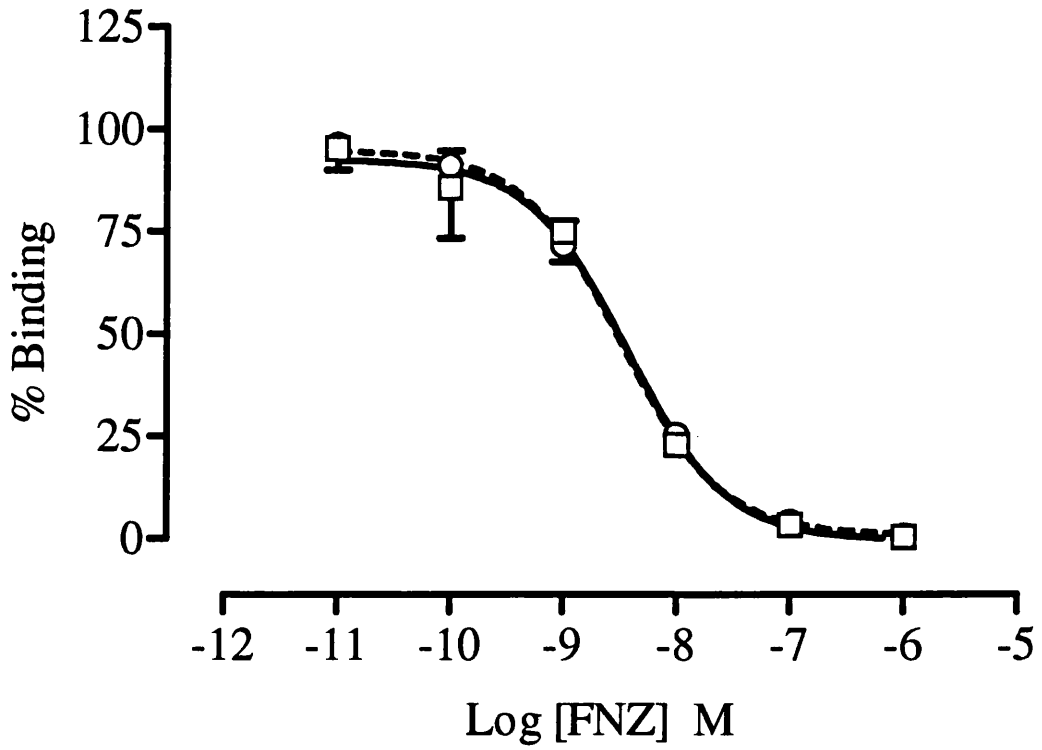
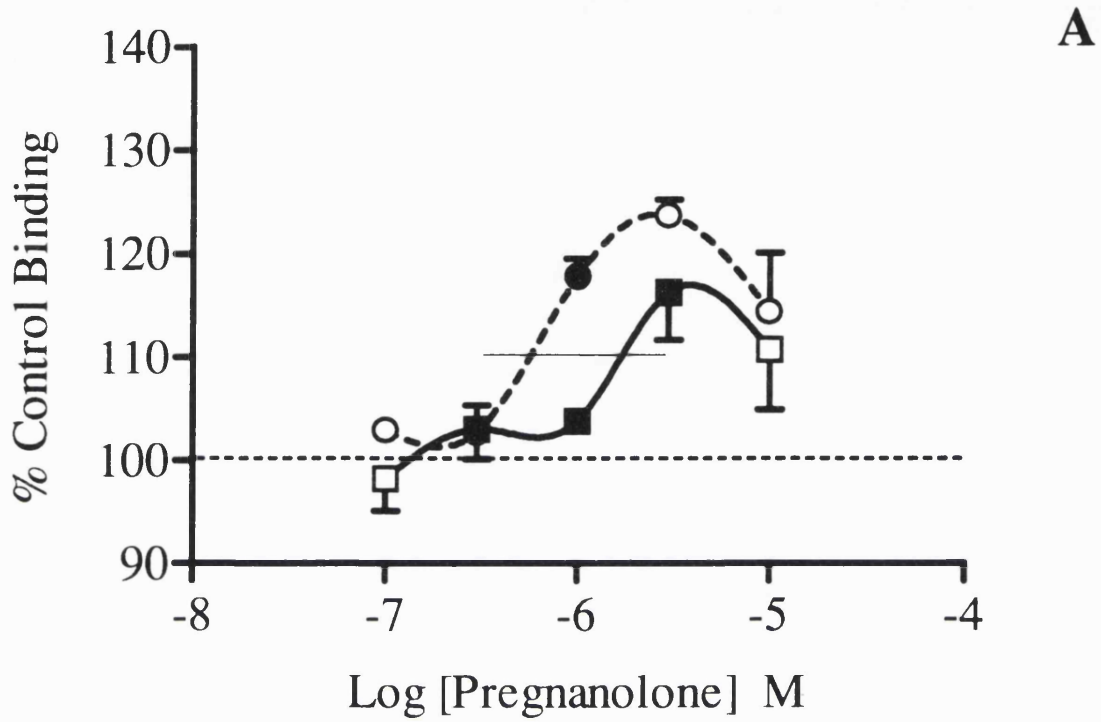


Figure 3.1 [^{3}H]-FNZ displacement by unlabelled FNZ in cholesterol enriched (squares) and unenriched (circles) whole brain membranes from in-house. Upon cholesterol enrichment [^{3}H]-FNZ control binding was significantly reduced from 563.54 ± 15.50 fmol.mg^{-1} of tissue protein in unenriched membranes to 462.40 ± 11.33 fmol.mg^{-1} of tissue protein in enriched membranes (P = 0.0019). The control binding in cholesterol enriched and unenriched membranes was thus set at 100%. The K_d of [^{3}H]-FNZ binding was 2.66 nM and 2.25 nM in cholesterol enriched and unenriched membranes, respectively. This would mean that with an assay concentration of 1 nM [^{3}H]-FNZ the likely occupancy of the binding sites would be 27 % and 31 % for the cholesterol enriched and unenriched membranes, respectively. The cholesterol content was significantly higher in the cholesterol enriched membranes (5.367 ± 0.16 µmol cholesterol mg^{-1} protein) compared to the unenriched (2.090 ± 0.15 µmol cholesterol mg^{-1} protein) (n = 3, P = 0.0001). Data are the means of 4 experiments ± s.e.mean carried out on a single batch of membranes.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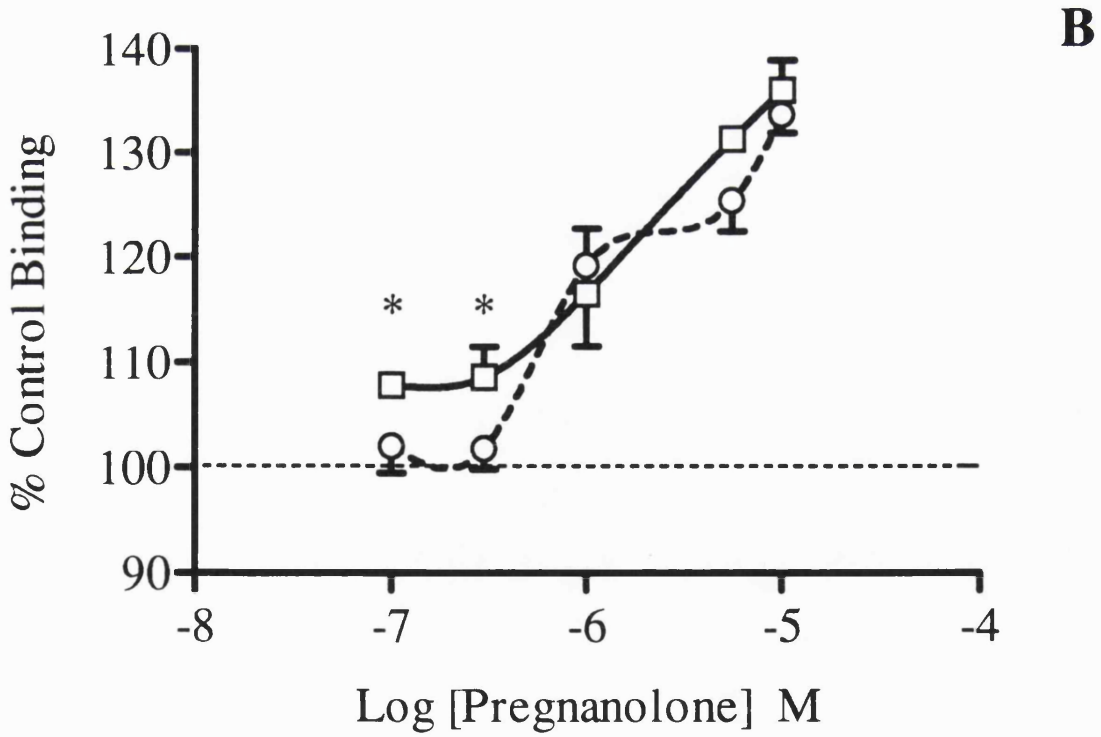
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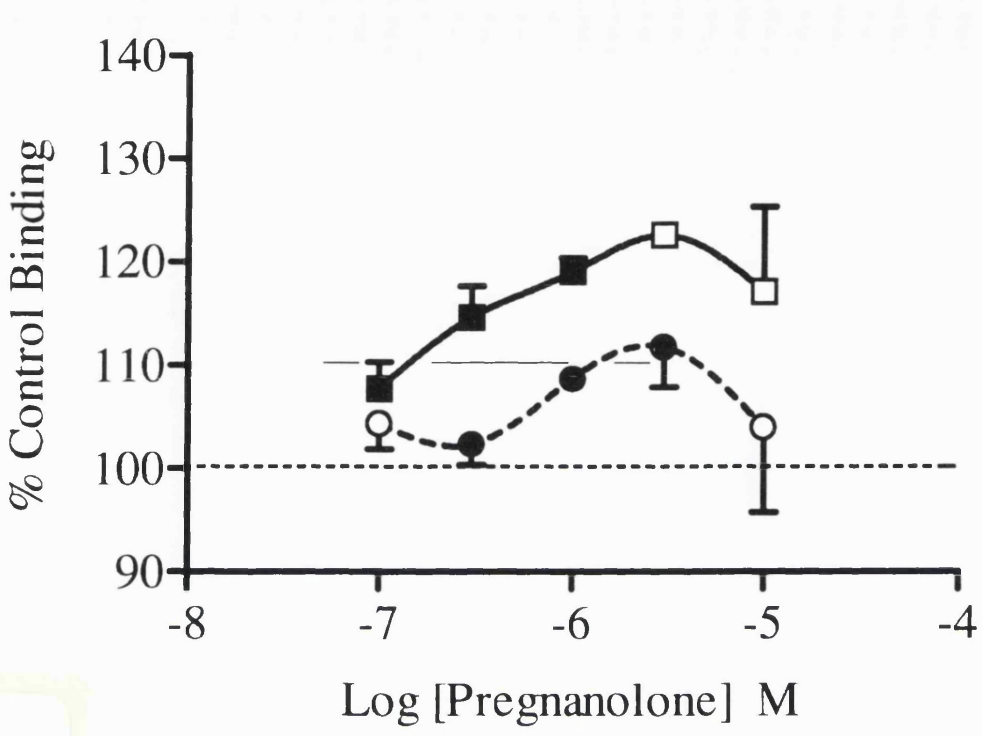

3.2 Potentiation of [^{3}H]-FNZ binding by pregnanolone in cholesterol enriched) and unenriched (circles) membranes from (A) cerebral cortex, (B) cerebellum spinal cord of in-house rats. Cholesterol contents of the membranes are shown in 2. Data are the means of 3 experiments ± s.e.mean except for the cortex where n led points are those used to determine the lateral displacement of the curves at the ıdicated by the horizontal line. For cerebral cortex membranes (A), cholesterol ent gave a shift of 0.506 ± 0.028 log units to the right (mean ± s.e.mean) and a depression in the curve (P = 0.0118 by ANOVA). For spinal cord membranes ɔlesterol enrichment gave a shift of 0.926 ± 0.052 log units to the left (mean ± n). * significant difference between enriched and unenriched by Student's *t* test P

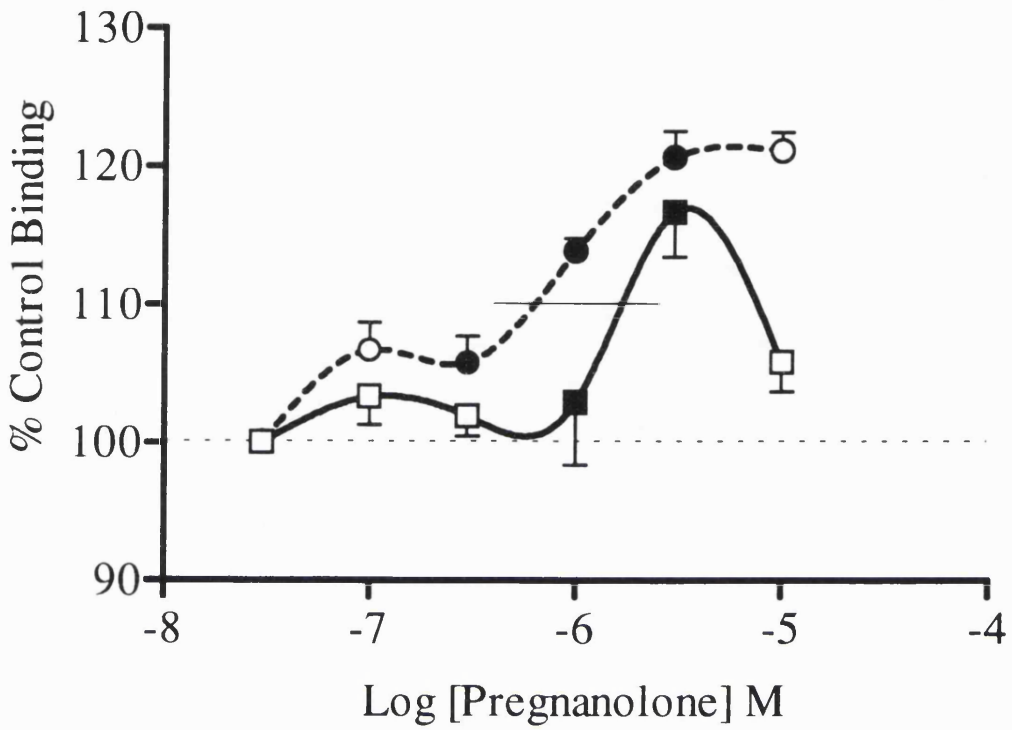

Figure 3.3 Potentiation of [^{3}H]-FNZ binding by pregnanolone in cholesterol enriched (squares) and unenriched (circles) whole brain membranes from in-house. The cholesterol content was significantly higher in the cholesterol enriched membranes (2.928 ± 0.13 µmol cholesterol mg^{-1} protein) compared to the unenriched (1.219 ± 0.04 µmol cholesterol mg^{-1} protein) (n = 3, P = 0.0002). Data are the means of 4 experiments ± s.e.mean carried out on a single batch of membranes. Filled points are those used to determine the lateral displacement of the curve at the level indicated by the horizontal line. Cholesterol enrichment of whole brain membranes gave a shift of 0.492 ± 0.102 log units to the right (mean ± s.e.mean)(P = 0.0001). Comparing the two curves by two way ANOVA showed a significant difference between the binding in the cholesterol enriched and unenriched membranes (P < 0.0001).

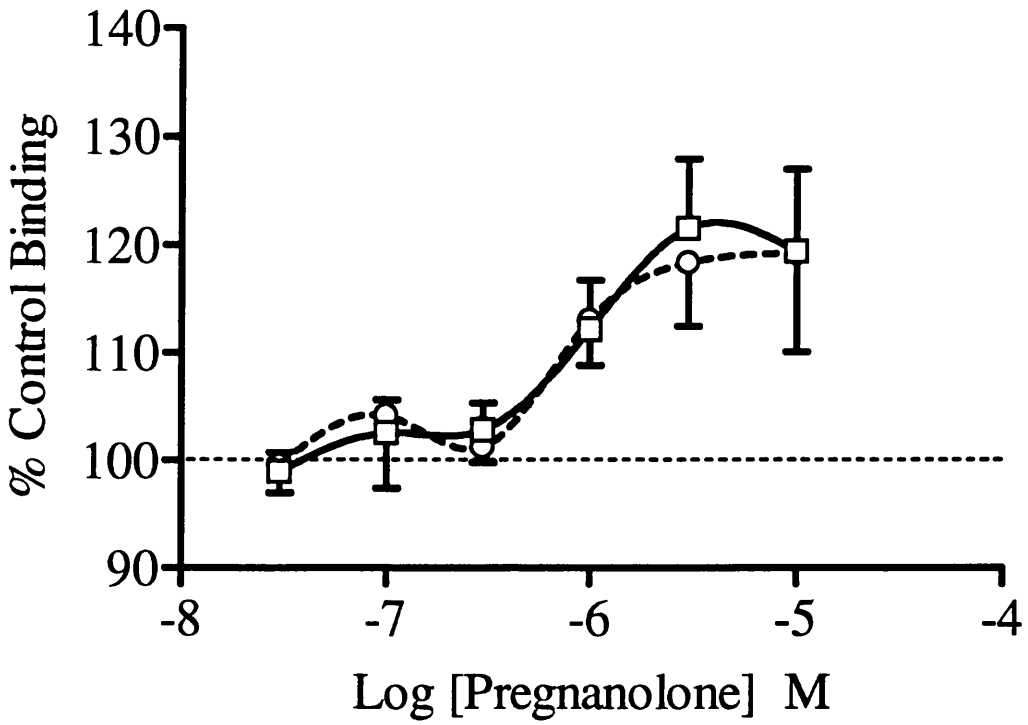

Figure 3.4 Potentiation of [^{3}H]-FNZ binding by pregnanolone in phosphatidylcholine incubated (squares) and control (circles) cerebral cortex membranes from in-house. Data are the means of 5 experiments ± s.e.mean.

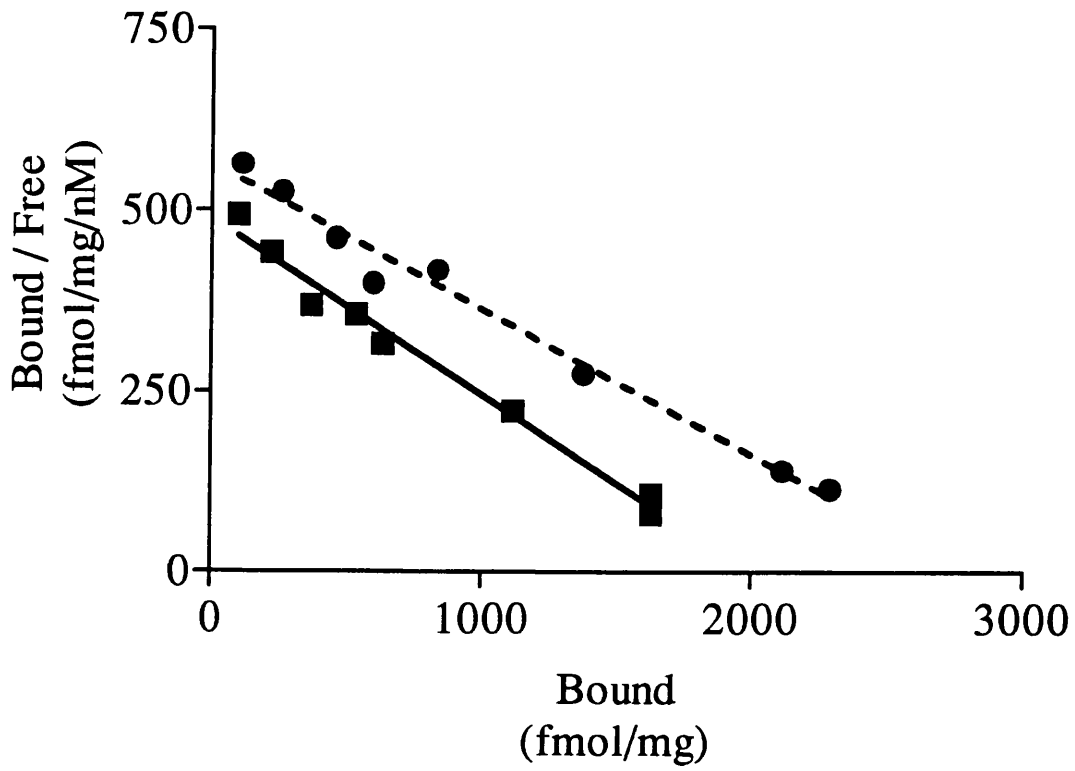

Figure 3.5 Scatchard plot showing the influence of cholesterol enrichment upon [^{3}H]-FNZ binding in the presence of 0.45% DMSO. The symbols show the binding in cholesterol enriched (squares) and unenriched (circles) cerebral cortex membranes (from Harlan U.K.). The cholesterol content was significantly higher in the cholesterol enriched (4.172 ± 0.21 μmol cholesterol mg^{-1} protein) compared to the unenriched cerebral cortex membranes (3.074 ± 0.11 μmol cholesterol mg^{-1} protein) (n = 3, P = 0.0089). The maximal binding was 2879 and 2031 fmol mg^{-1} and the affinity was 5.293 and 4.233 nM for unenriched and cholesterol enriched membranes, respectively. The data are from a single experiment representative of 4 other experiments.

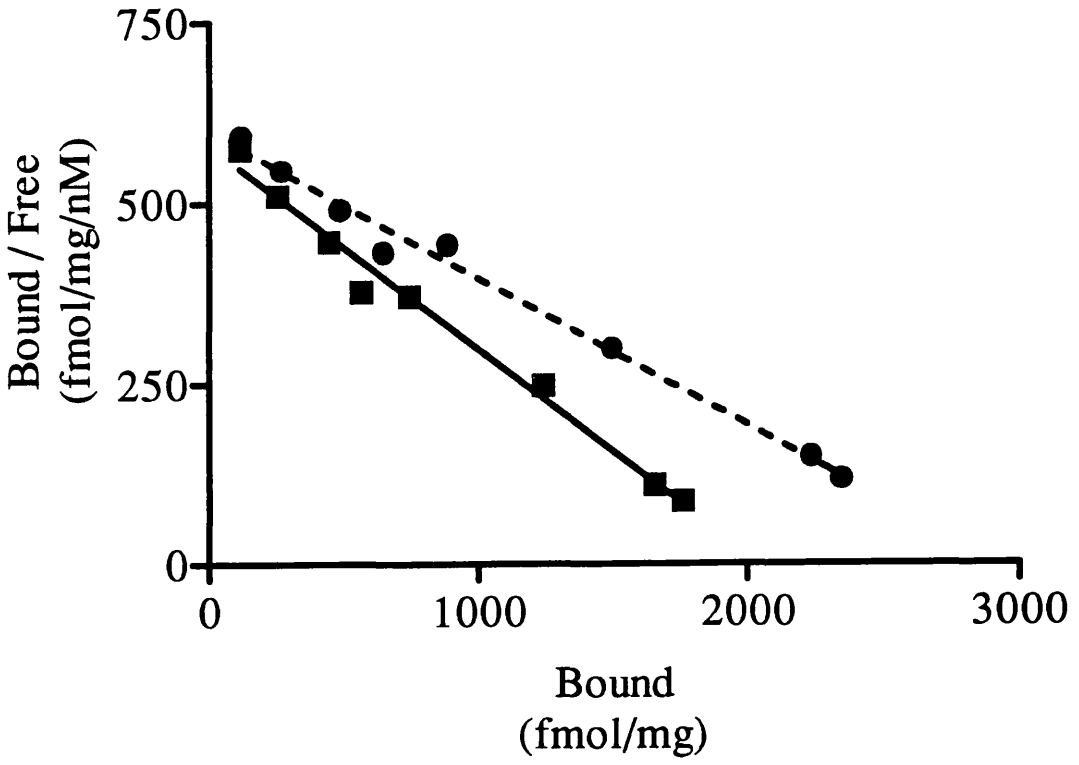

Figure 3.6 Scatchard plot showing the influence of cholesterol enrichment upon [^{3}H]-FNZ binding in the presence of 1 μM pregnanolone. The symbols show the binding in cholesterol enriched (squares) and unenriched (circles) cerebral cortex membranes (from Harlan U.K.). The cholesterol content was significantly higher in the cholesterol enriched (3.598 ± 0.03 μmol cholesterol mg^{-1} protein) compared to the unenriched cerebral cortex membranes (2.732 ± 0.30 μmol cholesterol mg^{-1} protein) (n = 3, P = 0.0448). The maximal binding was 2950 and 2075 fmol.mg^{-1} and the affinity was 4.904 and 3.617 nM for unenriched and cholesterol enriched membranes, respectively. The data are from a single experiment representative of 3 other experiments.

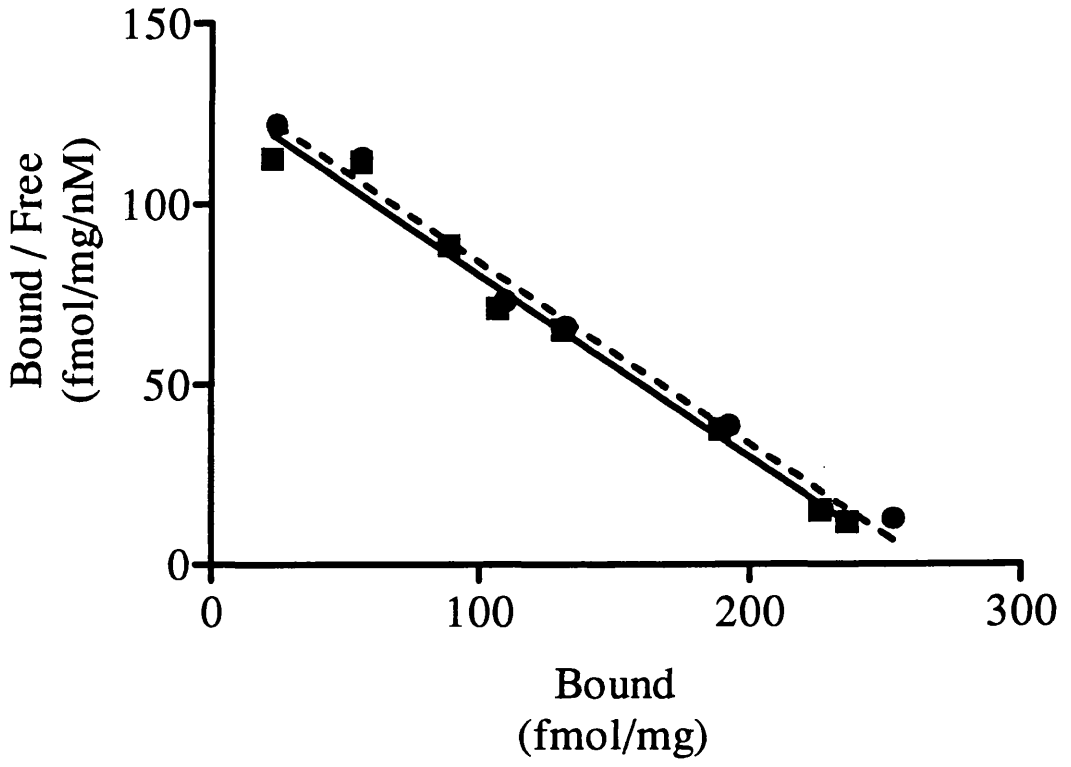

Figure 3.7 Scatchard plot showing the influence of cholesterol enrichment upon [^{3}H]-FNZ binding in the presence of 0.45% DMSO. The symbols show the binding in cholesterol enriched (squares) and unenriched (circles) spinal cord membranes (from Harlan U.K.). The cholesterol content was significantly higher in the cholesterol enriched (4.310 ± 0.06 µmol cholesterol mg^{-1} protein) compared to the unenriched spinal cord membranes (3.036 ± 0.05 µmol cholesterol mg^{-1} protein) ($n = 3$, $P < 0.0001$). The maximal binding was 270 and 258 fmol.mg^{-1} and the affinity was 2.081 and 1.960 nM for unenriched and cholesterol enriched membranes, respectively. The data are from a single experiment representative of 3 other experiments.

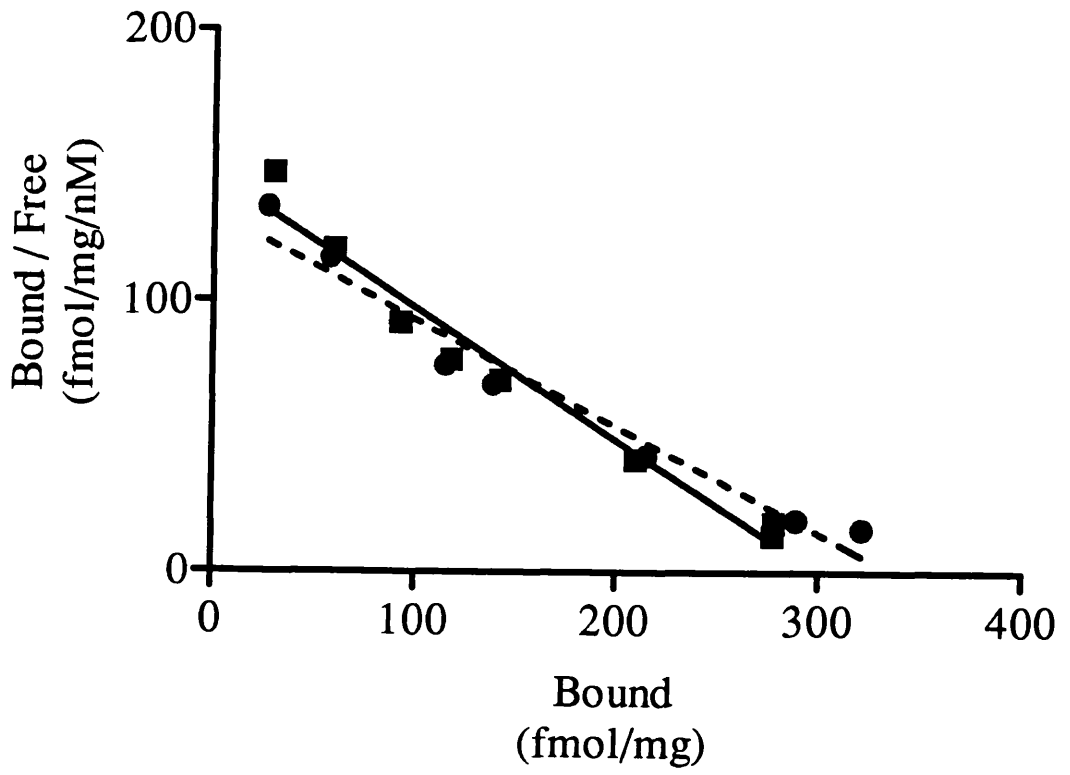

Figure 3.8 Scatchard plot showing the influence of cholesterol enrichment upon [^{3}H]-FNZ binding in the presence of 1 μM pregnanolone. The symbols show the binding in cholesterol enriched (squares) and unenriched (circles) spinal cord membranes (from Harlan U.K.). The cholesterol content was significantly higher in the cholesterol enriched (4.670 ± 0.07 μmol cholesterol mg^{-1} protein) compared to the unenriched spinal cord membranes (3.525 ± 0.04 μmol cholesterol mg^{-1} protein) (n = 3, P = 0.0001). The maximal binding was 359 and 315 fmol.mg^{-1} and the affinity was 3.105 and 2.407 nM for unenriched and cholesterol enriched membranes, respectively. The data are from a single experiment representative of 4 other experiments.

Table 3.1 Changes in the K_d and B_{max} of [^{3}H]-FNZ binding in the presence of cholesterol enriched or unenriched cortex and spinal cord membranes and incubated with 0.45% DMSO or 1 μM pregnanolone.

Incubated with:	B_{max} in cholesterol enriched membranes (fmol mg^{-1})	Significant difference	B_{max} in unenriched membranes (fmol mg^{-1})	K_d in cholesterol enriched membranes (nM)	Significant difference	K_d in unenriched membranes (nM)	n
Cerebral cortex membranes							
0.45% DMSO	2392 ± 145	S	3097 ± 140	5.02 ± 0.30	NS	5.59 ± 0.23	5
		S NS			S S		
1 μM pregnanolone	1972 ± 52	S	2767 ± 86	3.84 ± 0.12	NS	4.53 ± 0.27	4
Spinal cord membranes							
0.45% DMSO	224 ± 25	NS	239 ± 14	1.77 ± 0.22	NS	1.73 ± 0.25	4
		S S			S S		
1 μM pregnanolone	294 ± 8	S	360 ± 7	2.52 ± 0.13	S	3.22 ± 0.13	5

S: Significant difference by Student's *t* test ($P<0.05$). NS: No significant difference.
Red boxes show significance between rows and the yellow boxes show significance between columns.

Cerebral cortex: Pregnanolone increases the affinity of FNZ with no change in B_{max} in unenriched membranes which would account for the increase in [^{3}H]-FNZ binding in Figure 3.2A. Pregnanolone also increases the affinity of FNZ in enriched membranes but also decreases the B_{max} which would account for the lesser enhancement of [^{3}H]-FNZ binding seen in Figure 3.2A.

Spinal cord: Pregnanolone decreases the affinity of FNZ but increases B_{max} in unenriched membranes causing opposing influences in an experiment such as shown in Figure 3.2C which may account for the small enhancement of [^{3}H]-FNZ binding by pregnanolone. Pregnanolone also decreases affinity and increases B_{max} of FNZ in cholesterol enriched membranes, but the affinity reduction is only half that in unenriched membranes although the increase in B_{max} is also less. Under the conditions of the experiments shown in Figure 3.2C, this presumably allows for a greater enhancement of [^{3}H]-FNZ binding by pregnanolone.

Chapter 4

Influence of Cholesterol Enrichment on the Potentiation of [^{3}H]-FNZ Binding by Propofol and Muscimol

Chapter 4

Influence of cholesterol enrichment on the potentiation of [^{3}H]-FNZ binding by propofol and muscimol

4.1 Introduction

GABA, the major inhibitory neurotransmitter within the central nervous system binds to the $GABA_A$ receptor causing an influx of Cl^- ions hyperpolarizing the cell thus preventing an action potential. The binding site is created by the N-terminal extracellular domains of an α and a β subunit (Smith & Olsen, 1995). Muscimol, a natural product from the hallucinogenic mushroom *Amanita muscaria*, is a synthetic analogue of GABA that also acts at this binding site (MacDonald & Olsen, 1994). At least two molecules of GABA or muscimol must bind to the $GABA_A$ receptor for full activation of the native receptor. The binding properties of each of these two binding sites are such that they display high (nM) and low (μM) affinity states. The binding of [^{3}H]-FNZ *in vitro* is allosterically enhanced by GABA agonists (Sieghart, 1992; Richards *et al.*, 1991; Karobath & Sperk, 1979; Martin & Candy, 1978) after extensive washing of synaptic membranes with a freeze thaw step to remove endogenous GABA (Karobath & Sperk, 1979). This potentiation is via the low affinity GABA sites (Rabow *et al.*, 1995; Sieghart, 1992) and is dependent upon the presence of Cl^- ions (Olsen, 1982; Martin & Candy, 1978). The high affinity GABA sites may represent a desensitized form of the $GABA_A$ receptor.

Propofol (2,6-diisopropylphenol) an intravenous general anaesthetic (Langley & Heel, 1988) is a potent enhancer of GABA responses in electrophysiological studies (Hales & Lambert, 1991). Low concentrations of propofol (2-100 μM) potentiate GABA-evoked currents, increasing the frequency of opening of GABA channels, and reduce the rate of desensitization; intermediate concentrations (10-1000 μM) directly activate $GABA_A$ channels and induce receptor desensitization; high concentrations (600-10000 μM) inhibit receptor function (Orser *et al.*, 1994; Adodra & Hales, 1995). The enhancement of GABA evoked Cl^- currents may also be achieved by propofol prolonging the $GABA_A$ channel burst duration (Hales & Lambert, 1991). A distinct binding site has been proposed for propofol's interaction with the $GABA_A$ receptor at the extracellular domain of the protein (Concas *et al.*, 1991; Hales & Lambert, 1991). Propofol enhancement of [^{3}H]-FNZ binding requires the presence of Cl^- ions at a physiological concentration (Prince & Simmonds, 1992).

It was seen in Chapter 3 that cholesterol affects pregnanolone's modulation of [^{3}H]-FNZ binding. The effect of cholesterol enrichment upon muscimol and propofol potentiation of [^{3}H]-FNZ binding was investigated to see if this modulation is specific to the neurosteroid site or more generally at the $GABA_A$ receptor.

4.2 Methods

4.2.1 Membrane preparation

Membranes were prepared from the 'buffy coat' fraction of either whole brain, cerebral cortex, or spinal cord of male Wistar rats (130-200 g) as in Chapter 2 (Section 2.2.1).

4.2.2 Liposome preparation

Phosphatidylcholine (PC) and cholesterol (0.75 mg of each per ml of buffer added) liposomes were prepared as in Chapter 2 (Section 2.2.3).

4.2.3 Cholesterol enrichment of synaptic membranes

Cholesterol-enrichment of synaptic membranes was achieved by the method in Chapter 2 (Section 2.2.4).

4.2.4 Binding experiments

Experiments were performed to determine the effects propofol and muscimol had on [^{3}H]-FNZ binding to cholesterol-enriched and unenriched membranes. On the day of assay the membranes were thawed and centrifuged at 48400 g for 20 min at 4°C and the pellets resuspended in assay buffer to give a final protein concentration of about 1 mg ml^{-1}. Aliquots of membranes (100 µl) were preincubated with propofol 10 µM-3 mM, or muscimol 1 nM-0.1 mM in assay buffer at 37°C for 10 min. [^{3}H]-FNZ was added to give

a 1 nM concentration in a final volume of 0.5 ml and the samples were then incubated on ice for 60 min. The reaction was terminated with the addition of 2 ml ice cold wash buffer, followed by rapid filtration through Whatman GF-C filters using a Brandell Cell Harvester. The filter mat was washed a further four times with 2 ml wash buffer and the radioactivity determined by standard liquid scintillation techniques (Beckman 5801). Non-specific binding was determined by use of 1 μM FNZ and was in the region 5-10%. FNZ was dissolved initially in ethanol and propofol was dissolved in acetone. The final concentration of ethanol was less than 0.4% and acetone was 0.1%. The assay buffer contained 150 mM $NaCl^-$ which is essential for propofol potentiation of [^{3}H]-FNZ binding (Prince & Simmonds, 1992).

All chemicals and drugs were obtained from Sigma with the exception of [^{3}H]-FNZ (82.5 Ci $mmol^{-1}$ specific activity) which was obtained from Amersham International. Data were fitted using the Inplot package Prism (Graphpad Software). Statistical comparisons were made by Student's *t* test for independent samples and ANOVA.

4.3 Results

4.3.1 Influence of cholesterol enrichment on the potentiation of [^{3}H]-FNZ binding by muscimol

In the preceeding chapter it has been shown that cholesterol enrichment of whole brain synaptic membranes has no influence on cold FNZ displacement of [^{3}H]-FNZ (Figure 3.1). Therefore, the influence cholesterol enrichment had upon muscimol potentiation of [^{3}H]-FNZ binding could be studied directly. These experiments were

performed on whole brain and membranes from discrete regions of the CNS. Muscimol enhanced [^{3}H]-FNZ binding in unenriched membranes from whole brain and cerebral cortex in a simple concentration-dependent manner between 0.1 μM and 10 μM muscimol (Figures 4.1 and 4.2 respectively). The potency increase of 3.0 fold in cholesterol-enriched whole brain membranes (Figure 4.1) was associated with an apparent increase in the maximal effect of muscimol (the binding at 10^{-4} M muscimol was 150 ± 2.24% and 140.3 ± 3.83% of control [^{3}H]-FNZ binding for cholesterol enriched and unenriched, respectively. Values shown are the mean ± s.e.mean, n = 6, P = 0.05). In cholesterol-enriched cerebral cortex membranes, the potency of muscimol was increased 2.0 fold (Figure 4.2) with no change in the maximal binding (Table 4.1). At a concentration of 0.1 mM muscimol, a reduction of [^{3}H]-FNZ binding back to control levels was seen, which maybe due to desensitization of the receptor.

The muscimol enhancement of [^{3}H]-FNZ binding in the unenriched spinal cord membranes again followed a simple dose response curve between 1 μM and 0.1 mM. In the cholesterol enriched membranes the potency of muscimol was increased by 4.5 fold (Figure 4.3) (Table 4.1).

4.3.2 Influence of cholesterol enrichment on the potentiation of [^{3}H]-FNZ binding by propofol

The potentiating effects of propofol on [^{3}H]-FNZ binding were determined in whole brain, cerebral cortex and spinal cord membranes enriched with cholesterol.

In unenriched whole brain membranes the threshold concentration for enhancement of [^{3}H]-FNZ binding was 10 μM (Figure 4.4). The enhancement increased with propofol concentration up to 300 μM and then declined at higher concentrations. In the cholesterol-enriched membranes, there was no concentration-dependent enhancement of [^{3}H]-FNZ binding by propofol.

The unenriched cerebral cortex and spinal cord membranes showed a threshold concentration for enhancing [^{3}H]-FNZ binding of 10 μM (Figure 4.5 and Figure 4.6 respectively). A similar concentration dependence of the modulatory response of propofol was demonstrated in the cerebral cortex and spinal cord unenriched membranes as for the whole brain. In the cholesterol enriched cerebral cortex membranes the concentration dependent effect of propofol remained but was depressed to lower response levels, the depression between 30 μM and 1 mM being significant in an ANOVA ($P<0.0001$). A similar depression of the effect of propofol associated with cholesterol enrichment was seen in the spinal cord between 0.1 mM to 1 mM, which was also significant by ANOVA ($P<0.0001$). It should be noted that the cholesterol enrichment in the cerebral cortex and spinal cord membranes obtained from Harlan U.K. was less than half that in the whole brain membranes prepared from in-house rats.

4.4 Discussion

In the previous chapter (Chapter 3) cholesterol was seen to alter the neurosteroid pregnanolone's modulation of [^{3}H]-FNZ binding with different results depending on the

CNS region. If the cholesterol molecule was interacting exclusively at the neurosteroid site it would be expected to have little influence on muscimol or propofol potentiation of [^{3}H]-FNZ binding, since both muscimol and propofol have separate binding sites on the $GABA_A$ receptor (Concas *et al.*, 1991; Sieghart, 1992). The other alternative is that the cholesterol may have a general effect on the $GABA_A$ receptor. The results from muscimol and propofol modulation of [^{3}H]-FNZ binding seem to suggest the latter. Cholesterol enrichment of whole brain, cerebral cortex and spinal cord membranes increases the potency of muscimol to potentiate [^{3}H]-FNZ binding (Figures 4.1, 4.2 and 4.3), but reduces propofol's potentiation of [^{3}H]-FNZ binding by a reduction in the apparent maximal binding (Figures 4.4, 4.5 and 4.6).

The nature of cholesterol's general effect upon the $GABA_A$ receptor seems to be an alteration of the coupling between the modulatory sites. Cholesterol enrichment of whole brain membranes had no influence upon the affinity of FNZ for its own binding site (Figure 3.1) although there was a reduction in [^{3}H]-FNZ binding in cholesterol enriched membranes which might suggest that the number of benzodiazepine sites was reduced.

In comparing the modulators of [^{3}H]-FNZ binding it is apparent that pregnanolone showed opposite effects in the spinal cord and cerebral cortex membranes upon cholesterol enrichment (Figure 3.2), while muscimol showed the same effects in the spinal cord and cerebral cortex membranes upon cholesterol enrichment (Figures 4.2 and 4.3) as did propofol (Figures 4.5 and 4.6). The reason for this difference maybe due to neurosteroids having multiple binding sites in different CNS regions and/or upon the same receptor as discussed in Chapter 3 (Prince & Simmonds, 1992b; Gee & Lan, 1991),

whilst, propofol and muscimol interact with distinct binding sites for their modulation of [^{3}H]-FNZ binding (Concas *et al.*, 1991; Hales & Lambert, 1991; Rabow *et al.*, 1995; Sieghart, 1992). High and low affinity binding sites for muscimol do exist, but, muscimol only interacts with the low affinity sites to potentiate [^{3}H]-FNZ binding by increasing the affinity of the ligand (Tallman *et al.*, 1978; Martin & Candy, 1978). With only single binding sites for the interaction of muscimol or propofol with the $GABA_A$ receptor the effect of cholesterol enrichment upon their modulation of [^{3}H]-FNZ binding would therefore be likely to give consistent effects as seen in these results.

Cholesterol enrichment of the spinal cord and cerebral cortex membranes derived from Harlan U.K. rats seems to be rather low in these experiments (see Figures 4.2, 4.3, 4.5 and 4.6 for values of cholesterol content) compared to enrichment of whole brain membranes derived from in-house rats (see Figures 4.1 and 4.4 for values of cholesterol content) for the reasons discussed in Chapter 2. These different cholesterol enrichments seemed not to influence the results with muscimol but they could account for the greater reduction in the effect of propofol on whole brain membranes.

I have demonstrated that the interaction of cholesterol with the $GABA_A$ receptor is not restricted to the neurosteroid site. A more general interaction seems to occur with the cholesterol altering the coupling between the neurosteroid, GABA, or propofol sites with the benzodiazepine site.

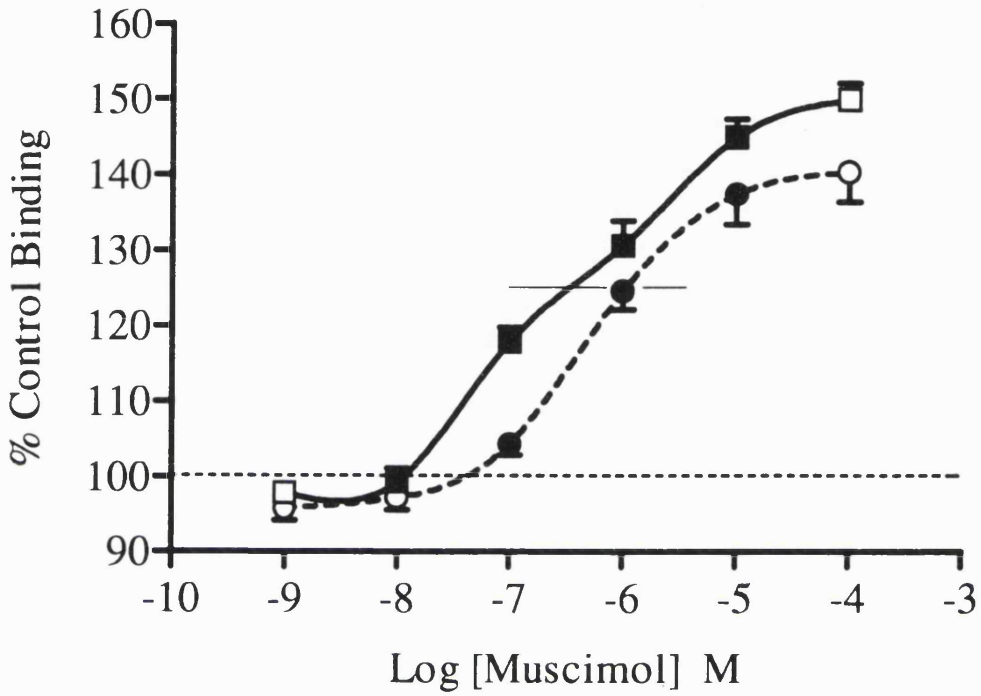

Figure 4.1 Potentiation of [^{3}H]-FNZ binding by muscimol in cholesterol enriched (squares) and unenriched (circles) whole brain membranes from in-house. The cholesterol content was significantly higher in the cholesterol enriched membranes (5.051 ± 0.19 µmol cholesterol mg^{-1} protein) compared to the unenriched (2.214 ± 0.07 µmol cholesterol mg^{-1} protein) (n = 3, P = 0.0002). Data are the means of 6 experiments ± s.e.mean carried out on a single batch of membranes. Filled points are those used to determine the lateral displacement of the curve at the level indicated by the horizontal line. Cholesterol enrichment gave a shift of 0.470 ± 0.190 log units to the left (mean ± s.e.mean) (P < 0.001).

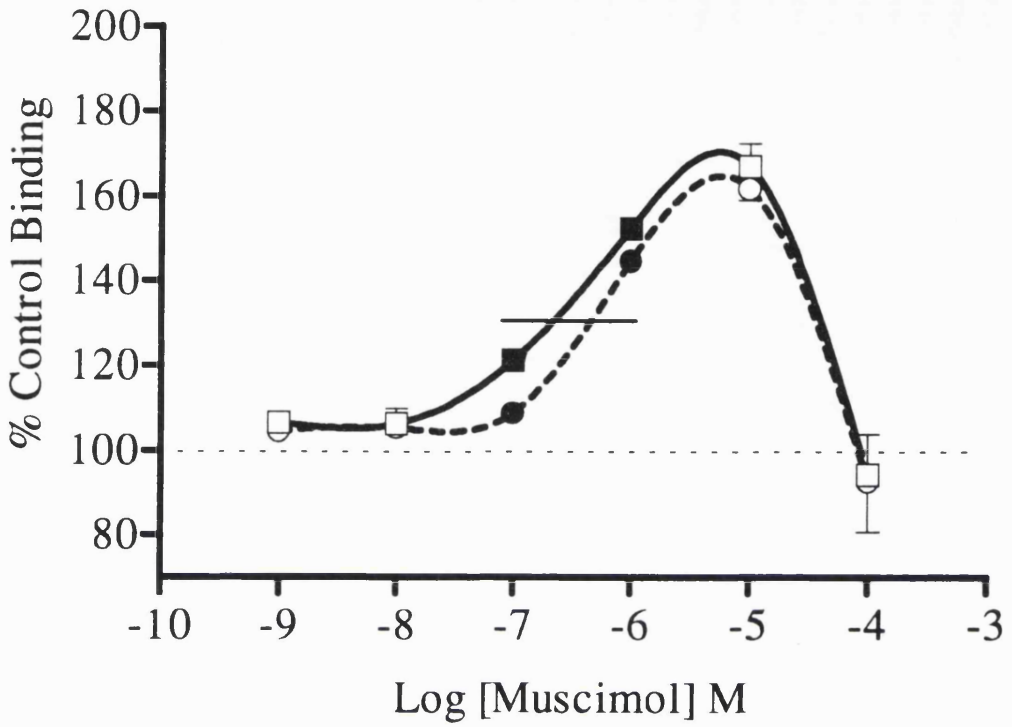

Figure 4.2 Potentiation of [^{3}H]-FNZ binding by muscimol in cholesterol enriched (solid and open squares) and unenriched (solid and open circles) cerebral cortex membranes from Harlan U.K. The cholesterol content was significantly higher in the cholesterol enriched membranes (3.081 ± 0.05 µmol cholesterol mg^{-1} protein) compared to the unenriched (2.114 ± 0.15 µmol cholesterol mg^{-1} protein) (n = 3, P = 0.0032). Data are the means of 4 experiments ± s.e.mean carried out on a single batch of membranes. Solid symbols are those used to determine the lateral displacement of the curve at the level indicated by the horizontal line. Cholesterol-enrichment gave a shift of 0.310 ± 0.058 log units to the left (mean ± s.e.mean) (P < 0.0001).

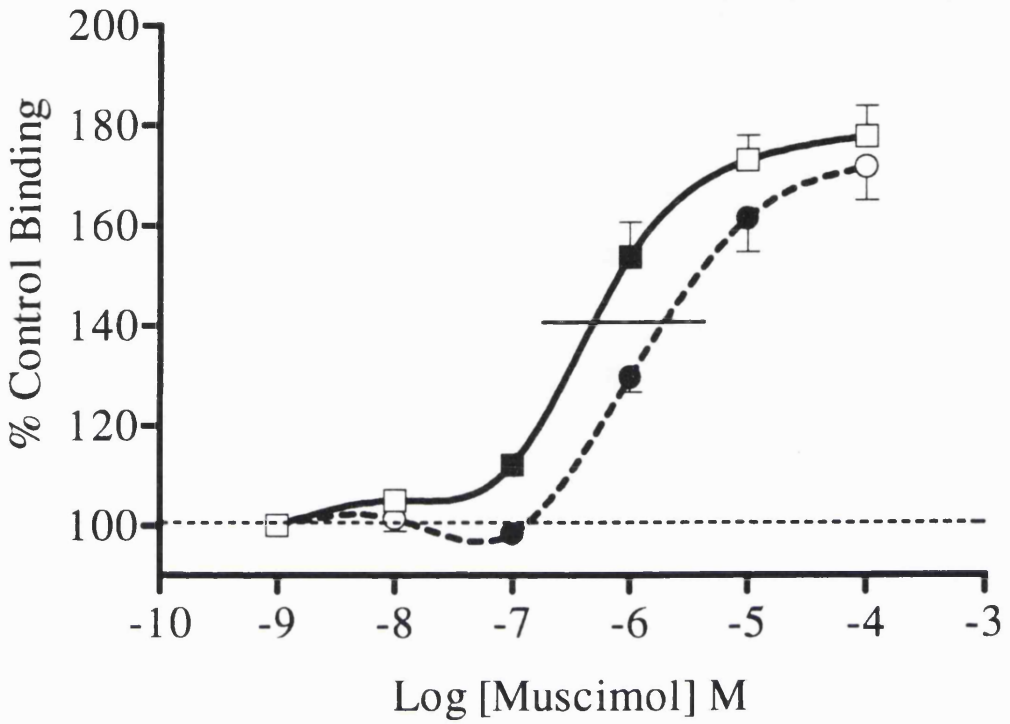

Figure 4.3 Potentiation of [^{3}H]-FNZ binding by muscimol in cholesterol enriched (solid and open squares) and unenriched (solid and open circles) spinal cord membranes from Harlan U.K. The cholesterol content was significantly higher in the cholesterol enriched membranes (4.310 ± 0.06 µmol cholesterol mg^{-1} protein) compared to the unenriched (3.036 ± 0.05 µmol cholesterol mg^{-1} protein) ($n = 3$, $P < 0.0001$). Data are the means of 3 experiments ± s.e.mean carried out on a single batch of membranes. Solid symbols are those used to determine the lateral displacement of the curve at the level indicated by the horizontal line. Cholesterol-enrichment gave a shift of 0.653 ± 0.144 log units to the left (mean ± s.e.mean) ($P < 0.0001$).

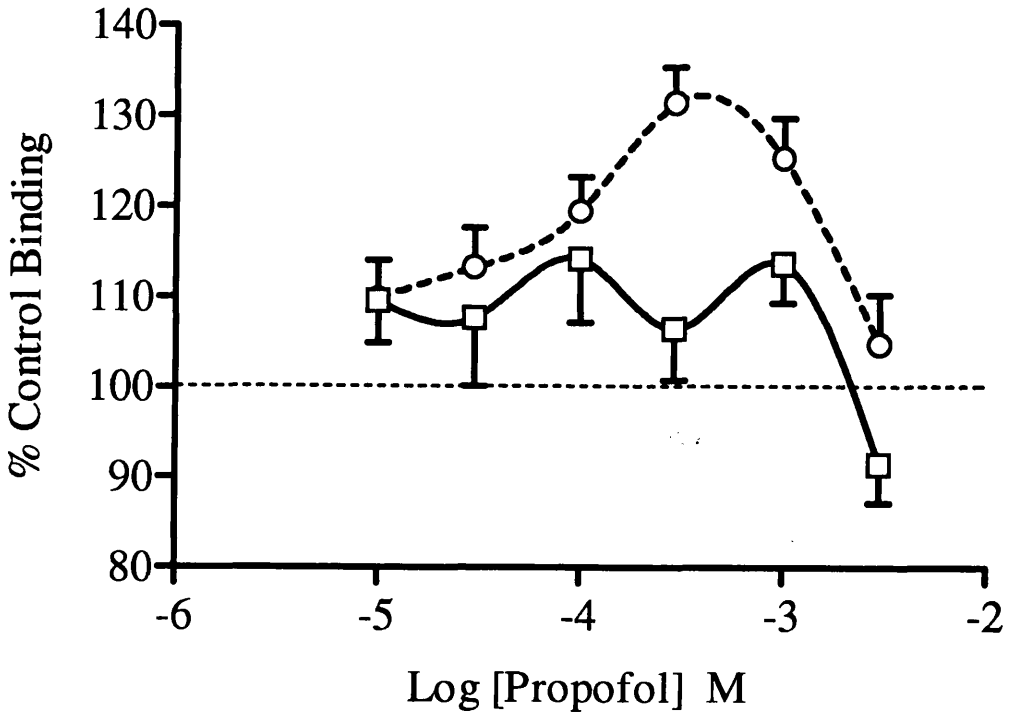

Figure 4.4 Potentiation of [^{3}H]-FNZ binding by propofol in cholesterol enriched (squares) and unenriched (circles) whole brain membranes from in-house. The cholesterol content was significantly higher in the cholesterol enriched membranes (4.170 ± 0.07 µmol cholesterol mg^{-1} protein) compared to the unenriched (1.764 ± 0.03 µmol cholesterol mg^{-1} protein) ($n = 3$, $P < 0.0001$). Data are the means of 5 experiments ± s.e.mean carried out on a single batch of membranes. The 0.1% (v/v) acetone used as the vehicle for propofol had no effect upon [^{3}H]-FNZ binding. Comparing the two curves by two way ANOVA showed a significant difference between the binding in the cholesterol enriched and unenriched membranes ($P = 0.013$).

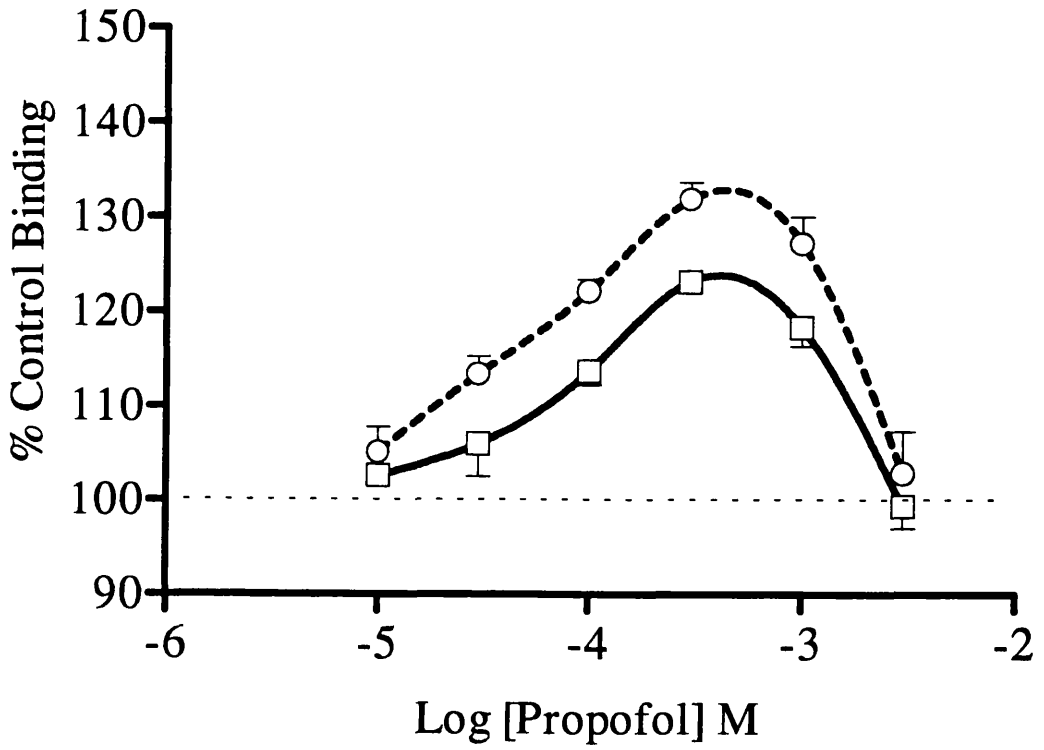

Figure 4.5 Potentiation of [^{3}H]-FNZ binding by propofol in cholesterol enriched (squares) and unenriched (circles) cerebral cortex membranes from Harlan U.K. The cholesterol content was significantly higher in the cholesterol enriched membranes (3.081 ± 0.05 µmol cholesterol mg^{-1} protein) compared to the unenriched (2.114 ± 0.15 µmol cholesterol mg^{-1} protein) (n = 3, P = 0.0032). Data are the means of 4 experiments ± s.e.mean carried out on a single batch of membranes. The 0.1% (v/v) acetone used as the vehicle for propofol had no effect upon [^{3}H]-FNZ binding. There is a significant difference between the enriched and unenriched curves determined by ANOVA (P < 0.0001).

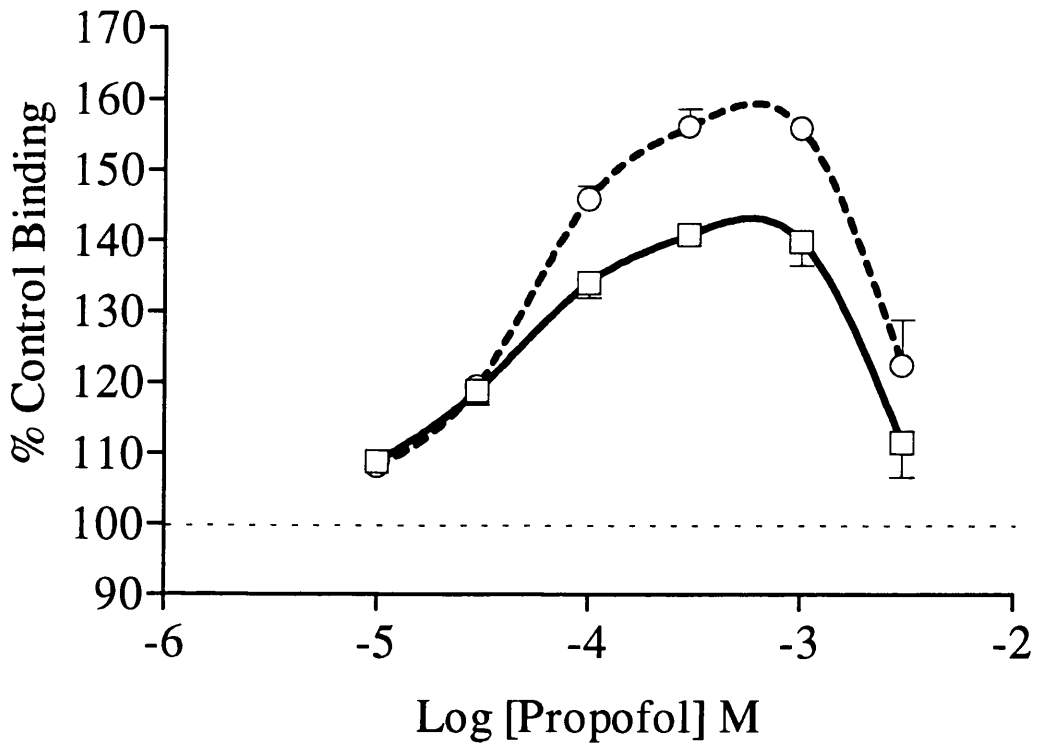

Figure 4.6 Potentiation of [^{3}H]-FNZ binding by propofol in cholesterol enriched (squares) and unenriched (circles) spinal cord membranes from Harlan U.K. The cholesterol content was significantly higher in the cholesterol enriched membranes (4.310 ± 0.06 µmol cholesterol mg^{-1} protein) compared to the unenriched (3.036 ± 0.05 µmol cholesterol mg^{-1} protein) ($n = 3$, $P < 0.0001$). Data are the means of 3 experiments ± s.e.mean carried out on a single batch of membranes. The 0.1% (v/v) acetone used as the vehicle for propofol had no effect upon [^{3}H]-FNZ binding. There is a significant difference between the cholesterol enriched and unenriched curves determined by ANOVA ($P < 0.0001$).

Table 4.1 Changes in the EC_{50} and B_{max} of muscimol potentiation of [^{3}H]-FNZ binding between cholesterol enriched or unenriched cerebral cortex and spinal cord membranes.

Cerebral cortex membranes						
B_{max} in cholesterol enriched membranes (fmol mg^{-1})	Significant difference	B_{max} in unenriched membranes (fmol mg^{-1})	EC_{50} in cholesterol enriched membranes (μM)	Significant difference	EC_{50} in unenriched membranes (μM)	n
946.92 ± 26.33	NS	891.86 ± 31.65	0.37 ± 0.08	S	0.60 ± 0.04	4
Spinal cord membranes						
209.32 ± 18.12	NS	224.53 ± 16.51	0.51 ± 0.08	S	1.49 ± 0.03	3

The values shown in the table are the mean and s.e.mean.

S: Significant by Student's *t* test ($P<0.05$). NS: No significant difference.

Cholesterol enrichment decreases the EC_{50} of muscimol to potentiate [^{3}H]-FNZ binding in cerebral cortex and spinal cord membranes. This would account for the increased potency of muscimol seen in Figures 4.2 and 4.3.

Chapter 5

Influence of Cholesterol Enrichment on the Modulation of [^{3}H]-Muscimol Binding

Chapter 5

Influence of cholesterol enrichment on the modulation of [^{3}H]-muscimol binding

5.1 Introduction

Modulation of the $GABA_A$ receptor occurs via an allosteric interaction with the GABA binding site (Sieghart, 1995). The modulation can be of a positive nature such as for propofol (Concas *et al.*, 1990, 1991) and pregnanolone (Harrison *et al.*, 1987; Simmonds, 1991; Peters *et al.*, 1988) which both enhance [^{3}H]-muscimol binding. Alternatively a negative modulation may occur as displayed by the plant convulsant picrotoxin. The active ingredient of picrotoxin, picrotoxinin partially inhibits GABA binding noncompetitively (Rabow *et al.*, 1995; Skerrit & Johnston, 1983). Electrophysiological studies show GABA induced Cl^- conductance responses are blocked upon application of picrotoxinin (Olsen, 1982; Bormann, 1988). The onset and recovery of the blockade produced by picrotoxinin depends on the presence of GABA. It was therefore concluded that picrotoxinin requires an open channel for blockade indicating the position of the picrotoxinin binding site to be within the channel (Inoue & Akaike, 1988; Inomata *et al.*, 1988). Work by Yoon *et al.* (1993) now questions this conclusion. Whatever the mechanism of this blockade it causes a reduction in the GABA-evoked average open and burst duration (Twyman *et al.*, 1989).

The modulation of GABA by pregnanolone, propofol and picrotoxinin is complicated by the existence of multiple binding affinities for GABA. Depending on the coupling of pregnanolone, propofol and picrotoxinin sites with the GABA high, low or very low affinity sites, there may be different effects of cholesterol enrichment upon the modulation of [^{3}H]-muscimol binding. In this chapter rat whole brain membranes have been enriched with cholesterol and the effects this has upon muscimol binding to the $GABA_A$ channel protein and the modulation of [^{3}H]-muscimol binding by picrotoxinin, propofol and pregnanolone described. FNZ modulation of [^{3}H]-muscimol binding in enriched and unenriched membranes was not studied because benzodiazepine stimulation of GABA binding is not easily observed and is sensitive to membrane manipulations and assay conditions (Skerrit *et al.*, 1982a).

5.2 Methods

5.2.1 Membrane preparation

Membranes were prepared from the whole brain of male Wistar rats (130-200g) as described in Chapter 2 (section 2.2.1).

5.2.2 Liposome preparation

Phosphatidylcholine and cholesterol (0.75 mg of each per ml of buffer added) liposomes were prepared as in Chapter 2 (section 2.2.3).

5.2.3 Cholesterol enrichment of synaptic membranes

Cholesterol enrichment of the whole brain membranes was achieved by the method in Chapter 2 (section 2.2.4).

5.2.4 Binding experiments ✳

On the day of assay the membranes were thawed and centrifuged at 48400 g for 20 min at 4°C and the pellets resuspended in assay buffer to give a final concentration of about 1 mg ml^{-1}. Aliquots of membranes (100 µl) were preincubated with muscimol 0.1 nM-10 µM, picrotoxinin 0.3 µM-0.1 mM, propofol 1 µM-0.3 mM or pregnanolone 30 nM-10 µM in assay buffer at room temperature for 10 min. [^{3}H]-muscimol was added to give a 1 nM concentration in a final volume of 0.5 ml and the samples were then incubated on ice for 60 min in the dark. The reaction was terminated with the addition of 2 ml ice cold wash buffer, followed by rapid filtration through Whatman GF-C filters using a Brandell Cell Harvester. The filter mat was washed a further four times with 2 ml wash buffer and the radioactivity determined by standard liquid scintillation techniques (Beckman 5801). Non-specific binding was determined by use of 1 mM GABA and was in the region 2-10%. Propofol was dissolved initially in acetone and pregnanolone was dissolved in DMSO. The final concentration of acetone was 0.1% and DMSO was 0.45%. [^{3}H]-muscimol was added to give a final concentration of 1 nM so as to bind only to the high affinity GABA binding sites. EDTA (1 mM) was included in the wash buffer

✳ (Modified from the method of Goodnough & Hawkinson, 1995).

to chelate divalent cations and preclude [^{3}H]-muscimol binding to the $GABA_B$ receptor (Harrison & Simmonds, 1984).

All chemicals and drugs were obtained from Sigma with the exception of [^{3}H]-muscimol (18.6 Ci mmol^{-1} specific activity) which was obtained from Amersham International.

5.3 Results

5.3.1 Influence of cholesterol enrichment on unlabelled muscimol displacement of [^{3}H]-muscimol

In initial experiments to determine the effect of cholesterol enrichment upon [^{3}H]-muscimol binding, displacement of [^{3}H]-muscimol binding by cold muscimol was studied in rat whole brain membranes. Muscimol binding was found to be unaffected by cholesterol enrichment (Figure 5.1).

5.3.2 Influence of cholesterol enrichment on the modulation of [^{3}H]-muscimol binding by picrotoxinin

The above result allowed an investigation of the influence of cholesterol enrichment upon the modulation of [^{3}H]-muscimol binding by picrotoxinin at the $GABA_A$ receptor. These experiments were carried out in rat whole brain membranes. In unenriched membranes the threshold concentration of picrotoxinin depression of [^{3}H]-muscimol binding was 1 μM (Figure 5.2). The depression in [^{3}H]-muscimol binding continued with increasing concentration in the range studied (0.3 μM-0.1 mM) and

appeared to have two components to the curve. This concentration affect relationship was compared to membranes enriched with cholesterol. Cholesterol enrichment though had no influence on picrotoxinin displacement of [^{3}H]-muscimol.

5.3.3 Influence of cholesterol enrichment upon propofol modulation of [^{3}H]-muscimol binding.

The effect cholesterol enrichment had upon propofol modulation of [^{3}H]-muscimol binding in rat whole brain membranes was investigated. The threshold concentration of propofol in unenriched membranes was 3 μM (Figure 5.3). The [^{3}H]-muscimol binding then increased in a dose dependent manner up to 30 μM. Above this concentration (30 μM-0.3 mM) the [^{3}H]-muscimol binding decreased giving a bell shaped dose response curve. Membranes that had been enriched with cholesterol showed a smaller increase in binding at a concentration of 10 μM and above.

5.3.4 Influence of cholesterol enrichment upon pregnanolone modulation of [^{3}H]-muscimol binding

The influence of cholesterol enrichment upon pregnanolone potentiation of [^{3}H]-muscimol binding was investigated. The threshold concentration for pregnanolone in unenriched membranes was 0.1 μM (Figure 5.4). The binding then increased in a dose dependent manner up to 3 μM above which a decrease was seen. In cholesterol enriched membranes a similar dose response curve was seen but the threshold concentration of

pregnanolone was 0.3 μM. The two curves were not found to be significantly different from one another.

5.4 Discussion

The experiments carried out in this chapter further the evidence for cholesterol affecting the coupling of modulatory sites on the $GABA_A$ receptor. Cold muscimol displacement of [^{3}H]-muscimol was unaffected by cholesterol enrichment (Figure 5.1), whilst propofol modulation of [^{3}H]-muscimol binding was depressed upon cholesterol enrichment (Figure 5.3) due to a decrease in B_{max}. This is in agreement with [^{3}H]-FNZ binding experiments in the previous chapters, where cold FNZ displacement of [^{3}H]-FNZ was unaffected by cholesterol enrichment (Figure 3.1), but propofol modulation of [^{3}H]-FNZ binding was depressed (Figure 4.4). These results tend to support the view that the coupling between the modulatory sites is affected by cholesterol enrichment, while displacement of the ligand is not. The general affect of cholesterol enrichment thus seems to be an alteration of the coupling between modulatory sites upon the $GABA_A$ receptor with no affect on the affinity of the radiolabel.

The poor cholesterol enrichment of the Harlan U.K. whole brain membranes used in the pregnanolone, propofol and picrotoxinin modulation of [^{3}H]-muscimol binding experiments, may have contributed to the reason why no effect of cholesterol was seen upon picrotoxinin or pregnanolone modulation of [^{3}H]-muscimol binding. With a higher cholesterol enrichment of in-house whole brain membranes, a reduction in pregnanolone's potentiation of [^{3}H]-FNZ binding has been seen (Figure 3.3). If a greater cholesterol

enrichment had been achieved in the Harlan U.K. whole brain membranes the effect of cholesterol on the [^{3}H]-muscimol binding studies may have been greater with a significant difference in the modulation of binding by pregnanolone or picrotoxinin compared to that in unenriched membranes. To investigate this possibility the pregnanolone and picrotoxinin modulation of [^{3}H]-muscimol experiments should be repeated in in-house enriched and unenriched whole brain membranes.

Using [^{3}H]-muscimol as the radioligand presents difficulties in interpreting the results for the following reasons. (i) GABA binds to high, low and very low affinity binding sites with K_d values in the low and high nanomolar range (Sieghart, 1995) which show similar drug specificity (Olsen *et al.*, 1984). To simplify this binding only 1 nM of [^{3}H]-muscimol was included in the binding assay so that binding was limited to the high affinity sites. (ii) The 'buffy coat' membrane preparation removes endogenous synaptic vesicles leaving synaptic membrane fragments in which the GABA carrier protein still remains. [^{3}H]-muscimol will bind to the carrier protein but not be transported. To prevent binding to the carrier protein a Na^+ free buffer is required (Olsen, 1982; Schumacher & McEwen, 1989). This precaution was not taken in the current experiments with the likely consequence that [^{3}H]-muscimol bound to both the GABA carrier protein and the high affinity GABA binding sites on the $GABA_A$ receptor. (iii) Cholesterol interacts specifically with the GABA carrier protein and stimulates GABA transport after the carrier has been reconstituted into phospholipid vesicles in a concentration dependent manner (Shouffani & Kanner, 1990). Enrichment of synaptic membranes with cholesterol is therefore likely to decrease [^{3}H]-muscimol binding to the carrier protein since

endogenous cholesterol is probably at sufficient levels to maintain activity of the protein and above 64 % mol cholesterol a decrease in its activity has been seen (Shouffani & Kanner, 1990). (iv) Propofol inhibits the specific high affinity GABA uptake process at clinically relevant concentrations (Mantz *et al.*, 1995). Therefore the depression in propofols modulation of [^{3}H]-muscimol upon cholesterol enrichment (Figure 5.3) may be due to a decrease in binding to the $GABA_A$ receptor caused by enrichment and a decrease in the binding to the GABA carrier protein caused by propofol. (v) A further problem is that [^{3}H]-muscimol will photolabel the GABA binding site. To prevent this irreversible binding the studies were carried out in the dark (Goodnough & Hawkinson, 1995). With the possible sources of error mentioned above whilst carrying out [^{3}H]-muscimol binding it becomes evident why care has to be taken when interpreting results.

The effect of cholesterol enrichment on the modulatory actions of propofol upon [^{3}H]-muscimol binding, may contribute to the hypothesis that cholesterol has a general effect upon the coupling between allosteric binding sites which was raised in Chapter 4. The effect of cholesterol upon coupling between modulatory sites may also be dependent on the degree of cholesterol enrichment which should be further investigated.

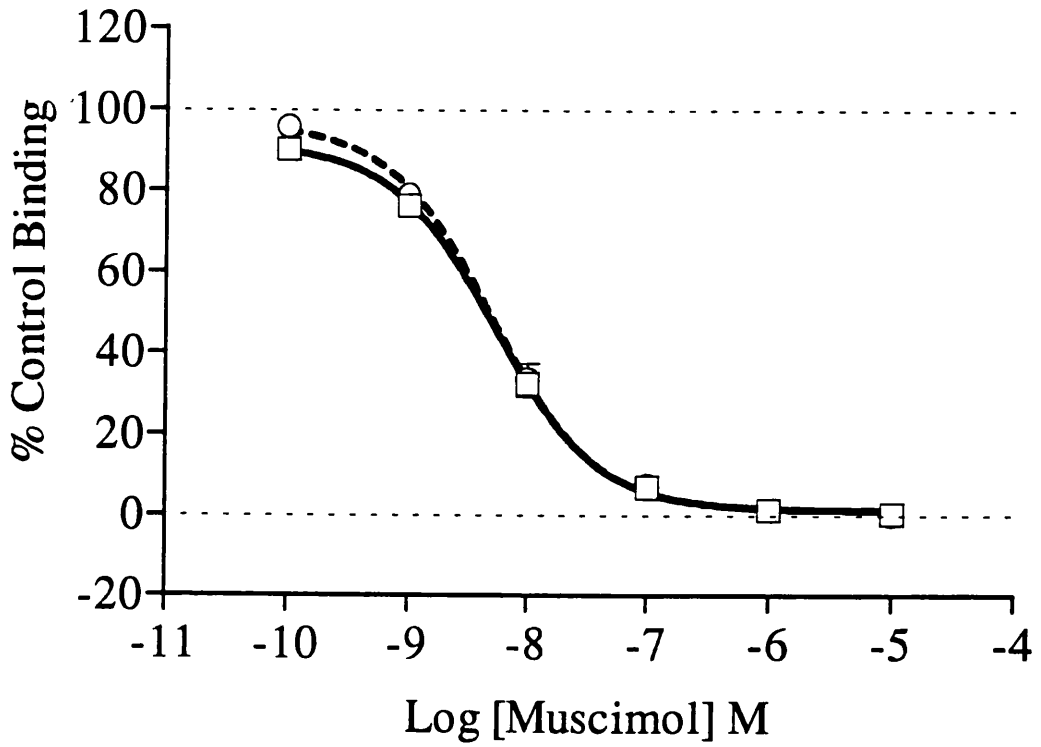

Figure 5.1 [^{3}H]-muscimol displacement by unlabelled muscimol in cholesterol enriched (squares) and unenriched (circles) whole brain membranes from in-house. No significant difference was seen between [^{3}H]-muscimol control binding of enriched (296.09 ± 48.09 fmol.mg^{-1} of protein) or unenriched membranes (230.76 ± 41.29 fmol.mg^{-1} of protein). The K_d of [^{3}H]-muscimol binding was 4.27 nM and 3.96 nM in cholesterol enriched and unenriched membranes, respectively. This would mean that with an assay concentration of 1 nM [^{3}H]-muscimol the likely occupancy of the binding sites would be 19 % and 20 % for the cholesterol enriched and unenriched membranes, respectively. The cholesterol content was significantly higher in the cholesterol enriched membranes (4.373 ± 0.09 µmol cholesterol mg^{-1} protein) compared to the unenriched (2.759 ± 0.08 µmol cholesterol mg^{-1} protein) (n = 3, P = 0.0002). Data are the means of 5 experiments ± s.e.mean carried out on a single batch of membranes. Were no error bars appear the error bar lies within the symbol.

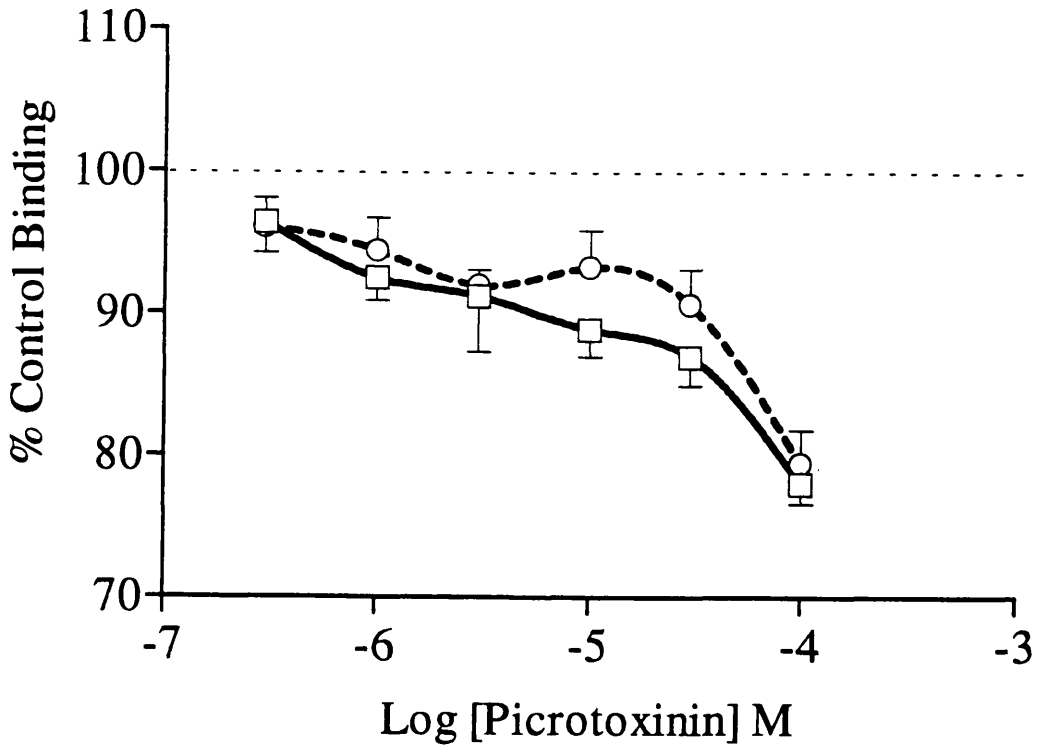

Figure 5.2 Modulation of [^{3}H]-muscimol binding by picrotoxinin in cholesterol enriched (squares) and unenriched (circles) whole brain membranes from Harlan U.K. The cholesterol content was significantly higher in the cholesterol enriched membranes (3.469 ± 0.14 µmol cholesterol mg^{-1} protein) compared to the unenriched (2.658 ± 0.17 µmol cholesterol mg^{-1} protein) (n = 3, P = 0.0218). Data are the means of 5 experiments ± s.e.mean carried out on a single batch of membranes.

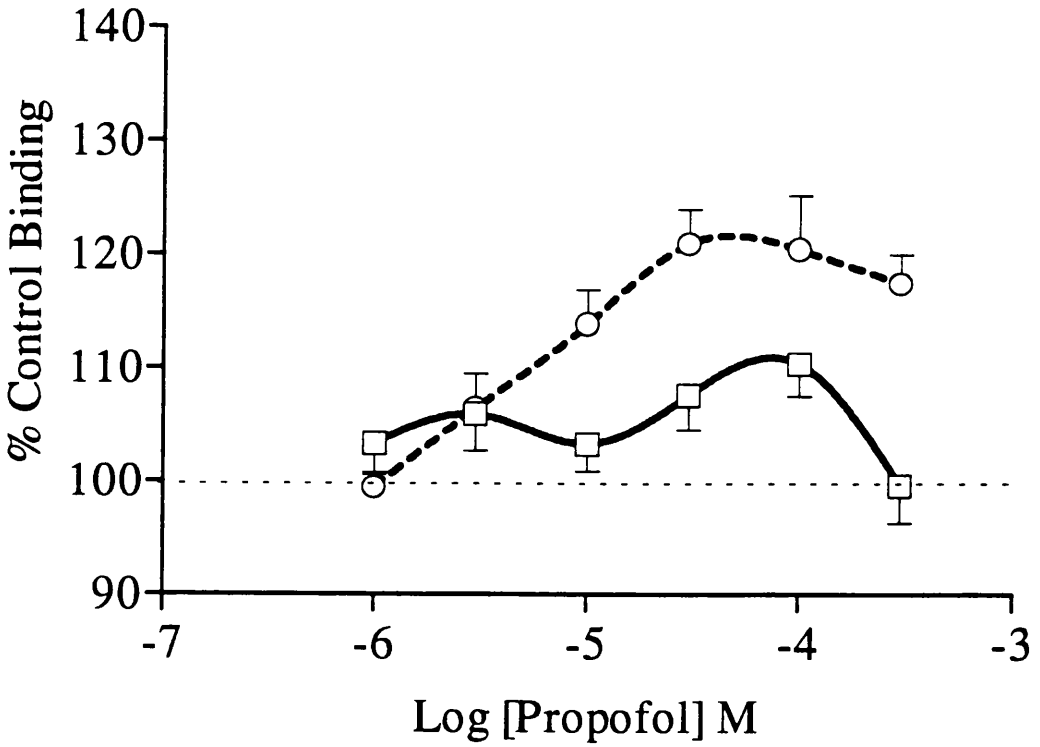

Figure 5.3 Potentiation of [^{3}H]-muscimol binding by propofol in cholesterol enriched (squares) and unenriched (circles) whole brain membranes from Harlan U.K. The cholesterol content was significantly higher in the cholesterol enriched membranes (3.469 ± 0.14 µmol cholesterol mg^{-1} protein) compared to the unenriched (2.658 ± 0.17 µmol cholesterol mg^{-1} protein) (n = 3, P = 0.0218). Data are the means of 5 experiments ± s.e.mean carried out on a single batch of membranes. The 0.1% (v/v) acetone used as the vehicle for propofol had no effect upon [^{3}H]-muscimol binding. Comparing the two curves by two way ANOVA showed a significant difference between the binding in the cholesterol enriched and unenriched membranes (P < 0.0001).

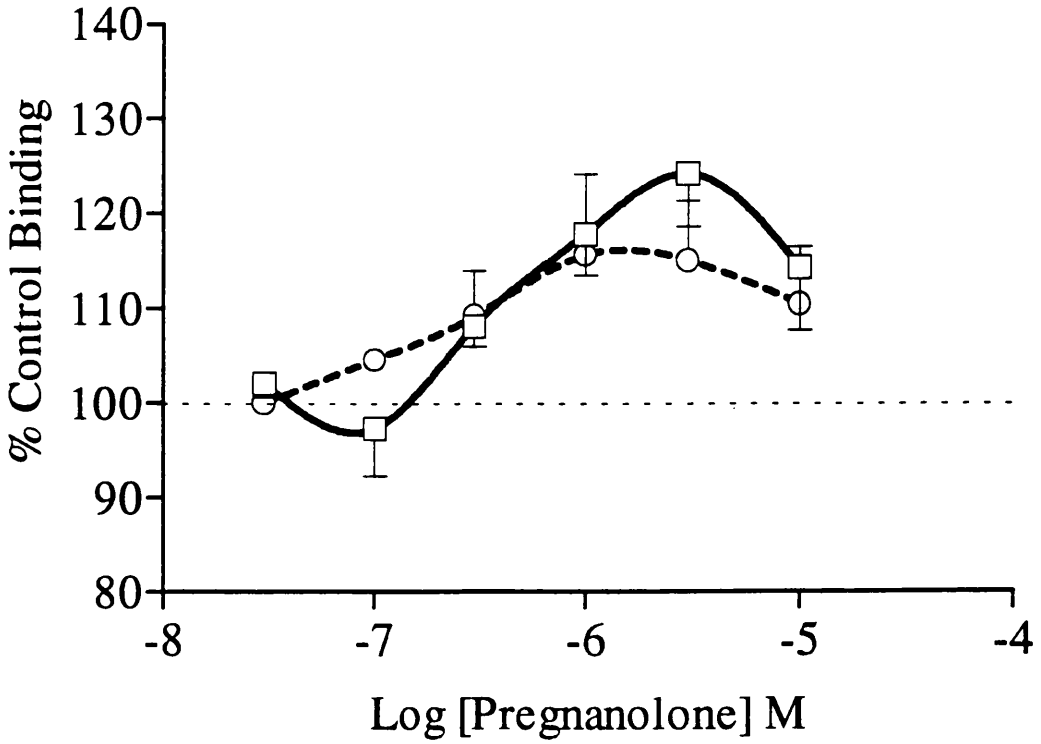

Figure 5.4 Potentiation of [^{3}H]-muscimol binding by pregnanolone in cholesterol enriched (squares) and unenriched (circles) whole brain membranes from Harlan U.K. The cholesterol content was significantly higher in the cholesterol enriched membranes (3.469 ± 0.14 µmol cholesterol mg^{-1} protein) compared to the unenriched (2.658 ± 0.17 µmol cholesterol mg^{-1} protein) (n = 3, P = 0.0218). Data are the means of 5 experiments ± s.e.mean carried out on a single batch of membranes. The 0.45% (v/v) DMSO used as the vehicle for pregnanolone had no effect upon [^{3}H]-muscimol binding.

Chapter 6

The Influence of Cholesterol Enrichment upon the Modulation of [^{3}H]-TBOB Binding

Chapter 6

The influence of cholesterol enrichment upon the modulation of [^{3}H]-TBOB binding

6.1 Introduction

Cage convulsants such as TBOB (t-butylbicycloorthobenzoate) and TBPS (t-butyl-bicyclophosphorothionate) as well as the plant convulsant picrotoxin act at a distinct binding site closely associated with the Cl^- ion channel of the $GABA_A$ receptor (Sieghart, 1992). These compounds block GABA evoked Cl^- currents by preventing conformational changes that occur at the ion channel to form an open Cl^- ionophore upon GABA binding (Macdonald & Olsen, 1994). At the single-channel level picrotoxin reduces the average open and burst duration (Twyman *et al.*, 1989).

Radioligands of the above compounds with high affinity have been developed allowing direct study of this site. Similar to the GABA and benzodiazepine binding sites the picrotoxin site is modulated allosterically by compounds acting at other modulatory sites. Modulation by muscimol (Im *et al.*, 1994), diazepam (Liljequist & Tabakoff, 1993) or alphaxalone (Sapp *et al.*, 1992; Turner *et al*, 1989) of [^{35}S]-TBPS binding has been shown to be biphasic in nature by potentiating binding at low and inhibiting this binding at higher concentrations. By contrast, propofol showed a monophasic inhibition of [^{35}S]-TBPS binding (Concas *et al.*, 1991) with increasing concentrations. In the present study the effect of cholesterol enrichment has been investigated on the coupling of the

modulatory sites for FNZ, muscimol, pregnanolone and propofol to the [^{3}H]-TBOB binding site.

6.2 Methods

6.2.1 Membrane preparation

Membranes were prepared from the whole brain of male Wistar rats as in Chapter 2 (section 2.2.1). In some binding studies, the membranes were used without being subjected to the procedure for cholesterol enrichment. Such membranes were designated as 'unconditioned' membranes.

6.2.2 Liposome preparation

Phosphatidylcholine (PC) and cholesterol liposomes (0.75 mg of each per ml of buffer added) were prepared as in Chapter 2 (section 2.2.3).

6.2.3 Cholesterol enrichment of synaptic membranes

Cholesterol enrichment of rat whole brain membranes was carried out as set out in Chapter 2 (section 2.2.4).

6.2.4 Binding experiments

On the day of assay the membranes were thawed and centrifuged at 48400 g for 20 min at 4°C and the pellets resuspended in assay buffer to give a final protein concentration of about 4 mg ml^{-1}. Aliquots of membranes (100 µl) were pre-incubated with picrotoxinin

1 nM-0.1 mM, FNZ 0.1 nM-10μM, muscimol 0.1 nM-10 μM, pregnanolone 30 nM-10 μM or propofol 0.3 μM-3 mM in assay buffer at room temperature for 10 min. [^{3}H]-TBOB was added to give a 9 nM concentration in a final volume of 0.5 ml and the samples were then incubated at room temperature for 30 min or 180 min (see legend of figures for incubation time applicable). The reaction was terminated with addition of 2 ml ice cold wash buffer, followed by rapid filtration through Whatman GF-C filters using a Brandell Cell Harvester. The filter mat was washed a further four times with 2 ml wash buffer and the radioactivity determined by standard liquid scintillation techniques (Beckman 5801). Non-specific binding was determined by use of 100 μM picrotoxinin and was in the region of 10-30%. FNZ was dissolved initially in ethanol, pregnanolone was dissolved in dimethylsulphoxide (DMSO) and propofol was dissolved in acetone. The final concentration of ethanol was less than 0.4%, DMSO was 0.45% and acetone was 0.1%. DMSO was present in all assay tubes for experiments using pregnanolone and acetone for experiments using propofol.

In experiments to determine if endogenous GABA leads to an increase of [^{3}H]-TBOB binding when incubated with FNZ, unconditioned membranes were incubated with or without 100 μM bicuculline.

All chemicals and drugs were obtained from Sigma with the exception of [^{3}H]-TBOB (25.0 Ci mmol^{-1} specific activity) which was obtained from Amersham International. Data were fitted using the Inplot package Prism (Graphpad Software).

Statistical comparisons were made by Student's *t* test for independent samples and by ANOVA.

6.3 Results

6.3.1 Influence of cholesterol enrichment upon picrotoxinin displacement of [^{3}H]-TBOB

To determine the influence of cholesterol enrichment upon [^{3}H]-TBOB binding, [^{3}H]-TBOB displacement by picrotoxinin (1 nM-0.1 mM) was measured in rat whole brain membranes. The effect of picrotoxinin was unchanged by cholesterol enrichment (Figure 6.1), but care should be taken with this interpretation since only a low enrichment was achieved.

6.3.2 Influence of cholesterol enrichment upon FNZ modulation of [^{3}H]-TBOB binding

The preceding result allowed an investigation of the influence cholesterol enrichment has upon the modulation of [^{3}H]-TBOB binding by FNZ. These experiments were carried out on rat whole brain membranes. In unenriched membranes a threshold concentration of 10 nM FNZ was required for enhancing [^{3}H]-TBOB binding. This enhancement increased upto 0.1 μM in a dose dependent manner. Above this concentration the enhancement of [^{3}H]-TBOB binding declined (Figure 6.2). In comparison, data for cholesterol enriched membranes showed less enhancement of [^{3}H]-TBOB binding at 0.1 μM FNZ and above.

Since it is clear from the literature that GABA influences [^{3}H]-TBOB binding it was material to determine if the FNZ modulation of [^{3}H]-TBOB binding was GABA

dependent. Unconditioned membranes were subjected to the same binding experiment conditions, however, in the presence or absence of 100 μM bicuculline. It was predicted that if endogenous GABA did cause the enhancement of [^{3}H]-TBOB binding this would be blocked by 100μM bicuculline. Alternatively, if bicuculline did not block the enhanced [^{3}H]-TBOB binding then this was due to the modulation by FNZ alone. Upon testing these predictions experimentally the results obtained were as shown in Figure 6.3. Again the threshold concentration for enhancement of [^{3}H]-TBOB binding was 10 nM FNZ. The enhancement increased with FNZ concentration upto 0.1 μM and then declined at higher concentrations as before. Addition of 100 μM bicuculline increased the enhancement of [^{3}H]-TBOB binding indicating that the enhancement of binding is due to the modulation by FNZ, and that, in the absence of bicuculline there was a GABA dependent component which contributed a reduction in [^{3}H]-TBOB binding.

6.3.3 Influence of cholesterol enrichment upon muscimol modulation of [^{3}H]-TBOB binding

The influence of cholesterol enrichment upon muscimol modulation of [^{3}H]-TBOB binding in rat whole brain membranes was investigated. In unenriched membranes a threshold concentration of 10 nM muscimol enhanced [^{3}H]-TBOB binding (Figure 6.4). This enhancement increased dose dependently upto 0.1 μM after which higher concentrations of muscimol caused a depression in the binding. In cholesterol-enriched

membranes, the enhancement in binding was much reduced leaving a more marked depression in the binding due to muscimol.

6.3.4 Influence of cholesterol enrichment upon pregnanolone modulation of [^{3}H]-TBOB binding

The effect of cholesterol enrichment upon pregnanolone modulation of [^{3}H]-TBOB binding was investigated in rat whole brain membranes. Unenriched membranes showed a threshold concentration of 0.1 μM pregnanolone for enhancing [^{3}H]-TBOB binding (Figure 6.5). This enhancement increased in a simple dose dependent manner at all concentrations studied. A similar curve was gained for the cholesterol enriched membranes, except that [^{3}H]-TBOB binding was less enhanced at a concentration of 1 μM pregnanolone and above.

6.3.5 The influence of cholesterol enrichment upon propofol modulation of [^{3}H]-TBOB binding

Propofol modulation of [^{3}H]-TBOB binding was studied in cholesterol enriched and unenriched rat whole brain membranes. In unenriched membranes the threshold concentration of propofol to enhance [^{3}H]-TBOB binding was 10 μM (Figure 6.6). The maximum enhancement of binding was at a concentration of 30 μM propofol, above which a depression in the [^{3}H]-TBOB binding was observed. Displacement of non-specific binding occurred at concentrations of 1 and 3 mM propofol. In cholesterol enriched

membranes a similar concentration profile was seen, except a small reduction in [^{3}H]-TBOB binding occurred at propofol concentrations of 10 and 30 μM compared to the unenriched membranes.

6.4 Discussion

The allosteric modulation of [^{3}H]-TBOB binding by $GABA_A$ receptor active ligands provides a convenient method to study the effect of cholesterol enrichment upon the coupling of these allosteric sites. This coupling maybe different to that for the benzodiazepine and GABA sites since their binding sites protrude in to the extracellular space. However, [^{3}H]-TBOB is believed to bind at a site on or near to the Cl^- ionophore, and thus its affinity will be sensitive to conformational changes in the channel (Squires *et al.*, 1983; Gee *et al.*, 1986). Cholesterol enrichment though had no influence on [^{3}H]-TBOB binding (Figure 6.1) allowing the present study to look at the effects of cholesterol enrichment of rat whole brain membranes upon FNZ, muscimol, pregnanolone and propofol modulation of [^{3}H]-TBOB binding. In all these experiments cholesterol enrichment altered the coupling of the allosteric sites, showing a reduction in the modulation of the [^{3}H]-TBOB binding even with poor cholesterol enrichment of the Harlan U.K. whole brain membranes (Figures 6.2, 6.4, 6.5 and 6.6). The nature of this reduction in the modulation of [^{3}H]-TBOB binding by FNZ, muscimol or propofol after cholesterol enrichment is as yet undefined. Cholesterol enrichment of the whole brain membranes may reduce the modulation of the modulator, cause a reduction in the enhanced binding or increase the depression of the binding. At present we have no

evidence to distinguish between any of the above scenarios therefore requiring further investigation to resolve this issue.

The unidirectional effect of cholesterol upon the coupling of the allosteric sites with the picrotoxinin site is in contrast to the coupling with the benzodiazepine site where the effects were bidirectional and the coupling with the GABA site where an effect was seen only with propofol (Table 7.1). The effect of cholesterol enrichment upon the coupling between the modulatory sites and various ligands thus seems to differ. The [^{3}H]-TBOB coupling experiment results presented in this chapter also give further evidence for cholesterol enrichment affecting the coupling between modulatory sites at the $GABA_A$ channel protein.

The displacement of non-specific as well as specific [^{3}H]-TBOB binding by 1 and 3 mM (Figure 6.6) propofol may indicate that propofol depresses the non-specific binding at these concentrations. To investigate this possibility the affect of propofol upon picrotoxinin blockage of [^{3}H]-TBOB binding should be determined. If propofol does alter the non-specific binding, a strong case for cholesterol enhancing the depression of [^{3}H]-TBOB binding cannot be made. An alternative explanation is that cholesterol enrichment may alter the non-specific [^{3}H]-TBOB binding resulting in an enhanced depression of the specific [^{3}H]-TBOB binding, but this will require further investigation.

When carrying out [^{3}H]-TBOB binding care must be taken in preparing the membranes. The membrane preparation must include a freeze thaw step followed by thorough washing of the membranes in order to remove endogenous GABA as in this study (see membrane preparation in methods section 2.2.1). Any GABA present will

complicate the binding study since it can itself reduce [^{35}S]-TBPS binding in a simple concentration dependent manner. Such a complication has been seen in the presence of endogenous or exogenously applied GABA which causes a simple monophasic reduction in [^{35}S]-TBPS binding when modulated by diazepam (Gee & Lan, 1991), muscimol (Gee, 1988; Ito & Ho, 1994; Maksay & Ticku, 1985; Maksay & Simonyi, 1986; Squires & Saederup, 1987), pregnanolone (Harrison *et al.*, 1987), or propofol (Concas *et al.*, 1991). Even blocking the GABA site with the antagonist bicuculline complicates matters since this will enhance [^{35}S]-TBPS binding (Simmonds, 1991). Such an enhancement of [^{3}H]-TBOB binding in the presence of 100 μM bicuculline when modulated by FNZ has been shown in the results (Figure 6.3). This experiment also demonstrated that the increase in [^{3}H]-TBOB binding was due to FNZ and not endogenous GABA. In the absence of endogenous or exogenously applied GABA biphasic modulation of [^{35}S]-TBPS or [^{3}H]-TBOB (in these results) by FNZ (Lijequist & Tabakoff, 1993; Im *et al.*, 1994) (Figure 6.2), muscimol (Im *et al.*, 1994) (Figure 6.4), alphaxalone (Turner *et al.*, 1989) or propofol (Figure 6.6) is seen. The absence of a depression in [^{3}H]-TBOB binding by pregnanolone (Figure 6.5) which was seen with alphaxalone modulation of [^{35}S]-TBPS binding (Turner *et al.*, 1989) maybe due to pregnanolone being less potent than alphaxalone so the threshold concentration for depression was not reached.

Previously questions have been asked about the enhancement of [^{35}S]-TBPS binding by the allosteric modulation by FNZ, muscimol, neurosteroids or propofol. The association rate of [^{35}S]-TBPS binding is rather slow and it was thought that compounds that enhance the action of GABA at the $GABA_A$ receptor may accelerate the approach to

equilibrium (Maksay & Simonyi, 1986). The slowness of this binding may be due to the convulsant binding site being localized at the Cl^- channel (Inoue & Akaike, 1988) making the site not readily accessible. Addition of compounds that alter the conformation of the $GABA_A$ receptor to an open state would facilitate access of [^{35}S]-TBPS to its binding site and enhance the association rate. Such a transient enhancement of [^{35}S]-TBPS (and therefore [^{3}H]-TBOB) binding by $GABA_A$ receptor positive allosteric compounds maybe found if the incubation period is not long enough to reach binding equilibrium. This may lead to incorrect interpretations of binding results (Sieghart, 1995). Such incorrect interpretations may manifest themselves as an enhancement of [^{3}H]-TBOB binding as in this study. However, this is unlikely because the incubation time for pregnanolone and propofol modulation of [^{3}H]-TBOB binding was 180 min which should allow binding equilibrium to be reached. The other incubation time for FNZ and muscimol modulation of [^{3}H]-TBOB binding was 30 mins. The results from these experiments do not appear to be incorrect interpretations though, because similar results in our laboratory have been gained with periods of 180 min incubation at room temperature upon membranes that have not been cholesterol enriched but extensively washed (Aniszewski & Simmonds, unpublished observations).

The influence of cholesterol enrichment upon the allosteric modulation of [^{3}H]-TBOB binding shows a comprehensive reduced enhancement by all the modulators tested which was not seen with the other ligands studied (Chapters 3, 4 and 5). This may indicate the differing coupling of the convulsant site with the allosteric agonists of the $GABA_A$ receptor compared to that between the GABA and benzodiazepine sites. A

further study should be made of the effect of cholesterol enrichment upon pregnanolone, muscimol, propofol or FNZ modulation of [^{3}H]-TBOB binding in specific CNS regions due to the differential effects upon pregnanolone modulation of [^{3}H]-FNZ binding in the cerebral cortex, cerebellum and spinal cord (Chapter 3).

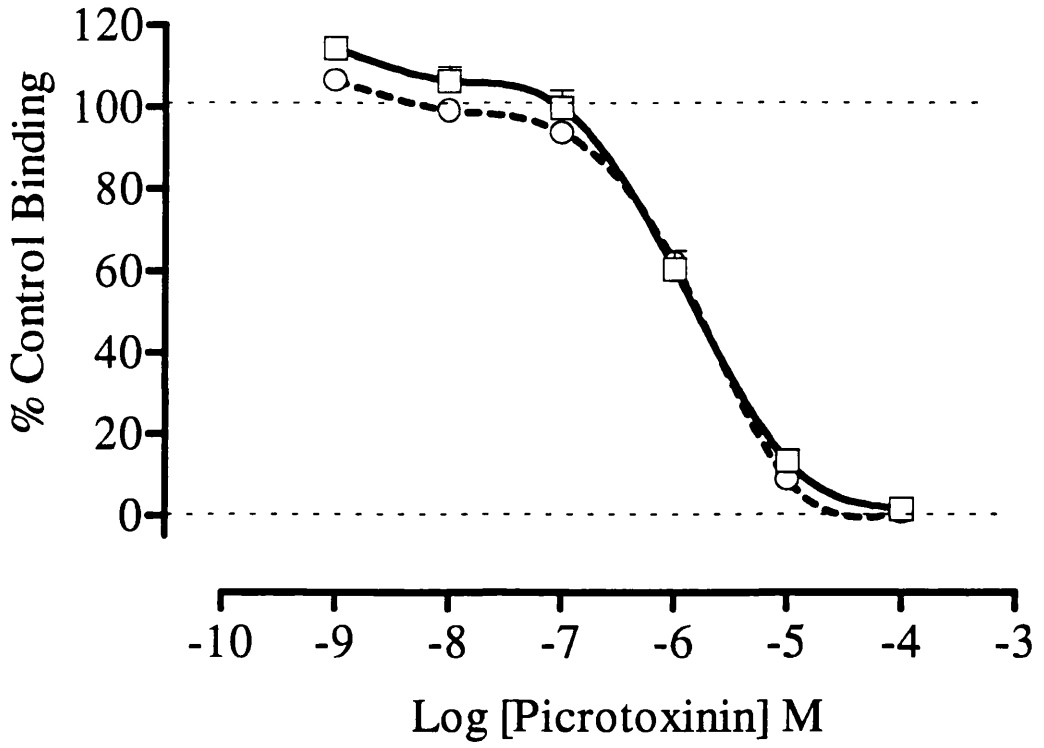

Figure 6.1 [^{3}H]-TBOB displacement by picrotoxinin in cholesterol enriched (squares) and unenriched (circles) whole brain membranes from Harlan U.K. No significant difference was seen between [^{3}H]-TBOB control binding in enriched (54.88 ± 10.98 fmol.mg^{-1} of protein) and unenriched membranes (105.00 ± 20.44 fmol.mg^{-1} of protein) after a 30 min assay incubation. The K_d of [^{3}H]-TBOB binding is approximately 30 nM (Rabow *et al.*, 1995) which means that with an assay concentration of 9 nM label the likely occupancy of the binding sites would be 23%. The cholesterol content was 4.475 ± 0.24 and 3.802 ± 0.23 µmol cholesterol mg^{-1} protein for cholesterol enriched and unenriched membranes, respectively. This difference in cholesterol content though was not significantly different. Data are the means of 3 experiments ± s.e.mean carried out on a single membrane batch. Where no error bar appears the error lies within the symbol.

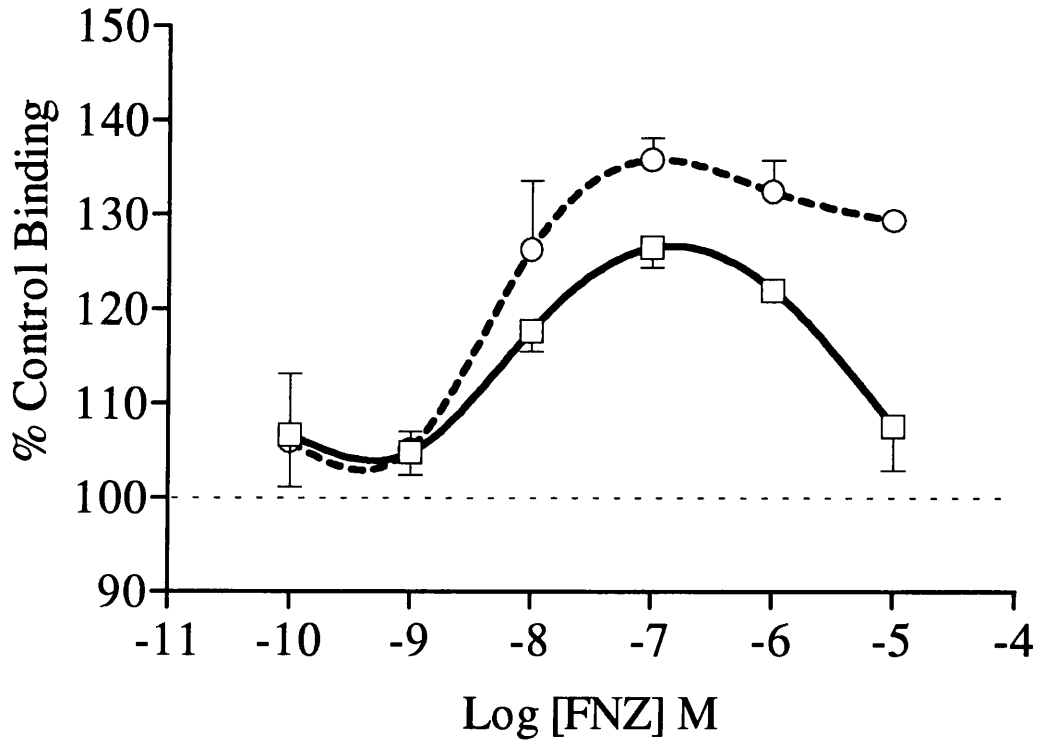

Figure 6.2 Modulation of [^{3}H]-TBOB by FNZ in cholesterol enriched (squares) and unenriched (circles) whole brain membranes from Harlan U.K. [^{3}H]-TBOB was incubated with the membranes for 30 min. The cholesterol content was 3.759 ± 0.34 and 2.796 ± 0.15 μmol cholesterol mg^{-1} protein for cholesterol enriched and unenriched membranes, respectively. This difference in cholesterol content though was not significantly different. Data are the means of 3 experiments ± s.e.mean carried out on a single batch of membranes. The 0.4 % (v/v) ethanol used as the vehicle for FNZ had no effect upon [^{3}H]-TBOB binding. Comparing the two curves by two way ANOVA showed a significant difference between the binding in the cholesterol enriched and unenriched membranes (P = 0.0017).

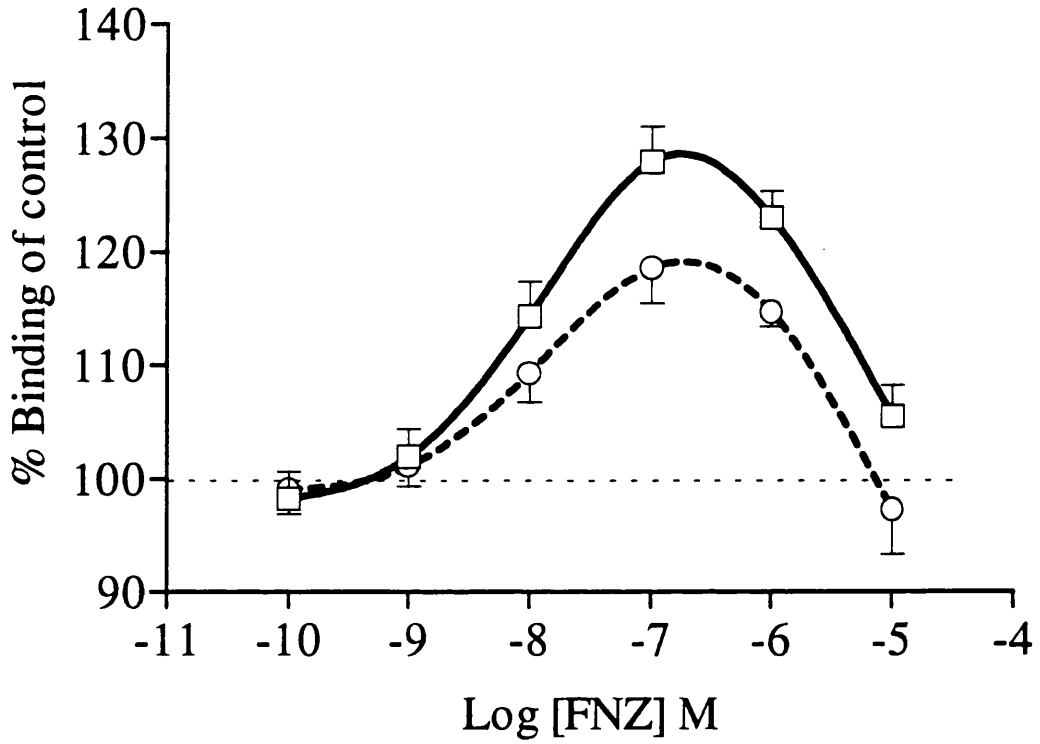

Figure 6.3 Modulation of [^{3}H]-TBOB binding by FNZ in the presence (squares) or absence (circles) of 100 μM bicuculline in 'unconditioned' whole brain membranes from Harlan U.K. [^{3}H]-TBOB was incubated with the membranes for 30 min. Data are the means of 6 experiments ± s.e.mean. Comparing the curves by two way ANOVA showed a significant difference between in the presence or absence of 100 μM bicuculline (P=0.0005).

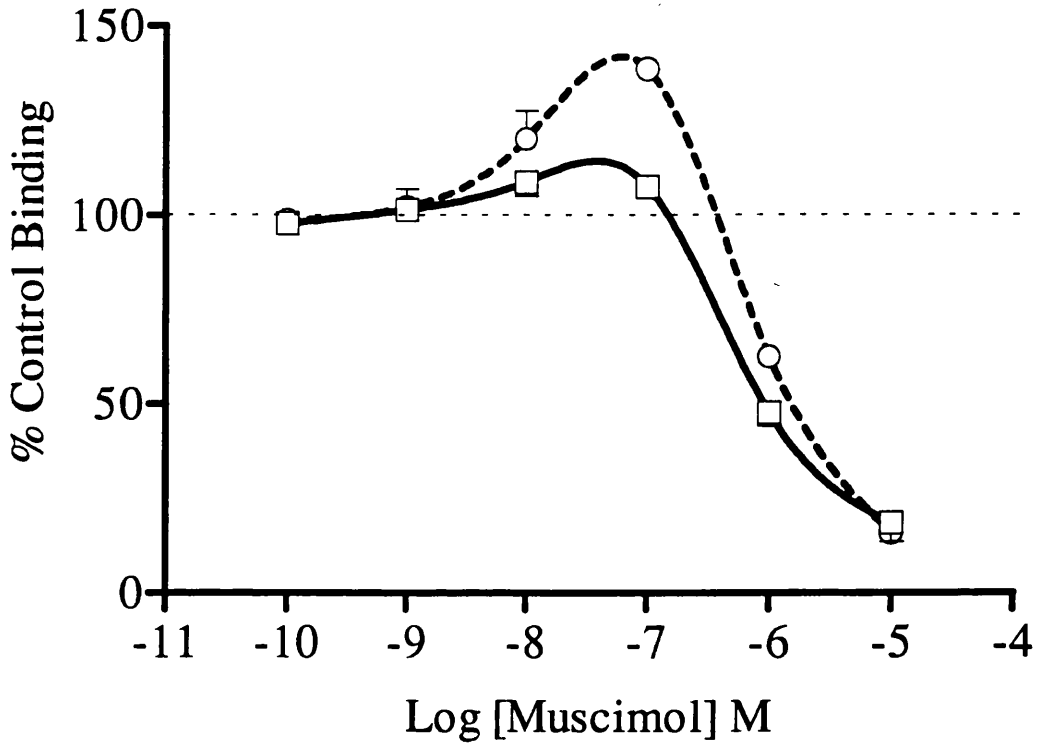

Figure 6.4 Modulation of [^{3}H]-TBOB binding by muscimol in cholesterol enriched (squares) and unenriched (circles) whole brain membranes from Harlan U.K. [^{3}H]-TBOB was incubated with the membranes for 30 min. The cholesterol content was significantly higher in the cholesterol enriched membranes (3.253 ± 0.08 µmol cholesterol mg^{-1} protein) compared to the unenriched (1.845 ± 0.05 µmol cholesterol mg^{-1} protein) (n = 3, P = 0.0001). Data are the means of 3 experiments ± s.e.mean carried out on a single batch of membranes. Comparing the two curves by two way ANOVA showed a significant difference between the binding in the cholesterol enriched and unenriched membranes (P = 0.0001). Where no error bars are shown the range of error is within the symbol.

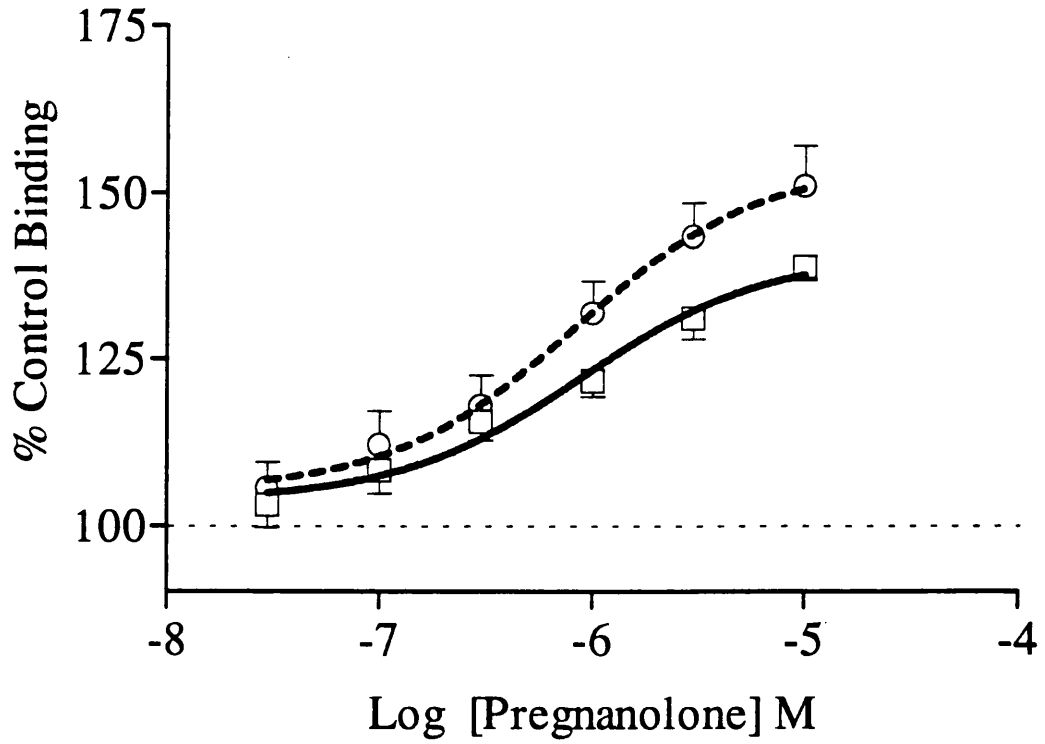

Figure 6.5 Modulation of [^{3}H]-TBOB binding by pregnanolone in cholesterol enriched (squares) and unenriched (circles) whole brain membranes from Harlan U.K. [^{3}H]-TBOB was incubated with the membranes for 180 min. This length of incubation though had no effect upon the membrane cholesterol content. The cholesterol content was significantly higher in the cholesterol enriched membranes (5.405 ± 0.19 µmol cholesterol mg^{-1} protein) compared to the unenriched (4.127 ± 0.18 µmol cholesterol mg^{-1} protein) (n = 3, P = 0.0079). Data are the means of 3 experiments ± s.e.mean carried out on a single batch of membranes. The 0.45 % (v/v) DMSO used as the vehicle for pregnanolone had no effect upon [^{3}H]-TBOB binding. Comparing the two curves by two way ANOVA showed a significant difference between the binding in the cholesterol enriched and unenriched membranes (P = 0.0056).

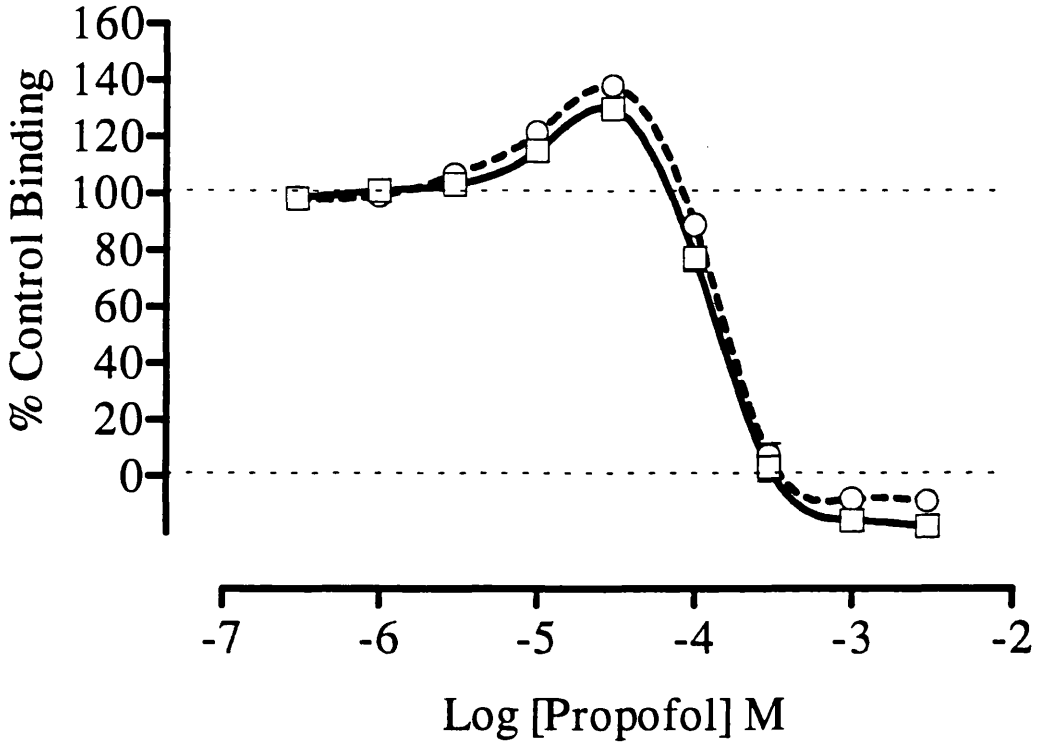

Figure 6.6 Modulation of [^{3}H]-TBOB binding by propofol in cholesterol enriched (squares) and unenriched (circles) whole brain membranes from Harlan U.K. [^{3}H]-TBOB was incubated with the membranes for 180 min. This length of incubation though had no effect upon the membrane cholesterol content. The cholesterol content was significantly higher in the cholesterol enriched membranes (3.283 ± 0.07 µmol cholesterol mg^{-1} protein) compared to the unenriched (2.128 ± 0.02 µmol cholesterol mg^{-1} protein) (n = 3, P = 0.0079). Data are the means of 3 experiments ± s.e.mean carried out on a single batch of membranes. The 0.1 % (v/v) acetone used as the vehicle for propofol had no effect upon [^{3}H]-TBOB binding. Comparing the two curves by two way ANOVA showed a significant difference between the binding in the cholesterol enriched and unenriched membranes (P < 0.0001).

Chapter 7

General Discussion

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General Discussion

7.1 Nature of cholesterol enrichment

Synaptic membranes have been shown to be easily enriched with cholesterol by spontaneous cholesterol transfer (Chapter 2). The site of this enrichment needs to be known in order to understand the effects of cholesterol enrichment upon the $GABA_A$ receptor. The likely sites of cholesterol enrichment are at the bulk fluid lipids (which will alter the viscosity of the lipid bilayer) (Shinitzy & Inbar, 1976), at specific domains of the membrane (leading to cholesterol rich domains), at the lipid-protein interface of the $GABA_A$ receptor and at hydrophobic pockets within the protein (Benga, 1985). If cholesterol interacts with the $GABA_A$ receptor protein, the level of cholesterol in unenriched synaptic membranes maybe insufficient to allow full saturation of the interface and hydrophobic pocket sites. Upon cholesterol enrichment the exogenous cholesterol would more fully saturate these sites. This could account for the altered coupling between the allosteric sites which was seen in the results chapters. Low levels of endogenous cholesterol in unenriched membranes may result from the loss of a labile component during membrane preparation. Such a labile component of the endogenous cholesterol may be possible since exogenous cholesterol has been found to be labile if enriched membranes are incubated in the presence of 1% BSA or are washed by centrifugation (Chapter 2).

Using radiolabelled cholesterol for enrichment, exogenous cholesterol was seen to mix entirely with the endogenous pool in the membranes, but this gives no indication of the sites that were enriched from the possibilities outlined above. The site of enrichment will therefore require further investigation to determine the mechanism of cholesterol's effect upon the $GABA_A$ receptor.

7.2 Site of action of cholesterol

From the alternatives above, the likely site of action of cholesterol upon the $GABA_A$ receptor following cholesterol enrichment is upon the protein channel itself at the interstices between the subunits. This site is likely because there is evidence that such binding sites occur at the nAChR (Jones & McNamee, 1988) which belongs to the same superfamily of ligand gated ion channels as the $GABA_A$ receptor and the subunits of both receptors show homology to one another throughout their length (Schofield *et al.*, 1987). Evidence for such an interaction of cholesterol with the $GABA_A$ receptor may come from Bristow & Martin (1987) who showed that after solubilization and reconstitution of the receptor into liposomes the functioning of the receptor was only maintained if the cholesterol analogue cholesteryl hemisuccinate and crude brain lipids were present. This evidence may therefore indicate that the effects of cholesterol are brought about by a specific interaction with the $GABA_A$ receptor rather than an alteration of the membrane's viscosity.

It is difficult to address the situation of whether cholesterol binding sites exist at the interstices between the subunits of the $GABA_A$ receptor as they do in the nAChR since

$GABA_A$ receptors are not expressed in membranes in such large quantities. Quenching experiments with brominated cholesterol (5,6-dibromocholestan-3β-ol) would quench the fluorescent tryptophan amino acids at the cholesterol binding sites if they exist (Jones & McNamee, 1988), but the interaction of cholesterol with proteins other than the $GABA_A$ receptor might also contribute to the quenching of tryptophan fluorescence. To overcome this situation the quenching of the tryptophan fluorescence due to brominated cholesterol should be determined in a cell lines expressing and not expressing $GABA_A$ receptors. If the fluorescence was lower in the cell line expressing $GABA_A$ receptors this may indicate that the cholesterol binding sites do exist, since the cholesterol molecule does not readily interact at the annulus of integral proteins due to its rigid planar structure (Benga, 1985).

7.3 Level of cholesterol content may control $GABA_A$ receptor function

With an influence of cholesterol upon the $GABA_A$ receptor the membrane cholesterol concentration maybe an important means of controlling the coupling between the receptor's allosteric sites. Therefore, the *in vivo* cholesterol content of synaptic membranes may regulate the functioning of the $GABA_A$ receptor.

During the membrane preparation of the synaptic membranes some of the endogenous cholesterol maybe lost since the *in vitro* cholesterol content of whole brain synaptic membranes could not be depleted by incubation with sphingomyelin : PC liposomes (Chapter 2). Such a depletion has been reported possible by Phillips *et al.* (1987) using pure PC liposomes. This inability to achieve cholesterol depletion may indicate that the membrane preparation procedure has already removed a labile cholesterol

component found *in vivo* that was exerting an effect upon the receptor. Upon cholesterol enrichment the cholesterol content may then be returned to the *in vivo* levels or above. If this *in vivo* level of cholesterol within synaptic membranes is as labile as it appears there may be a mechanism by which the functioning of the $GABA_A$ receptor is controlled by the cholesterol content.

To investigate if endogenous cholesterol does regulate the $GABA_A$ receptor the cholesterol content of membranes should be altered and the influence upon the receptor at different cholesterol concentrations determined. Different cholesterol contents of the membranes could be achieved by enriching them to a certain amount by different incubation periods or alternatively, expressing the neuronal $GABA_A$ receptor in different cell systems which will differ in their cholesterol and lipid content.

7.4 Cholesterol has a general effect upon the $GABA_A$ receptor

From the results it has been seen that there is no evidence for a specific interaction of cholesterol with the neurosteroid site but rather a general effect upon the coupling between modulatory sites (Table 7.1). Also the affinity of [^{3}H]-FNZ, [^{3}H]-muscimol or [^{3}H]-TBOB for their respective binding sites is unaffected by cholesterol enrichment of whole brain membranes. These effects upon the coupling of the modulatory sites may be brought about by one or more actions of cholesterol e.g. a conformational change of the receptor and/or stabilization of the α helices of the $GABA_A$ receptor. As yet the precise mechanism for the effect of cholesterol upon the coupling of the modulatory sites has not been determined, but an influence of cholesterol upon the nAChR tertiary structure has

been seen (Butler & McNamee, 1993; Fernandez-Ballester *et al.*, 1994) which maybe analogous to the $GABA_A$ receptor.

Upon cholesterol enrichment modulation of [^{3}H]-FNZ binding by the neurosteroid pregnanolone was seen to be affected differently in different CNS regions (Table 7.1) (Chapter 3). In the cerebral cortex the potency of pregnanolone to potentiate [^{3}H]-FNZ binding was reduced, whilst in the spinal cord an increase in pregnanolone's potency was seen. Meanwhile, in the cerebellum there was no influence of cholesterol enrichment upon the modulation of [^{3}H]-FNZ binding by pregnanolone except at the threshold concentration (Figure 3.2). These different effects are presumably due to different subunit constructs of the receptor in these different CNS regions contributing to the heterogeneity of neurosteroid binding sites. To address this situation recombinant receptors should be used to see the influence of cholesterol enrichment upon different subunit constructs.

All the studies carried out so far investigating the effect of cholesterol enrichment have been carried out *in vitro*. To see if similar results can be gained in a more physiological environment electrophysiology experiments should be performed after cholesterol enrichment of intact cells.

7.5 Potential clinical relevance of this work

Lowering the levels of cholesterol in the blood stream may be important in the reduction of heart disease, but recent studies have indicated that it may also increase the likelihood of death by suicide, accident or being murdered due to an increase in aggressive behaviour (Engelberg, 1992). Men that show such behaviour have on average 7 $mg.dl^{-1}$

less cholesterol than others with an average of 213 $mg.dl^{-1}$ (Kleiner, 1995). A possible explanation of this observation could be the association between low cholesterol and psychiatric illness which was suggested as long ago as 1935 by Brice, who presented evidence that schizophrenia was associated with low concentrations of cholesterol. Further evidence in support of cholesterol's involvement in psychiatric illness is that depressed people tend to have lower cholesterol levels than normal (Kleiner, 1995). This link may therefore depend upon the lipid neurochemistry.

It is interesting to speculate on possible neurochemical links between lowered cholesterol levels and the appearance of depression causing a higher risk of suicide. If brain neuronal membrane cholesterol is lowered by cholesterol-lowering drugs this will alter the amount of cholesterol surrounding the integral proteins within the membrane. Altering this cholesterol has been shown to have an influence on the functioning of integral proteins such as the upon the activity of Ca^{2+}-ATPase (Warren *et al.*, 1975), adenylate cyclase (Whetton *et al.*, 1983), 5'-nucleotidase (Whetton & Hously, 1983), the sodium and chloride coupled γ-aminobutyric acid transporter (Shouffani & Kanner, 1990), and the binding properties of the muscarinic ACh, β-adrenergic, serotonin receptors (Berstein *et al.*, 1989) and the coupling between the allosteric binding sites of the $GABA_A$ receptor (Table 7.1). Of the above receptors only the $GABA_A$ receptor is a ligand-gated ion channel which is the site of action of anxiolytic and anxiogenic drugs which have an affect upon mood. With an influence of cholesterol upon the coupling of the allosteric sites at the $GABA_A$ receptor, low in vivo levels of membrane cholesterol content may alter the functioning of the $GABA_A$ receptor which may lead to neurological and psychiatric

disease explaining the link between low cholesterol levels and depression. However, the classic theory for depression believes that there is a change in the level of neurotransmitters within the brain with no alteration of the number of receptors. It would be difficult to determine if a change in cholesterol content leads to depression since a labile cholesterol component exists (Chapter 2). Any attempt to measure the endogenous cholesterol content of membranes would require an extraction procedure which would lead to the labile component being lost.

The level of cholesterol within the frontal and temporal regions of the brain has been seen to reach a maximum at 20 years of age and to decrease linearly with increasing age upto 80 years, above which the changes in the membrane composition become more pronounced (Svennerholm *et al.*, 1994). These areas of the brain are involved in the early stages of dementia which is the most common disorder affecting the brain (Svennerholm & Gottfries, 1994). Perhaps the decrease in neuronal membrane cholesterol levels during ageing may contribute to the onset of dementia in a similar way to the use of cholesterol lowering drugs has been linked to depression. Evidence for this line of thought comes from the fact that a 30 % decrease in the molar cholesterol : phospholipid ratio in cell membranes during ageing or age related diseases alters the coupling and affinity properties of muscarinic, D_1, D_2, α_1-Adrenergic, α_2-Adrenergic and β-Adrenergic receptors (Roth *et al.*, 1995). Furthermore, increasing the neuronal membrane cholesterol can reverse the changes in signal transduction of the mAChR that are observed in Alzheimer's disease and normal ageing (Joseph *et al.*, 1995). This therefore seems to indicate a critical role for cholesterol to maintain the functioning of CNS neurotransmitter receptors.

The effects of cholesterol upon integral proteins seems to indicate a general influence of cholesterol upon proteins embedded within the plasma membrane of synaptic membranes. If such a general effect does occur the effect of cholesterol upon other excitatory and inhibitory neurotransmitter receptors should be investigated since the role of cholesterol content within the membrane may have a clinical importance. If such a clinical importance is found a requirement to know the *in vivo* level of synaptic membrane cholesterol present becomes a necessity since it will alter the potency of drugs at their specific binding sites.

7.6 In conclusion

My thesis has shown that membrane cholesterol influences the modulatory actions of the neurosteroid pregnanolone at the $GABA_A$ receptor. This influence of cholesterol enrichment upon neurosteroid modulation is also brain region dependent. The effect of cholesterol enrichment upon modulation though is not just limited to the neurosteroid site, but also effects the modulation of the benzodiazepine, GABA and propofol sites upon the $GABA_A$ receptor.

As well as the effects of cholesterol upon the $GABA_A$ receptor presented here, other researchers have shown that cholesterol influences the functioning of other integral membrane proteins. Therefore the cholesterol molecule appears to be an important modulator of integral membrane protein function.

	Label used for binding studies					
	[^{3}H]-FNZ				[^{3}H]-MUSC	[^{3}H]-TBOB
Drug used to modulate binding	WB	COR	CER	SP	WB	WB
FLUNITRAZEPAM	-	?	?	?	-	↓
PREGNANOLONE	↓	↓	-	↑	-	↓
MUSCIMOL	↑	↑	?	↑	-	↓
PROPOFOL	↓	↓	?	↓	↓	↓
PICROTOXININ	NA	NA	NA	NA	-	-

Key:

- \- No change in binding upon cholesterol enrichment.
- ? Experiment has not been done.
- ↑ Cholesterol enrichment increases the binding.
- ↓ Cholesterol enrichment decreases the binding.
- WB Rat whole brain synaptic membranes.
- COR Rat cerebral cortex synaptic membranes.
- CER Rat cerebellum synaptic membranes.
- SP Rat spinal cord synaptic membranes.
- NA Not applicable.

Table 7.1 Summary of binding experiments showing the effect of cholesterol enrichment.

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