MOLECULAR CHARACTERIZATION OF A NON-GLYCINERGIC STRYCHNINE BINDING SITE IN THE RODENT SPINAL CORD

BY

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Dedication

To my parents: for all the years of support and encouragement they have given me.

莉莉
一九九六年七月
Acknowledgements

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This thesis is my own account of investigations carried out by myself under the supervision of Jonathan Fry.
Abstract

Treatment of rat or mouse spinal cord membranes with the arginine specific reagent 2,3-butanedione reveals strychnine binding sites which are not located on the inhibitory glycine receptor and which display a specificity for use-dependent cation channel blockers. The binding properties of these binding sites do not survive solubilisation in a variety of detergents and so cannot be purified by conventional affinity chromatography. However, as a first step towards investigating the possible function of this binding site as an ion channel, procedures have been developed for its affinity labelling and purification.

Fractionation of rat spinal cord membranes on sucrose gradients showed the 2,3-butanedione induced binding of \(^{3}\text{H}\)-strychnine to be enriched in the myelin rather than in the synaptosomal or mitochondrial fractions. Further subfractionation of myelin located this binding site to the peraxolemmal myelin, as opposed to the axolemmal or compact myelin fractions.

Previous studies had shown quinacrine to be a high affinity ligand for the 2,3-butanedione induced \(^{3}\text{H}\)-strychnine binding site and therefore quinacrine mustard has been employed as an irreversible affinity label. Incubation of crude spinal cord membranes or purified myelin with the dichloroethylamino derivative of quinacrine inactivated at least 50% of subsequent \(^{3}\text{H}\)-strychnine binding. Subsequent solubilisation of such membranes in sodium dodecylsulphate followed by SDS polyacrylamide gel electrophoresis revealed a single fluorescent band at 23 kD. Both the inactivation of subsequent \(^{3}\text{H}\)-strychnine binding and the fluorescent labelling of this peptide were reduced if membranes were treated with quinacrine mustard in the presence of strychnine.

When employed with myelin membranes, affinity labelling with quinacrine mustard identifies a fluorescent peptide on SDS-PAGE in sufficient quantities for sequencing, which reveals the 2,3-butanedione induced strychnine binding site to be located on a previously undescribed isoform of proteolipid protein.
Abbreviations

4-AP  4-aminopyridine
5-HT  5-hydroxytryptamine
Ach  acetylcholine
B    bound ligand
BD   2,3-butanedione
$B_{eq}$  the amount of ligand bound at equilibrium
$B_{max}$  maximal number of binding sites
BSA  bovine serum albumin
CBBG coomassie brilliant blue G
cDNA  complementary DNA
CHAPS 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulphonate
CHD  1,2-cyclohexanediode
CNS  central nervous system
CPM  counts per minute
CQS  camphorquinone-10-sulphate
DRG  dorsal root ganglion
DPM  disintegrations per minute
DTT  dithiothreitol
EAE  experimental autoimmune encephalomyelitis
EEG  electroencephalography
EDTA  ethylenediaminetetraacetic acid
F    concentration of free ligand
GABA $\gamma$-amino-futyric acid
GAR  goat antibody to rabbit $\gamma$-globulin
GAR-HRP  goat anti-rabbit IgG horseradish peroxidase conjugate
HEPES N-2-hydroxyethylpiperazine-N'-ethanesulphonic acid
IC$_{50}$ concentration of displacer which inhibits 50% of control binding
Kobs observed association rate constant
$K_D$  equilibrium dissociation constant
$K_{off}$  dissociation rate constant
K\textsubscript{on} association rate constant
MAG myelin associated glycoprotein
MBP myelin basic protein
NMDA N-methyl-D-aspartic acid
PAGE polyacrylamide gel electrophoresis
PAP horseradish peroxidase and rabbit anti-horseradish peroxidase
PB phosphate buffer
PBS phosphate buffered saline
PEG polyethylene glycol
PEI polyethylenimine
PG phenylglyoxal
PLP proteolipid protein
PMSF phenylmethane sulphonyl fluoride
SDS-PAGE sodium dodecyl sulphate-polyacrylamide gel electrophoresis
T thiosulphate
TEA tetraethylammonium
TEMED N,N,N',N'-tetramethylethylenediamine
TTX tetrodotoxin
QM quinacrine mustard
UV ultraviolet

**Single letter code for amino acids**

A alanine
C cysteine
D aspartic acid
E glutamic acid
F phenylalanine
G glycine
H histidine
<table>
<thead>
<tr>
<th>Symbol</th>
<th>Amino Acid</th>
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<tbody>
<tr>
<td>I</td>
<td>isoleucine</td>
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<tr>
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<td>W</td>
<td>tryptophan</td>
</tr>
<tr>
<td>Y</td>
<td>tyrosine</td>
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CHAPTER ONE

INTRODUCTION

1.1 Strychnine and the glycine receptor

Strychnine, an alkaloid from the nut of the Indian tree *Strychnos nux vomica*, has been in use as a rat poison for over four hundred years. It is a powerful convulsant, apparently acting primarily by blocking spinal inhibition in low doses (Sherrington, 1905). There has been much work presenting evidence explaining its convulsant properties by a blockade of spinal postsynaptic inhibition (Bradley *et al*., 1953; Eccles *et al*., 1954). Curtis *et al*., (1968 a,b, 1971) showed that strychnine selectively blocks the effects of glycine in spinal cord and brain stem without affecting those of γ-aminobutyric acid (GABA), noradrenaline or dopamine. These data were used as further support for the hypothesis that glycine is the natural postsynaptic inhibitory transmitter in spinal cord. The binding of [³H]-strychnine to rat spinal cord and brain stem homogenates was first described by Young and Snyder (1973). It was shown that [³H]-strychnine could bind to synaptic membrane factions isolated from the spinal cord and brain stem, and appeared to represent a specific interaction with the same postsynaptic receptor to which glycine normally bound to produce its inhibitory action. Strychnine binding was saturable and of high affinity. Later this ligand was used to investigate in detail the distribution of glycine receptors in the central nervous system by means of autoradiographic
techniques (Probst et al., 1986; Bristow et al., 1986; Glendenning and Baker, 1988). The distribution of [³H]-strychnine binding within CNS approximately parallels the distribution of endogenous glycine in CNS. [³H]-strychnine has been used as a ligand for studying the purification of the glycine receptor protein (Pfeiffer et al., 1982) and the cloning of the cDNA coding for this receptor.

The kinetics of [³H]-strychnine binding to rat spinal cord membrane homogenates have been investigated by Young and Snyder (1974a,b). Saturation experiments show a $K_D$ of 6 nM and $B_{\text{max}}$ of 1.8 pmoles/mg, which is in agreement with results obtained from mouse spinal cord membranes in our laboratory (Phelan, et al. 1989; O'Connor, et al. 1996 in press). The displacement of [³H]-strychnine binding in rat spinal cord membranes by different compounds is also comparable with results obtain in mouse spinal cord membrane from our laboratory. (Young and Snyder, 1973, 1974a; Phelan, et al. 1987).

1.2 Non-glycinergic strychnine sensitive sites

The selectivity of strychnine as a specific ligand for inhibitory glycine receptors has been questioned on the basis of its actions at sites not associated with glycine. For example, strychnine inhibits not only glycine-induced Cl⁻ currents but also γ-aminobutyric acid (GABA) induced Cl⁻ currents in a competitive manner in a concentration range between 0.1 μM and 100 μM.
(Aibara et al., 1991; Shirasaki et al., 1991). The inhibitory effects of strychnine on GABA and glycine-induced responses were studied in the rat dissociated dorsal root ganglion cells (Aibara et al., 1991) and hippocampal CA1 pyramidal neurons (Shirasaki et al., 1991) in whole-cell mode by using the conventional patch-clamp technique. Moreover, at the higher concentration, strychnine inhibits the responses of the receptors activated by acetylcholine (Phillis and York, 1967; Faber and Klee, 1974), noradrenaline (Phillis and York, 1967), 5-hydroxytryptamine (Phillis and York, 1967; Faber and Klee, 1974), histamine (Groul and Weinreich, 1979), dopamine (Faber and Klee, 1974) or N-methyl-D-aspartate (NMDA) (Bertolino and Stefano, 1988). However, these effects are only observed with high doses of strychnine, relatively low concentrations of strychnine, administered electrophoretically (5-10 nA; 2×10^{-10} mM) or i.v. (0.2 mg kg^{-1}) blocked the effects of glycine without affecting those of GABA, noradrenaline or dopamine (Certis et al., 1971).

In addition, strychnine has also been shown to interact directly with a number of cation channels. Earlier experiments had shown that strychnine has some effects on the action potentials in toad myelinated nerve fibres (Maruhashi et al., 1956). In 1974, Shapiro and co-workers reported that strychnine effects both sodium and potassium currents in squid giant axon membranes (Shapiro et al., 1974) and in the frog node of Ranvier (Shapiro, 1977). This block is a voltage and time-dependent block of both sodium and potassium channels. The quaternary derivative of strychnine, N-methylstrychnine, causes a similar block only when applied intracellularly. It is concluded that tertiary strychnine must
cross the axon membrane and block from the axoplasmic side in the same fashion as the local anesthetic quaternary amines (Shapiro, 1977).

Aibara and co-workers showed that at high concentrations over $10^{-5}$ M, strychnine could induce potassium currents in both rat dorsal root ganglion (DGR) cells (Aibara et al., 1991) and hippocampal CA1 pyramidal neurones (Ebihara & Akaike, 1992). The strychnine induced outward potassium current was antagonized by potassium channel blockers such as Ba$^{2+}$, tetraethylammonium (TEA)-chloride, and 4-aminopyridine (4-AP) in a concentration-dependent manner. There is good evidence that strychnine-induced potassium currents do not result from either the activation of potassium conductance by intracellular Ca$^{2+}$ or the activation of ATP-sensitive potassium channels. The concentration response curves for outward current response were bell-shaped and a near maximum response occurred at $10^{-4}$ M strychnine. A transient 'hump' current appeared immediately after the wash-out of external solutions containing strychnine higher than $10^{-4}$ M. Such decreases of the peak current in the presence of strychnine may be due to blockade of strychnine activated potassium channels by the strychnine molecule itself, in the same fashion as strychnine blocks voltage-dependent potassium channels (Shapiro, 1977). The hump immediately after the wash-out of high concentrations of strychnine is probably due to activation of undesensitized receptors by strychnine, released from strychnine channel block. In addition, guanosine-5'-O-3-thiotriphosphate (GTPγS) suppressed the strychnine induced potassium current, suggesting that GTP binding proteins are involved in this
strychnine response.

The effects of strychnine on *aplysia* R₂ neurones were studied by using simultaneous intracellular recording of the soma and axon action potentials (Gola and Ducreux, 1984), the results indicated that strychnine selectively prolongs the axon spike, particularly in the proximal axon. The different effects of strychnine on the soma and on the axon were assumed to result from a selective blockage of the V-dependent potassium channels which would predominate in the axon, whereas calcium activated potassium channels would predominate in the soma.

In rat cerebral cortex, it has long been proposed that the strychnine spiking activity recorded during EEG measurements is due to an increased calcium conductance through calcium channels (Ramos, 1974). More recently, O’Neill and co-worker have reported that strychnine can interact with voltage-dependent calcium channels (VDCC), particular the 'L'-subtype, likely through binding to allosteric sites that are linked to specific dihydropyridine (DHP) calcium channel regulatory sites (O’Neill and Bolger, 1989).

All of these results suggest that strychnine has multiple pharmacological actions in CNS.

1.3 The discovery of the 2,3-butanedione (BD) induced [³H]-strychnine binding site in rodent CNS
1.3.1 The principle of chemical modification of proteins

Chemical modification is of special importance in probing the active-site structure of receptor and enzyme proteins. When a chemical reagent is placed in contact with the protein and a chemical reaction occurs, this reagent will modify amino acid side-chains in the protein and will produce changes in some measurable property of the protein. Ideally, the reagent should be of sufficient selectivity to react with only one kind of residue. This specific derivatization is then correlated to the protein property under consideration, so that a function can be proposed for the modified residue (Glazer, 1976). Of the 20 natural amino acids, only those possessing a polar side-chain are normally the object of chemical modification (Riordan, 1979).

There are two main types of approach to chemical modification. One is based on the use of group-specific reagents. The reagents in this approach must be sufficiently reactive to modify an amino acid side-chain, and at the same time be sufficiently specific to react with only one type of side-chain. The second type of protein modification employs affinity labels. These compounds are chemically reactive analogues of protein ligands. Owing to this structural similarity, they show affinity to the ligand binding site. By means of their reactive group, these analogues can form a covalent bond with the protein in the vicinity of the binding site (Cohen, 1970).

1.3.2 The effect of 2,3-butanedione modification of mouse spinal cord
membranes

As indicated above, polar amino acid residues which can show nucleophilicity at a certain pH range, can be subjected to chemical modification by group-specific reagents. One of the basic amino acids, arginine, possesses a highly basic guanidino group. Procedures for the modification of arginine residues in protein are based upon their reaction with aldehydes and with 1,2- or 1,3-dicarbonyl compounds. The aldehyde adducts are labile, while condensation with dicarbonyl compounds leads to the formation of relatively stable heterocyclic rings (Yankeelov, 1971). One of these reagents, 2,3-butanedione was introduced by Yankeelov (Yankeelov et al., 1970) and made popular as an arginine modifier by Riordan, who observed that borate buffer stabilizes the arginine-butanedione complex (Riordan, 1979).

Protein modifying reagents have been widely used in attempts to characterize the interaction between strychnine and glycine at the inhibitory glycine receptor protein (O'Connor, 1992 and 1996 in press). Typically, such studies investigate the effects of group-specific reagents on the ability of glycine and/or strychnine to inhibit [3H]-strychnine binding or investigate the ability of these ligands to protect against inactivation of the binding site(s) by protein modifying reagents. However, modification of mouse spinal cord membranes with the arginine-specific reagent 2,3-butanedione (BD) revealed the surprising result of an approximate tenfold increase rather than a decrease in subsequent [3H]-strychnine binding (O'Connor, 1989; and in press, 1996). This BD-
induced strychnine binding was specific in that it could be inhibited by unlabelled strychnine but not glycine. This increase was also seen in strychnine and glycine protected membranes. Indeed, the presence of excess strychnine as a so-called protecting ligand enhanced the effect of BD on subsequent $[^3]H$-strychnine binding. Modification of mouse spinal cord membranes with other membrane permeable arginine specific reagents phenylglyoxal (PG) and p-hydroxyphenylglyoxal (pPG) caused an increase in $[^3]H$-strychnine binding, but not cyclohexanedione (CHD) (O'Connor, 1992, 1996). It was reported by Falke and Chan (1986) that the differential reactivity of a functional arginine residue in Band 3 protein by PG and CHD, only planar PG molecule can gain access to the residue which the non-planar CHD molecule cannot. Interestingly, both BD and pPG are also planar molecules. Moreover, the membrane impermeable arginine selective reagent camphorquinone-10-sulphate (CQS) does not have a BD like effect (O'Connor, 1992). The common reactivity of the membrane permeable arginine specific reagents indicates that modification of the arginine residues within the membrane is responsible for the increase in the $[^3]H$-strychnine binding in mouse spinal cord.

1.4 Preliminary characterization of the 2,3-butanedione-induced strychnine binding site

The distribution of the BD-induced strychnine binding site in rat has been examined by O'Connor (1992). The BD-induced strychnine binding is greatest in tissues with high $[^3]H$-strychnine binding activity prior to BD treatment, and
therefore appears to parallel distribution of the glycine receptor, being high in the spinal cord and brain stem and low in the cerebellum, with little or no increase seen in non-neural tissues, e.g. lung, heart and kidney.

The BD-induced strychnine binding site is displaceable by strychnine but not by glycine and the following evidence suggests that this binding site does not reside on the previously characterised synaptic inhibitory glycine receptor complex (O'Connor, 1992).

(a) The BD-induced strychnine binding could be induced to a similar degree in spinal cord membranes from the mutant mouse *spastic*, despite the mutant having a reduced tissue density of glycine receptors, as low as 20 % of the value seen in normal littermates (Becker *et al.*, 1986).

(b) The[^H]-strychnine binding activity of glycine receptors affinity purified by the method of Pfeiffer *et al.*, 1982 does not show an increase after BD treatment.

(c) The BD-induced strychnine binding site has a lower affinity for strychnine (K_D of approximately 11 μM), and higher capacity (B_max of approximately 4 nmol/mg). Although this binding site appears to co-exist in normal spinal cord membranes with the high affinity (K_D=6 nM) and low capacity (B_max=1 pmol/mg) strychnine binding sites (glycine receptors) previously identified (Pfeiffer *et al.*, 1985; Becker *et al.*, 1986).
1.5 **Pharmacology of BD-induced strychnine binding site**

In order to identify this additional strychnine binding site, a range of compounds have been screened for their ability to displace the BD-induced \[^3\text{H}\]-strychnine binding (O'Connor and Fry, 1991; Dennis, 1992). The results show that only strychnine and closely related alkaloids inhibited \[^3\text{H}\]-strychnine binding to BD-treated membranes. However, the structure-activity relations for this inhibition were different to those observed for inhibition of \[^3\text{H}\]-strychnine binding to glycine receptor (Phelan *et al.*, 1989). The affinity of strychnine derivatives for BD-induced strychnine binding site was decreased by substitution with an amino group at position 2 or by the opening of the lactam ring III, in contrast to their binding at the glycine receptor (e.g. isostrychnic acid and diaboline, see Figure 1.5).

As mentioned in section 1.2, strychnine has been shown to act as an open channel blocker at both potassium and sodium channels at the frog node of Ranvier (Shapiro, 1977 a,b), acting via the same mechanism as quaternary amine local anaesthetics. In view of this, other such channel blockers were tested for their ability to inhibit BD-induced \[^3\text{H}\]-strychnine binding site. Many drugs were found to be effective in this respect, including several local anaesthetics (e.g. quinacrine and dibucaine) and use-dependent cation channel blockers, with IC$_{50}$ values in the \(\mu\text{M}\) range (O'Connor and Fry, 1990). The binding is not displaceable by the sodium channel blocker benzocaine, but is displaced by some potassium channels blockers (e.g. 9-aminoacridine, 4-
Figure 1.5: Comparison of IC_{50} values for inhibition of [^3H]-strychnine (6 nM) binding to untreated and 2,3-butanedione treated mouse spinal cord membranes by strychnine and structurally related alkaloids, numbered as follows: 1. strychnine, 2. 2-nitrostrychnine, 3. 2-aminostrychnine, 4. brucine, 5. bromodesoxyisostrychnine, 6. thebaine, 7. laudanosine, 8. boldine, 9. vincamine, 10. vomicine, 11. N-methyl strychnine, 12. Weiland-Gumlich aldehyde, 13. isostrychnic acid, 14. N-oxystrychninic acid, 15. cacotheline, 16. diaboline, 17. brucine-N-oxide, 18. gelsemine. The IC_{50} values were obtained from linear least squares fits of Hill plots from two experiments each performed in triplicate; vertical and horizontal bars indicate 95% confidence limits. The Spearman rank correlation coefficient for all compounds was 0.197 (p > 0.05) and for compounds nos. 1 to 7 was 0.705 (p > 0.05). A regression line has been drawn through the IC_{50} values for compounds 1 to 7 (O'Connor et al., in preparation).
aminopyridine and TEA). It has thus been suggested that the BD-induced strychnine binding site may be located on a potassium channel or a protein which interacts with one. All such compounds are generally basic amines. Related, but quaternised structures (e.g. QX-223, QX-314 and N-methylstrychnine) have been shown to be far less effective displacers of the BD-induced strychnine binding. This may reflect a necessity for the drug to interchange freely between charged and uncharged forms to effectively equilibrate with the binding site, since it must cross both hydrophobic and hydrophilic barriers, acting at a site located on a membrane spanning region of the peptide. Drugs acting at the BD-induced strychnine binding site therefore appear to act by the same mechanism proposed for the channel blocking actions of strychnine (Shapiro, 1977 a,b.) and quaternary amine local anaesthetics (Hille, 1977 a,b.; see also section 1.6).

1.6 Molecular mechanisms of local anesthesia

Local anaesthetics are a class of similar chemicals that reversibly block peripheral and central nerve pathways following regional administration. Despite the continuous clinical use of local anesthesia since the phenomenon was first described by Koller and Freud (Vandam, 1987) more than a century ago, a molecular basis for the action of local anesthetics on nerve has been established only within the past 20 years.

It is widely believed that the block of nervous conduction by local anaesthetic
follows largely from a reduction in the sodium conductance (Hille, 1966),
although the mechanism of this block of sodium conductance has not yet been
explained satisfactorily. It is believed that local anesthetic drugs act on
excitable tissues by combining with a specific membrane receptor to block
sodium channels (see Hille, 1977).

1.6.1 Structure of channels

The sodium channel was the first channel to be purified. Isolation of sodium
channels began when radioactive TTX and STX became available to identify
the protein purified (Henderson and Wang, 1972). When first purified from the
electric organs of electric fish, the sodium channels proved to comprise a
single peptide chain. This single peptide chain has large hydrophobic regions,
probably in α-helical conformations that span the membrane, interspersed with
hydrophilic regions that presumable either line the Na⁺-conducting pore of the
channel or the aqueous, polar interfaces of the membrane (Noda et al., 1984).

The cloned sodium ion channel consists of 1829 amino acids, with a calculated
molecular weight of 208,321. There are 4 internally homologous domains of
215-272 amino acids, each consisting of 6 proposed membrane spanning
segments. One of the internally homologous segments (S4) that is thought to
traverse the membrane has an unusual structure; every third amino acid is
either a lysine or arginine (positive charge). This is postulated to be the
voltage sensor. Biophysical studies suggest 4-6 gating charges cross the
Figure 1.6.1: Proposed transmembrane looping of the principle subunits of voltage gated channels: Internal repeats are labelled I, II, III, and IV. [Modified from Catterall, 1988]
membrane consistent with a helical turn of the S4 domain (Noda et al., 1984).

The potassium and calcium channels have similar structures. The potassium channel subunit is unusual in that it is essentially a single domain of the sodium channel (Figure 1.6.1).

1.6.2 **Mechanisms of use-dependent channel block by local anesthetics**

Local anesthetics block the excitability of nerve by blocking sodium channels (Taylor, 1959). In myelinated nerve fibres, the block is enhanced by prolonged depolarization or by repetitive depolarization, and at least part of the block is removed by prolonged hyperpolarization (Khorodove et al., 1974, 1976; Courtney, 1974, 1975; Hill, 1977, b). The accumulation of inhibition with repetitive stimulus has been called use-dependent block (Courtney, 1975) or phasic block.

Most clinical local anesthetics are tertiary amines which ionize with $pK_a$'s in the range 7.5-9.0 to give an equilibrium mixture of protonated cations and neutral free amine molecules. The cation predominates at low pH, and the amine at high pH.

Permanently charged N-methyl or N-ethyl derivatives of tertiary amine anesthetics definitely do not reach their receptor when applied to the outside of an axon, but block potently when applied to the inside (Frazier et al., 1970;
Figure 1.6.2: Hypothesis for block by local anesthetics: (A) Na-channel states and transitions with charged drug molecules. Charged (Hydrophilic) drug may come and go only while the gate is open. (B) Neutral (hydrophobic) drug can bind and unbind even when the gate is closed. (C) Two pathways exist for drug to reach its receptor in the pore. The hydrophillic pathway is closed when the gate is closed. Extracellular Na⁺ and H⁺ ions can reach bound drug molecules through the selectivity filter. [After Schwarz et al., 1977]
The drug-receptor reaction with these cations takes place only when the sodium channel is open, as if quaternary drugs must pass through the gates at the inner mouth of the channel to reach a binding site within the channel (Figure 1.6.2.c). It develops only when the depolarizing pulses are large enough to open sodium channels. The rate of block is proportional to the number of channels opened. On the other hand, amine or permanently neutral local anesthetics act rapidly when applied outside, probably because they diffuse freely through the cell membrane in the neutral form (Hille, 1977a,b). The more lipophilic drug molecules have access to the receptor at any time and show little extra accumulation during repetitive stimulation because they leave the receptor rapidly during the interval between pulses. Less hydrophobic drug forms may move only slowly in the hydrophobic pathway between pulses and must wait for the infrequent opening of the channel to provide a hydrophilic pathway. They show larger cumulative effects of repetitive stimulation. Schwarz and co-workers (1977) have shown that the charged and neutral drugs block sodium channels by acting on a single site within the channel, closely tied to the inactivation gating system and yet accessible to protons in the external medium.

The changes of inactivation of sodium channels might be physically interpreted as in Figure 1.6.2 (C). On the axoplasmic end of the pore is the inactivation gating domain, followed by a relatively wide vestibule with an anesthetic receptor. When the inactivation gating system is open, binding to the receptor is not very firm, but when the system is closed the receptor is modified and
the binding is stronger.

The idea proposed here of a modulated receptor with alternate hydrophobic and hydrophilic pathways is probably applicable to other cases. Another example of a drug-receptor interaction varying with the state of a channel and probable having a hydrophilic and hydrophobic reaction pathway is the block of potassium channels by the strychnine and the quaternary derivative of strychnine, N-methylstrychnine (Shapiro, 1977 a,b). The N-methylstrychnine acting via the same mechanism as quaternary amines: the drug-receptor reaction requires open gates and the drug does not reach its receptor when applied to the outside of an axon but blocks potently when applied to the inside.

1.6.3. **Nature and locus of the local anesthetic binding site**

Despite advances in the study of local anesthetic interactions with sodium channels, a number of clinically important questions remain unanswered. For example, although blockade of sodium channels is clearly paramount for peripheral nerve block, the mechanism of local anesthetic action in epidural and spinal anesthesia may be more complex. Studies to date have merely indicated the presence or absence of signal transmission through the spinal cord or have studied the time-dependent kinetics of 'wash-in' and 'wash-out' of local anesthetics in spinal cord regions or spinal roots (Bromage *et al.*, 1981)
Many membrane-associated proteins other than sodium channels are affected by local anesthetics. Therefore, increasing attention is being focused on the possibility that membrane protein can provide the anaesthetic receptor site in excitable membranes and couple it to the functional alterations produced.

Greenberg and Tsong (1982, 1984) have given strong support to the membrane protein hypothesis by showing that in axonal membranes the functionally relevant binding sites for local anesthetics are located in membrane proteins, proteins that are unlikely to be the sodium channels. The fluorescent local anesthetic drug, quinacrine, was found to bind specifically to an axonal membrane fraction derived from bovine corpus collosum. The binding site could be detected in membranes prepared from other brain regions whereas membranes derived from non-nervous tissue did not bind quinacrine appreciably. The binding was saturable and high affinity, with the Scatchard parameters of $K_d = 0.6$ to $1.2 \mu M$ and $B_{max}$ of $2$ to $4$ nmol/mg membrane protein. The binding site appeared to be an integral membrane protein. First, energy transfer was detected between tryptophan fluorescence of membrane proteins and the bound quinacrine. Second, 90 to 95% of the quinacrine binding was abolished by trypsin treatment. Moreover, treatment with phospholipases C and D did not cause a significant reduction in quinacrine binding. Importantly, the equilibrium binding of quinacrine to axonal membranes was found to be displaced by several local anesthetics according to their nerve blocking potencies. These results suggested strongly the existence of a local anesthetic receptor in mammalian axonal membranes. The quinacrine
binding site was solubilized with the detergent sodium cholate. An affinity chromatography column of quinacrine coupled to sepharose beads was made, and detergent-solubilized membranes were bound and eluted specifically with lidocaine, a local anesthetic. SDS-polycrylamide gel electrophoresis revealed a small protein with a molecular weight about 16-kD. This protein is presumed to be a putative local anesthetic receptor. The receptor concentration in the axonal membranes was estimated to be 2-5 nmol/mg protein, assuming a 1:1 of quinacrine binding, it should constitute 3-8% of the total axonal membrane proteins, consistent with the finding that it is the major protein constituent of the axonal membranes.

However, in addition to sodium channels, many other membrane-associated proteins are affected by local anesthetics. These include adenylate cyclase (Voeikov and Lefkowitz, 1980), guanylate cyclase (Richelsin et al., 1978), calmodulin-sensitive protein (Tanaka and Hidaka, 1981), the ion-pumping enzymes Na+/K+-ATPase (Henn and Sperelakis, 1968, Andersen, 1968) and Ca2+/mg2+-ATPase (Roufogalis, 1973), phospholipase A2 and phospholipase C (Waite and Sisson, 1972).

As mentioned above, K+ channels have been shown to be affected by strychnine. In addition, these potassium channels have been reported to be affected by local anesthetics. In the squid axon, a novel potential-independent, TEA-insensitive "leakage" potassium current is blocked by general anesthetics (Haydon et al., 1988). In peripheral myelinated axons of Xenopus laevis, the
TEA-insensitive flickering potassium channel has been found to contribute to the resting potential of not only the nodal but also the internodal regions and the local anesthetic bupivacaine seems to be the most potent inhibitor of this channel (Koh *et al.*, 1992).

It seems likely, therefore, that local anesthetics will bind to sites other than sodium channels and that binding to these other sites may contribute to their actions. Therefore, the actions of local anesthetics within the spinal cord should include their effects on a variety of ion channels and other membrane related activities, such as second-messenger systems, effects that may contribute for example to spinal and epidural anesthesia.

The present work describes the characterisation and localization of a novel strychnine binding site in the mammalian CNS which also bindings local anaesthetics. This site resides in the periaxolemmal myelin where if an ion channel it could have a role in K⁺ buffering.
CHAPTER TWO

MATERIALS AND METHODS

2.1 Equipment and materials

2.1.1 Equipment

Cell Harvester: 24 well (Brandel M-24R)

Centrifuges: High Spin 21 (M.S.E.Spin 21)
              J2-MC (Beckman)
              L7-65 Ultracentrifuge (Beckman)
              Optima™ TL Ultracentrifuge (Beckman)
              Portable laboratory centrifuge (Beckman microfuge 12)

Electrophoresis apparatus:
              Protean II Slab Cell (Bio-Rad Laboratories)
              Mini-Protean 2 Cell (Bio-Rad Laboratories)

Electrofocusing power supply: 2197 (L.K.B. Instrument Ltd.)

Filtration manifold: 12 well (Millipore UK Ltd.)

Fluorescence spectrometer: 3000 (Perkin-Elmer)

Gel Slab Drier: 486 (Bio-Rad Laboratories)

Homogenisers:
              Dounce homogenizer (40 ml, 15 ml)
              Polytron tissue (Prob PTA 10S): Kinematica Gmbh (Northern
Supply Ltd.)

Liquid Scintillation Spectrometer: Tri-Carb Model-D3375 (Packard)

Peristaltic pumps:

2132 Microprepex (L.K.B.Instruments Ltd.)

P1 (Pharmacia Ltd.)

Protein blotting apparatus: Mini Trans-Blot Cell (Bio-Rad)

pH Meter: Model 109 (Corning-EEL)

Safety light: (Ilford)

Shaker table: R100 Rotatest (Luckham)

Sonicator: Soniprep 150 (M.S.E.)

Spectrophotometer: Pye Unicam SP6-550 UV/VIS (Unicam/Phillips)

Ultraviolet lamps:

Hand-held: UVGL-58 (Cam.Lab.Ltd.)

Light Box: LKB 2001 MACROVL Transluminator

Vortex mixer: Scientific Industries Vortex Genie 2

2.1.2 Consumables

Dialysis tubing: (Pharmacia Ltd.)

Glass fibre filters (Whatman GF/B):

Cut into sheets (30x5cm)

Discs (25 mm diameter)

Nalgene centrifuge Tubes: 50 ml, 14 ml (Nalgene)

Plastic petri dishes: 3.5 cm, 5 cm diameter (Falcon)
Polypropylene centrifuge tubes: 12 ml, 30 ml (Sarstedt)
Polypropylene microcentrifuge tubes: 1.5 ml (Sarstedt)
Polystyrene conical tubes: 50 ml (Falcon)
Scintillation insert vials: 5 ml (Sterilin Ltd.)
Ultra-Clear centrifuge tubes: 12 ml (Beckman)
Universal containers: (Gibco Ltd.)

2.1.3 **General chemicals and reagents**

Acetic Acid (BDH Chemicals Ltd.); amido black (BDH Chemicals Ltd.); acrylamide (Bio-Rad Laboratories or BDH Electran); albumin (Bovine Serum, Fraction V; Sigma Chemical Co. Ltd.); ammonium sulphate (Enzyme grade; Fisons Plc); ammonium persulphate (Bio-Rad Laboratories); bacitracin (Sigma Chemical Co. Ltd); bovine-γ-globulin (Labile Enzyme Free, Fraction II; Miles Laboratory Ltd.); bromophenol blue (BDH Chemicals Ltd.); 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulphonate (CHAPS, Sigma Chemical Co. Ltd.); cholate (Sigma Chemical Co. Ltd.); Coomassie blue R and G-250 (Sigma Chemical Co. Ltd.); dithiothreitol (DTT, Sigma Chemical Co. Ltd.); ethanol (Absolute Alcohol A.R., James Burrough F.A.D. Ltd.); ethanolamine (Sigma Chemical Co. Ltd.); ethylenediaminetetraacetic acid (Disodium Salt, EDTA, BDH Chemicals Ltd); N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic Acid (HEPES, Sigma Chemical Co. Ltd.); mercaptoethanol (Sigma Chemical Co. Ltd.); methanol (BDH Chemicals Ltd.); molecular weight standards (Dalton Mark VII-L, Sigma Chemical Co. Ltd.);
phenylmethane sulphonyl fluoride (PMSF, Sigma Chemical Co. Ltd.);
polyethylene glycol 1500 (PEG, BDH Chemicals Ltd.); polyethylenimine
(Sigma Chemical Co. Ltd.); scintillation cocktails (Ecoscint H, National
Diagnostics); sodium dodecyl (lauryl) sulphate (BDH Electran); soybean
trypsin inhibitor (Sigma Chemical Co. Ltd.); sucrose (BDH Chemicals Ltd.);
N,N,N',N'-tetramethylethylenediamine (TEMED, Bio-Rad Laboratories); tris-
(hydroxymethyl)-methylamine (Aristar, BDH Chemicals Ltd.); triton X-100
(Sigma Chemical Co Ltd.).

All other chemicals (not listed) were A.R. grade and purchased from BDH
Chemicals or Sigma Chemical Co. Ltd.

2.1.4 **Drugs and radiochemicals**

Boldine was kindly synthesised by Prof. G.A.R. Johnston and Ms. H. Tran,
University of New South Wales, Sydney, Australia. dibucaine (Sigma
Chemical Co. Ltd.); glycine (Sigma Chemical Co Ltd.); isostrychnic acid
(Aldrich Chemical Co. Ltd.); N-5-Azido-2-nitrobenzoyloxsuccinimide (Sigma
Chemical Co. Ltd.); N-methylstrychnine was kindly synthesised by Dr.
V.M. O'Connor, University College London. quinacrine (Sigma Chemical Co.
Ltd.); quinacrine mustard (Sigma Chemical Co Ltd.); mustin (Sigma Chemical
Co. Ltd.); strychnine (Benzene Ring-³H, specific activity 23 Ci/mmol, 24
Ci/mmol and 24.5 Ci/mmol) was purchased from New England Nuclear;
strychnine hemi-SO₄ (Sigma Chemical Co Ltd.); vincamine (Aldrich Chemical
2.1.5 General buffers

Commonly used buffers and buffered solutions are given below.

Phosphate buffered saline (PBS): a solution containing NaCl (100 mM) and Na$_2$HPO$_4$ (41 mM), adjusted to pH 7.4 with NaH$_2$PO$_4$ (200 mM).

PBS/BSA: PBS as above, containing 0.1 % (w/v) bovine serum albumin.

PBS/BSA/T: PBS as above, containing BSA (0.1 %w/v) and sodium thiosulphate (10 mM).

Phosphate buffer (PB): a solution containing Na$_2$HPO$_4$ (0.1 M or 0.2 M) adjusted to pH 7.4 or pH 7.7 with NaH$_2$PO$_4$ (0.2 M).

Tris-citrate: a solution containing tris-(hydroxymethyl)-methylamine (50 mM) adjusted to pH 7 with saturated citric acid.

Buffer A: a solution containing sucrose (0.32 M), MgCl$_2$ (1 mM), CaCl$_2$ (50 μM) and HEPES (5 mM, adjusted to pH 7.4 with NaOH).

Buffer B: a solution containing sucrose (0.32 M), CaCl$_2$ (50 μM), and HEPES (5 mM, adjusted to pH 7.4 with NaOH).

Lysis buffer: a solution containing tris-(hydroxymethyl)-methylamine (5 mM) and CaCl$_2$ (50 μM) adjusted to pH 8 with HCl.

Gradient buffer: a solution containing HEPES (5 mM, adjusted to pH 7.4 with NaOH) and CaCl$_2$ (50 μM).

2.2 General methods
2.2.1 **Animals**

All rats were of the Wistar Strain or Sprague-Dawley and were bred in the animal house, University College London. Normal mice were bought in as adult and of the TO strain. Adult male mice (16 weeks or older) were used in all experiments.

2.2.2 **Dissection of spinal cord tissue**

Mice or rats were killed by cervical dislocation and decapitated immediately. Laminectomy was performed and the spinal cord removed. Mouse spinal cords were wrapped in aluminium foil and frozen in liquid nitrogen and stored at -70°C until required. Rat spinal cords were wrapped and frozen individually in liquid nitrogen and stored at -70°C or placed in centrifuge tubes at 4°C for subcellular fractionation immediately.

2.2.3 **Preparation of crude membranes**

The tissue was weighed quickly while still frozen and placed in polypropylene centrifuge tubes. Samples were homogenized at 4°C in approximately 20 volumes (tissue weight/volume) ice-cold tris-citrate (50 mM, pH 7) with a Polytron, speed 10 for 10 seconds. The polytron probe was rinsed with 4 mls tris-citrate between samples and the wash buffer added to the homogenized tissue which was then centrifuged (27,000xg, 30 min, 4°C). The supernatant
was discarded and the pellet was washed three times by resuspending in 20 volumes tris-citrate by sonicration (amplitude 25 microns, 10 seconds) at 4°C and the centrifuging as before. The pellet was finally resuspended in exactly 20 volumes Tris-citrate buffer by sonicration for 20 seconds and stored at -70°C until needed.

2.2.4 Subcellular fractionation

2.2.4.a Preparation of myelin

All the following procedures were slightly modified from that described by Whittaker et al. (1964) and were carried out at 4°C. Rat or mouse spinal cord was thawed and minced. Homogenization was performed by 5 up and down strokes of loose pestle followed by 20 up and down strokes of tight pestle with a Dounce homogenizer; using 4 volumes of buffer A. The homogenates were then diluted to 10% (w/v) in buffer B. The homogenate was then centrifuged (1,000xg, 10 min). The resultant pellet, designated as P₁ fraction, contained nuclei and cell debris. The supernatant was recentrifuged again (15,000xg, 20 min). The pellet of this centrifugation was resuspended in buffer B (2.5 ml/g), then recentrifuged as described above; the resultant pellet, designated as P₂ fraction, contained myelin, mitochondria and synaptosomes. The supernatants from these two centrifugations were pooled and designated as S fraction, containing microsomes and soluble cytosol.
The P₂ fraction was resuspended in a lysis buffer (2.5 ml/g) with 10 strokes of the homogenizer and lysed for 30 min, then 48% w/w sucrose was added to the resuspended material to give a final concentration of sucrose of 34% w/w. The sample was then gently transferred to centrifuge tubes and over layered by the same volumes of 28.5% w/w sucrose and 2ml of buffer B. The discontinuous sucrose gradient was centrifuged (19,000xg, 2 hours). After the centrifugation, 3 subfractions were obtained: the band between the buffer B and 28.5% w/w interface, as myelin fraction; the band between the 28.5%-34% w/w sucrose interface as a synaptic plasma membrane fraction; the pellet on the bottom of the tubes contained mainly mitochondria, as the mitochondrial fraction.

2.2.4.b Subfractionation of myelin

The subfractionation of myelinated axons was performed according to the method of Sapirstein et al., 1992.

Again, all the following procedures were carried out at 4°C. Rat or mouse spinal cord was thawed and minced. 1 g of minced spinal cord was transferred to a Dounce homogenizer containing 37 ml of a buffered salt-sucrose solution (0.9 M sucrose, 150 mM NaCl, 10 mM sodium HEPES and protease inhibitor mixture, (bacitracin: 25 μg/ml; soybean trypsin inhibitor: 25 μg/ml; EDTA: 1mM; KCl: 1M; dithiothreitol: 5mM; phenylmethane sulphonyl fluoride (PMSF): 0.1 mM, pH 7.5). A smooth homogenate was obtained with 5 strokes
of loose pestle followed by 20 strokes of tight pestle. The resulting whole homogenate solution was centrifuged (82,500xg, 25 min). Keeping the pellet as pellet A, the floated membranes were rehomogenized in sucrose (0.85 M) and recentrifuged. The pellet was pellet B, the floating membrane pads were resuspended in sodium HEPES (10 mM, pH 7.4) containing EDTA (5 mM) and protease inhibitor mixture, the membranes were then osmotically shocked in this buffer for 1.5 hours. After homogenizing again, the membranes were gently layered on a discontinuous gradient of 15%, 24%, 28%, 32%, and 37% sucrose and centrifuged for 12-15 hours (82,500xg) in a Beckman SW 24 rotor, the myelin, sedimenting at the 15%-24% sucrose interface and the crude axolemma sedimenting at the 28%-32% sucrose interface. The fractions obtained by this procedure were purified further as described below.

The myelin was osmotically shocked with distilled water for 30 min and centrifuged twice (12,000 x 20 min) to remove residual microsomal contaminants and layered over sucrose (0.75 M). The material was centrifuged in SW-24 rotor (75,000 x 1.5 hours), the band floating on 0.75 M sucrose was taken as purified myelin.

The crude axolemma was lysed (30 min, 5 mM Tris-HCl, pH 8.3, containing 0.1 mM EDTA), 1/4 volume of 40% w/w sucrose was then added and then it was layered over a discontinuous gradient of 0.65 M, 0.8 M, 1.0 M sucrose and centrifuged (75,000 x, 1.5 hours). The band floating on 0.8 M sucrose
was taken as the periaxolemmal myelin and the band on 1.0 M sucrose was
taken as the axolemmal fraction.

2.2.5 Treatment of membrane preparations with the protein modifying reagent
2,3-butanedione

The spinal cord membranes prepared as described in sections 2.2.3 and 2.2.4
were centrifuged (27,000xg, 30 min, 4°C). The supernatant was discarded and
the pellet resuspended in 40 volumes PBS. The reagent 2,3-butanedione (80
mM or 160 mM) was added to the treated samples and an equal volume of
PBS to the untreated samples. After incubation (40 min, 20-21°C) on a shaker
table, the reaction was terminated by the addition of PBS/BSA (0.1 % w/v; 160
vols of tissue wt./vol).

Samples of membrane were washed according to the following procedure after
2,3-butanedione treatment and before being assayed whenever they had been
incubated with other reactants (affinity labels, protecting ligands, etc).

The samples were first pelleted by centrifugation (27,000xg 4°C, 30 min) and
the supernatant discarded. The pellet was resuspended in 160 volumes of PBS
and sonicated (amplitude 25 microns, 4°, 10 s). The process was repeated four
times before the final resuspension in exactly 20 volumes of PBS, samples
were then sonicated twice. Equivalent membrane preparations were pooled,
stirred, divided into individual sample as appropriate and stored at -70°C until
needed. Samples were washed just before being centrifuged (2700xg, 4°C, 30 min) and resuspended in exactly 40 volumes of PBS.

2.2.6 \[^3H\]-Strychnine binding assays

Unless otherwise stated, 100μl samples of the sample to be assayed were placed in small plastic tubes in triplicate, to which were then added \[^3H\]-strychnine (final concentration 6 nM), the volume of each assay sample being made up to 1 ml with PBS. Tubes containing, in addition, an excess of unlabelled strychnine (2x10^4 M) or quinacrine (1x10^4 M) were used to measure nonspecific binding. The contents of the tubes were mixed by vortexing and incubated for at least 1.5 hours at 4°C. Incubations were terminated by filtration under vacuum through glass fibre filters using a filtration manifold to separate membrane-bound radioligand from that remaining free in solution. Filters were soaked for at least 30 min prior to use in polyethylenimine (0.1% w/v in PBS) to minimise non-specific binding to the filters. Radioligand binding to filters was routinely controlled by filtration of blanks containing PBS instead of experimental samples. Filters were first washed in 5 ml PBS. The samples were diluted with 4 ml PBS and filtered. The filters were finally washed twice with 5 ml buffer to ensure effective removal of free and non-specifically bound radioligand. The portions of the filters retaining the radioactivity from each sample were punched out into 3 ml of liquid scintillant (Ecoscint H), and the activity of each sample determined using a liquid scintillation counter.
2.2.7 Inhibition of $[^3]$H-strychnine binding by unlabelled drugs

Crude membrane was treated with BD and incubations were set up as described in 2.2.6, but in the presence of increasing concentrations (1 mM - 3.162x10^4 mM) of the following unlabelled drugs: strychnine hemi-\(\text{SO}_4\), quinacrine, boldine, vincamine, glycine at only one concentration (1 mM), N-methylstrychnine or isostrychninic acid. Blanks contained the appropriate solvent dilution instead of the drug.

2.2.8 Association/dissociation kinetics of BD-induced $[^3]$H-strychnine binding site

2.2.8.a The association of $[^3]$H-strychnine binding to BD-induced binding sites

Crude membrane was treated with BD and pretreated with isostrychninic acid (10^{-3}M, 30 min, 4°C) incubations were set up as described in 2.2.6 but incubations were taken at various time intervals after addition of $[^3]$H-strychnine (0-2 hours) for filtration through pre-washed glass-fibre filters.

2.2.8.b The dissociation of bound $[^3]$H-strychnine from BD-induced binding site

Crude membrane was treated with BD and incubations were set up as described in 2.2.5 and incubated for more than 2 hours on ice. At time zero, either an excess of unlabelled strychnine (0.1 mM) was added or the samples were diluted 50-fold. The samples were then taken for filtration at various time
intervals (0-1.5 hours) thereafter. For both the onset and offset experiments, blanks containing the above reagent but no membrane were included.

2.2.9 Liquid scintillation counting

The glass fibre filters from the filtration of samples were transferred to 5 ml scintillation insert vials. The scintillation fluid was added to the samples. The radioactive content of the samples were measured by counting for 10 min in a liquid scintillation spectrometer. The background level of the radioactivity was estimated by measuring the vials containing scintillation fluid alone.

The scintillation spectrometer was calibrated to count efficiency by counting a series of $[^1H]$-hexadecane standards quenched by adding increasing volumes of chloroform and this quench correction was held in the memory of the spectrometer. The counts per minute (CPM) were converted to disintegrations per minute (DPM) by estimating the counting efficiency in each vials.

2.2.10 Photoaffinity labelling of rat spinal cord membranes

2.2.10.a Photolabelling rat spinal cord membranes with $[^1H]$-strychnine

BD treated membranes (20 volumes) were incubated with 6 nM $[^1H]$-strychnine in PBS (>4 hrs, 4°C), and unlabelled quinacrine (0.1 mM) was added for measurement of nonspecific binding. Blank incubation were also set up
containing no membranes. The membranes were then decanted into plastic culture dishes (3.5-5 cm diameter) and illuminated at 4°C with short wave length light (nominal peak 254 nm, 300 μw/cm²) at a distance of 15.2 cm from an ultraviolet lamp for 30 min. Non-irradiated samples were run in parallel as controls. The membranes were then pipetted into test tubes and the reaction dishes rinsed with 1.5 ml of unlabelled strychnine (0.4 mM), which was then added to the incubation. After an overnight chase (4°) with unlabelled strychnine, the membranes were processed for filtration.

2.2.10.b Filtration of photolabelled membranes

Samples of the membrane incubation mixtures were dispensed in triplicate into assay tubes. Presoaked glass fibre filters (0.1% v/v PEI, 30 min) were used to filter samples as described above. The samples were diluted with 5 ml of PBS, filtered and washed twice with 5 ml PBS. The irreversible incorporation of [³H]-strychnine into the irradiated membranes was estimated by subtracting the level of [³H]-strychnine from non-irradiated samples.

2.2.10.c Solubilization of the photolabelled BD-induced binding sites in rat spinal cord

The BD-induced binding sites were solubilized from the photolabelled membrane as described in 2.2.10.a. The photolabelled membrane was incubated in PBS, containing Triton X-100 (0.5% v/v-2% v/v), or CHAPS (0.5% w/v-2% w/v), or -cholate (0.5% w/v-2% w/v) and a mixture of
protease inhibitors (25 μg/ml bacitracin, 25 μg/ml soybean trypsin inhibitor, 1 mM EDTA, 1 M KCl, 0.1 mM PMSF, and 5 mM dithiothreitol). The final protein concentration was between 0.5-1 mg/ml. The mixture was agitated gently for 15 min at room temperature, then held on ice and mixed at intervals during a 1 hour period. The supernatant, following centrifugation of this mixture (100,000 xg, 1 hour, 4°C), was used as a source of solubilized BD-induced binding sites. The membrane pellet was used to measure the activity of the [3H]-strychnine binding.

2.2. Precipitation of the photolabelled and solubilised BD-induced [3H]-strychnine binding sites in rat spinal cord

The detergent-extracted samples (60-120 μg; 10 volumes) were incubated with either PEG (15% w/v-40% w/v) or ammonium sulphate (30%-80% saturation) and bovine γ-globulin (100 μg/ml) at 4°C in PBS in a total volume of 1 ml. The incubation mixture was kept on ice for 30 min to allow complete precipitation and then diluted with either 5 ml of PEG (10% w/v) or ammonium sulphate (50% saturation) in PBS and immediately filtered through PEI presoaked glass fibre filters.

All samples were set up in triplicate, and all binding data corrected for non-specific binding which was determined in the presence of quinacrine (10 mM).
2.2.11 Affinity labelling of the BD-induced $[^3]$H-strychnine binding sites with quinacrine mustard

2.2.11.a Affinity labelling of BD-induced $[^3]$H-strychnine binding sites using quinacrine mustard

The quinacrine mustard was prepared in dilute acid to delay the formation of the aziridinium ion, after the method of Creech and O'Connell (1981, also see Figure 5.1.2). The mustard was prepared at a concentration of 2 mM in 1 mM HCl. Either untreated and BD-treated membrane preparation or subcellular fractions were thawed as normal and resuspended in exactly 40 volumes of PB (0.1 M, pH 7.7) instead of PBS. Quinacrine mustard (1μM-100μM) was then added to all samples except the solvent control which received the HCl (1 mM) alone. All samples were then vortexed and the reaction stopped at an appropriate time by the addition of approximately 160 volumes of PBS/BSA/thiosulphate, vortexing and allowing samples to equilibrate at room temperature for a further 10 min. Sample were then washed 4 times in 160 volumes PBS and then assayed for $[^3]$H-strychnine binding activity as in 2.2.6, with quinacrine and/or strychnine as the displacing ligand.

2.2.12 Protection against quinacrine mustard labelling of the BD-induced $[^3]$H-strychnine binding site

BD-treated membrane preparation or subcellular membrane fractions were
incubated with strychnine (10 mM), quinacrine (1 mM) and glycine (10 mM) individually (30 min, at 4°) before being labelled with quinacrine mustard. The concentrations of the protecting drugs were 1,000-10,000 fold higher than that of the quinacrine mustard.

2.2.13 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Denaturing polyacrylamide gel electrophoresis was performed according to the method of Laemmli (1970).

2.2.13.a Buffers and solutions (all stored at 4°)

Reservoir Buffer: Tris-(hydroxymethyl)-methylamine (Tris; 0.025 M), glycine (0.192 M), pH 8.3.

Upper Reservoir Buffer: Same as the reservoir buffer but containing in addition of sodium dodecyl sulphate (SDS; 0.1%).

Stacking gel buffer: Tris (0.5 M) adjusted to pH 6.8 with 1 M HCl.

Resolving gel buffer: Tris (1.5 M) adjusted to pH 8.8 with 1 M HCl.

Acrylamide: 30% stock containing 29.2% acrylamide and 0.8% N,N'-methylene-bis-acrylamide. (stored in dark).

Stacking Gel: (20 ml/slab gel or 5 ml/mini slab gel) final concentrations: Acrylamide (3% v/v), Tris/HCl, (0.125 M; pH 6.8), ammonium persulphate (0.075%).

Resolving Gel: (45 ml/slab gel or 5 ml/mini slab gel) Final concentrations:
Acrylamide (10% v/v), Tris-HCl (0.375 M; pH 8.8), SDS (0.1% w/v), ammonium persulphate (0.075%).

Master mix: Diluted with sample 2:1 v/v in Tris/HCl (0.05 M; pH 6.8) to give final concentrations: 2-mercaptoethanol (2-ME, 5% v/v), SDS (2% w/v), ethylenediaminetetraacetic acid (EDTA, 10 mM; pH 7.0), glycerol (30% v/v), bromophenol blue (0.01% w/v).

The stacking and resolving gel were degassed for 15 minutes and the polymerisation catalyst N,N,N',N'-tetramethylenediamine (TEMED) added immediately prior to use. The TEMED final concentration was 0.1% in the stacking gel and 0.08% in the resolving gel.

2.2.13 b Preparation of gels

Resolving Gel:
Freshly prepared resolving gel was pipetted between an electrophoretic plate sandwich, overlayed with 0.1% SDS and left to polymerize (>1 hour; 20-21°C).

Stacking Gel:
The SDS overlay was removed, the gel surface dried and rinsed with freshly prepared stacking gel. Stacking gel was then pipetted above the resolving gel and the spacer comb carefully inserted into the gel. After allowing the gel to polymerize (>1 hr), the spacer was removed, the wells raised with upper reservoir buffer and the gel apparatus was mounted on a cooling core with a
2.2.13.c Preparation and loading of the samples for SDS-PAGE

Protein samples were pelleted and resuspended in SDS (2%) followed by a dilution with master mix (1:2). The samples were then applied to the stacking gel, for each well the maximum protein was 100 µg in a volume of 60 µl. The molecular weight markers were also diluted in master mix (1:2) and loaded. Upper and lower reservoir buffers were added and the sample run at constant current (90 mA, 300 V) through stacking and (60 mA, 200 V) resolving gels. If running overnight, 10 mA and 30 V were used through the stacking gel during night time and then 60 mA and 200 V the next day. After electrophoresis the gel was viewed under UV light to show the fluorescence bands (see 5.2.4); the gel was either stained or cut and stained.

2.2.13.d Gel staining

Coomassie Blue R-250:
SDS-PAGE gel was fixed in methanol (40% v/v), acetic acid (10% v/v) for 30 minutes (changed once) followed by staining for 4 hours in solution of Coomassie Blue R-250 (0.25% w/v) in methanol (40% v/v), acetic acid (10% v/v), destained in a large excess of methanol (40%), acetic acid (10%) until the background was clear and stored in acetic acid (7%).
Coomassie Blue G-250:
SDS-PAGE gel was stained in 80 ml stain solution (10% w/v (NH₄)₂SO₄, 2% v/v H₃PO₄, 0.1% w/v CBBG-250 and 20 ml methanol) for more than 4 hours followed by destaining in distilled water until the background was clear and stored in (NH₄)₂SO₄ (10% w/v).

2.2.13.e Preparation of samples for sequence analysis

Gels were stained with coomassie brilliant blue G-250 (0.25% w/v final concentration in 30% v/v methanol and 10% v/v acetic acid) for 8 min, then destained in methanol (30% v/v) and acetic acid (10% v/v) for 4 min. Peptide bands were cut out on the light box and stored at -70°C for further sequence analysis.

2.2.14 Fluorescence measurement of quinacrine mustard labelled peptides

The fluorescent bands of the gel were cut out under UV light and eluted in 1 ml Tris/HCl buffer (5 mM, pH 8.8) for more then 2 hours. Fluorescence was measured in an Perkin-Elmer (3000) spectrofluorimeter at room temperature. Cuvettes (1.5 ml) were filled with 1 ml gel eluate or reference solution of 1 μM quinacrine. The excitation wavelength used was 350 nm, the emission wavelength used was 500 nm. In all experiments the background signal was first recorded however it appeared to be negligible. The reference
solution was measured between the samples and used to reset sensitivity of instrument.

2.2.15 Western blotting

2.2.15.a Materials

Nitrocellulose Paper
Blotting Buffer: Tris (0.125 M), glycine (0.1 M) and methanol (20% v/v), adjusted to pH 8.8 with HCl.
HRP Colour Development Solution: 4-Chloro-1-napthol (60 mg) was dissolved in methanol (20 ml) just before use, then this was mixed with PBS (100 ml) containing 30 % H₂O₂ (60 μl) at room temperature immediately before use.
Amidoblack Staining Solution: Amidoblack (0.1%) dissolved in distilled water.
Amidoblack Destaining Solution: Methanol (30% v/v) and Acetic acid (10% v/v).
Blocking Solution: BSA (3% w/v) and methiolate (0.01% w/v) dissolved in PBS (100 ml).
Antibody Buffer: BSA (2% w/v) and Methiolate (0.01% w/v) dissolved in PBS (100 ml).
GAR-HRP (Goat Anti-Rabbit LgG Horseradish Peroxidase Conjugate)
PAP (Horseradish Peroxidase and Rabbit Anti-horseradish Peroxidase)
Primary Antibody: Mouse MAG (gift from Professor R. Mirsky, Dept. Anatomy and Developmental Biology, UCL)
Mouse MBP (as above)

Hudson PLP (as above)

Neurofilament (Sigma Chemical Co. Ltd)

Secondary Antibody: Anti-Mouse Ig G (Bio-Rad Laboratories)

GAR (Goat antibody to rabbit γ-Globulin) (as above)

2.2.15.b *Method*

The blotting sandwich was assembled within the blotting cassette, taken in order: Scotch-brite pad, chromatography paper, nitrocellulose sheet, polyacrylamide gel, chromatography paper, and Scotch-brite pad. Care was taken to avoid any air bubbles between the gel and the nitrocellulose. Then it was connected to the power supply. The cathode was on the gel side. Blotting took place overnight at 0.1 A, 40 V. Next morning the current was turned up to 0.15 A for 1 hour.

2.2.15.c *Staining Western blots*

The nitrocellulose sheet was taken out from blotting sandwich, the molecular weight standard lanes were cut off and stained in amidoblack staining solution for 2 hours, follow by destaining in amidoblack destaining solution until the background was clear.

Before visualisation of protein bands with labelled antibodies, it was necessary
to block the remaining protein-binding sites on the nitrocellulose. Bovine serum albumin (BSA, 3%) was used for blocking. The nitrocellulose blot was cut into strips and washed in PBS (10 min), then they were placed in the blocking solution and gently shaken for more than 1 hour at room temperature.

2.2.15.d Blocking protein-binding sites

Dilution of antibody: Mouse MAG, Mouse MBP, Hudson PLP and Neurofilament antibodies at 1:1000 in the antibody buffer respectively. The blot strips were briefly rinsed with distilled water and immersed in the antibody solution respectively. After being gently mixed on a shaker, they were left overnight at room temperature. The strips were then divided into two groups:

Group A: After a brief rinse with distilled water, the strips were incubated in Anti-mouse Ig G (1:100 in antibody buffer) for 2 hours, then rinsed as before, following by incubation in GAR-HRP (1:500 in antibody buffer) for 2 hours, then rinsed again.

Group B: After a brief rinse with distilled water, the strips were incubated in GAR (2.5 units/10 ml) for 2 hours, then rinsed as before followed by incubation in rabbit PAP (1:100 in antibody buffer) for 2 hours, then rinsed again.
The strips from group A and B were washed thoroughly by immersing in excess PBS for 10 min on the shaker, repeating the wash step twice. During the final wash the HRP colour development solution was prepared. After brief rinse with distilled water, the strips were immersed in the HRP reagent and mixed on the shaker in dark until bands developed sufficient colour, the strips were then rinsed with distilled water, dried and wrapped in foil.

2.2.16 Determination of protein concentrations

The protein concentration of samples was assayed by the dye-binding method of Bradford (1976).

2.2.16.a Dye binding method

The method is based on the fact that binding of protein to the dye Coomassie Brilliant Blue G-250 causes a shift in the absorption maximum of the dye from 465 nm to 595 nm. The increase of the absorption at 595 nm can be measured and used to calculate the protein concentration.

Coomassie brilliant blue G-250 (150 mg) was dissolved in ethanol (final concentration 7.125% w/v) and left overnight. To this was added phosphoric acid (final concentration 10.2% v/v), the mixture being brought up to 2 litres with distilled water and left to cool, filtered and stored in a dark bottle at room temperature.
2.2.16.b Macro assay:

Samples (10 μl-50 μl) were added to quadruplicate glass tubes containing 100 μl of water. Corresponding blanks contained the same volume of either distilled water or sample buffer. BSA (50 μl) was added to the fourth tube of each assay sample. The sample tubes containing standard protein were used as an internal standard and the blank tubes containing standard protein were used as an external standard, to check that there was nothing present in the sample which would interfere with the assay of the standard. Coomassie brilliant blue reagent 5 ml was added to each tube and the contents of each vigorously mixed by vortexing. The absorbance of each sample at 595 nm was then read in a spectrophotometer within 1 hour. Blanks containing water or the appropriate buffer instead of experimental samples were assayed to check for possible effects of reagents on the dye.

After subtracting buffer blank absorbance, the protein concentration (μg/μl) was calculated.

2.2.16.c Micro assay:

A micro assay was used for samples containing 1-10 μg protein. The assay was performed using only 20 μl water, 5 μl samples, 5 μl BSA and 1 ml Coomassie brilliant blue. The absorbance was measured as previously described.

2.2.17 Analysis of binding data
2.2.17.a Principles of receptor ligand interactions:

In the simplest situation, one molecule of ligand (L) interacts with one molecule of receptor (R) to reversibly form a complex (RL).

\[ R + L \rightarrow RL \] ..............................1

For a receptor binding experiment, both radioactive ligand and unlabelled ligand were used, so the above equation can be represented by:

\[ R + L + I = RL + RI \] ..............................2

\( R \) = concentration of free binding sites
\( L \) = concentration of free radioactive ligand
\( I \) = concentration of unlabelled ligand
\( RL \) = concentration of the receptor bound with L
\( RI \) = concentration of the receptor bound with I

2.2.17.b Saturation and inhibition experiments

In a saturation experiment, the receptor concentration is held constant and RL is determined at equilibrium as a function of L. So Equation 2 becomes:

\[ R + L = RL \]

The equilibrium dissociation constant \( K_D \) is defined as:

\[ K_D = \frac{R \times L}{RL} \] ..............................3

Since the total receptor concentration \( R_T \) must be the sum of the free and bound concentrations

\[ R_T = R + RL \] ..............................4

The Rosenthal relationship is derived from Equation 3 and 4:

\[ \frac{RL}{L} = \frac{R}{K_D} = \frac{-RL}{K_D} = \frac{R_T}{K_D} \]

The parameters in this equation are usually represented by a different set of symbols: \( RL = B \) (Bound); \( L = F \) (Free); \( R_T = B_{\text{max}} \) (maximal number of binding sites).
So the $B_{\text{max}}$ is defined as:

$$B_{\text{max}} = B + (B/F)K_D$$ ..............................................5

$B_{\text{max}} = $ maximum number of binding sites

$B =$ concentration of receptors bound by ligand

$F =$ concentration of free radioactive ligand

$K_D =$ equilibrium dissociation constant

Experimental data were analyzed by non-linear least square fits to estimates of $K_D$ and $B_{\text{max}}$.

In the inhibition experiments, the $IC_{50}$ has been used to describe the potency of unlabelled ligand for the receptor site. The following equilibrium can be derived from the equilibrium represented in 2:

$$B = \frac{B_{\text{max}} x F}{F + K_D (1 + I/K)}$$ ..............................................6

When $I = IC_{50}$, the equilibrium in 6 can be represented as:

$$IC_{50} = K_D (1 + F/K_D)$$ ..............................................7

$IC_{50} =$ concentration of displacer which inhibits 50% of control binding

A computerised linear least squares method was used to fit Hill plots and estimate $IC_{50}$ for the compounds with 95% confidence limits.

2.2.17.c Association and dissociation of binding

Results for association and dissociation of strychnine specific binding to BD-treated spinal cord membranes were fitted by non-linear least squares. For both
association and dissociation, the best fit was to a model with two sets of binding sites. The equation fitted for association was $B = B_{eq1}(1-e^{-k_{obs1}t}) + B_{eq2}(1-e^{-k_{obs2}t})$, where $B$ is the amount of strychnine bound at time $t$, $B_{eq}$ the amount bound at equilibrium and $k_{obs}$ the observed association rate constant. The equation fitted for dissociation was $B = B_{eq1}e^{-k_{off1}t} + B_{eq2}e^{-k_{off2}t}$, where $k_{off}$ is the dissociation rate constant. Estimates of the true association rate constant $K_{on}$ where made from the equation $K_{obs} = (K_{on}.L + K_{off})$, where $L$ is the concentration of the ligand $[^3H]$-strychnine.
CHAPTER THREE

PHARMACOLOGICAL CHARACTERISATION OF THE 2,3-BUTANEDIONE INDUCED STRYCHNINE BINDING SITES IN RAT AND MOUSE SPINAL CORD MEMBRANES

3.1 Introduction

As mentioned in Chapter One, treatment of mouse spinal cord membranes with the arginine specific protein modifying reagent 2,3-butanedione (BD) induced an approximate tenfold increase in $[^3]H$-strychnine binding (which is not located on the glycine receptor) (O'Connor, 1989). The increased binding can be displaced by strychnine but not by glycine and may be induced to a similar degree in the mutant mouse spastic in which the density of glycine receptors has been reduced to as low as 20% of the value seen in normal littermates (Becker, 1986). Furthermore, the $[^3]H$-strychnine binding activity of purified glycine receptors is decreased by BD-treatment rather than increased. This binding site has a lower affinity for strychnine than the glycine receptor site, ($K_D = 11 \mu M$ for the BD-induced site compared to 6 nM for the glycine receptor site), it presents an approximately 1000 fold higher concentration ($B_{max} = 4 \text{ nmol/mg protein}$ for the BD-induced binding site compared to 1 pmol/mg protein for glycine receptor site). Assuming this BD-induced binding site has a 1:1 molar ratio of bound strychnine, this would comprise about 10% of total membrane protein. In addition to its actions at the glycine receptor,
Strychnine has many non-glycinergic actions, it has been found for example to act as an open channel blocker at both potassium and sodium channels at the frog node of Ranvier (Shapiro, 1977a, b). In view of this, a range of drugs have been screened for their ability to displace the BD-induced $[^3]$H-strychnine binding (Dennis, 1992; O'Connor, 1992). Results show that many drugs were found to be effective in this respect, including several local anaesthetics, antiarrhythmics and use-dependent cation channel blockers (O'Connor, et al., 1991).

In this study, rat and mouse spinal cord membranes were used to further investigate the specificity of the BD-induced binding site and the kinetics of $[^3]$H-strychnine binding to provide further information for purification.

It is known in the BD-treated spinal cord membranes that there are not only BD-induced strychnine binding sites, but also glycine receptors. Preliminary experiments have indicated that BD treatment has affected the ability of glycine to inhibit total $[^3]$H-strychnine binding to the glycine receptor and reduced the total $[^3]$H-strychnine binding to purified glycine receptors. So, $[^3]$H-strychnine binding to the BD-treatment membranes consists of a component of the modified inhibitory glycine receptor and the BD-induced binding site. It is clear that any analysis of $[^3]$H-strychnine binding data needs to consider that there are two distinctive binding sites rather than one homogenous site.

3.2 The effect of BD treatment on $[^3]$H-strychnine binding of the rat spinal
**cord membranes**

The rat spinal cord membranes were prepared as 2.2.3 and treated with 80 mM arginine specific protein modifying reagent 2,3-butanedione (BD) as 2.2.5. After washing as in 2.2.5, the samples were then assayed for protein content as in 2.2.16.a and for $[^{3}H]$-strychnine binding activity as in 2.2.6. with strychnine as the displacing ligand.

The results are shown in Figure 3.2. In the BD-treated rat spinal cord membranes, there was about 10 fold increase in $[^{3}H]$-strychnine binding in comparison with the untreated membranes. This result is in agreement with that obtained from the mouse spinal cord.

### 3.3 Comparison of the inhibition of $[^{3}H]$-strychnine binding to the BD-induced strychnine binding site in rat spinal cord membranes by different ligands

Several ligands, which included glycine, strychnine and its structurally related alkaloids: N-methystrychnine and isostrychninic acid, non-strychnos alkaloids: boldine and vincamine, local anaesthetic: quinacrine and dibucaine, the structures of which are shown in Figure 3.3.a, were tested for their ability to inhibit $[^{3}H]$-strychnine binding to BD-treated rat spinal cord membranes. The ligands were chosen due to the fact that they have been previously screened for inhibition of $[^{3}H]$-strychnine binding to the BD-induced binding site in mouse spinal cord membranes in our laboratory (O'Connor, 1992 and Dennis, 1992).
Figure 3.3.a: Structures of glycine, strychnine, N-methystrychnine, isostrychninic acid, boldine, vincamine, quinacrine and dibucaine.
Figure 3.2: Effect of 2,3-butanedione on subsequent $[^3]$H-strychnine binding to rat spinal cord membranes: Rat spinal cord membranes were treated (T) with BD at 80 mM in phosphate buffered saline (PBS, pH 7.4 at 20°C) for 40 min. Untreated (U) membranes received PBS only. The reaction was terminated by the addition of PBS (160 vols) containing bovine serum albumin (0.1% w/v). The membranes were then washed four times with PBS before being incubated with $[^3]$H-strychnine (6 nM, 40°C, >2hrs), unlabelled strychnine (2x10^-4M) was used as the inhibiting ligand to enable estimates of strychnine specific binding. The binding is expressed as fmole/mg protein. Results were obtained from eight experiments (mean±s.e.m.), each of them performed in triplicate.
This experiment was carried out to confirm that the pharmacology of the BD-induced binding site found in the rat spinal cord membranes is the same as that in the mouse spinal cord membranes.

BD-treated rat spinal cord membranes were incubated with $[^3H]$-strychnine (6 nM) and various concentrations (from $10^{-4}$M to $3.162 \times 10^{-8}$M) of unlabelled compounds at 4°C overnight (see 2.2.8). Inhibition of specific binding, which represents binding displaced by $10^{-4}$ M quinacrine, is shown in Figure 3.3.b.

For each compound the concentration required to inhibit 50 % of $[^3H]$-strychnine binding (IC$_{50}$) was calculated from the least squares fit of a Hill plot of the data (Table 3.3). Quinacrine was the most potent ligand and it was approximately three fold more potent than strychnine itself. The third most potent ligand was dibucaine. Boldine was less potent than dibucaine but slightly more potent than vincamine. Glycine, N-methylstrychnine and isostrychninic acid, where their IC$_{50}$ values were more than $10^{-4}$ M, were the least potent ligands. Hill coefficients were close to one for most of the compounds except for strychnine which was significantly less than one and boldine which was greater than one.

3.4 **Saturation analysis of unlabelled strychnine binding to the BD-induced strychnine binding site**

Attempts to saturate and define the $B_{max}$ and $K_D$ of the BD-induced strychnine
<table>
<thead>
<tr>
<th>DRUG</th>
<th>I.C.\textsubscript{50} (M)</th>
<th>95% CONF. LIMITS</th>
<th>HILL COEFF.</th>
<th>95% CONF. LIMITS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quinacrine</td>
<td>0.26 \times 10^{-6}</td>
<td>0.17 - 0.37 \times 10^{-6}</td>
<td>1.04</td>
<td>0.77 - 1.30</td>
</tr>
<tr>
<td>Strychnine</td>
<td>0.72 \times 10^{-6}</td>
<td>0.33 - 1.55 \times 10^{-6}</td>
<td>0.22</td>
<td>0.14 - 0.29</td>
</tr>
<tr>
<td>Dibucaine</td>
<td>1.74 \times 10^{-6}</td>
<td>0.83 - 3.64 \times 10^{-6}</td>
<td>0.67</td>
<td>0.44 - 0.90</td>
</tr>
<tr>
<td>Boldine</td>
<td>8.96 \times 10^{-6}</td>
<td>5.60 - 14.32 \times 10^{-6}</td>
<td>1.81</td>
<td>0.85 - 2.76</td>
</tr>
<tr>
<td>Vincamine</td>
<td>19.28 \times 10^{-6}</td>
<td>10.41 - 35.72 \times 10^{-6}</td>
<td>0.91</td>
<td>0.41 - 1.40</td>
</tr>
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<td>Glycine</td>
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<tr>
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<td>Isostrychninic acid</td>
<td>&gt;10^4</td>
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Table 3.3: IC\textsubscript{50} and Hill coefficients for inhibition of BD-induced [\textsuperscript{3}H]-strychnine binding to rat spinal cord membranes: BD-treated rat spinal cord membranes were incubated with [\textsuperscript{3}H]-strychnine (6 nM, 4\textdegree C, overnight) in presence of drug dilutions, with unlabelled quinacrine (10\textsuperscript{4} M) used as the displacing ligand to estimate of quinacrine specific binding. Results from one experiment performed in triplicate.
binding site in BD-treated mouse spinal cord membranes with increasing concentrations of labelled strychnine (0.1-33 nM) were unsuccessful in our laboratory (O'Connor, 1992), this was primarily due to the range of the labelled ligand, [³H]-strychnine concentrations were insufficient to saturate at this low affinity, high capacity strychnine binding site. However, for estimating $B_{\text{max}}$ and $K_{D}$, the experiments that involve saturation of specific binding sites could be done either by an increase in the concentration of labelled ligand or increasing the concentration of unlabelled ligand in presence of a single concentration of labelled ligand (Bowery et al., 1983). In this study, the data of unlabelled strychnine inhibition of [³H]-strychnine binding to BD-induced strychnine binding site (see section 3.3) was used to further analyze to estimate the $B_{\text{max}}$ and $K_{D}$ of the BD-induced strychnine binding site.

INPLOT was the curve fitting program which could use crude binding data to provide the estimates of $B_{\text{max}}$ and $K_{D}$. When the data was analyzed, it best fitted a one site model with the $B_{\text{max}}$ of 13 nmol/mg and the $K_{D}$ of 71 μM (Figure 3.4; Table 3.4), indicating that the BD-induced strychnine binding site was of lower affinity and higher capacity than the glycine receptor ($B_{\text{max}} = 1$ pmol/mg; $K_{D} = 6$ nM). These results confirmed the earlier report of the low affinity and high capacity of the BD-induced strychnine binding site in mouse spinal cord membranes (O' Connor, 1992).

3.5. **Association and dissociation of specific [³H]-strychnine binding to the BD-treated mouse spinal cord membranes**
Figure 3.4, Table 3.4: Determining equilibrium binding parameters of $[^\text{H}]$-strychnine binding to BD-treated rat spinal cord membranes: BD-treated rat spinal cord membranes were incubated with increasing concentrations of unlabelled strychnine together with $[^\text{H}]$-strychnine (6 nM, 4°C, overnight), unlabelled quinacrine ($10^4$ M) was used as the displacing ligand to estimate quinacrine specific binding. Results from one experiment performed in triplicate. Results were analyzed by considering it as a saturation binding assay by increasing concentrations of labelled strychnine. In plot of data is shown on Figure 3.4 and the estimates of $K_D$ and $B_{\text{max}}$ derived from nonlinear least square fits of data are indicated on Table 3.4.

<table>
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<td>0.7 n mole/mg</td>
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<td>$K_D$</td>
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<td>0.74 μM</td>
<td>69.66-73.13 (μM)</td>
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Table 3.4:
Binding parameters were estimated by studying the association and dissociation of specific $[^3H]$-strychnine binding to 2,3-butanedione treated mouse spinal cord membranes (in presence of $10^{-4}$ M isostrychninic acid to prevent binding at the inhibitory glycine receptor; see 2.2.8). Figure 3.5.a shows the onset of binding, which equilibrates within 1.5 hrs (at 6 nM; 4°C). Dissociation of $[^3H]$-strychnine binding from 2,3-butanedione-treated membranes is shown in figure 3.5.b and was more rapid when initiated by the addition of excess unlabelled strychnine than by dilution of the membranes. Non-linear least squares analyses gave best fits with two site models for both association and dissociation. When weighted by their relative abundance, these give an average observed association rate constant ($K_{obs}$) of $2.50 \times 10^{-3}$ s$^{-1}$ and an average dissociation rate constant ($K_{off}$) of $2.14 \times 10^{-3}$s$^{-1}$ for dissociation initiated by dilution of the incubation and of $10.65 \times 10^{-3}$s$^{-1}$ for dissociation initiated by the addition of excess unlabelled strychnine. If these average values are then used to estimate an average true association rate constant ($K_{on}$) the equation $K_{obs} = (K_{on} \cdot L + K_{off})$, then a value of $6.0 \times 10^{-4}$ M$^{-1}$s$^{-1}$ is obtained with the $K_{off}$ by dilution. The $K_{off}$ after addition of excess unlabelled strychnine is too fast for this calculation. The above average $K_{off}$ by dilution and estimated $K_{on}$ give an overall dissociation constant of 0.04 $\mu$M, lower than that obtained from the equilibrium binding assay with mouse (11 $\mu$M; O'Connor, 1992) or rat (71 $\mu$M, Table 3.4).

3.6 Discussion
Figure 3.5: Association and dissociation of strychnine-specific $[^3H]$-strychnine binding to 2,3-butanedione treated mouse spinal cord membranes. All incubations included isostrychnic acid ($10^{-4}$ M) to prevent binding at the glycine receptor. Binding is plotted as a percentage of the maximal binding at equilibrium (2797.45 fmol/mg protein). For association, (A) binding was measured at different times after addition of $[^3H]$-strychnine (6 nM). For dissociation (B), binding to membranes previously equilibrated with $[^3H]$-strychnine (6 nM) was measured either after addition of excess unlabelled strychnine ($2\times10^{-4}$M, •) or after 50-fold dilution of the incubation (○). Each point is the mean ± SEM from 4 experiments performed in triplicate. Curves drawn are the non-linear least squares fits of the results, which gave a best fit to a two site model for both association and dissociation. Observed association rate constants ($K_{obs}$) were $0.58±0.09\times10^3$s$^{-1}$ (91±4%) and $14.11±7.07\times10^3$s$^{-1}$ (14±4%). Dissociation rate constants ($K_{off}$) after addition of excess unlabelled strychnine were $18.63±5.96\times10^3$s$^{-1}$ (52±11%) and $2.01±0.68\times10^3$s$^{-1}$ (48±11%) whereas after dilution of the incubation they were $0.48±0.05\times10^3$s$^{-1}$ (73±5%) and $6.61±2.14\times10^3$s$^{-1}$ (27±5%); all fitted values ± approximate standard error).
The ligand binding properties of the BD-induced binding were investigated by comparing the potency of local anesthetics, strychnos and non-strychnos alkaloids to inhibit \[^{3}\text{H}]-\text{strychnine}\) binding to BD-treated rat spinal cord membranes. Quinacrine was the most potent ligand and it was approximately three fold more potent than strychnine itself. The order of potency for inhibition of \[^{3}\text{H}]-\text{strychnine}\) binding by unlabelled ligands were as follows: quinacrine > strychnine > dibucaine > boldine > vincaine. Glycine, N-methylstrychnine and isostrychninic acid, which IC\(_{50}\) values of more than 10\(^{-4}\) M, were the least potent ligands. These results confirm that previously reported in mouse spinal cord membranes (O'Connor, 1992 and Dennis, 1992). Most compounds have Hill coefficients close to one suggesting that the BD-induced binding site for these compounds do not interact cooperatively. However, the Hill coefficient of strychnine was significantly less than one which could indicate the presence of multiple strychnine binding sites in BD-treated membranes. This result was also in agreement with the earlier report from mouse spinal cord membranes (O'Connor, 1992, Dennis, 1992). The rat spinal cord membranes were used for subsequent purification in order to obtain more material and the pharmacological specificity of their BD-induced strychnine binding sites was similar to those of the mouse.

Further evidence for multiple strychnine binding sites in BD-treated spinal cord membranes came from measurements of the association and dissociation of \[^{3}\text{H}]-\text{strychnine}\) binding from mouse spinal cord. for both association and dissociation, the best fit was to a two site models. However, association of strychnine binding to BD-treated spinal cord membranes was dominated (91\%)
by a site with a slow onset ($t_{1/2} 1204 \text{ s}$). Dissociation of binding when initiated by dilution of the incubation was also dominated (73%) by a slow process ($t_{1/2} 1434 \text{ s}$). By contrast, dissociation of binding initiated by the addition of excess unlabelled strychnine was much faster, with dissociation almost complete within 600 s. How can these observations be rationalised into a model of the binding site? The quaternary derivative N-methylstrychnine is inactive at the binding site (see Table 3.3), suggesting that strychnine must become uncharged at some point and diffuse into the lipid membrane to gain access to the site. Oil/water partition coefficients for strychnine are high (Mackerer et al, 1977) and it would be expected to diffuse rapidly into the membrane. Nevertheless, comparison of the potencies of alkaloids structurally related to strychnine at this binding site indicate that the protonatable N19 is essential and suggest that strychnine must regain a positive charge here before it is bound. Thus, the slow onset of binding may reflect strychnine regaining a proton and partitioning into a binding site buried within the membrane. Once bound there, its dissociation would be slow unless flushed out by an excess of unlabelled strychnine. This provides the most consistent explanation for the results; negative cooperativity seems unlikely because the Hill coefficients for inhibition of binding by unlabelled strychnine are less than unity. The ability of unlabelled strychnine to speed the dissociation of $[\text{H}]-\text{strychnine}$ also explains why the equilibrium dissociation constant $K_D$ obtained by isotopic dilution is higher (Mouse 10.9 $\mu$M, O'Connor 1992; Rat 71 $\mu$M, Table 3.4) than the dissociation constant (0.04 $\mu$M) given by the average association and dissociation (by dilution) rate constants. Indeed, the 2,3-butanedione induced
binding site appears to have a higher affinity than would be suggested from equilibrium binding assays.

Data presented in this Chapter confirms the earlier observations that 2,3-butanedione induced strychnine binding site is not associated with glycine receptor. The increased strychnine binding is displaceable by strychnine and local anesthetics such as quinacline or dibucaine, but not by glycine. N-methylstrychnine, a compound which carries a permanent positive charge has been shown to be a far less effective displacer of the BD-induced \[^3H\]-strychnine binding. This presumably reflects a necessity for the drug to interchange freely between charged and uncharged forms to effectively equilibrate with the binding site, since it must cross both hydrophobic and hydrophilic barriers, acting at a site located on a membrane spanning region of the peptide. Drugs acting at the BD-induced strychnine binding site therefore appear to act by the same mechanism proposed for the channel blocking actions of strychnine (Shapiro, 1977 a,b.) and quaternary amine local anaesthetics (Hille, 1977 a, b.). This BD-induced strychnine binding site has a low affinity (\(K_D=11 \, \mu M\) in mouse spinal cord, \(K_D=71 \, \mu M\) in rat spinal cord) and high capacity (\(B_{\text{max}}=4 \, \text{nmol/mg protein in mouse spinal cord,}\) \(B_{\text{max}}=13 \, \text{nmol/mg protein in rat spinal cord}\)). This value for \(B_{\text{max}}\) is in agreement with that obtained for a previously identified local anaesthetic binding site (\(B_{\text{max}}=5 \, \text{nmol/mg protein,}\) Greenberg and Tsong, 1982). In their study, quinacline was found to bind specifically to the axonal membrane fraction prepared from bovine corpus callosum and the fluorescent intensity of
bound quinacrine was enhanced 20 to 25 % compared to free quinacrine. The quinacrine binding was of high affinity with an apparent $K_d$ of 0.6 to 1.2 $\mu$M for quinacrine. Also quinacrine bound to axonal membranes is displaced effectively by several clinically useful local anesthetics. So it appears that existence of a local anesthetic binding in axonal membranes and the BD-induced strychnine binding site shows many similarities to this local anesthetic binding site. In order to carry out a definitive characterization of this BD-induced strychnine binding site, it has been solubilised and purified as described in Chapter Four.
CHAPTER FOUR

THE SOLUBILIZATION OF THE PHOTOAFFINITY LABELLED 2,3-BUTANEDIONE INDUCED STRYCHNINE BINDING SITE FROM RAT SPINAL CORD

4.1 Introduction

One of the fundamental approaches in understanding the biochemistry of any drug or neurotransmitter binding site is to solubilize it in its recognition property. There are three detergents that have successfully been used to solubilize the $[^3H]$-strychnine binding of the inhibitory glycine receptor from mammalian spinal cord membranes: 1). Triton X-100 (Pfeiffer et al., 1982), a non-ionic detergent which tends to break weak protein-protein interactions and strip away endogenous lipids, 2). sodium cholate (Graham et al., 1985; Garcia-Calveo et al., 1989), an anionic detergent and 3). 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulphonate CHAPS (O’Connor, 1992), a zwitterionic detergent, which is capable of preserving easily disrupted protein-protein interactions.

As described in Chapter 1, strychnine has been used as a specific ligand in solubilization and purification of the inhibitory glycine receptor. As a first step, Pfeiffer and Betz (1981) solubilised the glycine receptor from rat spinal cord membranes using non-ionic detergent Triton X-100. The glycine receptor
was then purified to homogeneity on an affinity matrix consisting of 2-
aminostrychnine coupled to agarose beads (Pfeiffer et al., 1982).

Previously experiments set up to attempt to solubilise the BD-induced
strychnine binding site in our laboratory have been unsuccessful (O'Connor,
1992; Dennis, 1992), because the BD-induced strychnine binding site appeared
to be sensitive to detergents. Nevertheless, CHAPS appeared to be the most
effective detergent; at 0.1% final concentration of CHAPS, there was about
4.8% bound [3H]-strychnine found in the solubilized fraction in comparison
with the unsolubilised membranes (Dennis, 1992). However, only 1% bound
[3H]-strychnine was found when cholate was used as the detergent (O'Connor,
1992). In addition, Triton X-100 (0.1% v/v-2% v/v) appeared to inactivate the
strychnine binding, causing about a 95% reduction of the total [3H]-strychnine
bound when compared to the control value.

In view of the above problems in solubilising [3H]-strychnine binding activity
from BD-treated membranes, it was decided to evaluate solubilisation of this
binding site after prior labelling with an affinity ligand. Covalent coupling of
ligands to their binding sites using various affinity crosslinking reagents has
been used in the identification, purification and characterisation of many
neurotransmitter receptors (Zisapel and Sokolovsky, 1977). In particular, the
synthesis of photolabile derivatives of ligands for photoaffinity-labelling
procedures are suited for this purpose (Bayley and Knowles, 1977). It has been
reported that [3H]-strychnine has been irreversible bound to the glycine
receptor of rat spinal cord membranes upon ultraviolet illumination (Graham
et al., 1981, 1983). For this reason, the solubilization of the photoaffinity
labelled BD-induced strychnine binding site is to be investigated in this
Chapter.

4.2 Photolabelling the \(^3\text{H}\)-strychnine binding site in untreated and BD
treated rat spinal cord membranes

Untreated and BD treated rat spinal cord membranes were prepared as in 2.2.5
and photoaffinity labelled with \(^3\text{H}\)-strychnine (6 nM) as 2.2.10, the samples
were then filtered in the same way as in 2.2.10.b.

When the rat spinal cord membranes were incubated with 6 nM \(^3\text{H}\)-
strychnine and then illuminated with UV light, both strychnine displaceable
and quinacrine displaceable \(^3\text{H}\)-strychnine became bound irreversibly to the
untreated and BD treated membranes (Figure 4.2). With non-irradiated control
samples, \(^3\text{H}\)-strychnine was not incorporated irreversible into the membranes,
so the binding was lower in comparison to the irradiated samples. In untreated
membranes, the strychnine displaceable \(^3\text{H}\)-strychnine bound to the glycine
receptor; however, in the BD-treated membranes the quinacrine displaceable
\(^3\text{H}\)-strychnine only bound to the BD-induced strychnine binding site. This
pharmacological specificity indicates that strychnine can be bound irreversibly
to both glycine receptor and the BD-induced strychnine binding site in rat
spinal cord membranes upon UV illumination. Thus, the photoaffinity-labelling
Figure 4.2: Irreversible incorporation of \[^{3}\text{H}]\)-strychnine into rat spinal cord membranes upon ultraviolet illumination: Untreated (U) and BD-treated (T) rat spinal cord membranes were incubated with \[^{3}\text{H}]\)-strychnine (6 nM, 4°C). Samples labelled in the presence of unlabelled strychnine (2x10⁻⁴M) or of quinacrine (10⁻⁴ M) were used to estimate strychnine (Us, Ts) or quinacrine (Uq, Tq) specific binding. The incubation mixtures were either photoaffinity-labelled (irradiated, ■; nominal peak 254 nm, 30 μw/cm² at 15 cm) or unlabelled (non-irradiated, □), then they were chased with an excess of unlabelled strychnine (2x10⁻⁴M, 4°C, overnight) and filtered. The binding is expressed in fmole. Results were obtained from one experiment performed in triplicate.
procedure may offer a valuable tool for evaluating the solubilization of the BD-induced strychnine binding site.

4.3 Attempt to solubilize the photoaffinity labelled BD-induced $[^3H]$-strychnine binding site in rat spinal cord with different detergents

BD-treated rat spinal cord membranes were photolabelled with $[^3H]$-strychnine as in 2.2.10.a and solubilized in Triton X-100 (1.5% v/v) as in 2.2.10.c. Polyethylene glycol 1500 (PEG) (15% w/v) was added to the solubilized membrane preparation and the binding precipitated as in 2.2.10. The samples were then filtered in the same way as in 2.2.10.b. The BD-induced strychnine binding activity in the solubilized membrane preparation, membrane pellet and solubilized precipitate were measured.

The effects of Triton X-100 on the photoaffinity labelled $[^3H]$-strychnine binding site in the BD-treated rat spinal cord are shown in Figure 4.3. Triton X-100 increased the specific $[^3H]$-strychnine binding in the solubilized membrane preparation, so the $[^3H]$-strychnine binding activity in it was higher than that in the photolabelled membranes (control value). There was only 7% of $[^3H]$-strychnine binding found in the remaining membrane pellet in comparison with the control value, indicating that most of the BD-induced strychnine binding site had been successfully solubilized. However, only 7% of this $[^3H]$-strychnine binding was recovered in the PEG precipitated fraction. Thus, it appeared that the BD-induced strychnine binding site had not been
Figure 4.3: Solubilization of photolabelled $[^3]H$-strychnine binding site from BD-treated rat spinal cord membranes using Triton X-100: Photolabelled BD-treated rat spinal cord membranes were incubated with Triton X-100 (1.5% v/v) and a mixture of protease inhibitors. The sample was agitated for 15 min in room temperature and held on ice for 1 hour. After centrifugation (100,000 xg, 1 hour, 4°C) the supernatant was used as a source of solubilized membrane preparation and the pellet was used as the membrane pellet. Part of the solubilized membrane precipitate was further incubated with PEG (15% w/v) and bovine $\gamma$-globulin (100 $\mu$g/ml) at 4°C for 30 min, then diluted with PEG (10% w/v) as the solubilized preparation. All of the samples were then filtered and the binding is expressed as fmole/mg protein. Results were obtained from one experiment performed in triplicate.
4.3.1 Comparison of the ability of PEG and ammonium sulphate to precipitate the solubilized photoaffinity labelled $[^3H]$-strychnine binding site in BD-treated rat spinal cord

In view of the insufficient precipitation of the BD-induced strychnine binding site during the 15 %w/v PEG procedure in the previous experiment, it was decided to compare the ability of PEG and ammonium sulphate to precipitate the solubilized photoaffinity labelled BD-induced strychnine binding site.

BD-treated rat spinal cord membranes were photolabelled with $[^3H]$-strychnine as in 2.2.10.a and solubilized in Triton X-100 (1.5% v/v) as in 2.2.10.c. A range of PEG (to 15%-40% w/v) or ammonium sulphate (to 30%-80% of saturation) concentrations were then added to solubilized membrane preparations as in 2.2.10.d. The samples were then filtered in the same way as in 2.2.10.b. The BD-induced strychnine binding activity was then measured in the whole solubilized preparation, the remaining membrane pellet and the solubilized precipitate.

Figure 4.3.1 shows the results of the PEG and ammonium sulphate precipitation of the BD-induced strychnine binding site. The precipitate obtained by PEG (15% w/v - 40% w/v) showed little binding activity. By contrast, the precipitate obtained by ammonium sulphate (30% -80% of
Figure 4.3.1: Comparison of the ability of PEG and ammonium sulphate to precipitate the solubilized photoaffinity labelled $[^3]H$-strychnine binding site in the BD-treated rat spinal cord: Triton X-100 (1.5% v/v) extracted photoaffinity labelled BD-induced strychnine binding site proteins were incubated with either PEG (■; 15% w/v-40% w/v) or ammonium sulphate (□; 30%-80% of saturation) in the presence of bovine $\gamma$-globulin (100 $\mu$g/ml) at 4°C for 30 min, then diluted with either PEG (10% w/v) or ammonium sulphate (50% of saturation) and immediately filtered. Results were obtained from one experiment performed in triplicate.
saturation) had recovered about 50% of the initial $[^3\text{H}]-$strychnine binding activity. So the ammonium sulphate appeared to be a better precipitator for the BD-induced strychnine binding site.

4.3.2 Ability of different detergents to solubilise the photoaffinity labelled $[^3\text{H}]-$strychnine binding site in BD-treated rat spinal cord membranes

In an attempt to find the optimal conditions to solubilise the BD-induced strychnine binding site, several detergents were examined for their ability to solubilize photolabelled BD-induced binding sites as well as for their tendency to inhibit $[^3\text{H}]-$strychnine binding to the BD-induced strychnine binding site.

BD-treated rat spinal cord membranes were photolabelled with $[^3\text{H}]-$strychnine as in 2.2.10.a and solubilized in Triton X-100 (0-2% v/v), CHAPS (0-2% w/v) or sodium cholate (0-2% w/v) as in 2.2.10.c. Ammonium sulphate (50% of saturation) was added to precipitate the membrane preparation as in 2.2.10.d. The samples were then filtered in the same way as in 2.2.10.b. The BD-induced strychnine binding activity was then measured in the whole solubilized preparation, the remaining membrane pellet and the solubilized precipitate.

The effects of increasing Triton X-100, CHAPS and sodium cholate concentrations on the solubilization of photolabelled BD-induced $[^3\text{H}]-$strychnine binding sites in the rat spinal cord are shown in Table 4.3.2 The
binding of [³H]-strychnine to the BD induced strychnine binding site was little
detected in the solubilized precipitate with 0.5% of both CHAPS and sodium
cholate, although more than 85% of the binding activity was detected in the
solubilized membrane preparation. With 2% of Triton X-100, CHAPS and
sodium cholate, there was more than 100% of the binding site activity
appearing in the solubilized membrane preparation but only 13%, 10% and
0.9% of binding activity respectively were recovered in the corresponding
solubilized precipitate. This may again be because of the incomplete
precipitation of the solubilized binding site by the procedure. However, Triton
X-100 appeared to be a better detergent than CHAPS and sodium cholate for
solubilization of the BD-induced strychnine binding site.

Previous experiments in our laboratory have shown that the BD-induced
binding site lost more than 90% of its binding ability if Triton X-100 (0.1%-2%) was used in the binding medium (Dennis, 1992). Figure 4.3.2
demonstrates the concentration-dependent inhibition of [³H]-strychnine binding
to the BD-induced strychnine binding site by various detergents. Triton X-100
was a strong inhibitor, whereas CHAPS and sodium cholate were relatively
weak inhibitors. These results provided the limiting detergent and the
concentration values allowed for each of the detergents in the binding medium.
Since CHAPS yielded the better recovery of [³H]-strychnine binding activity
in the solubilized precipitate than cholate, and at the concentration of 0.25%
there was more than 75% of [³H]-strychnine binding activity remaining, it
therefore appeared to be the detergent of choice for solubilization of the BD-
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Table 4.3.2 Effect of detergents on solubilization of photolabelled [³H]-strychnine binding site in the BD-treated rat spinal cord: Photolabelled BD-treated rat spinal cord membranes were incubated with Triton X-100 (0-2% v/v), CHAPS (0-2% w/v) or sodium cholate (0-2% w/v) with a mixture of protease inhibitors. The mixture was agitated for 15 min in room temperature and held on ice for 1 hour. After centrifugation (100,000 xg, 1 hour, 4°C) the supernatant was used as a source of solubilized preparation and the pellet was used as the membrane pellet. Part of the solubilized membrane preparation was further incubated with ammonium sulphate (50% of saturation) and bovine γ-globulin (100 μg/ml) at 4°C for 30 min as the solubilized precipitate. All of the samples were filtered and the binding is expressed as a % of the original photolabelled membrane. Results were obtained from one experiment performed in triplicate.
**Figure 4.3.2: Effects of different concentration of detergents on $[^3]$H-strychnine binding in BD-treated mouse spinal cord membrane:** BD-treated mouse spinal cord membranes were incubated with detergents (final concentration: 0.1-2% w/v) and $[^3]$H-strychnine (6 nM) overnight at 4°C, with unlabelled strychnine (10⁴ M) used as the displacing ligand to enable estimates of strychnine specific binding. The results is expressed as % of distilled water control. Results were obtained from one experiment performed in triplicate (After Dennis, 1992).
induced strychnine binding site.

4.3.3 Solubilization of the photoaffinity labelled [³H]-strychnine binding site in the untreated and BD-treated rat spinal cord with CHAPS

Untreated and BD-treated rat spinal cord membranes were photolabelled with [³H]-strychnine as in 2.2.10.a and solubilized in CHAPS (0.25% and 0.75% w/v) as in 2.2.10.c. Ammonium sulphate (50% of saturation) was added to solubilized membrane preparation for precipitation as in 2.2.10.d. The samples were then filtered in the same way as in 2.2.10.b. The BD-induced strychnine binding activity was then measured in the whole solubilized preparation, the remaining membrane pellet and solubilized precipitate.

The effects of CHAPS on the solubilization of photolabelled [³H]-strychnine binding sites in the rat spinal cord are shown in Figure 4.3.3 The yield of [³H]-strychnine binding to the quinacrine displaceable [³H]-strychnine binding site was only 0.4% with 0.75% CHAPS, although more than 55% of the binding activity was detected in the whole solubilized membrane preparation. With 0.25% of CHAPS, [³H]-strychnine binding to the quinacrine displaceable [³H]-strychnine binding site was not detected in the solubilized precipitate. However, in the untreated sample 2% of the strychnine displaceable [³H]-strychnine binding site were recovered in the solubilized precipitate with both 0.75% and 0.25% of CHAPS. These results indicate that some of the photolabelled glycine receptors have been solubilized by CHAPS. For the BD-
Figure 4.3.3: Solubilization of the photoaffinity labelled $[^3]H$-strychnine binding site in the rat spinal cord using CHAPS: Photolabelled untreated (Us, strychnine specific; Uq, quinacrine specific) and BD-treated (Ts, strychnine specific; Tq, quinacrine specific) rat spinal cord membranes were incubated with CHAPS (0.25% w/v or 0.75 w/v) and a mixture of protease inhibitors. The mixture was agitated for 15 min at room temperature and held on ice for 1 hour. After centrifugation (100,000 xg, 1 hour, 4°C), the supernatant was used as a source of solubilized membrane preparation and the pellet was used as the membrane pellet. Part of the solubilized membrane preparation was further incubated with ammonium sulphate (50% of saturation) and bovine $\gamma$-globulin (100 $\mu$g/ml) at 4°C for 30 min as the solubilized precipitate. All of the samples were filtered and the binding is expressed as a % of the original photolabelled membrane control. Results were obtained from one experiment performed in triplicate.
induced strychnine binding site, the yield of solubilization was very low when using CHAPS.

4.4 Discussion

Preservation of binding properties in soluble binding sites appears to depend upon the choice of an appropriate detergent. The first approach in the present studies was to establish which detergent would provide the most efficient vehicle to solubilize the photolabelled BD-induced strychnine binding site. All three detergents gave a good yield of solubilised radioactivity from the photolabelled membranes. However, the recovery of this radioactivity upon precipitation with ammonium sulphate was low, with a maximum recovery of only 13% in 2% v/v Triton X-100.

In an attempt to achieve soluble active strychnine binding sites, there is a need not only to examine the ability of detergents to solubilize the binding site but also their tendency to inhibit $[^{3}\text{H}]$-strychnine binding to the binding site. Previous work showed that Triton X-100 is a strong inhibitor of $[^{3}\text{H}]$-strychnine binding to the BD-induced binding site, even at 0.1% w/v, more than 95% of BD-induced $[^{3}\text{H}]$-strychnine binding activity was inhibited. CHAPS and sodium cholate were less potent as inhibitors of $[^{3}\text{H}]$-strychnine binding, but their yield of solubilized BD-induced strychnine binding sites were also very low at concentrations below those which inhibit binding. With CHAPS (0.75%), although more than 55% of $[^{3}\text{H}]$-strychnine binding activity
was recovered in the whole solubilized membrane preparation in comparison with the photolabelled membrane control, there was only 0.35% of \(^{3}\text{H}\)-strychnine binding activity still remaining with the final solubilized precipitate.

A major problem in purifying the BD-induced strychnine binding site for molecular characterisation has been the loss of functional activity of this binding site which occurs upon solubilisation in detergent, rendering impossible any attempt at affinity chromatography.

The failure to sufficiently optimise this solubilization procedure for active binding sites prompted the use of affinity labelling as alternative approach to the isolation of the BD-induced strychnine binding site and this is described in Chapter Five.
CHAPTER FIVE

AFFINITY LABELLING OF THE 2,3-BUTANEDIONE-INDUCED STRYCHNINE BINDING SITE IN RAT SPINAL CORD MEMBRANES

5.1 Introduction

As described in Chapter Four, binding properties of the 2,3-butanedione induced strychnine binding site do not survive solubilization in a variety of detergents and so it cannot be purified by conventional affinity chromatography. Affinity labelling has therefore been used as an alternative approach to the isolation of the binding site.

Labelling of a binding site may be achieved by using a reactive ligand analogue, or affinity label. Although this approach inactivates the binding site, inhibiting radioligand binding and preventing functional study, the presence of a ligand, especially one crosslinked to the receptor, may stabilise the binding site during solubilization. Furthermore, the presence of an affinity label may allow detection of the ligand binding subunit of the protein in the gel of sodium dodecyl sulfate polyacrylamide (SDS-PAGE) electrophorogram, by use of suitable properties of the label such as fluorescence, or by use of antibodies directed against the affinity label.
Quinacrine mustard: a fluorescent affinity labelling reagent

Quinacrine has been reported to have a local anesthetic-like action (Grunhagen, et al., 1977; Adams and Feltz., 1980a, b; Volpi, et al., 1981) and to bind specifically to local anesthetic binding proteins in axonal membranes (Greenberg and Tsong, 1982). As described in Chapter Three for the BD-induced binding site, quinacrine is the highest affinity ligand that has been found. Derivatives of quinacrine (the azido analogue quinacrine-N₃ and the alkylating derivative quinacrine mustard) have been employed as fluorescent affinity-labelling reagents (Lauffer et al., 1979; Oxford and Hudson, 1982; Müller et al., 1982). Quinacrine mustard, one of these derivatives, belongs to the group of β-haloalkylamines, which have been widely employed in receptor pharmacology as irreversible syntropic antagonists (Jenkinson, 1991). In this form of drug antagonism, the antagonist forms a covalent bond with its binding site. The dissociation rate for the antagonist is negligible since this would involve the cleavage of a covalent bond. The action of such compounds relies on the structure $R_1R_2NCH_2CH_2X$, where $X$ is a halogen atom which acts as a leaving group. Quinacrine mustard itself is a bi- mustard, containing two such reactive groups (Figure 5.1.2). In aqueous solution, the haloalkylamine group cyclises to form an unstable aziridinium ion. For quinacrine mustard, this may have a greater affinity for the BD-induced strychnine binding site which it is to irreversibly label, since it may then form an ionic bond with its target. As the quinacrine mustard reaches the binding site, the aziridinium ring opens before the molecule dissociates from the target. A reactive intermediate
Figure 5.1.2 The structures of the two principle ligands for the BD-induced strychnine binding site, strychnine and quinacrine, together with the alkylating derivative quinacrine mustard.

is formed (possibly a carbonium ion) which covalently bonds with neighbouring nucleophilic groups such as -SH, -OH, =NH or -COOH. The aziridinium ion therefore reacts with fairly ubiquitous groups and may bind to nucleotides or lipids as well as proteins. Unreacted mustard may be quenched after affinity labelling the binding site by using nucleophilic groups such as the thiosulphate anion (Wormser, 1991). Chemical reagents such as sodium thiosulphate may thus be used to minimise non-specific labelling at very low affinity sites by addition to the preparation after specific labelling has occurred.
The following account describes attempts to irreversibly label the BD-induced strychnine binding site using this pharmacologically specific quinacrine mustard.

5.2 Affinity labelling the BD-induced strychnine binding site using quinacrine mustard

5.2.1 Optimizing the conditions for the incorporation of quinacrine mustard into the BD-induced strychnine binding site

5.2.1.a Attempt to label the BD-induced strychnine binding site with pre-cyclised quinacrine mustard

The quinacrine mustard was prepared at a concentration of 2 mM in 50% ethanol containing NaOH (2 mM) to facilitate the cyclisation of the mustard to form the active aziridinium ion, after the method of Dreyer, et al., 1986. This was diluted in PBS to give a 25 µM solution which was vortexed and left for 30 min at room temperature before use. A solution of solvent alone, containing ethanol, NaOH and PBS as above but no quinacrine was prepared as solvent control.

Untreated and BD-treated rat spinal cord membranes were prepared (see section 2.2.5) and each divided into 3 groups: the original untreated or BD-treated membranes, another with quinacrine mustard added to final
Figure 5.2.1.a: Labelling of the BD-induced strychnine binding site with pre-cyclised quinacrine mustard: BD-treated rat spinal cord membranes were incubated with (B) solvent (50 μM NaOH in 50% of ethanol) or (C) quinacrine mustard (QM, 1 μM) for 2 hours at room temperature, the reaction was terminated by the addition of 160 volumes of PBS/BSA/T. The samples were left for a further 10 min at room temperature before being washed at the same time with (A), the original membrane. [3H]-strychnine (6 nM) was incubated with each sample, with unlabelled strychnine (2x10^-4 M) used as the displacing ligand to enable estimates of strychnine specific binding, the binding is expressed as pmole/mg protein. Results were obtained from one experiment performed in triplicate.
concentration 1 μM and a third group with the same amount of solvent alone. The samples were vortexed and incubated for 2 hours at room temperature. The reaction was stopped by the addition of 160 volumes of phosphate buffered saline/bovine serum albumin (0.1% w/v) /sodium thiosulphate (10 mM) (PBS/BSA/T). The samples were vortexed again and left for a further 10 min at room temperature before being washed (4 times, 160 vols), assayed for protein content (see 2.2.16.b) and for [³H]-strychnine binding activity (see 2.2.6) with strychnine used as the displacing ligand.

In the untreated membrane there was very little difference in [³H]-strychnine binding between the solvent control and quinacrine mustard labelled samples (Data not show). This suggests that there was very little irreversible labelling of the strychnine binding site on the glycine receptor. That was probably because the quinacrine mustard has a similarly low affinity as quinacrine to the glycine receptor. But in BD-treated membranes (Figure 5.2.1.a), there was also little difference in [³H]-strychnine binding between quinacrine mustard labelled membranes and solvent control value.

The lack of irreversible labelling suggests that the affinity label was not effectively incorporated into the BD-induced strychnine binding site during the incubation. It has been speculated (see Chapter 1) that ligands can only gain access to the BD-induced strychnine binding site through the lipid membrane, rather than through the pore of its putative channel. The evidence for this obtains from previous observations of neutral and permanently quaternised
strychnine derivatives showing that the ligands which cannot undergo an interconversion between charged and uncharged forms have a low affinity for this binding site (O'Connor, 1992, see also Chapter One). The present results are consistent with this evidence because they have employed quinacrine mustard which has been cyclised and charged before being added to membranes.

5.2.1.b Attempt to label the BD-induced strychnine binding site with quinacrine mustard in its uncharged state

To enable the quinacrine mustard to diffuse into the lipid bilayer, the mustard must be added in a predominantly inactive, uncharged form. It was hoped that once the quinacrine mustard had diffused into the binding site, spontaneously cyclising mustard molecules would react with the site to cause irreversible labelling. In view of this, it was decided to modify the preparation of the quinacrine mustard in order to delay cyclisation until it had been added to the membranes.

In this experiment, only BD-treated membranes were used and quinacrine mustard was prepared in dilute acid to delay the formation of aziridinium ion, after the method of Creech and O'Connell (1981). The mustard was prepared at a concentration of 2 mM in 1 mM HCl, the acidic environment favouring the presence of the inactive, un cyclised form of the quinacrine mustard. All other steps were carried out as in 5.2.1.a.
Figure 5.2.1.b: Labelling of the BD-induced strychnine binding site with quinacrine mustard: BD-treated rat spinal cord membranes were incubated with (B) solvent (HCl, 50 μM) or (C) quinacrine mustard (QM, 1μM) for 2 hours at room temperature, the reaction was terminated by the addition of 160 volumes of phosphate buffered saline/bovine serum albumin/sodium thiosulphate (PBS/BSA/T). The samples were left for a further 10 min at room temperature before being washed at same time with (A), the original membrane. For binding assays, [3H]-strychnine (6 nM) was incubated with each sample, with unlabelled strychnine (2x10⁻⁸M) used as the displacing ligand to enable estimates of strychnine specific binding. Binding is expressed as pmole/mg protein. Results were obtained from one experiment performed in triplicate.
There was a decrease in $[^3\text{H}]$-strychnine binding in quinacrine mustard labelled membrane compared to the solvent control membrane (Figure 5.2.1.b). In this incubation condition (QM: $1 \mu\text{M}$, 2 hours, room temperature), it represents a 46% reduction in $[^3\text{H}]$-strychnine binding compared to the control value. Irreversible binding appears to have occurred.

5.2.1.c Optimizing the pH for incorporation of quinacrine mustard into the BD-induced strychnine binding site

As mentioned above, it has been postulated that the ability of ligands to combine with the BD-induced strychnine binding site might be heavily dependent on their ability to undergo interconversion between charged and uncharged forms (O'Connor, 1992) in a similar manner to the mechanism proposed for the actions of local anesthetic amines (Hille, 1977a, b.) and channel blockers (Shapiro, 1977a, b.). It was therefore likely that the activity of such ligands would be sensitive to pH, and so the effect of varying pH was investigated.

Two parallel competition assays were set up, one at pH 7.4 as normal and another at pH 7.7, one of the $pK_a$ values of quinacrine. BD-treated membrane preparation (see 2.2.5) was thawed as usual, but resuspended in 40 volumes of PB (0.2M) at either pH 7.4 or pH 7.7, as appropriate instead of PBS. Quinacrine mustard was prepared at an initial concentration of 2 mM in HCl (1 mM), more dilute solutions being achieved by serial dilution in 1 mM HCl.
Figure 5.2.1.c: Effect of pH condition on the labelling of BD-induced strychnine binding site with quinacrine mustard: BD-treated rat spinal cord membranes were resuspended in PB, pH 7.4 (■) or pH 7.7(□) and incubated with solvent (HCl, 50 μM) or quinacrine mustard (1 μM, 10 μM or 100μM) for 2 hours at room temperature, the reaction was terminated by the addition of 160 volumes of PBS/BSA/T. The samples were left for a further 10 min at room temperature before being washed at the same time with original membrane. [3H]-strychnine (6 nM) was incubated with each sample, with the unlabelled quinacrine (10^4M) used as the displacing ligand to enable estimates of quinacrine specific binding, the binding is expressed as pmole/mg protein. Results were obtained from one experiment performed in triplicate.
Other solutions were prepared in the appropriate phosphate buffer (pH 7.4 and pH 7.7). All other steps were carried out as in 5.2.1.b. A binding assay was set up as in 2.2.6, with quinacrine as the displacing ligand.

At both pH 7.4 and pH 7.7 there was a decrease in $[^3]$H-strychnine binding in those membranes pre-treated with quinacrine mustard (Figure 5.2.1.c) and this decrease was concentration dependent. The slightly more alkaline pH 7.7 environment gave both increased $[^3]$H-strychnine binding initially and a greater inhibition of binding by quinacrine mustard relative to the samples incubated at pH 7.4. The optimum conditions for labelling of the BD-induced strychnine binding site appear to be at pH 7.7 and quinacrine mustard at a concentration of 10 $\mu$M. This represents a 97% reduction in $[^3]$H-strychnine binding compared to the solvent control value.

5.2.1.d The time course of quinacrine mustard labelling of BD-induced strychnine binding site

To investigate the time course of the quinacrine mustard incorporation into the binding site, the quinacrine mustard was prepared as in 5.2.1.c. The BD-treated rat spinal cord membrane preparation (see 2.2.5) was thawed as normal but resuspended in 40 volumes of PB (0.1 M, pH 7.7) instead of PBS. Two experiments (A and B) were set up. Quinacrine mustard (1 $\mu$M) was added to all tubes containing 0.9 ml of treated membrane preparation except the solvent control which received solvent HCl (50 $\mu$M) alone. All samples were then
Figure 5.2.1.d: Effect of length of incubation with quinacrine mustard on subsequent strychnine binding to BD-treated membranes: BD-treated rat spinal cord membranes (in 0.1 M, pH 7.7 PB buffer) were incubated with solvent (HCl, 50 μM) or quinacrine mustard (1 μM) at room temperature (A: □) and at 4 °C (B: ■), the reaction was stopped at differing times (from 0 to 360 min) by addition of 160 volumes of PBS/BSA/T. The samples were left for a further 10 min at room temperature before being washed. [3H]-strychnine (6 nM) was incubated with each sample, with unlabelled quinacrine (10 μM) used as the displacing ligand to enable estimates of quinacrine specific binding, the binding is expressed as % of solvent control. Results were obtained from one experiment performed in triplicate.
vortexed, experiment A was incubated at room temperature and experiment B was incubated at 4°C. The reaction was stopped at differing times by adding approximately 160 volumes of PBS/BSA/T. (For the time zero sample, PBS/BSA/T was added before the quinacrine mustard.) After vortexing, the quenched samples were left at room temperature for 10 min and washed as in 2.2.5. They were then assayed for protein content as in 2.2.17.a and for [3H]-strychnine binding activity (see 2.2.6), with quinacrine as the displacing ligand.

Lower binding was observed at time zero in both experiments A and B compared to the solvent control (Figure 5.2.1.d). This is may because the thiosulphate would have taken a finite time to inactivate quinacrine mustard molecules, although thiosulphate was present in large excess, some quinacrine mustard bound to the BD-induced binding site was still expected. In the experiment A, there is a clear reduction in [3H]-strychnine binding over time, suggesting increased occupancy of BD-induced binding site with quinacrine mustard. There was about 77% reduction in [3H]-strychnine binding at 240 min and at that point it appeared to have reach the minimum.

In experiment B, the incubation was carried on 4°C in order to minimise protein degradation. There was also a clear reduction in [3H]-strychnine binding over time, and by 240 min it reached the minimum. There is a 75% reduction in [3H]-strychnine binding at 240 min compared to the solvent control. This is similar to the result shows in experiment A. So the
temperature did not make clear a difference in the effect of quinacrine labelling to the BD-induced strychnine binding site.

5.2.2 Checking of the incorporation of mustine into BD-induced strychnine binding site

In view of the previous experiments, a large proportion of the BD-induced strychnine binding sites appear to have been irreversibly labelled. In order to obtain further evidence that this labelling is due to quinacrine mustard specific binding to BD-induced strychnine binding site rather than mustard non-specific binding, the non-specific mustard mustine (2-chloro-N[2-chloroethyl]-N-methylamine) was used. Also, it was thought that if problems arose with later experiments in the non-specific incorporation of quinacrine mustard, then mustine could be used to block reactive non-specific sites.

BD-treated membrane preparation (see 2.2.5) was thawed as usual, and resuspended in 40 volumes of PB (0.1 M, pH 7.7). Two small polypropylene centrifuge tubes were set up, each containing 1 ml of treated membrane preparation, to one was added mustine (10 μM) in HCl (50 μM) and to the other was added the HCl alone. The samples were vortexed and incubated (2 hours, room temperature). The reaction was stopped by adding 160 volumes of PBS/BSA/T, leaving for a further 10 min at room temperature. They were then washed as in 2.2.5, assayed for protein content as in 2.2.16.a and for [\(^3\)H]-strychnine binding activity as in 2.2.6 with quinacrine as the displacing
Figure 5.2.2: The incorporation of mustine into BD-induced strychnine binding site: BD-treated rat spinal cord membranes were resuspended in PB (0.1 M, pH 7.7) and incubated with (B) solvent alone (HCl, 50 μM) or (C) mustine (1 μM) for 2 hours at room temperature. The samples were left for a further 10 min at room temperature before being washed at the same time with (A), the original membrane. [3H]-strychnine (6 nM) was incubated with each sample, with unlabelled quinacrine (10^4 M) used as the displacing ligand to enable estimates of quinacrine specific binding. The binding is expressed as pmoles/mg protein. Results were obtained from one experiment performed in triplicate.
There was no reduction observed in $[^3\text{H}]$-strychnine binding in mustine treated membrane compared to the solvent control value (Figure 5.2.2), suggesting that mustine could not occupy the BD-induced strychnine binding site. This finding indicates that the reduction of $[^3\text{H}]$-strychnine binding by quinacrine mustard was due to the irreversible occupancy of the binding site by quinacrine mustard rather than the mustard non-specific binding. Also mustine could be used as a pre-treatment in further labelling experiments to reduce non-specific incorporation of the quinacrine mustard.

5.2.3 Comparison of quenching agents used to stop the quinacrine mustard reaction

In the experiment shown in Figure of 5.2.1.d, lower binding was observed at time zero compared to the solvent control, indicating that the speed of quinacrine mustard diffusion into and reaction with the binding site was faster than the speed of thiosulphate quenching it, although thiosulphate was present in excess. In order to get a better quenching effect, several quenching agents were investigated here.

Four quenching agents were used in this experiment: Thiosulphate, dithiothreithol, mercaptoethanol and ethanolamine. All of them were dissolved in PBS in the presence of unlabelled strychnine (at a final concentration of
Figure 5.2.3: Comparison of different quenching agents used to quench the quinacrine mustard reaction: BD-treated rat spinal cord membranes were incubated with quinacrine mustard (QM+) or without quinacrine mustard (QM-, in which sample received solvent HCl only). After incubation (0 min for A and 10 min for B), Thiosulphate (10 mM), dithiothreithol (10 mM), mercaptoethanol (10 mM) and ethanolamine (10 mM) in the presence of unlabelled strychnine (12.5 mM) were added and the samples were then washed immediately. For binding assays, [\textsuperscript{3}H]-strychnine (6 nM) was incubated with each sample, with unlabelled strychnine (2x10^4M) used as the displacing ligand to enable estimates of strychnine specific binding. The binding is expressed as pmole/mg protein. Results were obtained from one experiment performed in triplicate.
12.5 mM). It was speculated that unlabelled strychnine might be useful in occupying the BD-induced strychnine binding site which had not been labelled by quinacrine mustard in order to prevent further labelling. BD-treated membrane preparation (see 2.2.5) was thawed as usual and resuspended in 40 volumes of PB (0.1 M, pH 7.7). Two groups of experiments (A and B) were set up and the membranes were incubated with or without quinacrine mustard (10 μM). For group A, quenching agents were added before quinacrine mustard (time zero); for group B, quenching agents were added after 10 min incubation (10 min). The samples were immediately washed (see 2.2.5), assayed for protein content (see 2.2.16.b) and for [³H]-strychnine binding activity (see 2.2.6) with strychnine as the displacing ligand.

In group A (Figure 5.2.3), there was clear reduction in [³H]-strychnine binding in all of the samples compared to their solvent control, suggesting that none of these four quenching agents was successful in completely quenching the quinacrine mustard on the time zero. The degrees of the reduction in [³H]-strychnine binding was very similar (from 46% to 61%). In the group B, quenching agents were added after incubation (10 min, 4 °C) of quinacrine mustard. Here there was also a decrease in [³H]-strychnine binding in all of the samples compared to their solvent control. Again the degrees of the reduction was very similar (from 49% to 52%). So there was almost no difference in the effect of quenching quinacrine mustard for all four of these quenching agents, although 10 min incubation with ethanolamine alone appear to have decreased binding.
5.2.3.a Checking the effect of thiosulphate on the BD-induced strychnine binding site

In order to check whether thiosulphate as a quenching agent has any effect on the activity of BD-induced strychnine binding site, PBS, BSA (0.1% w/v), unlabelled strychnine (10 mM) and thiosulphate (10 mM) have been used in different combinations of incubations with BD-treated membranes.

Treated membrane preparation (see 2.2.5) was thawed as normal and resuspended in 40 volumes PBS. The preparation was then divided into samples, each containing 1 ml of membrane preparation. PBS, BSA, unlabelled strychnine and thiosulphate were added in different combinations as shown in Figure 5.2.3.a, vortexed and left for 10 min at room temperature. Then the samples were washed as in 2.2.5, assayed for protein content as in 2.2.16.a and for [3H]-strychnine binding activity as in 2.2.6, with strychnine as the displacing ligand.

There was little variation in [3H]-strychnine binding observed here (Figure 5.2.3.a). The binding of which incubated with PBS/BSA was slightly higher compared to the PBS control, this was probably due to the stabilisation of the binding site by BSA. There was no marked decrease in [3H]-strychnine binding in the samples incubated with PBS/BSA/strychnine/thiosulphate or PBA/strychnine/thiosulphate in comparison with the PBS control, suggesting that there was no side effect of thiosulphate on BD-induced strychnine binding.
Figure 5.2.3.a: Effect of thiosulphate on the BD-induced strychnine binding site:
BD-treated rat spinal cord membranes were incubated with either PBS/strychnine(12.5 mM), PBS/BSA, PBS, PBS/BSA, thiosulphate(10mM)/BSA/strychnine(12.5mM) or thiosulphate(10mM)/strychnine(12.5mM) respectively for 10 min at room temperature. After washing, [3H]-strychnine (6 nM) was incubated with each sample, with unlabelled strychnine (2x10^{-4}M) used as the displacing ligand to enable estimates of strychnine specific binding. The binding is expressed as pmole/mg protein. Results were obtained from one experiment performed in triplicate.
site and confirming that unlabelled strychnine had been washed away. So
thiosulphate was chosen for further experiments as a quenching agent for
quinacrine mustard labelling BD-induced binding site.

5.2.4 Sodium dodecyl sulfate polyacrylamide gel electrophoresis of peptides
from quinacrine mustard labelled mouse spinal cord membranes

Quinacrine mustard reduced the level of [3H]-strychnine binding by up to 97%
(5.3.1.c). This appears to be due to irreversible binding of mustard to the BD-
induced strychnine binding site rather than simply reflecting the presence of
high-affinity but non-covalent forces between the ligand and the binding site.
To prove this, quinacrine mustard labelled peptide has been resolved on an
SDS-polyacrylamide gel.

Untreated and BD-treated mouse spinal cord membrane preparations (see
2.2.5) were thawed as normal and resuspended in 40 volumes of PB (0.1 M,
pH 7.7). reaction tubes were set up, each containing a 1.2 ml untreated or BD-
treated membrane. Quinacrine mustard was dissolved in HCl (1 mM) and
added to all tubes to final concentrations of 10 μM, 50 μM and 100 μM
respectively but solvent control tubes received HCl (50 μM) only. After
incubation (10 min, 4°C), 160 volumes of PBS/BSA/T was added to each tube
to stop the reaction. The samples were vortexed and washed as in 2.2.6. A
protein assay (2.2.16.a) and a binding assay were then set up (2.2.6), with
quinacrine as the displacing ligand. In parallel, a portion of each sample was
solubilised in 2% SDS and loaded on a 10% SDS polyacrylamide gel for electrophoresis (see 2.2.14.c). Two gels were run at the same time, gel A has been photographed under the ultraviolet light box and stained in coomassie brilliant blue-G 250 (CBB-G 250) and the fluorescence bands in the gel B have been cut out to quantify the degree of quinacrine mustard labelling (see below).

From the binding assay results (Figure 5.2.4.a), there was a decrease in [³H]-strychnine binding in quinacrine mustard labelled BD-treated membrane compared to the solvent control. The degree of reduction corresponds to the concentration of quinacrine mustard. When quinacrine mustard was used at a concentration of 10 μM, it caused a 56% reduction in [³H]-strychnine binding. When the concentration of quinacrine mustard was 100 μM, the reduction of the binding increased to 80%. By contrast, there was no clear decline in [³H]-strychnine binding in quinacrine mustard labelled untreated membranes compared to their solvent controls. It appeared to be that BD-induced strychnine binding site had been specifically affinity labelled by quinacrine mustard.

The gel A picture is shown on Figure 5.2.4.b. In the UV fluorescence picture, there was a labelled fluorescence band at 23 kD with an increased concentration of quinacrine mustard. At the concentration of 10 μM quinacrine mustard, there appeared a very weak fluorescent labelled band, but at the concentration of 100 μM, a clear fluorescent band could be seen. The quinacrine mustard
Figure 5.2.4.a: Untreated and BD-treated mouse spinal cord membranes labelled with different concentration of quinacrine mustard: Untreated (U:■) and BD-treated (T:□) mouse spinal cord membranes were incubated with quinacrine mustard (either 0, 10 μM, 50 μM or 100 μM) for 10 min at 4°C. The reaction was stopped by the addition of 160 volumes of PBS/BSA (0.1% w/v)/thiosulphate (10 mM). For subsequent binding assays, [3H]-strychnine (6 nM) was incubated with each sample, with unlabelled quinacrine (10^4M) used as the displacing ligand to enable estimates of quinacrine specific binding. The binding is expressed as pmole/mg protein. Experiments have repeated four times, results were obtained from one experiment performed in triplicate.
Figure 5.2.4.b: Electrophoretic profile of untreated or BD-treated mouse spinal cord membranes labelled with quinacrine mustard:
Untreated (U) and BD-treated (T) mouse spinal cord membranes were incubated with quinacrine mustard (either 0, 10 μM, 50 μM or 100 μM) for 10 min at 4°C. The reaction was stopped by the addition of 160 volumes of PBS/BSA/T. The samples were washed, solubilized in 2 % SDS then mixed with "master mix" (1:2 v/v). Electrophoresis was carried out on 10 % acrylamide gel using 100 μg protein per sample. A. represents gel after being stained in CBBG-250 and B. represents the same gel under UV light.
can not only label BD-treated membrane but also labels the untreated membrane. This gel has also been stained in CBB-G 250 (see 2.2.13.d) to show the total peptide distribution. The labelled fluorescent band corresponds to the major peptide in the band at 23 kD, indicating that the 23 kD peptide has been irreversibly labelled by fluorescent quinacrine mustard. If the samples were boiled before being loaded into the gel, not only the labelled fluorescent band disappeared under the UV light but also the 23 kD peptide band on protein stain gel disappeared too (see 6.5).

In order to quantify the degree of quinacrine mustard labelling, the fluorescent bands (on gel B) have been cut out and their fluorescence eluted and measured (see 2.2.15). There was an increase in fluorescence which corresponded with an increase in the concentration of quinacrine mustard used as a label in both untreated and BD-treated membranes (Figure 5.2.4.c, data is from four experiments). Thus, unlike the situation with the reversible [³H]-strychnine ligand binding, irreversible labelling with quinacrine mustard reveals binding in both untreated and BD-treated membranes. This is the same as shown qualitatively on the gel A UV fluorescent picture in Figure 5.2.4.b. This is probably because in the untreated membrane, the BD-induced strychnine binding site is normally in a very low affinity state to strychnine which is reversibly binding and cannot be detected by the [³H]-strychnine binding assay. However BD treatment increases the affinity of this binding site to strychnine, so that it can be detected by the [³H]-strychnine binding assay. By contrast, quinacrine mustard was able to irreversibly label this binding site in both
Figure 5.2.4.c: Labelling of a 23 kD peptide in untreated and 2,3-butanedione treated mouse spinal cord membranes by increasing concentrations of quinacrine mustard (10, 50 and 100 μM, 10 min, 4 °C): The portion of the gel corresponding to 23 kD was cut out and eluted for the direct measurement of fluorescence (Abs 350 nm; Em 500 nm). Fluorescence is expressed as quinacrine equivalents and is the mean ± s.e.m. from four experiments. Student's paired t-test for significance of difference between untreated and 2,3-butanedione treated membranes: P < 0.05.
untreated membrane and BD-treated membrane. That may be the explanation as to why a quinacrine mustard labelled fluorescence band was observed in both untreated and BD-treated membrane loaded gel. The fluorescence from the BD-treated sample was higher than that from untreated sample.

5.3 An investigation of the ability of strychnine, glycine or quinacrine to protect against inactivation of $[^3H]$-strychnine binding in BD-treated spinal cord membranes by quinacrine mustard

During the next protection experiments, some samples were preincubated with protecting ligands: strychnine, glycine or quinacrine in order to investigate whether or not the quinacrine mustard induced reduction in $[^3H]$-strychnine binding was due to the irreversible labelling of the BD-induced site or to degradation of membrane protein or other reasons. If the former, it was expected that strychnine and quinacrine would act as competitive inhibitors of quinacrine mustard labelling, and thereby protect amino acid residues in the binding site.

5.3.1 Ability of strychnine, glycine or quinacrine to protect against quinacrine mustard labelling of the BD-induced strychnine binding

Both untreated and BD-treated membranes were used in order to show that any variations in $[^3H]$-strychnine binding activity could be ascribed to the BD-induced site rather than the glycine receptor.
Reaction tubes were set up, each containing a 2 ml sample of membrane preparation, untreated or BD-treated as appropriate (see 2.2.5). Strychnine (final concentration 1 mM), quinacrine (final concentration 1 mM) or glycine (final concentration 10 mM) were added separately for investigation as protecting ligands, the remaining unprotected sample receiving PBS only. The tubes were vortexed and left for 30 min at room temperature. Then the samples were labelled with quinacrine mustard (see 2.2.11.b). After the samples had been washed, they were then assayed for protein content (see 2.2.16.b) and for strychnine binding activity (see 2.2.6) with strychnine as the displacing ligand.

In BD-treated membranes, there was more than a 50% decrease of [³H]-strychnine binding in the unprotected sample which had been incubated with quinacrine mustard compared to those that had not (Figure 5.3.1). This suggests as before that the BD-induced strychnine binding site had been irreversibly labelled. If unlabelled strychnine and quinacrine had successfully protected the BD-induced binding site, preventing irreversible labelling by quinacrine mustard then the subsequent [³H]-strychnine binding in protected samples should be higher than those in the unprotected samples. However there was little difference in [³H]-strychnine between the unprotected samples and the strychnine protected samples. There are two possible explanations for this. Firstly, the protecting concentration of strychnine and/or quinacrine might not be high enough; the binding site is known to be a low affinity high capacity site (O'Connor, 1992; see also Chapter 3), and any unprotected site exposed
Figure 5.3.1: Protection against quinacrine mustard labelling of the BD-induced strychnine binding site by strychnine, glycine and quinacrine: BD-treated mouse spinal cord membranes were preincubated either with no ligand (PBS for solvent control and unprotected), or strychnine (1 mM: strychnine protected), or glycine (10 mM, glycine protected) or quinacrine (1 mM, quinacrine protected) for 30 min at room temperature. The samples were then labelled with quinacrine mustard (10 µM, 10 min, 4 °C) and washed. [³H]-strychnine (6 nM) was then incubated with each sample, with unlabelled strychnine (2x10⁻⁴M) used as the displacing ligand to enable estimates of strychnine specific binding. The binding is expressed as pmole/mg protein. Results were obtained from one experiment performed in triplicate.
to the quinacrine mustard would have been irreversible labelled by it. The second possibility is that strychnine and/or quinacrine may not have been adequately washed out of the binding site by the procedure detailed in 2.2.6, in which case they would have still been present to inhibit [³H]-strychnine binding during the binding assay. The [³H]-strychnine binding in the glycine-protected samples was the same, as expected. The glycine did not show any protection against quinacrine mustard labelling, because of its low affinity to this binding site (see Chapter Three).

5.3.2 The time course for washout of strychnine and quinacrine from the BD-induced strychnine binding site

In view of the results obtained in the last experiment, it was decided to check that protecting ligands were being completely removed before subsequent measurements of [³H]-strychnine binding activity to the BD-induced strychnine binding site.

Incubation tubes were set up, each containing 2.5 ml BD-treated membrane preparation (see 2.2.5). Strychnine or quinacrine (both final concentration: 10 mM) were added to different tubes and the control tubes received the solvent (PBS) alone. Samples were vortexed and incubated for 30 min at room temperature. Then 1.25 ml of each sample was removed and kept at 4 °C as unwashed samples for later binding assay. The rest of samples were washed from one to six times respectively. They were then assayed for protein content.
(see 2.2.16.b) and for $[^3]$H-strychnine binding activity (see 2.2.6), with strychnine as the displacing ligand.

There was a small decrease in $[^3]$H-strychnine binding activity of the control sample after the washing, this might have occurred because part of BD-induced strychnine binding site had denatured. (Figure 5.3.2). In strychnine incubated samples, there was an increase in the $[^3]$H-strychnine binding after only one wash. It was shown that after two washes, the unlabelled strychnine had been completely removed and $[^3]$H-strychnine binding activity had reached the same levels as the control samples. By contrast, the quinacrine had not been washed out at all, even after washing six times. So the reason for the failure of the protection experiment in 5.3.1, for strychnine may be due to the fact that the concentration of strychnine was not high enough to protect the binding site, however for quinacrine it may because the quinacrine could not be washed out from the binding site.

5.3.2.a Optimizing the conditions for quinacrine washed out from the BD-induced strychnine binding site

It has been shown that quinacrine could not be washed out from the BD-induced binding site by normal washing procedures (5.3.2). The ability of ligands to interact with the recognition site is affected by the pH of either the receptor protein and/or the chemical properties of the ligands. For this reason, a series of washing buffers were tried over a range of pH values.
Figure 5.3.2: Washing out of strychnine and quinacrine from the BD-induced strychnine binding site: BD-treated rat spinal cord membranes were treated with either no ligand (control: ■), strychnine (10 mM, □) or quinacrine (10 mM, ♦) for 30 min at room temperature. The samples were then washed from zero to six times respectively. For binding assays, [$^3$H]-strychnine (6 nM) was incubated with each sample, with unlabelled strychnine (2x10^{-4}M) used as the displacing ligand to enable estimates of strychnine specific binding. The binding is expressed as fmole/mg protein. Results were obtained from one experiment performed in triplicate.
BD-treated membrane preparations (see 2.2.5) were incubated (30 min, 4°C) either with quinacrine (10 \( \mu \text{M} \)) in a PB buffer (0.1 mM, pH 7.7) or with PB buffer alone as solvent control. They were then washed twice in the appropriate phosphate/citric acid buffer at pH from 4 to 7 respectively and resuspended in PBS. They were then assayed for protein (see 2.2.16.b) and for \([^3\text{H}]\)-strychnine binding activity (see 2.2.6) with quinacrine as the displacing ligand.

After the samples were washed in the pH of 5, 6 and 7 washing buffers, it was found that there was very little change in the \([^3\text{H}]\)-strychnine binding between them, not only in the control samples but also in the quinacrine pre-incubated samples (Figure 5.3.2.a). There was about a 72% - 74% reduction in the \([^3\text{H}]\)-strychnine binding activity in the samples which were incubated with quinacrine compared to their control. However after the samples were washed in pH 4 washing buffer, there was an increase in the \([^3\text{H}]\)-strychnine binding activity in both the control sample and quinacrine treated sample. This may have been because in the acidic environment the BD-induced strychnine binding site had been activated. Also in the washing condition of pH 4, there was only 30% of \([^3\text{H}]\)-strychnine binding activity reduction in the sample incubated with quinacrine compared to the control. This suggests that the acidic environment not only activates the binding site but also favours quinacrine dissociation from the binding site.
Figure 5.3.2.a: Effect of pH on quinacrine wash out from BD-induced strychnine binding site: BD-treated rat spinal cord membranes were incubated either with quinacrine (10 μM, □) or PB buffer (solvent control, ■) for 30 min at 4 °C. The samples were then washed twice in a phosphate citric acid buffer, with a pH from 4 to 7 respectively and then re-suspended in PBS for binding assay. For these binding assays, [3H]-strychnine (6 nM) was incubated with each sample, with unlabelled quinacrine (10^4M) used as the displacing ligand to enable estimates of quinacrine specific binding. The binding is expressed as pmole/mg protein. The specific binding of original membrane was 1.052 pmole/mg protein. Results were obtained from one experiment performed in triplicate.
5.3.2.b Optimisation of quinacrine wash out from BD-induced strychnine binding site at pH 4

Since the last experiment which shows that a washing buffer of pH 4 was the best condition for washing quinacrine out from BD-induced strychnine binding site, a further experiment was performed to optimise the wash out of quinacrine from the BD-induced binding site at this pH. BD-treated membrane preparation (see 2.2.5) was incubated with quinacrine as in 5.3.2.a but the membranes were then washed in an appropriate pH 4 phosphate citric acid buffer from 0 to 6 times respectively. Membranes were then assayed for protein (see 2.2.16.b) and for $[^3H]$-strychnine binding activity (see 2.2.6) with quinacrine as the displacing ligand.

There was increased $[^3H]$-strychnine binding activity in both the control samples and the quinacrine preincubated samples with an increased number of washes (5.3.2.b). The ratio of the binding in the quinacrine preincubated sample compared to the binding in control sample was clearly increased by increased washing. With no washing, there was only 10.82% of binding in the quinacrine preincubated sample compared to the binding of its control, but after washing 6 times, there was a 90.53% of binding in the quinacrine preincubated sample compared to the binding of its control. This demonstrates that about 90% of the bound quinacrine had been washed out from the BD-induced strychnine binding site after washing 6 times in pH 4 washing buffer.
Figure 5.3.2.b: Washing out of quinacrine from BD-induced strychnine binding site: BD-treated rat spinal cord membranes were incubated with either quinacrine (10 μM, □) or PB buffer alone (0.1 M, pH 7.7, ■) for 30 min at 4 °C. The samples were then washed in phosphate citric acid buffer (160 vols; pH 4) from 0 to 6 times respectively. For the binding assays, [3H]-strychnine (6 nM) was incubated with each sample, with unlabelled quinacrine (10^4 M) used as the displacing ligand to enable estimates of quinacrine specific binding. The sample is expressed as fmole/mg protein. Results were obtained from one experiment performed in triplicate.
5.3.3 Attempt to use quinacrine as a protecting ligand to protect against quinacrine mustard labelling of the BD-induced strychnine binding site

In this experiment, quinacrine has been used to act as a protecting ligand to protect against the quinacrine mustard labelling the BD-induced strychnine binding site.

BD-treated membrane preparation (see 2.2.5) was thawed as normal and resuspended in 40 volumes of PB (0.1 mM, pH 7.7). Membranes were then incubated with quinacrine (10 μM, 30 min, 4 °C) before being labelled by quinacrine mustard (see 2.2.11.b). After washing 6 times in phosphate citric acid buffer (pH 4), the samples were resuspended in PBS, assayed for protein content (2.2.16.b) and for [³H]-strychnine binding activity (see 2.2.6) with quinacrine as the displacing ligand.

In the unprotected samples, there was about an 18 % reduction of [³H]-strychnine binding in quinacrine mustard labelled samples compared to unlabelled samples (Figure 5.3.3). This is far less then the normally observed 50 % reduction of [³H]-strychnine binding in the same labelling condition but with samples washed in PBS (pH 7.4). This was probably due to the acidic environment increasing the activation of the binding site (see Figure 5.3.2). The [³H]-strychnine binding in the quinacrine-protected sample was the opposite of that expected. Quinacrine should compete for the BD-induced strychnine binding site, preventing irreversible labelling by quinacrine mustard.
Figure 5.3.3: Protection of BD-induced strychnine binding site by quinacrine: Quinacrine (10 μM) was added to the BD-treated rat spinal cord membrane 30 min before the addition of either quinacrine mustard: □ (10 μM, 10 min, 4°C, ) or solvent: ■ (50 μM HCl, 10 min, 4°C, ). Unprotected samples received PBS instead of quinacrine. The reaction was terminated by the addition of 160 volumes of PBS/BSA/T, and the samples were washed 6 times with a pH 4 phosphate citric acid buffer before a final resuspension in PBS. For the binding assays, [³H]-strychnine (6 nM) was incubated with each sample, with unlabelled quinacrine (10⁻⁴M) used as the displacing ligand to enable estimates of quinacrine specific binding. The binding is expressed as pmole/mg protein. Results were obtained from one experiment performed in triplicate.
and hence preventing the associated reduction in [$^3$H]-strychnine binding activity, the quinacrine-protected samples actually showed lower [$^3$H]-strychnine binding than the unprotected samples. There are two possible explanations for this. Firstly, quinacrine may still not have been adequately washed out of the binding site by washing six times in phosphate citric acid buffer (pH 4), in which case it would still be present to inhibit [$^3$H]-strychnine binding during the assay. The second possibility is that the binding of ligands at BD-induced binding site is greatly facilitated by the presence of quinacrine (see Discussion). So it looks like quinacrine cannot be used as a protecting ligand for the BD-induced strychnine binding site in binding assay.

5.3.4 Attempt to use strychnine as a protecting ligand to protect against quinacrine mustard labelling of the BD-induced strychnine binding site

Section 5.3.2 shows that strychnine could be washed out from a BD-induced strychnine binding site. The reason for it failing to act as a protection ligand in experiment 5.3.2 was probably due to the low protection concentration which had been used. Unlabelled strychnine is a reversible ligand binding with low affinity (see Chapter 3) to the BD-induced strychnine binding site. In contrast, the irreversible quinacrine mustard binds and forms a covalent bond. Thus, a large excess of reversible strychnine is required to protect a binding site from the irreversible ligand quinacrine mustard.

5.3.4.a Optimizing conditions for strychnine as a protecting ligand

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In this experiment, 10 mM strychnine was used to protect against quinacrine mustard labelling. BD-treated membrane preparation (see 2.2.5) was used, and unlabelled strychnine (10 mM) was added 30 min (4 °C) before the membranes were incubated with quinacrine mustard (10 μM; 2.2.12.b). The reaction was stopped at two different incubation times: 10 min and 20 min. After washing, the samples were assayed for protein content (see 2.2.16.b) and for [3H]-strychnine binding activity. Strychnine was used as the displacing ligand.

As shown in Figure 5.3.4.a, irreversibly labelling was observed in the strychnine unprotected samples. There was a reduction of 48% and 52% after 10 min and 20 min incubation respectively in comparison with their solvent controls. Protection was shown at both the 10 min and 20 min incubation samples, the binding difference between the unprotected and strychnine protected in quinacrine mustard labelled samples was larger in the 10 min than that in the 20 min incubation time. This indicates that 10 min is the best quinacrine mustard incubation time to demonstrate strychnine protection. If the incubation time is any longer than this, it would increase the chances of the quinacrine mustard to irreversible bind to the binding site, and if shorter than 10 min, there would be less than 50% of quinacrine mustard incorporated into the binding site.

5.3.4.b Ability of strychnine to protect against quinacrine mustard labelling of the BD-induced strychnine binding site
Figure 5.3.4.a: Effect of QM incubation time on protection of BD-induced strychnine binding site by strychnine: Strychnine: S(+), 10 mM was added to the BD-treated rat spinal cord membrane 30 min before the addition of either quinacrine mustard: QM(+), (10 μM, 10 min, 4°C), or solvent alone: QM(-), (50 μM HCl, 10 min, 4°C, ). Unprotected samples: S(-) were received PBS instead of strychnine. The reaction was terminated after 10 or 20 min incubation by the addition of 160 volumes of PBS/BSA/thiosulphate (10 mM). For the binding assays, [\(^3\)H]-strychnine (6 nM) was incubated with each sample, with unlabelled quinacrine (10aising M) used as the displacing ligand to enable estimates of quinacrine specific binding. The binding is expressed as pmole/mg protein. Results were obtained from one experiment performed in triplicate.
BD-treated membrane preparation (see 2.2.5) was thawed as normal and incubated with strychnine (10 mM, 30 min, 4°C). Then the samples were labelled with quinacrine mustard for 10 min at 4°C (see 2.2.13) and washed. A protein assay (see 2.2.16.b) and a binding assay was set up (see 2.2.7) with quinacrine as the displacing ligand. At the same time, portions of the sample were prepared and loaded on a 10% SDS-PAGE gel (see 2.2.13).

There were two experiments done here. Experiment A is shown in Figure 5.3.4.b.1. In the binding assay results, there was a decrease in [3H]-strychnine binding activity in unprotected samples, of about 75% reduction in comparison with the solvent control. By contrast, in the sample which was strychnine protected before it was labelled by quinacrine mustard, there was only a 50% reduction of [3H]-strychnine binding in comparison with the solvent control. So the strychnine has effectively protected the BD-induced strychnine binding site against the quinacrine mustard labelling. The same samples were run on 10% SDS-PAGE gel, and the gel viewed under the UV light. Fluorescence bands observed and cut out. The fluorescence has been eluted from these gel slices and read using a spectrofluorimeter (see 2.2.14). The result shows that there was higher fluorescence in the strychnine unprotected sample in comparison with to the strychnine protected sample. The rest of gel was stained in CBB-G250 and the molecular weight of the fluorescence band corresponded to a peptide in 23 kD position. This indicated that this 23 kD peptide had not only been affinity labelled by the quinacrine mustard but also successfully protected by the unlabelled strychnine.
Figure 5.3.4.b.1: Protection of BD-induced strychnine binding site by strychnine:
Strychnine (10 mM) was added to the BD-treated rat spinal cord membrane 30 min before the addition of either quinacrine mustard: □ (10 μM, 10 min, 4°C, ) or solvent alone: ■ (50 μM HCl, 10 min, 4°C, ). Unprotected samples received PBS instead of protected ligand. The reaction was terminated by the addition of a 160 volumes of PBS/BSA/T and the samples were washed. A: Binding of [3H]-strychnine (6 nM) with unlabelled quinacrine (10 μM) used as the displacing ligand to enable estimates of quinacrine specific binding. The binding is expressed as pmole/mg protein. Data was obtained from one experiment and performed in triplicate. B: Portions of the same samples were washed and applied to 10 % SDS-PAGE gel. The fluorescent labelled peptide band at 23 kD was cut and the fluorescence has been eluted in 1ml Tris/HCl buffer (5 mM, pH 8.8) for at least 2 hours. The measurements were preformed using 350 nm excitation wavelength and 500 emission wavelength. Results are expressed as pmole of quinacrine fluorescence.
In experiment B, the binding assay results were similar to experiment A (Figure 5.3.4.b.). Thus, in the unprotected sample, quinacrine mustard treatment caused a 57% reduction in [³H]-strychnine binding, but in the strychnine protected sample, there was only an 11% reduction. Again, this shows the binding site has been both affinity labelled and protected. As in experiment A, portion of these samples were solubilized and run on an SDS PAGE gels. This gel was then cut into 1 cm slices and the fluorescence eluted and measured (2.2.14). Result are shown in Figure 5.3.4.b.2. In the sample which was labelled with quinacrine mustard, with no protection there was a labelled fluorescence material at 23 kD, however when with strychnine protection, the strychnine prevented the fluorescence labelling and reduced the fluorescence to the gel background which was seen in the samples without quinacrine mustard labelling. This again confirmed that the quinacrine mustard has specifically labelled a peptide at 23 kD and that strychnine has effectively protected this labelling.

5.3.5 Ability of strychnine, glycine, and quinacrine to protect against quinacrine mustard labelling of the BD-induced strychnine binding site

With the benefit of the above preliminary experiments, a full investigation was made of the ability of strychnine, glycine and quinacrine to protect quinacrine mustard labelling of BD-induced strychnine binding site. BD-treated mouse spinal cord membrane preparations (see 2.2.5) were thawed as normal and resuspended in PB (0.1 mM, pH 7.7). The membranes received either
Figure 5.3.4.b.2: Protection of BD-induced strychnine binding site by strychnine:
Strychnine (10 mM) was added to the BD-treated rat spinal cord membrane 30 min before the addition of either quinacrine mustard: \( \square \) (10 \( \mu \)M, 10 min, 4\(^\circ\)C, ) or solvent alone: ■ (1 mM HCl, 10 min, 4\(^\circ\)C, ). Unprotected samples received PBS instead of strychnine. The reaction was terminated by the addition of a 160 volumes of PBS/BSA/T then samples were washed. A: Binding of \( ^{3}H\)-strychnine (6 nM) with unlabelled quinacrine (10\(^{-4}\)M) used as the displacing ligand to enable estimates of quinacrine specific binding. The binding is expressed as fmole/mg protein. Results obtained from one experiments performed in triplicate. B: Portions of the same samples were solubilised in 2% SDS and applied to 10% SDS-PAGE gel. After electrophoresis, the gel was cut into 1 cm slices and fluorescence was eluted in 1ml Tris/HCl buffer (5 mM, pH 8.8) for at least 2 hours. The measurements were formed using 350 nm excitation wavelength and 500 emission wavelength in a spectrofluorimeter at room temperature. Results are expressed as mean, \( n=3 \).
strychnine (10 mM) or glycine (10 mM) or quinacrine (1 mM) or solvent only (30 min, 4\(^\circ\) C) before they were labelled with or without quinacrine mustard (see 2.2.11.b). After washing, the membranes were assayed for protein content (see 2.2.16.b), \(^{3}\text{H}\)-strychnine binding activity (6 nM) with quinacrine as the displacing ligand (see 2.2.6), and were run on 10 % SDS-PAGE gel (see 2.2.13).

The binding of \(^{3}\text{H}\)-strychnine to BD-treated membranes with or without previous quinacrine mustard labelling is shown on Figure 5.3.5.a. The quinacrine mustard labelling condition was chosen to cause approximately a 50% decrease in \(^{3}\text{H}\)-strychnine binding in unprotected membranes. When using strychnine (10 mM) as a protecting ligand in quinacrine mustard labelling processes, the strychnine significantly prevented the labelling. In contrast, glycine provided no significant protection. It was not possible to evaluate the effectiveness of quinacrine as a protecting ligand, since quinacrine cannot be washed out from the binding sites by the normal washing procedure after the labelling processes, so it was not possible to measure \(^{3}\text{H}\)-strychnine binding activity.

When the labelled membranes were solubilized in SDS and resolved in SDS-PAGE gels, there was a fluorescent labelled peptide at about 23 kD. The 23 kD fluorescence band was cut out and its fluorescence has been eluted and measured (see 2.2.14). This was the method used to quantify the degree of the labelling. The results are shown as a percentage of the control fluorescence.
eluted from the 23 kD band in the unprotected membrane (Figure 5.3.5.b). When using strychnine as protecting ligand, it has less quinacrine mustard labelling at 11.25% fluorescence of the unprotected control. Glycine was unable to prevent this labelling, it shows 82% fluorescence of the control, but quinacrine was able to prevent the labelling and its fluorescence was only 19% of the control. Again these results are consistent with the [³H]-strychnine binding results. So it appears to be that the BD-induced strychnine binding site has been affinity labelled with quinacrine mustard.

5.4. Discussion

Quinacrine mustard, one of the derivatives of quinacrine, has been successfully employed as a fluorescent affinity-labelling reagent to label the BD-induced strychnine binding site in this study. Interestingly, the ability of the quinacrine mustard to combine with the BD-induced binding site is heavily dependent on its ability to undergo interconversion between charged and uncharged forms. If the quinacrine mustard was prepared with NaOH (2 mM) to facilitate the cyclisation of the mustard to form the active aziridinium ion, then no affinity labelling was observed. The only way for quinacrine mustard to irreversible label this binding site is to prepare it in an acidic environment (HCl, 1 mM), in order to delay cyclisation until it reaches the binding site. This confirms the earlier observations of neutral and permanently quaternised strychnine derivatives made in this laboratory (O’Connor and Fry, 1992; O’Connor, 1992; Chapter Three), those compounds which were quarternerized and carried
Figure 5.3.5: Protection of BD-induced strychnine binding site by strychnine, glycine and quinacrine: Strychnine (10 mM), glycine (10 mM) and quinacrine (1 mM) were added to the BD-treated rat spinal cord membrane 30 min before the addition of either quinacrine mustard: ■ (10 μM, 10 min, 4°C,) or solvent alone: □ (50 μM HCl, 10 min, 4°C,). Unprotected samples received PBS instead of protecting ligand. The reaction was terminated by the addition of a 160 volumes of PBS/BSA/T and the samples were washed. A: [³H]-strychnine (6 nM) was incubated with each sample, with unlabelled quinacrine (10⁴M) used as the displacing ligand to enable estimates of quinacrine specific binding. The binding is expressed as percent of control binding. Control specific binding was 2466.78 pmoles/mg protein. B: The same samples were washed and applied to 10% SDS-PAGE gel. The 23 kD fluorescence labelled peptide band was cut out and the fluorescence eluted in 1ml Tris/HCl buffer (5 mM, pH 8.8) for at least 2 hours. The measurements were performed using 350 nm excitation wavelength and 500 emission wavelength. Results are expressed as % of control. Control quinacrine fluorescence (1.23x10⁷ M) which was obtained by using a 23 kD fluorescence labelled peptide in an unprotected sample loaded gel. Data was shown as mean ± standard error of the mean, n=4. Student’s paired t-test: #p > 0.02 and *p < 0.01 for significance of difference from unlabelled control; †p < 0.05 for significance of difference from no protecting ligand.
a permanent positive charge appear to be inactive for the BD-induced strychnine binding site. It has been speculated that the BD-induced binding site lies within membrane rather than being exposed to the extracellular or intracellular face of the membranes, the finding to emerge from this study supports the hypothesis that the binding is only accessible from within the membrane itself by the uncharged ligands since charged, polar molecules are hydrophilic and have a low affinity for the hydrophobic lipid environment of the membrane.

Another finding of this study was that prebound quinacrine could not be removed from the BD-induced strychnine binding site by the normal washing procedure. This suggests that the off-rate of quinacrine from this site appears to be very low. A modified wash protocol has been tried in which membranes were washed 6 times in a pH 4 phosphate citric acid buffer. In these conditions, the dissociation rate of quinacrine increased and 90% of bound quinacrine has been washed out. However the washing procedure itself has a significant effect on the binding of the [³H]-strychnine; there was a large increased binding in both quinacrine protected membranes and solvent control membranes. One explanation for this is that the acidic environment increased the affinity of the BD-induced binding site. Even under these conditions, it did not remove the quinacrine completely from the binding site, so quinacrine is not a suitable protection ligand to be use for assays employing [³H]-strychnine binding, although it could be shown to protect against fluorescent labelling of the 23 kD peptide by quinacrine mustard.
In view of the above, strychnine had to be used as the protecting ligand. However, problems occur when using a low-affinity, reversible ligand such as strychnine to protect against the irreversible ligand quinacrine mustard. Whereas a reversible ligand dissociates after binding, the irreversible ligand gets to the binding site and then forms a covalent bond. In order to get efficient protection a large excess (10 mM) of strychnine was required to protect the binding site from the quinacrine mustard. Also to get both affinity labelling and protection the incubation conditions with the quinacrine mustard were critical. The incubation condition of quinacrine mustard 10 \mu M for 10 min at 4 \degree C was chosen to cause an approximately 50% reduction in \(^{3}\text{H}\text{-strychnine}} binding so that protection could be demonstrated.

As shown in the present Chapter, quinacrine cannot be washed out from the BD-induced strychnine binding site in rat or mouse spinal cord membranes at physiological pH. Thus, inactivation of \(^{3}\text{H}\text{-strychnine}} binding to these membranes by quinacrine mustard cannot of itself be taken as proof of irreversible labelling of the binding site. This proof came from solubilisation of the quinacrine mustard labelled membranes and resolution of their component peptides on SDS-PAGE gels. Although many peptide bands were revealed by the protein staining, only one had been labelled by the quinacrine mustard and this labelling was pharmacologically specific, being prevented by quinacrine and strychnine but not glycine.

As mentioned in Chapter One, many drugs were found to be effective at
inhibiting [³H]-strychnine in the BD-induced strychnine binding site, including several local anaesthetics, antiarrhythmics and use-dependent cation channel blockers (O'Connor and Fry, 1992). A previously identified local anaesthetic binding site (Greenberg and Tsong, 1982, 1984) shows many similarities to the BD-induced binding site and may be identical. In the study of Greenberg and Tsong (1982), the fluorescent local anesthetic drug, quinacrine, was found to bind specifically to the axonal membrane fraction prepared from bovine corpus callosum, which is also potent displacer of BD-induced [³H]-strychnine binding (Dennis, S.M., 1992). The local anaesthetic site appears to have a low affinity ($K_d = 0.6-1.2 \mu M$) and high capacity ($B_{\text{max}} = 5-10 \text{ nmole/mg protein}$) for quinacrine. These values are in agreement with those obtained for BD-induced strychnine binding site (see Chapter 3). The quinacrine was not displaceable from the local anaesthetic binding site by tetrodotoxin, but was displaced by the potassium channel blockers TEA and 3,4-diaminopyridine, again a similar result to that obtained for the BD-induced strychnine binding site (O'Connor and Fry, 1991; Chapter Three). Greenberg and Tsong (1984) solubilised the local anesthetic binding site with the detergent sodium cholate. This solubilised material was then applied to an affinity chromatography column of quinacrine coupled to sepharose beads. After specific elution with lidocaine, a local anesthetic. SDS-polyacrylamide gel electrophoresis revealed a small protein with a molecular weight about 16 kD. This value is somewhat lower than the estimate of the 23 kD obtained or the BD-induced binding site in the present study. This may be due to the proteolysis during their purification process and/or the problem with calibration of low molecular weight peptide bands in SDS-
PAGE. The local anaesthetic binding protein was isolated from bovine corpus callosum (Greenberg and Tsong, 1982). This is the brain region richest in white matter and the amount of BD-induced strychnine binding in various tissues appears to parallel their white matter content, being highest in the spinal cord and brain stem (O'Connor, 1992). This raises the possibility that the BD-induced site resides on a protein which is associated with myelinated neurons.

As far as other attempts to label CNS peptides with quinacrine mustard are concerned, it has been reported that quinacrine mustard could block the agonist-induced ion flux of acetylcholine receptor-rich membrane vesicles (Lauffer et al., 1979). However, acetylcholine still bound to the quinacrine mustard labelled membrane vesicles, so it appeared that quinacrine mustard did not block the agonist binding site. The quinacrine mustard may therefore block the ion permeability responses of the membranes. Ohta and co-workers have reported that quinacrine mustard had an inhibitory effect on depolarization induced calcium uptake by synaptosomes similar to that of quinacrine (Ohta et al., 1985). In their studies, the subcellular localization of the proteins labelled with quinacrine mustard has been examined. The myelin-rich, the microsomal and the mitochondrial fractions were obtained from rat cerebral cortices. In the myelin-rich fraction, only one fluorescent band was found, corresponding to an apparent molecular weight of 26 kD. This labelled site may correspond to the BD-induced strychnine binding site. There were not any fluorescent bands found in the case of the microsomal and the...
mitochondrial fractions. To obtain information about the subcellular location of the BD-induced strychnine binding site, a series of experiments was performed which will be described in the next Chapter.
CHAPTER SIX

SUBCELLULAR FRACTIONATION AND PURIFICATION OF THE BUTANEDIONE-INDUCED STRYCHNINE BINDING SITE FROM RAT SPINAL CORD

6.1 Introduction

For the purification of the membrane-bound BD-induced strychnine binding site, the approach described in this Chapter consists of irreversibly labelling the binding site with a specific fluorescent ligand: quinacrine mustard (see Chapter 5), followed by solubilization of the membrane in a detergent and subsequent purification of the labelled peptide. Since the covalent bond formed between the ligand and the binding site is stable to detergent solubilization, the binding site-ligand complex can easily be detected after solubilization by its fluorescence. In order to get high purification yields, the distribution of the BD-induced strychnine binding site within the central nervous system becomes of fundamental importance.

The technique of us density-gradient centrifugation (Whittaker et al., 1964), has permitted the isolation of certain morphological structures in relatively pure form. The first neuronal cell fraction to be isolated was the pinched-off nerve ending or synaptosome (Whittaker et al., 1964). When nerve tissue is homogenized in isotonic sucrose of low ionic strength, the myelin is peeled off
the axon and forms loose vesicles and the presynaptic nerve endings are snapped from their attachments to form discrete particles or synaptosomes. When the homogenate is separated by differential centrifugation, myelin and synaptosomes are found predominantly in the crude mitochondrial fraction (P2), although a variable amount also comes down with the nuclear fraction (P1). The crude mitochondrial fraction is centrifuged on a sucrose step gradient, typically having steps of 0.32 M, 0.8 M and 1.2 M sucrose. Crude myelin will layer out at the 0.32 M-0.8 M sucrose interface, synaptosomes will layer out at the 0.8 M-1.2 M sucrose interface and mitochondrial will layer out at the bottom of the tube.

If ions are present in the initial homogenization medium, then the myelin sheath is preserved on the axon and the crude myelin layer will actually consist of myelinated axons (DeVries et al., 1972). Centrifugation of this white matter homogenate yielded a floating layer of myelinated axons, then the purified myelinated axon fraction was subjected to an osmotic shock, the shocked myelinated axons were separated on a discontinuous gradient sucrose. Two membrane fractions were collected, the crude myelin and the crude axolemma. The crude myelin was further osmotically shocked in distilled water and centrifuged on 0.75 M sucrose to get purified myelin. The crude axolemma was separated on a three-step discontinuous gradient consisting of 0.65, 0.8 and 1.2 M sucrose. Two membrane fractions were collected, the first at the 0.8 sucrose (termed the periaxolemmal fraction), the second at the 1.0 M sucrose (termed the axolemmal fraction).
The following account describes attempts to isolate and purify the BD-induced strychnine binding site and in particular the combination of subcellular fractionation and SDS polyacrylamide gel electrophoresis (PAGE) as a means of purification.

6.2 Binding of $[^3H]$-strychnine to untreated and BD-treated fractions of rat spinal cord homogenate

To clarify functions of the BD-induced strychnine binding in spinal cord membranes and to get purified material for sequence analysis, it is important to analyze the subcellular distribution of this binding site in the spinal cord.

In this study, rat spinal cord homogenate was used to prepare the $P_1$ pellet, representing the nuclei fraction of the homogenate and the $P_2$ pellet. Further differentiation of the $P_2$ or the mitochondrial pellet, into its subcellular fractions of myelin, synaptic plasma membranes and mitochondria, was achieved by utilizing a discontinuous sucrose gradient as detailed in 2.2.4.a. $P_1$, $P_2$ fractions and the subfractions of $P_2$ were either left untreated or treated with BD as in 2.2.5. The fractions were then assayed for $[^3H]$-strychnine binding activity with both strychnine and quinacrine as the displacing ligand. (see detail in 2.2.6).

Untreated and BD-treated fractions were used and both strychnine and quinacrine displaced $[^3H]$-strychnine binding activity was measured in order to
determine the distribution of both the glycine receptor and the BD-induced binding site. $[^3H]$-strychnine binding activity was always higher in the $P_2$ fraction than the $P_1$ fraction (Figure 6.2.1). In the untreated fractions, the $[^3H]$-strychnine binding levels in $P_2$ were about 7.5 or 4.3 fold higher than those in $P_1$ with strychnine or quinacrine as displacing ligand respectively. In the BD-treated fractions, the $[^3H]$-strychnine binding levels in $P_2$ were about 6.5 and 12 fold higher than those in $P_1$ with strychnine or quinacrine as displacing ligand respectively. So it is quite clear that the majority $[^3H]$-strychnine binding in both untreated and BD-treated membrane was found in the $P_2$ fraction.

Further fractionation of the $P_2$ fraction on the discontinuous sucrose gradient produced the subfractions of myelin, synaptic plasma membranes and mitochondria. The distribution of $[^3H]$-strychnine binding is shown in Figure 6.2.2. In untreated subfractions, there was negligible quinacrine displaced $[^3H]$-strychnine binding compared to the strychnine displaced $[^3H]$-strychnine binding. This suggested that $[^3H]$-strychnine was mainly bound to the glycine receptor in the untreated preparations. In the mitochondria subfraction, containing the majority of strychnine displaced $[^3H]$-strychnine binding, the level of the binding was about 34 fold higher than those in the myelin subfraction and 2.2 fold higher than those in the synaptic plasma membranes. By contrast, in BD-treated subfractions, strychnine displaced $[^3H]$-strychnine binding revealed that $[^3H]$-strychnine bound to both the glycine receptor and the BD-induced strychnine bind site. When using quinacrine as the displacing
Figure 6.2.1: Distribution of [³H]-strychnine binding in the untreated and BD-treated fractions of rat spinal cord homogenate: Untreated (U) and BD-treated (T) rat spinal cord homogenate nuclei fraction (P1) and mitochondrial fraction (P2) were incubated with [³H]-strychnine (6 nM), with unlabelled strychnine (2x10⁻⁶M, Us and Ts) or quinacrine (10⁻⁶M, Uq and Tq) as the displacing ligand to enable estimates of strychnine or quinacrine specific binding. Binding is expressed as fmole/mg protein. Results were obtained from one experiment performed in triplicate.
Figure 6.2.2: Distribution of $[^{3}H]$-strychnine binding in the untreated and BD-treated subfractions of rat spinal cord homogenate: Untreated (U) and BD-treated (T) rat spinal cord homogenate subfractions: myelin, synaptic plasma membrane and mitochondria were incubated with $[^{3}H]$-strychnine (6 nM), with unlabelled strychnine ($2 \times 10^{-5}$ M, Us and Ts) or quinacrine ($10^{-4}$ M, Uq and Tq) as the displacing ligand to enable estimates of strychnine or quinacrine specific binding. Binding is expressed as fmole/mg protein. Results were obtained from five experiments (mean ± s.e.m.) and each of them performed in triplicate.
ligand, the $[^3]H$-strychnine binding was shown mainly located in the myelin subfraction. The levels of the binding was 6.3 fold higher than those in synaptic plasma membranes subfraction and 3.8 fold higher than those in mitochondria subfraction. These findings suggest that the BD-induced strychnine binding site was concentrated in the myelin subfraction.

6.3 Binding of $[^3]H$-strychnine to untreated and BD treated subfractions of rat spinal cord myelin

Previous experiments have shown that the BD-induced strychnine binding site was concentrated in the myelin fraction of rat spinal cord. Further fractionation of myelinated axons to isolate enriched subfractions of pure myelin, periaxolemmal myelin and axolemma were used here to identify the subcellular localization of the BD-induced strychnine binding site.

Myelin, periaxolemmal and axolemmal enriched fractions were purified from rat spinal cord. These membrane fractions were all isolated from the same preparation by the procedure of Sapirstein, et al., (see 2.2.4.b). The subfractions were treated either with or without BD as in 2.2.5 and then assayed for $[^3]H$-strychnine binding activity with both strychnine and quinacrine as the displacing ligand. (see detail in 2.2.6).

The distribution of $[^3]H$-strychnine binding is shown in Figure 6.3. In the untreated subfractions, there was very low $[^3]H$-strychnine binding observed
Figure 6.3: Distribution of [3H]-strychnine binding in the untreated and BD-treated subfractions of rat spinal cord myelinated axon: Untreated and BD-treated rat spinal cord myelinated axon subfractions: myelin, periaxolemmal myelin and axolemma were incubated with [3H]-strychnine (6 nM), with unlabelled strychnine (2x10^4M, Us and Ts) or quinacrine (10^4M, Uq and Tq) as the displacing ligand to enable estimates of strychnine or quinacrine specific binding. Binding is expressed as fmole/mg protein. Results were obtained from three experiments (mean±s.e.m.) and each of them performed in triplicate.
with both strychnine and quinacrine as the displacing ligand. This suggested that there are very few glycine receptors or strychnine-specific strychnine binding site detectable in the untreated subfractions of purified myelin, periaxolemmal myelin and axolemma. By contrast, in BD-treated subfractions, quinacrine-specific [³H]-strychnine binding in periaxolemmal myelin subfraction was shown to be increased in comparison with the untreated control. The amount of quinacrine-specific [³H]-strychnine binding in periaxolemmal fractions was about 9 fold higher than those in the myelin subfractions and 3.5 fold higher than those in the axolemma subfraction. These findings suggest that the majority of BD-induced strychnine binding site was concentrated in periaxolemmal subfraction. Western blotting has been used to check identity of the subfractions (data not shown).

6.4 A modified procedure for polyacrylamide gel electrophoresis of myelin protein

In the previous Chapter, it was shown that the BD-induced strychnine binding site has been successfully affinity labelled by quinacrine mustard. By investigating the subcellular distribution of BD-induced binding site, there was a suggestion that this site may be enriched in the myelin fraction. A series of experiments were performed to affinity label the BD-induced binding site in the myelin fraction using quinacrine mustard. The results of [³H]-strychnine binding assay showed that quinacrine mustard (10 μM, 4°C, 10 min) has affinity labelled the BD-induced binding site by reduction of 70 % of the [³H]-strychnine binding in comparison to the solvent control (Fig 6.5.a), but when
Figure 6.4: Electrophoretic profile of BD-treated rat spinal cord myelin labelled with quinacrine mustard: BD-treated rat spinal cord myelin fraction was incubated with quinacrine mustard (10 μM, 10 min, 4°C). The reaction was stopped by the addition of 160 volumes of PBS/BSA (0.1% w/v)/Thiosulphate (10 mM). The samples were washed, solubilized in 2% SDS, mixed with "master mix" (1:2 v/v, with or without 2-mercaptoethanol-BME) and then unboiled or boiled for 3 min. Electrophoresis was carried out on 14% acrylamide gel using 100 μg protein per sample. Lane (a), (b) and (d) are samples were boiled for 3 min, Lane (c) and (e) are samples were unboiled, lane (a-c) with 5% BME and (d-e) without BME.
the labelled material was solubilized in SDS and resolved in SDS-PAGE gel, initial experiments revealed no peptide band to be seen labelled by fluorescent quinacrine mustard.

The routine procedure for analysis involved solubilizing the sample in SDS (2%) and diluting 1 vol of this sample in 2 vol of concentrated sample solvent consisting of: 4 mM of Tris-HCl at pH 6.8, 3% (w/v) SDS, 7.5% (v/v) BME, 15 mM EDTA, 45% (v/v) glycerol and 0.015% (w/v) bromophenol blue. Following standard procedures (Laemmli, 1970) the sample was heated to 100°C prior to electrophoresis in order to disrupt possible metastable aggregates. However, there was no labelled fluorescent peptide could been seen in the gel by using the standard procedures. Therefore different conditions were tried in the present experiment. The procedure was modified by omitting BME from the sample solvent for some of the samples, and the samples were either heated or unheated prior to electrophoresis.

It was interesting to see that there was no 23 kD band in the boiled samples. The result was shown on the total protein stain of the gel (Figure 6.4). The 23 kD peptide was completely excluded from the SDS-PAGE gel after the myelin fraction sample was boiled. By contrast, in the unboiled sample, there was the 23 kD peptide band. The samples treated with or without BME did not reveal any differences in both the fluorescence and peptide distribution on the gel.
quinacrine mustard

Rat spinal cord homogenate fractions: myelin, synaptic plasma membranes and mitochondria were prepared as 2.2.4 and treated with BD as 2.2.5, the samples were then labelled with quinacrine mustard (see 2.2.11.b). After being washed, the samples were then assayed for protein content as in 2.2.16.a and for $[^3]$H-strychnine binding activity as in 2.2.6 with quinacrine as the displacing ligand. Portions of these samples were also solubilized in SDS, resolved in 10 % SDS-PAGE gel.

The binding assay results are shown on Figure 6.5.a. The majority of quinacrine displaceable $[^3]$H-strychnine binding was concentrated on the myelin fraction, this result confirms that the BD-induced binding site is enriched in myelin rather than synaptic plasma membrane or mitochondria. In quinacrine mustard labelled fractions, there was a decrease in $[^3]$H-strychnine binding in all three fractions compared to their solvent control. In this incubation condition (QM: 10 $\mu$M, 10 min, 4°C), the myelin fraction shows a 70 % reduction in $[^3]$H-strychnine binding compared to the control value, while the synaptic plasma membrane fraction and mitochondria fraction represent 32 % and 43 % reduction in $[^3]$H-strychnine binding respectively in compared with their solvent control. These indicate that quinacrine mustard has irreversibly labelled the BD-induced binding site in all three of fractions.

In the SDS-PAGE gel, as found with crude spinal cord membranes (see 5.2.4),
Figure 6.5.a: Effect of quinacrine mustard on subsequent strychnine binding to BD-treated rat spinal cord homogenate subfractions: BD-treated rat spinal cord homogenate subfractions: myelin, synaptic plasma membrane and mitochondria were incubated with solvent (HCl, 50 µM, ■) or quinacrine mustard (10 µM, □) for 10 min at 4 °C. The reaction was stopped by addition of 160 volumes of PBS/BSA (0.1% w/v)/thiosulphate (10 mM). For binding assays, [³H]-strychnine (6 nM) was incubated with each sample, with unlabelled quinacrine (10 mM) used as the displacing ligand to enable estimates of quinacrine specific binding. Binding is expressed as fmole/mg protein. Results were obtained from one experiment performed in triplicate.
a 23 kD peptide was irreversibly labelled by fluorescent quinacrine mustard. However, this labelled 23 kD peptide band was only seen on the myelin fraction. Figure 6.5.b shows the SDS-PAGE gel stained with CBBG-250. On the total protein staining of the gel, the peptide band of 23 kD molecular weight was the only one appearing in the myelin fraction, there was not any corresponding peptide band that could been seen in the synaptic plasma membrane fraction or the mitochondria fraction. These results confirm the earlier observation from the binding assay that the BD-induced strychnine binding site is mainly located in the myelin fraction rather than synaptic plasma membrane fraction or mitochondria fraction. The results also show that BD treatment has not altered general peptide pattern.

6.6 Optimizing the conditions for quinacrine mustard labelling of purified myelin

Myelin fraction preparation (see 2.2.4) was thawed and the protein concentration of this sample was adjusted to 0.2 mg/ml with PBS. The sample was then treated with BD (100 mM). After twice washing, the sample was resuspended in 40 volumes of PB (0.1 M, pH 7.7). The incubations were set up. Quinacrine mustard was dissolved in HCl (1 mM) and added to all tubes to final concentrations from 1 μM to 100 μM, respectively. Solvent control tubes received 50 μM HCl only. After incubation (10 min, 4°C), 160 volumes of PBS/BSA (0.1% w/v)/thiosulphate (10 mM) was added to stop the reaction. The samples were vortexed and washed as in 2.2.6. A protein assay
Figure 6.5.b: Total protein stain of SDS-PAGE gel loaded with untreated and 2,3-butanedione (BD) treated fractions of rat spinal cord homogenate: Untreated or BD-treated rat spinal cord homogenate subfractions: myelin (MYL), synaptic plasma membrane (SPM) and mitochondria (MIT) were incubated with quinacrine mustard (10 μM, 10 min, 4°C). The reaction was stopped by addition of 160 volumes of PBS/BSA (0.1% w/v)/Thiosulphate (10 mM). The sample were washed, solubilised in 2% SDS then mixed with "Master mix" (1:2 v/v). Electrophoresis was carried out on 10% acrylamide gel using 100 μg protein per sample. The gel was then stained in coomassie brilliant blue G-250.
(2.2.16.a) and a binding assay were set up (2.2.6), with quinacrine as the displacing ligand. In parallel, a portion of the samples were solubilized in 2% SDS and loaded on a 10% SDS polyacrylamide gel for electrophoresis (see 2.2.13.c).

From the binding assay results (Figure 6.6.a), there was a decrease in $[^3H]$-strychnine binding in the quinacrine mustard labelled BD-treated myelin fraction compared to the solvent control with increasing concentrations of quinacrine mustard. When quinacrine mustard was used at a concentration of 1 $\mu$M, it caused a 24% reduction in $[^3H]$-strychnine binding. When the concentration of quinacrine mustard was 100 $\mu$M, the reduction of the binding increased to 66.5%. It appeared to be that BD-induced strychnine binding site in the myelin fraction had been specifically affinity labelled by the quinacrine mustard.

The gel picture is shown in Figure 6.6.b. In the UV fluorescence picture, there was a labelled fluorescence band at 23 kD at increased intensity with an increase in concentration of quinacrine mustard. In the concentration of 10 $\mu$M quinacrine mustard, there was a very weak fluorescence band, but in the concentration of 100 $\mu$M, a clear fluorescence band could be seen. This gel has also been stained in coomassie brilliant blue G-250 (CBBG-250, see 2.2.14.d) to show the distribution peptide. The labelled florescence band corresponds to the major peptide in the band at 23 kD. Again, these results indicating that the 23 kD peptide has been irreversibly labelled by florescent
Figure 6.6.a: Effect of quinacrine mustard concentration on subsequent [³H]-strychnine binding to BD-treated rat spinal cord myelin subfraction: BD-treated rat spinal cord myelin subfractions were incubated with quinacrine mustard (either 1 μM, 10 μM, 20 μM, 50 μM or 100 μM) for 10 min at 4 °C. The reaction was stopped by the addition of 160 volumes of PBS/BSA/thiosulphate. For binding assays, [³H]-strychnine (6 nM) was incubated with each sample, with unlabelled quinacrine (10⁴M) used as the displacing ligand to enable estimates of quinacrine specific binding. Binding is expressed as fmole/mg protein in % of solvent control (126.17 fmole/mg). Results were obtained from one experiment performed in triplicate.
Figure 6.6.b: Electrophoretic profile of untreated or BD-treated rat spinal cord myelin labelled with quinacrine mustard: Untreated (U) and BD-treated (T) rat spinal cord myelin fraction was incubated with quinacrine mustard (either 0, 1 μM, 10 μM, 20 μM, 50 μM or 100 μM) for 10 min at 4°C. The reaction was stopped by the addition of 160 volumes of PBS/BSA/Thiosulphate. The samples were washed, solubilized in 2% SDS then mixed with "master mix" (1:2 v/v). Electrophoresis was carried out on 10% acrylamide gel using 100 μg protein per sample. A. represents gel after being stained in CBBG-250 and B. represents the same gel under UV light.
6.7 Ability of strychnine to protect against quinacrine mustard labelling of myelin fractions from rat spinal cord homogenates

A BD-treated myelin fraction was prepared (see 2.2.4.a and 2.2.5) and incubated with strychnine (10 mM, 30 min, 4 °C). The samples were then labelled with quinacrine mustard (see 2.2.13) and washed. A protein assay (see 2.2.16.b) and a binding assay were set up (see 2.2.6) with quinacrine as the displacing ligand.

The results are shown in Figure 6.7. There was a decrease in $[^3]$H-strychnine binding activity in unprotected samples, of about 35 % of the reduction in comparison with the solvent control. By contrast, in the sample which was strychnine protected before it was labelled by quinacrine mustard, there was slightly increase in $[^3]$H-strychnine binding in comparison with the solvent control, this was probably due to the stabilisation of the binding site by the occupying strychnine. Results presented here confirm the earlier observation with crude spinal cord membranes (see Chapter 5) that strychnine could effectively protect against the quinacrine mustard labelling of the BD-induced strychnine binding site.

6.8 Discussion
Figure 6.7: Protection of BD-induced strychnine binding site against quinacrine mustard by strychnine in rat spinal cord myelin: Strychnine (10 mM) was added to the BD-treated rat spinal cord myelin fraction 30 min before the addition of either quinacrine mustard: □ (10 μM, 10 min, 4°C, ) or solvent alone: ■ (50 μM HCl, 10 min, 4°C, ). Unprotected samples received PBS instead of strychnine. The reaction was terminated by the addition of 160 volumes of PBS/BSA (0.1% w/v)/thiosulphate (10 mM) and the samples were washed. For the binding assays, [³H]-strychnine (6 nM) was incubated with each sample, with unlabelled quinacrine (10⁻⁴M) used as the displacing ligand to enable estimates of quinacrine specific binding. The binding is expressed as fmole/mg protein. Experiments repeated three times and results were obtained from one experiment performed in triplicate.
The results presented in this Chapter, using sucrose density gradient fractionation of the rat spinal cord homogenates, show that the majority of BD-induced strychnine binding sites were present in the $P_2$ fraction. When the $P_2$ fraction was further fractionated on discontinuous sucrose gradients, the binding assay results showed that BD-induced strychnine binding was enriched in myelin and depleted in synaptic plasma membrane and mitochondria. This has been confirmed by the treatment of all of the three fractions: myelin, synaptic plasma membrane and mitochondria with quinacrine mustard, having resolved them on an SDS-PAGE gel. The molecular weight of this labelled peptide is about 23 kD, this is in excellent agreement with the estimate obtained from crude spinal cord membranes for the BD-induced strychnine binding site (see Chapter Five). The 23 kD peptide only appeared on the myelin fraction sample on the ultraviolet light and the total protein stained gel. The BD-induced strychnine binding site in the myelin fraction also may be protected by strychnine. All of these points indicate that the BD-induced strychnine binding site is enriched in the myelin fraction rather than the synaptic plasma membrane fraction or mitochondria fraction. The fractionation of rat spinal cord homogenate into cellular components was done utilizing the standard procedure for subcellular fractionation of neuronal tissue.

Further subcellular fractionation of myelinated axons on a discontinuous density gradient gives rise to three membrane fractions: the myelin fraction, the periaxolemmal myelin fraction and the axolemmal fraction. Results presented in this Chapter suggest that the BD-induced strychnine binding site
is highly enriched in periaxolemmal myelin fraction. The periaxolemmal myelin fraction contains fragments of lipid-rich myelin which attached to less dense axonal membrane and is, therefore, intermediate in density between the myelin fraction and the axolemma fraction. The proportion of nodal and internodal axolemma of the fractions may also be different. The nodal axolemma contains a higher density of intramembranous particles relative to that of the internodal axolemma (Schnapp and Mugnaini, 1977) and consequently may have a greater density than that of the internodal axolemma. Ritchie and Rogart (1977) have presented evidence that nodal axolemma is more enriched in sodium channels than the internodal axolemma of the myelinated axon. Therefore, the increased binding of tetrodotoxin (DeVries et al., 1978) and saxitoxin (DeVries et al., 1981) in the axolemma-enriched fraction relative to the periaxolemmal enriched fraction supports the idea that the more dense axolemma-enriched fraction may have a higher content of nodal axolemma. So results presented in this Chapter indicates that the BD-induced strychnine binding site is enriched in the myelin which is attached to the axonal membrane and also that it may be found mainly in internodal periaxolemmal myelin.

As mentioned in Chapter One, drugs specific for the BD-induced strychnine binding site appear to exert their actions by mechanisms analogous to that proposed for the actions of local anaesthetic amines (Hille, 1977 a, b.) and channel blockers (Shapiro, 1977 a,b.). Incidentally, in oligodendrocytes from the mouse spinal cord, "leakage" potassium channels of varying conductances
ranges were observed with single-channel recording (Kettenmann, et al, 1984). These channels have recently been characterized further, with both whole-cell and single-channel recording (Barres et al, 1988). The resting conductance of optic nerve oligodendrocytes in culture results from two types of inwardly rectifying potassium channels, having conductances of 30 and 120 pS. There are also reports of outward potassium currents in oligodendrocytes (Sontheimer & Kettenmann, 1988; Barres et al, 1990). Patch clamp studies on paranodal myelin of PNS (Wilson, et al., 1990) suggest that the ionic milieu of the axon may in part be regulated by ion channels, in particular the potassium channel activity in the myelin complex.

The electrophysiological voltage-clamp experiments show that the acute demyelination treatments uncover axonal membranes previously covered by the myelin and that this exposed membrane contains potassium channels. (Chiu and Ritchie, 1980, 1981). These potassium channels could contribute to potassium accumulation in the space under the myelin during axonal activity. For the CNS, there is considerable experimental evidence that glial cells participate in ionic homeostasis by temporarily storing potassium ions that have been released by neurons (Barres et al., 1990; Walz, 1989). However, the exact mechanism is still unclear. The BD-induced strychnine binding site appears to have a low affinity and high capacity for strychnine, so the presence of the BD-induced strychnine binding site of ion channel activity in periaxolemmal myelin may play a important role in buffering potassium ions during axonal firing.
Another finding of this study was that if the myelin samples were placed in boiling water for 3 min prior to electrophoresis, the 23 kD peptide of the BD-induced strychnine binding site was excluded from the gel. Characterization of the polypeptide composition of membrane protein by polyacrylamide gel electrophoresis in a buffer containing SDS has been a standard procedure for many years (Shapiro, et al., 1967). When using such a system, it is generally assumed that subjecting the sample to a protein solvent consisting of a buffer containing SDS and BME to reduce disulphide bonds and also bringing the sample to the boil will disrupt metastable aggregates. This assumption may lead to operational difficulties in the study of certain membrane proteins. It has been reported that when myelin samples were boiled prior to electrophoresis, over 90% of the 23 kD proteolipid protein (PLP) was excluded. However, under the same conditions, the amount of material in the myelin basic protein remained constant (Morell et al., 1975). Most prominent of the peculiar properties of PLP is its solubility in organic solvents (Folch and Lees, 1951). Aqueous solutions of PLP can be obtained by stepwise dialysis from organic solvents into solutions of increasing polarity, or by the use of detergents. It is possible that under these conditions the protein is somehow not completely denatured. If the protein is subjected to more severe denaturing conditions such as repeated flash evaporation from chloroform-methanol it forms a precipitate which can not be resolubilized either in an organic solvent or in water, even in the presence of detergents (Morell et al., 1975). It is possible that boiling is sufficient to completely denature PLP which then precipitates out of the
detergent containing solution and remains at the gel interface during electrophoresis. This "complete" denaturation might correspond to the dissociation of residual lipid. Another possibility is that at a lower temperature there exists an SDS-protein complex in which the protein retains some structure. At 100°C this SDS-protein complex becomes destabilized due to the sulfhydryl groups becoming accessible for reduction by mercaptoethanol, leading to the complete unfolding of the polypeptide chains and aggregation. What needs to be determined now is whether the quinacrine mustard labelled 23 kD peptide of the BD-induced binding site is the same protein (PLP) or is it a different protein which behaves similarly to the PLP, the results of the sequence analysis should answer this question.
APPENDIX

A PARTIAL AMINO ACID SEQUENCE FOR THE
BD-INDUCED STRYCHNINE BINDING SITE

The BD-induced strychnine binding site has been affinity labelled with quinacrine mustard and visualised as a fluorescent peptide of 23 kD on SDS-PAGE (see Chapter 5). This fluorescent label provides a convenient way of monitoring purification of the component peptide. Furthermore, the subcellular fractionation studies have shown this binding site to be enriched in the myelin rather in the synaptosomal or mitochondrial fractions of rat spinal cord. A combination of subcellular fractionation and SDS-PAGE has enabled me to purify µg quantities of this peptide.

Myelin fraction was prepared as 2.2.4.a and treated with BD (160 mM) as 2.2.5. The sample was then solubilized in 2 % SDS and applied to 10 % SDS-PAGE gel. After electrophoresis, the gel was then stained in CBBG-250 (0.25 % v/v), methanol (45 % v/v) and acetic acid (10 % v/v) for 8 min, then destained in methanol (30 % v/v) and acetic acid (10 % v/v) for 4 min. The 23 kD peptide band was cut out on the light box and has been sequenced by N.Totty and J. Hsuan in the Ludwig Institute for cancer research, London. The amino acid compositions and sequences are summarized in Figure P 1.
1 GLLLECCARCL VGAPFASLVA TGLCFFGVAL FCGCGHEALT GTEKLIETYF LIETYF

51 SKNYQDYEYL INVIH AFQYV IYGTASFFFL YGALLAEGF YTTGAVRQIF QIF

101 GDYKTTICGK GLSATVTGGQ KGRGSRGQH0 AHSLERVCHC LGKWLGHPDK GDYK GLSATVTGGQ K

151 FVGITYALT VWWLFACSA VPVYIYFNTW TTCQSIAPS KTSASIGSLC

201 ADARYMYGVL PWNAPGKVCG SNLLSICKTA EFQMTFHLFI AAFVGAATL

251 VSLLTFMIAA TYNFAVLKLM GRGTKF

Figure P 1: Amino acid sequences of BD-induced strychnine binding site: The three segments of amino acid sequences (bold) cleaved from the 23 kD peptide band of the BD-induced strychnine binding site are shown alongside. The 276 amino acid sequences of proteolipid protein (PLP) were obtained from Norway rat (Dautigny et al., 1985). The peptide segment of T^{116}-K^{150} (underline) is missing in DM_{20}. The sequences of the BD-induced binding site are identical with the sequence L^{45}-K^{52}, Q^{98}-K^{104} and G^{110}-K^{121} of rat PLP.
CHAPTER SEVEN

DISCUSSION

The present research concerns a non-glycinergic strychnine binding site first discovered by V.M. O’Connor and J.P. Fry in rodent spinal cord membranes. Treatment of rat or mouse spinal cord membranes with the arginine specific reagent 2,3-butanedione (BD) revealed a strychnine binding site which is not located on the inhibitory glycine receptor. As mentioned in Chapter 3, this site displays the pharmacological properties of a cation channel, binding use-dependent cation channel blockers such as local anaesthetics and quinacrine, in addition to strychnine. Kinetic studies presented in Chapter Three suggest the BD-induced strychnine binding has a lower affinity ($K_d=71 \ \mu M$) and higher capacity ($B_{\text{max}}=13 \ \text{nmol/mg}$) than the inhibitory glycine receptor ($K_d=6 \ \text{nM}; B_{\text{max}}=1 \ \text{pmol/mg}$). Attempts to solubilise the BD-induced strychnine binding activity were unsuccessful (Chapter Four), because the binding properties of the recognition site do not survive solubilization in a variety of detergents. Thus, it cannot be purified by conventional affinity chromatography. However, as a first step towards the molecular characterisation of this binding site, procedures have been developed in Chapter 5 for its affinity labelling and purification.

Previous studies had shown quinacrine to be a high affinity ligand for the 2,3-butanedione induced [$^3\text{H}$]-strychnine binding site and therefore quinacrine mustard has been employed as an irreversible affinity label. Incubation of crude 2,3-butanedione treated spinal cord membranes with the dichloroethylamino derivative of quinacrine inactivated at least 50% of subsequent [$^3\text{H}$]-strychnine binding. Subsequent
solubilization of such membranes in sodium dodecylsulphate followed by SDS polyacrylamide gel electrophoresis (SDS-PAGE) revealed a single fluorescent band at 23 kD. Both the inactivation of subsequent [³H]-strychnine binding and the fluorescent labelling of this peptide were reduced if membranes were treated with quinacrine mustard in the presence of strychnine. Moreover, using this irreversible mustard as a ligand, it was possible to show labelling in untreated spinal cord membranes, establishing that this binding site is not an artefact of 2,3-butanedione treatment.

Fractionation of rat spinal cord membranes on sucrose gradients (Chapter 6) showed the 2,3-butanedione-induced binding of [³H]-strychnine to be enriched in the myelin rather than in the synaptosomal or mitochondrial fractions. Further subfractionation of myelinated axons located this binding site to the periaxolemmal myelin, as opposed to the axolemmal or compact myelin fractions.

When employed with myelin membranes, the quinacrine mustard affinity labelling procedure also identified a fluorescent peptide of 23 kD on SDS-PAGE. This fluorescent label therefore provides a convenient way of monitoring purification of the component peptide. A combination of subcellular fractionation and SDS-PAGE has enabled purification of µg quantities of this peptide for sequencing, which reveals the 2,3-butanedione induced strychnine binding site to be located on a previously undescribed isoform of proteolipid protein (PLP, see Appendix).

Myelin proteolipid protein (PLP) is the most abundant protein component and the major integral membrane protein in the myelin of the tetrapod (amphibia, reptilia,
aves, and mammalia) central nervous system (CNS) and accounts for more than 50% of myelin membrane protein of CNS. It is one of the most hydrophobic proteins known, insoluble in aqueous solution but soluble in organic solvents like mixtures of chloroform and methanol (Folch-Pi and Lees, 1951), even after all its bound lipids (except some covalently linked fatty acids) have been removed. PLP awaited its structural elucidation for 30 years due to these unusual hydrophobic properties (Stoffel, et al., 1982). When the myelin proteolipid protein was solubilised in aqueous solutions containing sodium dodecylsulphate (SDS), the electrophoresis of proteolipids by SDS-polyacrylamide gel electrophoresis (PAGE) shows two main bands- PLP and DM<sub>20</sub>. The polypeptide chain of PLP consist of 276 amino acid residues. Furthermore, PLP and DM<sub>20</sub> share complete sequence identity except for a peptide segment of 35 amino acids (116-150 of PLP sequence) that is missing from DM<sub>20</sub>. (Nave et al., 1987; Macklin et al., 1987; Simons et al., 1987). The amino acid sequence of PLP shows high similarities (99%-100% conservation) between cow (Lees et al., 1983; Stoffel et al., 1983), rat (Dautigny et al., 1985; Milner et al., 1985), Man (Diehl et al., 1986) and mouse (Macklin et al., 1987) proteins. Since the peptide segment of T<sup>116-K<sup>150</sup></sup> (underline) is missing from DM<sub>20</sub>, so the BD-induced strychnine binding site is not located on DM<sub>20</sub>. Furthermore, the molecular weigh (23 kD) of BD-induced strychnine binding site is smaller than that of the PLP (28 kD, Kitagawa et al., 1993), so the BD-induced strychnine binding site appears to be a previously undescribed isoform of PLP. As yet, it is not possible to conclude whether this isoform differs in amino acid sequence and/or acylation from the 28 kD PLP band.

The 276-amino acid polypeptide contains approximately 50 % hydrophobic amino acids and is distinctly structured into four hydrophobic and five hydrophilic domains.
The hydrophobicity is further enhanced by the presence of covalently linked long-chain fatty acids (Stoffyn and Folch-Pi, 1971).

The recent molecular genetic analysis of mutations in PLP genes has identified some functions of PLP. For example, the mutation carried by the *jimpy* mouse has been found to result from single-base missense changes that alter specific amino acid residues within the PLP protein. These mutations have been observed in inherited, X-linked myelination disorders that together make up Pelizaeus-Merzbacher disease (PMD) in humans (see Nave, 1996). The feature of these mutations is the widespread degeneration and death of oligodendrocytes that is typically associated with the hypomyelinating phenotype. However, another mouse PLP mutation, which results in hypomyelination but not in oligodendrocyte cell death, has now been analyzed at the molecular level (Schneider *et al.* 1992). These studies have suggested that PLP, or its alternatively spliced variant DM-20, plays a role not only in early oligodendrocyte progenitor development but also in oligodendrocyte differentiation in the proper formation of the intraperiod line of CNS myelin during its final elaboration and compaction.

A possible ionophoric function of the proteolipid protein has been suggested by Lin and Lees (1982). Dicyclohexylcarbodiimide (DCCD), which is known to bind preferentially to a proteolipid subunit of proton-translocating systems and thereby to inhibit proton translocation, specifically binds to the proteolipid in myelin. A DCCD-inhibitable proton transport could be demonstrated in liposomes reconstituted with the myelin PLP apoprotein. Furthermore, the proteolipid protein participation in the movements of ions in model membrane systems has been explored. Tosteson and
Figure 7.1: Proposed model of the topology of the proteolipid protein (PLP) in the plasma membrane of oligodendrocytes and the myelin membrane as suggested by the amino acid sequence and the determination of the state of all 14 cysteine residues (circles). The region which is deleted in DM-20 is indicated by bars. [After Weimbs and Stoffel, 1992]
Sapirstein (1981) have found that the proteolipids isolated from bovine kidney plasma membrane interact irreversibly with bilayers made from diphytanoylphosphatidylcholine. The incorporated proteolipids from channels which are more selective for cations than anions and which are about three times more permeable to K\(^+\) than to Na\(^+\). In addition, a voltage-dependent conductance change could be observed upon incorporation of the proteolipid apoprotein from brain white matter into planar lipid bilayers (Ting-Beal et al., 1979). Helynck and coworkers (1983) have studied the effects of the different fractions of total bovine brain proteolipids obtained on the electrical properties of lipid bilayer membranes. Single channel events were observed at low protein concentrations, in particular with one of the final homogeneous apoproteolipids of molecular mass 24 kD. Incidentally, this value is close to the estimated molecular weight of 23 kD obtained from the BD-induced strychnine binding site identified in the present study.

Also with regard to possible ion channel properties of PLP, Kitagawa and co-worker have demonstrated that PCR primed with degenerate oligonucleotides corresponding to common segments of rat PLP revealed three novel mRNAs in the brains of two elasmobranchs which they term the DM gene family. (Kitagawa et al., 1993). As a family, the expressed molecules in brain tissue are not only highly homologous to each other, but bear similarities with segments of channel-forming regions of the nicotinic acetylcholine receptor (nAChR) and the glutamate receptor (GluR) macromolecular complexes. It has been proposed that one or more four hydrophobic domain polypeptides eventually evolved in cells to assemble a primitive pore or channel of these ligand gated channels (Hille, 1992). It may be envisioned, analogously, that in oligodendrocyte, the proteolipid subunits, of which at least some have a strong
Frankenhaeuser and Hodgkin (1956) first suggested that potassium ions released by axons during neuronal activity could be temporarily accumulated in the periaxommal space between the myelin sheath and the axon. Since extracellular potassium is known to have a profound influence on nerve membrane potential and excitability, efficient potassium clearance is crucial for nerve function. However, in myelinated axons, 99.9% of the axonal surface is insulated by the myelin sheath and potassium channels are known to be present on the internodal axolemma (Black et al., 1990; Waxman and Ritchie, 1985; Bostock et al., 1981), where they could contribute to potassium accumulation in the space under the myelin. It has been suggested that the leakage resistance of the paranodal and internodal region of myelin membrane is very high (Chiu and Ritchie, 1980; Roper and Schwarz, 1989), so the potassium channels need to operate underneath the internodal myelin which faces the internodal axolemma (Baker et al., 1987; Bowe et al., 1985). Potassium clearance by pumps is unlikely since (Na^+ + K^+)-ATPase activity is apparently absent on the myelin membrane and the paranodal, internodal axolemma (Ariyasu et al., 1985). A consequence of the internodal axonal potassium channels being activated during a normal impulse would be a momentary loading of the internodal periaxonal space with potassium ions. It has long been proposed that the glial cells in the CNS have an important role in buffering the potassium released by active neurons into the extracellular space (Orkand, et al., 1966). However, the exact mechanism remains unclear. For the CNS, there is considerable experimental evidence that glial cells participate in ionic homeostasis by temporarily storing potassium ions that have been released by neurons (Barres et al.,
1990; Walz, 1989). Newman (1985) has reported that glial cells have an inward rectifier potassium channel and this is the only potassium channels they possess which is open at normal membrane resting potentials. The inward rectifier potassium channel is found in a high density in oligodendrocytes from brain of lambs (Soliven et al., 1988) and the optic nerve of cat (Barres et al., 1988). A transient outward potassium channel was also found to be present in retinal glial cells, mouse and rat astrocytes and in oligodendrocytes (Newman, 1985; Bevan and Raff, 1985; Nowak et al., 1987; Soliven et al., 1988; Barres et al., 1988). The present research has identified an isoform of proteolipid protein in oligodendrocytes as a binding site for use-dependent cation channel blockers in mouse and rat central nervous system myelin. This binding site is enriched in periaxolemmal myelin where if it forms a channel it may play a important role in buffering potassium ions during axonal firing.

The buffering role of PLP may well have clinical relevance in central demyelinating disease. Multiple sclerosis (MS) is the prototypical demyelinating disease and current evidence suggests that the etiology of multiple sclerosis and other demyelinating diseases involves both viral and autoimmune factors (Johnson, 1984). A particularly well studied model for multiple sclerosis is experimental autoimmune encephalomyelitis (EAE), which can be induced by immunization of laboratory animals with CNS tissue, with myelin basic protein (MBP; Alvord et al., 1984) or with myelin proteolipid protein (PLP; Cambi et al., 1983). Cambi and coworkers have reported that immunization of rabbits with the proteolipid apoprotein leads to the development of a chronic, progressive or relapsing form of EAE (Cambi et al, 1983). However, the epitopes in myelin proteolipid which cause the encephalitogenic response
are not known Interestingly, Shaw and coworkers (1986) have compared the putative extramembrane segments of the PLP and a number of viral proteins, several of which infect humans, the results shows strong sequence similarities between them. These similarities are even more striking than those reported previously between viral proteins and the encephalitogenic MBP (Shaw et al., 1986). It has been proposed (Jahnke et al., 1985; Fujinami and Oldstone, 1985) that EAE-like diseases can arise when virus-evoked antibodies and/or sensitized T-cells cross react with homologous amino acid sequences (epitopes) in MBP. However, since MBP is located entirely on the cytoplasmic face of the cell membrane and is therefore within the oligodendroglial cell, it would be expected to be relatively inaccessible to immune surveillance. On the other hand, PLP projects both on the cytoplasmic face and also on the outer surface of the membranes. By analogy with Schwann cell function in peripheral myelinated nerves (Chiu, 1991), defects in potassium buffering in the paranodal region of CNS axons should cause the myelin sheath to shrink and might expose this potential antigenic site on PLP. Recognition of this site by antiviral antibodies or sensitized T-cells could induce a cascade of immunological events leading to oligodendrocyte cell destruction. In addition, the virus-evoked antibodies or sensitized T-cells could cross react with the potential antigenic sites of PLP which are located on the outer surface of the cell membrane. In the process, MBP would be released, which might result in further inflammatory consequences. My research has identified an isoform of PLP with possible channel function which, if a site of specific immunological attack, would be expected to be an early cause of demyelination. Furthermore, if drugs could be found which enhanced the channel function of this peptide and improved potassium buffering then they might be expected to delay the progression of demyelinating disease.
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