CHARACTERIZATION OF STRYCHNINE BINDING SITES IN THE RODENT SPINAL CORD

BY

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This thesis is dedicated to the select band of people,
including the most recent arrivals and the sorely missed departed,
that I hold closest in my affections.
"At the end of the day" they make It all worthwhile.
Remember, in the words of the famous actor whose name at present escapes me;
"Nobody said it would be easy".
Although my own feeling is that Nobody was probably wrong.

Thesis abstract

The convulsant alkaloid strychnine is a selective and highly potent antagonist at postsynaptic receptor for the inhibitory neurotransmitter glycine . These properties have led to the extensive use of strychnine as a ligand to probe the postsynaptic glycine receptor. Despite the recent increased understanding of the molecular structure of this receptor protein there is still much dispute as to the nature of the interaction between glycine and strychnine. In an attempt to more clearly define this interaction the experiments described, combine the techniques of protein modification and ligand binding. These investigations also revealed a novel [^{3}H]-strychnine binding site in the rodent CNS and attempts were made to characterize this phenomenon.

The results described suggest that glycine is a fully competitive inhibitor of [^{3}H]-strychnine binding and its reported action as a partial competitive inhibitor is an artefact of the assay conditions. The disruption of [^{3}H]-strychnine binding by residue selective protein modifying reagents suggests some overlap in the strychnine and glycine binding sites at the receptor. Protection studies confirm this and the results are best explained by overlapping yet conformationally distinct recognition sites for strychnine and glycine. Experiments which describe protein modification and ligand protection of strychnine binding antisera highlight possible congruence in the molecular recognition at the antisera and the receptor. This is of interest in the light of proposed models of the strychnine binding site at the postsynaptic glycine receptor.

Modification of spinal cord membranes by the arginine selective reagent 2,3-butanedione (BD) reveals a low affinity and high capacity [^{3}H]-strychnine binding site which is not detectable in untreated membranes. This binding site showed a similar distribution in the CNS as the high affinity site. However, experiments using affinity purified glycine receptor and crude membrane preparations from the mutant mouse *spastic* indicated that the BD-induced binding site is not located on the postsynaptic glycine receptor.

Competition studies revealed that [^{3}H]-strychnine binding sites in untreated and BD-treated membranes have different structural determinants. The ability to effectively inhibit [^{3}H]-strychnine binding to the BD-induced site by cation channel blockers is in accord with reports that strychnine can interact with various cation channels to open or block them. In addition several compounds that inhibit the BD-induced [^{3}H]-strychnine binding can also modulate the reaction of BD with spinal cord membranes if present during the treatment, suggesting a conformational dependent modification. Upon exposure to ultraviolet light [^{3}H]-strychnine is specifically incorporated into a low molecular weight peptide in BD-treated membranes in addition to the ligand binding subunit of the inhibitory glycine receptor, which is the only peptide photolabelled in untreated membranes. The significance of this biochemical and pharmacological characterization of this previously undescribed strychnine binding site is presently unclear. However, the uneven distribution in the CNS and the interaction with important therapeutic agents; local anaesthetics and anti-arrhythmics, indicate the possible biological importance of the novel strychnine binding site.

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

ABBREVIATIONS

2-AS	2-aminostrychnine
5-HT	5-hydroxytryptamine
Ach	acetylcholine
B	bound ligand
BD	2,3-butanedione
B_{max}	maximal number of binding sites
BSA	bovine serum albumin
CAD	cationic amphipathic drug
cDNA	cloned deoxyribonucleic acid
CHAPS	3-[(3-Cholamidopropyl)-dimethylammonio]-1-propanesulphonate
CHD	1,2-cyclohexanedione
CNS	central nervous system
cpm	counts per minute
CQS	camphorquinone-10-sulphate
D-600	methoxy verapamil
DEP	diethylpyrocarbonate
dpm	disintegrations per minute
DSA	diasotized sulphanilate
DT	diazonium tetratzole
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
FI	flurescein isothiocyanate
GABA	γ-amino-N-butyric acid
IAA	iodoacetamide
IC_{50}	concentration of displacer which inhibits 50% of control binding
Ig	immunoglobulin
Iso-THAZ	5,6,7,8-tetrahydro-4H-isooxazolo[3,4]azepin-3-ol
k_{+1}	association rate constant
k_{-1}	dissociation rate constant
kd	kilodalton
K_D	equilibrium dissociation constant
K_I	equilibrium inhibition constant
KLH	keyhole limpet haemocyanin
M	merthiolate
mAb	monoclonal antibody
mRNA	messenger ribonucleic acid
NA	noradrenaline
NBS	N-bromosuccinimide
NEM	N-ethylmaleimide
NMDA	N-methyl-D-aspartic acid
PAGE	polyacrylamide gel electrophoresis
PBB	phosphate buffered borate
PBS	phosphate buffered saline
PCR	polymeraze chain reaction
PEG	polyethylene glycol
PEI	polyethylenimine

PG	phenylglyoxal
PLP	pyridoxal phosphate
PMSF	phenylmethane sulphonyl fluoride
pPG	p-hydroxy-phenylglyoxal
PTC	phosphatidylcholine
SBTI	soya bean trypsin inhibitor
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
spa	spastic
TBPS	t-butylbicyclophosphorothionate
TCA	trichloroacetic acid
TEA	tetraethylammonium
TEMED	NNN',N-tetramethylethylenediamine
TNBS	trinitrobenzosulphonic acid
TNM	tetranitromethane
UV	ultraviolet

Single letter code for amino acids

A	alanine
C	cysteine
D	aspartic acid
E	glutamic acid
F	phenylalanine
G	glycine
H	histidine
I	isoleucine
K	lysine
L	leucine
M	methionine
N	asparagine
P	proline
Q	glutamine
R	arginine
S	serine
T	threonine
V	valine
W	tryptophan
Y	tyrosine

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Chapter One

Introduction

1.1 The convulsant alkaloid strychnine, found in the seeds of the indian plant *Strychnos nux vomica* has found use not only as a poison but also as a tonic and a stimulant. Indeed, as recently as the 1991 World Athletic Championships a sprinter was disqualified for attempting to use this drug as a performance enhancer. Perhaps she should have heeded the advice of Stevenson Macadam (1856); "Take this poison, or that, or the other, but as you value your life my good fellow; don't try strychnine." Fortunately these sentiments were not heeded by scientists whose investigations of the biological action of this alkaloid have helped to focus our present understanding of the structure and organization of the inhibitory glycine receptor: the primary site through which the effects of strychnine are exerted. The following introduction highlights the approaches that have established this understanding then considers the secondary actions of strychnine at so called "non-specific sites". The structure of this alkaloid first synthesized by Woodward and colleagues (1954) is shown in fig 1.1.

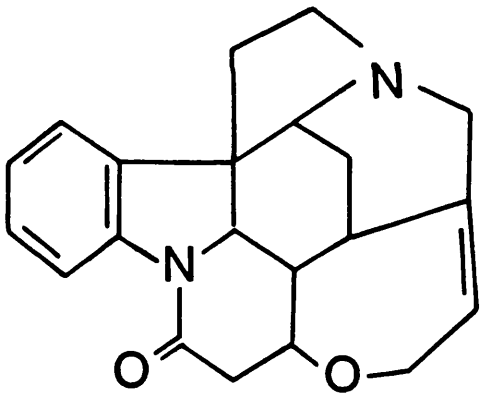

Figure 1.1: The structure of strychnine

1.2 Actions of strychnine on CNS inhibition

The toxic and associated convulsant properties of strychnine had long been recognised (see Dusser de Barenne, 1933) when Sherrington observed (1905) and later analyzed (Owen and Sherrington, 1911) the ability of the alkaloid to convert an inhibitory spinal reflex to an excitatory one. Although a mixed inhibitory and excitatory activity of afferent stimulation to the muscle were acknowledged, it was suggested that this action

related to strychnine causing a prepotency of the excitatory activity or a conversion of the inhibitory to an excitatory mode (Owen and Sherrington, 1911), the increased excitatory activity underlying the convulsant properties of strychnine.

Later, using stimulation of 1a and cutaneous afferents to evoke a direct inhibition recorded in the motorneurone, Bradley and colleagues (1953) showed that this inhibition could be depressed by the administration of strychnine. These authors also discussed the lack of effect of strychnine on monosynaptic excitatory reflexes and concluded that activity of strychnine resided in its ability to depress motoneurone inhibition. Subsequent experiments have confirmed the strychnine sensitivity of other forms of motorneurone inhibition (see Curtis and Johnston, 1974). These effects underlie the disturbed motor behaviour associated with strychnine intoxication. However, other behavioural manifestations have been associated with strychnine and some of these probably underlie its use as a tonic and a stimulant. These include heightened acuity of perception, a hyperalgesia and increased respiratory rate, bradycardia and hypertension (see Zarbin *et al.*, 1981). Such effects, often more obvious at subconvulsive doses, can be detected prior to and subsequent to a convulsive episode (Beyer *et al.*, 1988). They are manifestations of strychnine's activity that extend beyond depression of spinal mechanisms of motor inhibition but they remain less well understood. Strychnine depression of neuronal inhibition at loci associated with nociception (Yokoto *et al.*, 1979; Ishida, 1977), vision (Frumkes *et al.*, 1981), audition (Wu and Oertel, 1986) and respiratory control (Murakoshi and Otsuka, 1985) have been reported. Yet it is the concentration of strychnine binding sites in regions of CNS associated with such functions that have more clearly defined possible explanation for the non-motor disturbances associated with strychnine poisoning (Zarbin *et al.*, 1981; see later). Although, the sensory disturbances are not directly involved in convulsant activity, treatment of subconvulsive doses of strychnine can precipitate convulsions upon presentation of auditory or visual stimulation. This is probably caused by evoking hyperexcitable sensori-motor reflexes (Ascher and Gachelin, 1967) and is consistent with the early work of Dusser de Brenne (1933) which showed that the mechanisms underlying strychnine induced tetanic contractions ultimately resided in intrinsic spinal mechanisms.

In discussing strychnine depression of direct inhibition, Bradley and colleagues (1953) postulated that the alkaloid was either competing with the inhibitory transmitter

at the postsynaptic membrane or disrupting the process underlying inhibition further downstream. At the time, the process was considered to involve an outward movement of cations, a possibility which has since been refined (see later). Subsequently it was shown that strychnine did not depress the discharges of the inhibitory pathway (Eccles *et al.*, 1954) which argued strongly that its action was at the inhibitory synapse. Indeed this specificity for the inhibitory synapse was important in establishing credentials of the putative transmitter glycine as having a physiological function.

1.3 Establishing glycine as an inhibitory transmitter in CNS

Initially the depressant action of glycine when applied to spinal neurons (Curtis and Watkins, 1960) was considered non-specific because of the general nature of this effect (see Aprison, 1990). However, the possibility that it had a role as a spinal inhibitory transmitter was revived by observations that glycine was unevenly distributed throughout the spinal cord (Aprison and Werman, 1965; Graham *et al.*, 1967; Johnson, 1968). This idea gained further support when it was shown that selective loss of interneurons following temporary aortic occlusion showed significant correlation with a decreased glycine concentration in spinal cord. The anoxic condition that generated the interneuron cell loss was also associated with muscle dysfunction (Davidoff *et al.*, 1967). The association between glycinergic interneurons and muscle function was extended when it was noted that glycine concentration in different spinal cord segments reflected the extent of motor outflow from these regions. It was also suggested that the higher concentration of glycine in ventral spinal cord might reflect association with Renshaw cell and terminals of inhibitory interneurons onto motorneurones in grey matter (Johnson, 1968).

The above neurochemical observations prompted a more thorough investigation of the depressant effect of glycine on spinal neurones. The amino acid was shown to depress the activity of motorneurones, interneurons and Renshaw cells (Werman *et al.*, 1966; 1967a; 1967b; 1968; Ten Bruggencate and Engberg, 1968; see also references on strychnine depression of glycine). The action of glycine mirrored that of synaptically released transmitters resulting in a membrane hyperpolarization associated with an increased anion permeability (Coombes *et al.*, 1955).

The accumulated evidence made a strong case for evoking a physiological role for

glycine as an inhibitory neurotransmitter. However, it had very similar actions to another endogenous amino acid γ-aminobutyric acid (GABA; Curtis *et al.*, 1968a; 1968b). This caused investigators to be suspicious about the specificity of the effects and prompted studies exploiting the specificity of strychnine, described above, to probe inhibition evoked by putative transmitters GABA and glycine. Depression of motoneurones (Curtis *et al.*, 1967a; 1967b; 1968b; Larson, 1969), dorsal horn interneurons (Curtis *et al.*, 1967b; 1968a, Larson, 1969) and Renshaw cells (Curtis *et al.*, 1967b; 1968a) by glycine and ß-alanine was always strychnine sensitive. In contrast, in the initial studies strychnine was not observed to cause inhibition of the depression evoked by application of GABA. Therefore, the putative inhibitory amino acids were classified as "glycine-like" (α and ß amino acids) or "GABA-like" (γ and ω amino acids) on the basis of their strychnine sensitivity. The issue of high concentrations of strychnine inhibiting GABA evoked depression is discussed later (see section 1.7). The distinct specificity of neuronal inhibition evoked by GABA and glycine was further validated by studies that showed bicuculline was a selective GABA antagonist Curtis *et al.*, 1971b). The idea that glycine was an endogenous inhibitory transmitter was supported by two further experimental approaches. This included the demonstration of stimulated glycine release from both *in vivo* (Jordan and Webster, 1971) and *in vitro* (Shank and Aprison, 1970; Roberts and Mitchell, 1972, Mulder and Snyder; 1974) spinal cord preparations. While identification of a high affinity glycine uptake system additionally to low affinity uptake in regions of CNS which glycine was postulated to act as a transmitter provided an explanation as to how glycine levels in the synaptic cleft could be controlled to ensure efficient neurotransmission (Johnston and Iversen, 1971; Logan and Snyder, 1972).

Thus, using presynaptic location, identity of action with endogenous transmitter and release criterion (Aprison, 1990) along with pharmacological selectivity of the antagonism by strychnine, glycine was established as an inhibitory transmitter.

1.4 Use of strychnine as a molecular probe to study the inhibitory glycine receptor

The specificity of strychnine as an antagonist of the postsynaptic action of glycine led to the idea that the alkaloid could serve as a probe for the inhibitory glycine receptor. The initial experiments of Young and Snyder (1973) were the first steps to realizing this possibility. They demonstrated that [^{3}H]-strychnine bound to synaptic membranes of rat

spinal cord in a fashion that was saturable, high affinity and inhibited by glycine and related amino acids with potencies that paralleled their ability to elicit strychnine antagonised depression of spinal neurones. The details of this interaction between agonist, glycine and antagonist, strychnine is the subject of Chapter 3 and is not further discussed here.

The experimental approaches in which strychnine has been used are described below after considering the significance of identifying it as a high affinity and selective label for the inhibitory glycine receptor. Specifically this relates to the failure to identify an alternative ligand, most obvious in the inability to develop glycine or other agonists, to serve as suitable labels. Such approaches are hindered by an apparent low affinity of glycine for the receptor that is compounded by the tendency of the amino acid to interact with surfaces of laboratory consumables (Phelan, 1987). In addition, experimental conditions have not been developed which allow glycine to achieve a clear discrimination between the glycine receptor and other CNS recognition sites. These include the sodium dependent low and high affinity uptake systems that dominated in the initial experiments that attempted to use [^{14}C]-glycine as a label (De Feudis and Schiff, 1975). This interpretation is vindicated by experiments that show that the addition of sodium caused a 15 fold increase in [^{3}H]-glycine binding to spinal cord membrane (White *et al.*, 1989). Even when sodium is omitted from buffer, binding to a distinct recognition site from the inhibitory receptor appears to predominate in CNS (Bristow *et al.*, 1986). This latter site has now been identified as the NMDA cation channel protein (Johnson and Ascher, 1987; Bowery 1987). A component of [^{3}H]-glycine binding in spinal cord membranes appeared to be at the inhibitory receptor (White *et al.*, 1989); however, this was only a small proportion of total binding. Consequently, despite efforts to develop glycine as a label, [^{3}H]-strychnine remains the ligand of choice when investigating the inhibitory glycine receptor, with glycine displaced [^{3}H]-strychnine binding being used to assay for the glycine recognition site (see Chapter 3).

1.4.a Distribution of glycine receptors in the CNS

In their initial study (1973) Young and Snyder showed that the amount of [^{3}H]-strychnine bound to synaptic membrane fractions prepared from different regions of CNS, had a distinct caudo-rostral distribution. The predominance of [^{3}H]-strychnine binding to

brainstem and spinal cord membranes has since been confirmed in the pigeon (Le Fort *et al.*, 1978).

A similar gross caudo-rostral distribution was also observed in rat (Zarbin *et al.*, 1981) mouse (Frostholm and Rotter, 1985; White *et al.*, 1990) and human (Probst *et al.*, 1986) in studies that considered [^{3}H]-strychnine binding to tissue sections. There is a good agreement in the overall density of binding sites among the same structures from different species. In general somatic motor and sensory areas have the highest density of [^{3}H]-strychnine binding. Visceral afferent and efferent nuclei contain moderate amounts of binding. The majority of the reticular formation has high to moderate binding with decreased amounts in the more peripheral reticular nuclei. Similarly, the inferior and superior colliculi exhibit low labelling intensities. The density of binding in the diencephalon, limbic areas and telencephalic structures are low, an exception being specific labelling of the amygdala. In all cases, glycine-specific [^{3}H]-strychnine binding is absent in the cerebellum or white matter areas of the CNS. This distribution correlates well with both electrophysiological and neurochemical data described earlier on the distribution of the inhibitory glycine neurotransmitter system. This distribution also coincides with functional consequences of behaviours associated with strychnine intoxication (Zarbin *et al.*, 1981).

The availability of immunological probes for the inhibitory glycine receptor (see section 1.5.c) has led to the investigation of the distribution of immunoreactivity in the CNS. In general terms there is good agreement with the previously described distribution of [^{3}H]-strychnine binding in the CNS. However, two kinds of discrepancy were apparent. Firstly, glycine receptor immunoreactivity was described in cerebellum, olfactory bulb (van den Pol and Gorcs, 1988) and more recently cortex (see Betz, 1991); all regions in which there was no detectable [^{3}H]-strychnine binding (Zarbin *et al.*, 1981). Interestingly, in olfactory bulb and cerebellum, high affinity uptake (Halasz *et al.*, 1979; Wilkin *et al.*, 1981) and glycine immunoreactivity has been described, consistent with glycine acting as a transmitter. This could correspond to the expression of a receptor subtype which cannot be detected by [^{3}H]-strychnine binding techniques. Alternatively, the immunocytochemical approach may provide greater sensitivity than [^{3}H]-strychnine binding for detection of low amounts of receptor protein.

Another contrasting discrepancy is exhibited in spinal cord where the intensity of immunocytochemical labelling in substantia gelatinosa and trigeminal nucleus caudalis (Basbaum, 1988) was much lower than expected for the described density of [^{3}H]-strychnine binding site (Zarbin *et al.*, 1981). Basbaum (1988) rejected the idea that a receptor subtype which was not recognized by the monoclonal used in this study could underlie this discrepancy. This was based on the fact that the labelling was also low when a monoclonal specific for another subunit was used to probe receptor immunoreactivity. However, recent epitope mapping indicates the monoclonal antibody used in this study recognise a region of primary sequence that is distinct in different isoforms of the ligand binding subunit (Schröder *et al.*, 1991; see section 1.6). Also an isoform of the ligand binding subunit with a distinct epitope in this region has divergent putative cytoplasmic domains which might effect the ability to associate with the peripheral receptor subunit. Alternatively, the glycine receptor could be more sensitive to fixation in regions of spinal cord where unexpectedly immunoreactivity is low (Basbaum, 1988).

In addition, the subunit specific mAbs have been used to probe the cellular distribution of glycine receptors by immunoflurescance microscopy. These studies indicated that glycine receptors were concentrated in patches on cell soma with sparser labelling at dendrites, in rat ventral horn, goldfish Mauthner cell (Seitandiou, 1988) and rat ventral cochlear nucleus (Altschuler *et al.*, 1986). In contrast the pattern in Purkinje cells in cerebellum described a segregation of receptors to dendritic regions (Triller *et al.*, 1987).

A concentration of glycine receptor on soma of spinal cord cultures has been reported using a fluorescent form strychnine (Srinivasan *et al.*, 1990). In these studies fluorescent markers were coupled through an amino function at position 2 of strychnine. The resultant fluorescent compounds exhibited a high affinity interaction with the inhibitory glycine receptor comparable to strychnine. These studies also provided evidence that two populations of receptors existed, 50% having a high lateral mobility while the remaining receptors appeared to have a fixed positions. The fixed population is probably anchored by cytoskeletal interactions. Despite high lateral mobility which was diffusion limited, receptors were confined to soma suggesting some impermeable barrier which restrict entrance into other regions of the cell.

1.4.b Ionic modulation of [^{3}H]-strychnine binding

The association of [^{3}H]-strychnine with a postsynaptic mechanism that causes neuronal inhibition by increasing anion conductance has prompted several investigations of the ionic modulation of binding. Young and Snyder (1974a) initially stated that sodium, potassium or lithium chloride caused a marked increase in the ratio of specific to non-specific binding. This was postulated to occur through an activation of specific binding by an unmasking of buried sites and also by preventing the charged interactions between strychnine and synaptic membranes that caused non-specific binding. Thus assay buffers that included sodium chloride have been used to optimize detection of [^{3}H]-strychnine binding (Young and Snyder, 1974a). More recently, potassium thiocyanate has been used for similar reasons (Maksay, 1990).

The activation of [^{3}H]-strychnine binding by sodium salts of monovalent anions was confirmed and extended (Braestrup *et al*, 1986; Marvizón *et al*, 1986b; Marvizón and Skolnik, 1988). However these experiments suggest the activation is related to a direct modulation of the binding site rather than an unmasking of buried sites. This was indicated by a lower K_D without significant changes in B_{max} for [^{3}H]-strychnine when assays were performed in the presence of increasing concentrations of sodium chloride. Experiments indicated that the activation was dependent not only on the alkali earth metal cation (Braestrup *et al*, 1986) but also on the nature of the associated anion. Activation was seen with anions that had previously been shown to permeate the synaptic membrane when stimulated by transmitter; namely Eccles anions (Marvizón *et al*, 1986b). In addition, the concentration dependant activation of [^{3}H]-strychnine binding by increasing concentration of these salts was associated with Hill slope greater than one (Marvizón and Skolnik, 1988). A very similar activation was described for t-[^{35}S]-butylbicyclophosphorothionate (TBPS) binding, an anion channel blocker of the $GABA_A$ receptor. On this basis, it was postulated that strychnine interacts in a similar way to TBPS, and blocks the conductance pathway associated with the inhibitory glycine receptor (Marvizón and Skolnik, 1988).

The effect on the potency of glycine inhibition of [^{3}H]-strychnine binding by monovalent anions has also been investigated. Sodium, ammonium and choline salts of monovalent anions have been shown to decrease the potency of glycine (Braestrup *et al*,

1986; Muller and Snyder, 1978). This suggested that it is the nature of the anionic species that is important in determining the effect. Indeed, the ability to reduce the potency of glycine is particular to Eccles anions (Muller and Snyder, 1978). In contrast to the decreased potency of glycine and related agonists, strychnine, strychnos alkaloids and other receptor antagonists had an increased potency when sodium chloride was present in assays. This effect coincided with sodium chloride activation of [^{3}H]-strychnine binding, as indicated by an 80% fall in specific binding when choline chloride was substituted for sodium chloride (Braestrup *et al*, 1986). The discriminatory effect of sodium chloride on potencies of the agonist, glycine and the antagonist, strychnine argued against identity of their recognition sites. However, this sodium chloride shift could not be used to define agonist/antagonist profiles because the iso-THAZ class of glycine receptor antagonist were shown to have a decreased potency for inhibition of [^{3}H]-strychnine when assays were performed in the presence of sodium chloride (Braestrup *et al*, 1986).

The issue of ionic modulation of [^{3}H]-strychnine binding is more complex, as Young and Snyder (1974b) have shown that ammonium salts of monovalent anions inhibited binding. Moreover, the dependence of inhibition on the associated species being an Eccles anion led them to propose that this effect was independent of the ammonium cation. The postulated inhibition by anions was shown to be associated with a Hill slope greater than one, suggesting a positive co-operativity. This led Young and Snyder to propose that strychnine was associated with the glycine receptor at structures involved in conductance mechanisms.

Thus, Eccles anions would appear to activate or inhibit [^{3}H]-strychnine binding dependent on whether they are associated with sodium or ammonium cations, respectively. However, it has also been shown that sodium and alkali earth metal cations in absence of Eccles anions can at low concentrations inhibit [^{3}H]-strychnine binding, an effect not apparent at higher concentrations of these cations (Muller and Snyder, 1978). While, in contrast, to Young and Snyder's original proposal it has been shown that ammonium ions in the absence of Eccles anions have an inhibitory effect on [^{3}H]-strychnine binding (Marvizón *et al*, 1986b). In addition, this has been extended to show that the activation of [^{3}H]-strychnine by sodium chloride is inhibited by ammonium ions (Marvizón *et al*, 1986b).

The modulatory action of ions of [^{3}H]-strychnine described above might underlie the biphasic effect reported in presence of increasing concentrations of ammonium chloride for assays performed at 37°C (Phelan, 1987). At a low concentration which correspond to physiological concentrations of chloride, binding is potentiated but at higher concentrations it is inhibited as has been previously described (Young and Snyder, 1974b). When assays were performed at 4°C only an inhibition of [^{3}H]-strychnine binding by ammonium chloride at all concentrations was observed (O'Connor, unpublished). This could be explained by evoking a potentiation of the Eccles anion chloride at physiological temperatures and low concentrations of ammonium chloride which is overshadowed by an inhibitory action of the ammonium cation at higher concentrations or at all concentrations when assays are performed at 4°C. Interestingly, the potentiation of [^{3}H]-strychnine binding occurs over a concentration range at which the chloride binding site in the conducting pathway would become saturated (Bormann, 1990).

It should be pointed out that the conflicts in the action of Eccles anions depending on the associated cations have both been interpreted in favour of the idea that strychnine is interacting with the conductance mechanism of the inhibitory glycine receptor (Young and Snyder, 1974b; Marvizón and Skolnik; 1988). This would be consistent with strychnine acting down stream from the recognition sites of the inhibitory transmitter, one of the two possibilities initially proposed by Bradley and colleagues (1953, see earlier). This issue is further considered in Chapter 3.

1.5 Biochemical characterization of the inhibitory glycine receptor

1.5.a Solubilization and affinity purification of the inhibitory glycine receptor

The [^{3}H]-strychnine binding at rat spinal cord membranes was first solubilized using Triton X-100 (Pfeiffer and Betz, 1981). The solubilized binding activity had an affinity for [^{3}H]-strychnine and a relative potency for inhibition by glycine and related amino acids which was comparable to that exhibited by the membrane bound receptor. This indicated solubilization of the receptor in a functionally intact state, although the [^{3}H]-strychnine binding was sensitive to inactivation unless exogenous lipid was included.

Subsequently, the solubilized [^{3}H]-strychnine binding was purified by affinity

chromatography (Pfeiffer *et al*, 1982). The procedure depended on the immobilization, via an amino function at position two of the strychnine molecule, to an agarose support. This 2-aminostrychnine, which had been shown to be almost equipotent with strychnine at inhibitory [^{3}H]-strychnine binding and its derivatization to agarose was reported not to effect its high affinity interaction with the inhibitory glycine receptor (Pfeiffer *et al*, 1982). The detergent extracts were passed over 2-aminostrychnine-agarose, thoroughly washed before pharmacological specific elution of [^{3}H]-strychnine binding activity by high concentrations of unlabelled glycine. This approach led to at least a 1000-fold purification of [^{3}H]-strychnine binding activity. A chromatographic step using a wheat germ agglutinin was more efficient than protein precipitation procedures for removing unlabelled glycine, led to a further 2 fold increase in purification without effecting the associated peptide pattern of the purified fraction. This chromatographic step also indicated that the glycine receptor was a glycoprotein. When the purified fraction was analyzed by denaturing sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) three peptides were identified with apparent molecular weights of 48kd, 58kd and 93kd. The affinity chromatography procedure has since been applied to mouse (Becker, *et al*, 1986) pig (Graham *et al*, 1985) and rat spinal cord (García-Calvo *et al*, 1989). Although this has confirmed the general peptide pattern described above for purified glycine receptor, in rat when sodium cholate was the detergent, the detection of an associated peptide of approximate weight 93kd proved to be variable between preparations (García-Calvo *et al*, 1989).

1.5.b Photolabelling the inhibitory glycine receptor

In parallel to experiments that led to solubilization and affinity purification of the inhibitory glycine receptor, the intrinsic photosensitivity of strychnine was considered in attempts to develop it as a photoaffinity label. Pfeiffer and Betz (1981) first showed that spinal cord membranes incubated with [^{3}H]-strychnine and exposed to UV light irreversibly incorporated the ligand. In addition, the irreversible incorporation of ligand was prevented in incubations that contained excess unlabelled strychnine and glycine but not GABA. When the kinetics of photolabelling were more closely analyzed (Graham *et al*, 1983), the irreversible incorporation was shown to have the same affinity as reversible [^{3}H]-strychnine binding. Moreover the amount of ligand irreversibly incorporated into spinal cord membranes was identical whether or not the [^{3}H]-strychnine used was

subjected to pre-exposure to UV prior to incubation with spinal cord membranes. This indicated that strychnine was not decomposed by UV light in absence of membranes under conditions used for photolabelling and led to the suggestion that photoactivation required an energy transfer process from the glycine receptor protein, which possibly involved aromatic amino acids at or close to the strychnine recognition site (Graham *et al*, 1983).

When photolabelled membranes were solubilized and subjected to SDS-PAGE and analyzed by fluorography only a peptide of apparent molecular weight 48kd was labelled (Pfeiffer and Betz, 1981; Graham *et al*, 1983). This labelling was inhibited in membranes incubated with unlabelled strychnine or glycine during photolabelling procedures. Thus it was proposed that the smallest subunit of the purified glycine receptor (see section 1.5.a) harboured the antagonist binding site. Furthermore, trypsin treatment of photolabelled membranes indicated that an 11kd peptide could be removed without release of irreversibly incorporated [^{3}H]-strychnine binding. Thus, the 37kd fragment that retained irreversibly incorporated strychnine was inaccessible to proteases, suggesting it was membrane buried (Graham *et al*, 1983). It should be noted that this result does not exclude a direct or indirect role for portions of the putative extracellular 11kd peptide fragment in strychnine recognition as photoincorporation may not occur at all regions that make up the binding site.

The photolabelling procedures have since been repeated with mouse spinal cord membranes (Becker *et al*, 1986), purified pig inhibitory glycine receptor (Graham *et al*, 1985) and purified rat inhibitory glycine receptor reconstituted into liposomes (García-Calvo *et al*, 1989). All these studies confirm the pharmacologically specific, irreversible incorporation into the subunit with apparent molecular weight of 48kd. However, in purified pig receptor and reconstituted rat receptors, a weaker labelling of the 58kd subunit has been identified. The specificity of this labelling needs to be addressed but it has been taken to indicate either that in addition to the 48kd subunit portions of the 58kd subunit constitute antagonist binding site (Betz, 1987) or that the 58kd subunit has its own strychnine recognition site (Ruíz-Gómez *et al*, 1990).

1.5.c Antibodies specific for the inhibitory glycine receptor

A direct consequence of the affinity purification of the glycine receptor was the

development of receptor specific antibodies. This included a polyclonal antisera (AS-1; Pfeiffer; 1983) and a panel of 9 monoclonal antibodies (mAbs, 1-9; Pfeiffer *et al*, 1984). All these mAbs were able to remove glycine receptors from detergent solubilized preparation and precipitated fractions were shown to have the characteristic peptide pattern associated with the purified glycine receptor (Pfeiffer *et al*, 1984). However only 6 of the mAbs were able to recognise denatured subunits in western blots. Of these, four mAbs show subunit specificity, whereas the other two cross-react with other subunits: mAb4a with 48 and 58kd subunits; mAb7a with 48 and 93kd subunits. The cross reactivity and the similarity in peptide maps of protease treated iodinated subunit were first indications that subunits might exhibit structural similarities (Pfeiffer *et al*, 1986). In the case of 48 and 58kd subunits this postulate has been proven by subsequent structural and molecular characterization (see section 1.6). However, a proposed similarity between 93kd and other subunits is more difficult to reconcile (see later).

1.5.d Subunit associations in the inhibitory glycine receptor

The physical characteristics of affinity purified rat glycine receptor solubilized in Triton X-100 was analyzed by sucrose density gradient centrifugation and gel exclusion chromatography of the associated [^{3}H]-strychnine binding activity (Pfeiffer *et al*, 1982). These investigations calculated that the inhibitory glycine receptor had an apparent molecular weight of 250kd. Similar analyses performed on purified pig glycine receptor solubilized in sodium cholate gave different physical characteristics. However, this was considered to be due to differences in the detergent micelles and it was therefore concluded that the pig receptor had a similar molecular weight of 250kd. Based on these results and the peptide composition of the purified fractions, which suggested that the 48kd subunit was the most abundant, it was initially proposed that the receptor consisted of a tetramer. This tetramer was postulated to be made up of 2 x 48kd subunits associated with one each of the 58 and 93kd subunits (Betz, 1985).

Subsequent biochemical analysis indicated that under conditions used in the initial analysis, specifically the presence of the reducing agent dithiothreitol, the 93kd subunit disassociated from a core structure made up of 48 and 58kd subunits during sucrose density centrifugation and gel exclusion chromatography (Schmitt *et al*, 1987). The [^{3}H]-strychnine binding activity resided in the fractionated samples that contained this core

structure. The disassociation has been confirmed by subunit analysis of fractions from sucrose density gradient centrifugation with subunit specific mAbs (Becker *et al*, 1989). These studies also confirmed that DTT present during affinity purification by 2-amino strychnine or subunit specific antibodies does not effect association between 93kd and core structure.

Analysis of the 93kd subunit by immunofluorescence labelling using subunit specific mAbs has shown it to be closely associated with 48kd subunit labelling, opposite presynaptic structures (Triller *et al*, 1987). Ultrastructural localization of 93kd immunoreactivity has further indicated that it is associated with the cytoplasmic side of the plasma membrane (Triller *et al*, 1985). This is compatible with studies that indicate the dissociation of the 93kd subunit from synaptic membranes by chemical treatments that have previously been shown to remove membrane associated proteins (Schmitt *et al*, 1987). In addition, unlike the 58kd and 48kd subunits, the 93kd subunit shows no apparent glycosylation, consistent with its association to the cytoplasmic face of the plasma membranes (Graham *et al*, 1987; Schmitt *et al*, 1987). Yet, in addition to the immunocytochemical data listed above, it has been shown that the membrane association of 93kd subunit is specific for the core structure of the inhibitory glycine receptor. The 93kd subunit has been shown stoichometrically to co-distribute with the 48kd subunit, while the quantity of the 93kd subunit in different regions of CNS correlates with the regional distribution of [^{3}H]-strychnine binding (Becker *et al*, 1989).

The sedimentation of disassociated 93kd subunit into fractions on sucrose density gradient centrifugation indicates it is larger than the strychnine binding core associated with 58 and 48kd subunits (Schmitt *et al*, 1987; Becker *et al*, 1989) which has an estimated molecular weight of 250kd (see above). This indicates the 93kd peptide forms homo-oligomeric complexes. This property is analogous to the 43kd subunit that is specific for the nicotinic acetylcholine receptor found in *Torpedo* electric organ or muscle and has a cytoplasmic membrane association. The 43kd subunit has been postulated to play a role in synaptic organization of the nicotinic acetylcholine receptor which is compatible with its ability to bind to lipid and actin. The similar localization and receptor core association that the 93kd subunits of the glycine receptor exhibits have been taken to indicate that it has a similar role at glycinergic synapses (Schmitt *et al*, 1987; Becker *et al*, 1989).

The [^{3}H]-strychnine binding activity of the purified glycine receptor analyzed by sucrose density gradient centrifugation remains associated with a core structure made up of 48 and 58kd subunit. The core structure probably contains domains important in receptor function as it can be reconstituted, with an apparent absence of 93kd subunit, to give agonist stimulated anion fluxes in liposomes which were blocked by the antagonist strychnine (García-Calvo *et al*, 1989). This further indicates that the 93kd subunit has an ancillary role.

The structure of the integral membrane core of the glycine receptor has been analyzed by cross-linking studies of purified and membrane bound receptors, using protein modifying reagents with different specificities (Langosch *et al*, 1988). Despite the different systems that were cross-linked, a consistent pattern of adducts was apparent when analyzed by SDS-PAGE. These ranged from molecular weights of 95,000 up to a maximum of 260,000, which was the major band. When adducts were cleaved and probed on basis of size or immunoreactivity with receptor specific mAbs, they were shown to be made up exclusively of 48 and 58kd subunits. Exceptions were the 95kd adducts, which contained unreacted 93kd, or high molecular weight adducts that failed to enter the resolving gel. In the latter case these consisted of 48, 58 and 93kd subunits and are thought to arise through core structures that remain associated with the homoligomeric 93kd complexes described in sucrose density gradient centrifugation analysis (see above). The major adduct of 240-260kd was speculated to be a pentamer of 48 and 58kd that constituted the [^{3}H]-strychnine binding core. The minor adducts being dimers, trimers and tetramers of this core structure. Although densametry staining of purified glycine receptors resolved by SDS-PAGE indicated either a pentameric core made up of 4 x 48kd; 1 x 58kd subunits or 3 x 48kd: 2 x 58kd subunits, the latter stoichiometry was favoured. This was based on presence of a 116,000kd adduct made up of 58kd subunits. While it was also considered consistent with electrophysiological data that 2-3 glycine molecules bind to activate the anion channel, as each 48kd subunit was argued to bind a single agonist molecule. The existence of a pentameric functionally active core structure and an associated peripheral subunit has led to reinterpretation of the proposed structure of the inhibitory glycine receptor (Betz, 1987; 1988) and this is represented diagrammatically in figure 1.2. Moreover this pentameric arrangement points to a conserved quaternary structure for glycine and nicotinic acetylcholine receptors which Langosch and colleagues (1988) have argued extends to $GABA_A$ receptor in contrast with

previous speculations which suggested the latter has a tetrameric quaternary structure.

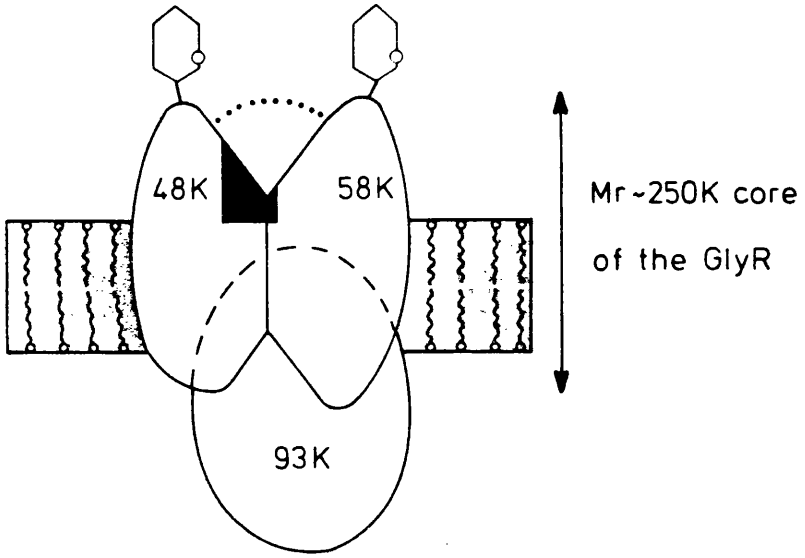

Figure 1.2: A model of the postsynaptic glycine receptor complex: The strychnine--binding core of the glycine receptor is assumed to be a 250 kd heteroligomer of the 48 and 58 kd polypeptides; only one copy of each subunit is shown. The postulated strychnine binding region is indicated in black and hexagonal extensions symbolize carbohydrate sidechains (from Betz, 1991)

1.5.e Developmental regulation of [^{3}H]-strychnine binding and inhibitory glycine receptor

Early studies postulated that there was an accumulation of the glycine receptor during postnatal development as assayed by increases in the amount of [^{3}H]-strychnine binding (Zukin *et al*, 1975;Benavides *et al*, 1981). However, subsequent analysis using receptor specific mAbs indicate a more subtle development which is oversimplified if probed by [^{3}H]-strychnine binding alone. Thus, Becker and colleagues (1988) observed that the quantified immunoreactivity to spinal cord membranes for mAbs 2b and mAbs 7a which are specific 48 and 93kd subunits respectively, paralleled the increases in [^{3}H]-strychnine binding during postnatal development. However mAb4a, which cross-react with 48 and 58kd subunits, detected high amounts of immunoreactivity in presence of low amount of [^{3}H]-strychnine binding. This was obvious in membranes from neonatal animals. The ability to purify equal quantities of mAb4a immunoreactivity from solubilized neonatal and adult membranes by a 2-aminostrychnine-agarose affinity

chromatography indicated that the both types of mAb4a reactivity could interact with glycine and strychnine. However one type of reactivity had too low an affinity for strychnine to enable detection by [^{3}H]-strychnine binding assays and its interaction with strychnine was only detected in the highly cross-linked environment of the agarose column. This feature was used to resolve components of mAb4a reactivity from neonatal membranes by application of a glycine concentration elution gradient in affinity chromatography. In this way two peaks of mAB4a reactivity were eluted from a 2 aminostrychnine-agarose column loaded with neonatal membranes. The second peak corresponded to the position of a single peak of mAb4a reactivity purified from adult tissue, which eluted at high glycine concentrations. This tends to indicate the second peak has a higher affinity for strychnine.

Western blot analysis of mAb4a reactivity in membranes from animals at different developmental stages showed that at birth a band with subunit molecular weight 49kd co-existed with 48kd subunit. During development and associated with increased [^{3}H]-strychnine binding the 49kd was depleted but 48kd subunit appeared to be accumulated. Sizes of the two isoforms was confirmed by differential affinity chromatography using mAb2a and 2-aminostrychnine columns. Further, although the western blotting studies confirmed 93kd expression was lower in neonate than adult it could associate with mAb4a immunoreactivity on the 49kd subunit. Also, sucrose density centrifugation indicated that the mAb4a reactivity detected had similar physical characteristics to core structure (see section 1.5.d) of purified adult glycine receptor. On this basis it was speculated that both 48 and 49Kd subunits form core structures. Although in the case of 49kd subunit no association with a 58kd subunit was apparent. This was the first direct indication that isoforms of receptor subunits of the glycine receptor existed. The disappearance of the strychnine insensitive receptor during postnatal development was associated with no change in total mAb4a immunoreactivity but an increase in [^{3}H]-strychnine binding. Thus a switch in isoform expression was postulated, a process that has precedent at the nicotinic acetylcholine receptor of muscle (see Becker *et al*, 1988).

In the *spastic* mouse, a mutant that suffers from motor disorder and a 80% decrease in its high affinity [^{3}H]-strychnine binding sites (White and Heller, 1982) the temporal expression of glycine receptor isoforms during development has been studied. These investigations are interesting as they indicate that the mutants exhibit the associated

neonatal immunoreactivity but fail to accumulate high affinity [^{3}H]-strychnine binding sites (see Becker, 1990).

Subsequently, Hoch and colleagues (1989) used differences in immunoreactivity and [^{3}H]-strychnine binding to dissect the nature of inhibitory glycine receptors in mouse and rat embryonic spinal cord cultures. These studies indicate that 90% of the surface membrane glycine receptors are associated with a 49kd subunit isoform and as a consequence, the cultures have low amounts of [^{3}H]-strychnine binding. This has been disputed (Srinivason *et al*, 1990) by studies that have used similar cultures but detected much higher amounts of [^{3}H]-strychnine binding. However the cultures used in this latter study were free from antibiotics, horse serum and nerve growth factor and it is interesting to speculate as to the significance of these differences in culture media on the expression of glycine receptor isoforms. Alternatively, the differences in amounts of [^{3}H]-strychnine binding might reflect early developmental events that are important in determining a switch from low to high affinity strychnine binding, because in the latter study 15 day old embryos, a day or two older than those of Hoch and colleagues, were used to make cultures.

1.6 Molecular Genetic Analysis of the inhibitory glycine receptor

1.6.a Primary structure of receptor subunits and their isoforms

Purification of subunits associated with the inhibitory glycine receptor provided impetus for molecular genetic analysis. Gas phase microsequenced, protease-digested peptide fragments were used to design corresponding oligonucleotides to probe rat spinal cord cDNA libraries. This led to cloning and deduction of the full amino acid sequence of the 48 and later the 58kd subunits, designated α1 and ß1 respectively (Grenningloh *et al*, 1987; Grenningloh *et al*, 1990a). There is a 47% amino acid sequence identity between α1 and ß1 which extends to 58% if isofunctional exchanges are considered (Grenningloh *et al*, 1990a). This indicates the two subunits result from a gene duplication and confirms the biochemical analysis described earlier which suggested homologies between these subunits. Using an expression cloning strategy a very similar cDNA clone with identical amino acid sequence to α1 has been identified (Akagi *et al*, 1991). The deduced amino acid sequence of α1 and ß1 subunits are shown in Figure 1.3.

Rat α1 sequences have since been used to probe a human cDNA library (Grenningloh *et al*, 1990b) and have identified two variants of the human glycine receptor. One variant has 99% amino acid identity to rat α1 and is designated as human α1 equivalent. The second full length clone has only 76% predicted amino acid sequence identify with rat or human α1 subunit and was designated α2. Similar homology screening of rat cDNA library using hybridization of clones to rat α1 but not ß subunit probes as basis for further analysis identified two additional α subunit variants in the rat (Kuhse *et al*, 1990b). The first despite sharing 99% amino acid sequence identity with human α2 exhibited a distinct pharmacological profile upon expression and functional assay and was designated rat α2* (Kuhse *et al*, 1990a). The other clone from rat by virtue of its 82 and 81% deduced amino acid sequence identity with rat α1 and α2* respectively, was designated α3 (Kuhse *et al*, 1990b). Subsequently when rat spinal cord cDNA libraries were screened using rat α2* probes an equivalent clone to human α2 was identified which unlike α2* was functionally similar to the human α2 variant (Kuhse *et al*, 1991).

The ability to map human α1 and α2 subunits onto different chromosomes indicates these variations in primary structure represent individual primary transcripts (Grenningloh *et al*, 1990b). However, variations in glycine receptor cDNA sequences have been identified to arise through mechanisms other than distinct genes. These mechanisms include allelic variation and alternative splicing.

The distinct pharmacological profile of rat α2* and human α2 upon expression of corresponding message in oocytes relates to the relative strychnine insensitivity of glycine evoked currents expressed by α2* message (Kuhse *et al*, 1990a). The codon that coded for one of 5 amino acids that were distinct in primary structure of human α2 and rat α2* was mutated from a glutamate to a glycine so the expressed protein would have the same amino acid as α2 at position 206 (see fig 1.3), the resulting current expressed by mutants exhibited strychnine sensitivity characteristic of currents expressed by α2 (Kuhse *et al*, 1990a). The identification of rat α2 sequences which like human α2 expressed characteristic strychnine sensitivity provided further insight into the genetic mechanism that gives rise to α2* (Kuhse, 1991). The cDNA sequences of α2 and α2* varied at only 2 nucleotide positions, the one variation in the coding region giving rise to the glutamate - glycine transformation at position 206. Based on almost complete sequence identity of α2 and α2* cDNA it was considered unlikely that distinct genes are responsible and

allelic variation is speculated to account for the distinct primary structures (Kuhse *et al*, 1991).

Another mechanism which gives rise to primary structure of inhibitory glycine receptors is alternative splicing. This has been identified for rat α1 and α2 subunits. In case of α2, two sequences were identified by probing rat cDNA library with α2* sequences and designated α2A and α2B (Kuhse *et al*, 1991). The α2A and α2B cDNA sequences were identical expect for a short sequence where identity dropped to 79%. However, of 22 amino acids coded for by this divergent sequence only two were different. Two exons were identified in mouse genomic library which had identical sequence to those divergent regions found in rat cDNA of α2A or α2B. The exons were separated by a small intron sequence and surrounded by proper splice acceptor and donor sequences.

In contrast, the alternative splice identified in rat α1 was caused by insertion of an additional piece of sequence (Malosio *et al*, 1991a). Thus, when rat α1 subunit sequence was used to probe rat cDNA spinal cord libraries, a cDNA was identified that contained a 24 base pair insert not identified in original α1 cDNA sequences. This insert encoded amino acids that constitute a putative phosphorylation site. The amino acid sequence was also identified by gas microsequencing of tryptic digested purified glycine receptors. Analysis of genomic DNA identified clones that contained a 24 base pair insert and this was shown to lie at exon/intron boundary

The primary structure of glycine receptor variants are summarized in Figure 1.3 and 1.4.

1.6.b Secondary structure predicted from primary sequence and homology with related receptors

In addition to the amino acid sequence homology between glycine receptor subunits, there is a fair degree of homology with $GABA_A$ receptor and to a less extent nicotinic acetylcholine receptor subunits (Barnard *et al*, 1987; Betz, 1990). This suggests that these ion channels evolved from a common ancestral gene and represent a super family of ligand gated ion channels (Barnard *et al*, 1987). The homology has been important in developing structural and functional models of these receptors. In contrast,

```
Rat α1 1    -----MGKSPGLLDYLWAWTLF--EFPSKEAD-------AARSAPKPMS-
Hum α1 1    -----MYSFNTLRLYLSGAIVFFSLAASKEAE-------AARSATKPMS-
Rat α2 1    MNRQLVNILTALFAFFLGTNHFREAFCKDHDSR------SGKHPSQTLS-
Hum α2 1    MNRQLVNILTALFAFFLETNHFRTAFCKDHDSR------SGKQPSQTLS-
Rat α3 1    MAH-VRHFRTLLSGFYFWEAALLLSLVATKETN------SARSRSAPMS-
Rat ß  1    MKFSLAVSFFILMSLLFEDACSKEKSSKKGKGKKKQYLCPSQQSAEDLAR

                                        2                   2
Rat α1 51   -PSDFLDKLMGRT-SGYDARIRPNFKGPPVNVSCNIFINSFGSIAETTMD
Hum α1 51   -PSDFLDKLMGRT-SGYDARIRPNFKGPPVNVSCNIFINSFGSIAETTMD
Rat α2 51   -PSDFLDKLMGRT-SGYDARIRPNFKGPPVNVTCNIFINSFGSVTETTMD
Hum α2 51   -PSDFLDKLMGRT-SGYDARIRPNFKGPPVNVTCNIFINSFGSVTETTMD
Rat α3 51   -PSDFLDKLMGRT-SGYDARIRPNFKGIPVDVVVNFFINSFGSIAETTMD
Rat ß  51   VPPNSTSNILNRLLVSYDPRIRPNFKGIPVDVVVNIFINSFGSIQETTMD

Rat α1 101  YRVNIFLRQQWNDPRLAY-NEYPD-DSLDLDPSNLDSIWKPDLFFANEKG
Hum α1 101  YRVNIFLRQQWNDPRLAY-NEYPD-DSLDLDPSNLDSIWKPDLFFANEKG
Rat α2 101  YRVNIFLRQQWNDSRLAY-SEYPD-DSLDLDPSMLDSIWKPDLFFANEKG
Hum α2 101  YRVNIFLRQQWNDSRLAY-SEYPD-DSLDLDPSMLDSIWKPDLFFANEKG
Rat α3 101  YRVNIFLRQKWNDPRLAY-SEYPD-DSLDLDPSMLDSIWKPDLFFANEKS
Rat ß  101  YRVNIFLRQKWNDPRLKLPSDFRGSDALTVDPTMYKCLWKPDLFFANEKS

                                                *           *
Rat α1 151  AHFHEITTDNKLLRISRNGNVLYSIRITLTLACPMDLKNFPMDVQTCIMQ
Hum α1 151  AHFHEITTDNKLLRISRNGNVLYSIRITLTLACPMDLKNFPMDVQTCIMQ
Rat α2 151  ANFHDVTTDNKLLRISKNGKVLYSIRLTLTLSCPMDLKNFPMDVQTCTMQ
Hum α2 151  ANFHDVTTDNKLLRISKNGKVLYSIRLTLTLSCPMDLKNFPMDVQTCTMQ
Rat α3 151  ANFHEVTTDNKLLRIFKNGNVLYSIRLTLTLSCPMDLKNFPMDVQTCTMQ
Rat ß  151  ANFHDVTQENILLFIFRDGDVLVSMRLSITLSCPLDLTLFPMDTQRCKMQ

                1                                       *
Rat α1 201  LESFGYTMNDLIFEWQEQGAVQVADGLTLPQF-ILKEEKDLRYCTKHYN-
Hum α1 201  LESFGYTMNDLIFEWQEQGAVQVADGLTLPQF-ILKEEKDLRYCTKHYN-
Rat α2 201  LESFGYTMNDLIFEWLSDGPVQVAEGLTLPQF-ILKEEKELGYCTKHYN-
Hum α2 201  LESFGYTMNDLIFEWLSDGPVQVAEGLTLPQF-ILKEEKELGYCTKHYN-
Rat α3 201  LESFGYTMNDLIFEWQDEAPVQVAEKIALPQF-LLKEEKELGYCTKHYN-
Rat ß  201  LESFGYTTDLLRFIWQSGDPVQL-EKIALPQFDIKKEDIEYGNCTKYYKG

                *
Rat α1 251  TGKFTCIEARFHLERQMGYYLIQMYIPSLLIVILSWISFWINMDAAPARV
Hum α1 251  TGKFTCIEARFHLERQMGYYLIQMYIPSLLIVILSWISFWINMDAAPARV
Rat α2 251  TGKFTCIEVKFHLERQMGYYLIQMYIPSLLIVILSWVSFWINMDAAPARV
Hum α2 251  TGKFTCIEVKFHLERQMGYYLIQMYIPSLLIVILSWVSFWINMDAAPARV
Rat α3 251  TGKFTCIEVRFHLERQMGYYLIQMYIPSLLIVILSWVSFWINMDAAPARV
Rat ß  251  TGYYTCVEVIFTLRRQVGFYMMGVYAPTLLIVVLSWLSFWINPDASAARV

Rat α1 301  GLGITTVLTMTTQSSGSRASLPKVSYVKAIDIWMAVCLLFVFSALLEYAA
Hum α1 301  GLGITTVLTMTTQSSGSRASLPKVSYVKAIDIWMAVCLLFVFSALLEYAA
Rat α2 301  ALGITTVLTMTTQSSGSRASLPKVSYVKAIDIWMAVCLLFVFAALLEYAA
Hum α2 301  ALGITTVLTMTTQSSGSRASLPKVSYVKAIDIWMAVCLLFVFAALLEYAA
Rat α3 301  ALGITTVLTMTTQSSGSRASLPKVSYVKAIDIWMAVCLLFVFSALLEYAA
Rat ß  301  PLGIFSVLSLASECTTLAAELPKVSYVKALDVWLIACLLFGFASLVEYAV

                                                        3
Rat α1 351  VNFV----SRQHKELLRFRR------------K-RRHHKDDEGG-EG---
Hum α1 351  VNFV----SRQHKELLRFRR------------K-RRHHKDDEGG-EG---
Rat α2 351  VNFV----SRQHKEFLRLRR------------RQKRQNKEEDVTRES---
Hum α2 351  VNFV----SRQHKEFLRLRR------------RQKRQNKEEDVTRES---
Rat α3 351  VNFV----SRQHKELLRFRRKRKNKTEAFALEKFYRFSDTDDEVRES---
Rat ß  351  VQVMLNNPKRVEAEKARIAK------------AEQADGKGGNAAKKNTVN
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Rat α1 401  ----RFNFSAYGMGPA-C---------LQAKDGISV--------------
Hum α1 401  ----RFNFSAYGMGPA-C---------LQAKDGISV--------------
Rat α2 401  ----RFNFSGYGMGH--C---------LQVKDGTAV--------------
Hum α2 401  ----RFNFSGYGMGH--C---------LQVKDGTAV--------------
Rat α3 401  ----RLSFTAYGMGP--C---------LQAKDGVVP--------------
Rat ß  401  GTGTPVHISTLQVGETRCKKVCTSKSDLRSNDFSIVGSLPRDFELSNYDC

Rat α1 451  --KGANNNNTTNPAPAPSKSPEEMRKLFIQRAKKIDKISRIGFPMAFLIF
Hum α1 451  --KGANNSNTTNPPPAPSKSPEEMRKLFIQRAKKIDKISRIGFPMAFLIF
Rat α2 451  --KAT----PANPLPQPPKDADAIKKKFVDRAKRIDTISRAAFPLAFLIF
Hum α2 451  --KAT----PANPLPQPPKDGDAIKKKFVDRAKRIDTISRAAFPLAFLIF
Rat α3 451  --KG-----PNHAVQVMPKSADEMRKVFIDRAKKIDTISRACFPLAFLIF
Rat ß  451  YGKPIEVNNGLGKPQAKNKKPPPAKPVIPTAAKRIDLYARALFPFCFLFF

Rat α1 501  NMFYWIIYKIVRREDVHNK
Hum α1 501  NMFYWIIYKIVRREDVHNQ
Rat α2 501  NIFYWITYKIIRHEDVHK
Hum α2 501  NIFYWITYKIIRHEDVHKK
Rat α3 501  NVIYWSIYL
Rat ß  501  NIFYWVIYKILRHEDIHHQQD
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Fig 1.3: Sequence alignment for α and ß glycine receptor subunits: α1 (rat and human), α2 (rat and human) and ß were subjected to AMPS alignment algorithm (Barton and Sternberg, 1987a; 1987b). The α3 was aligned manually using previously published alignment (Kuhse *et al.*, 1990b). N-terminal sequence in italics putative signal peptides. * * indicate Cysteines postulated to be disulphide linked. Underlined sequences indicate proposed transmembrane domains. Bold residues represent Arginine, Tyrosine and Histidine residues that are found in the α-subunit but are not conserved in the ß-subunit and are discussed further in Chapter 3. 1 Glycine at this position is a Glutamate in the α2*. 2.....2 position between which the alternatively spliced sequence PPVNVTCNIFINSFGSIAETTM is found in the α2B. 3 position in which 8 amino acid inset SPMLNLFQ is found in the α1-ins.

the more recently cloned subtypes of the cation channels gated by glutamate (Hollmann *et al*, 1989; Keinanen *et al*, 1990; Moriyoshi *et al*, 1991; Kumar *et al*, 1991) have no apparent sequence homology although postulated similarities in small portions may be a result of convergent evolution.

The hydropathy profile of glycine receptor subunits, in common with $GABA_A$ and nicotinic acetylcholine receptor subunits, predicts that there are four α helical domains of sufficient hydropathy and length to cross the membrane (Barnard *et al*, 1987; Betz, 1990; 1991). These transmembrane regions are termed M1 - M4. There is particular sequence homology in transmembrane sequences of the glycine receptor, the most divergent being those of the ß subunits (see figure 1.3). A transmembrane topology has been suggested (Grenningloh *et al*, 1987; Betz 1990; 1991) in which the large N-terminal that precedes M1 and smaller C-terminal subsequent to M4 are extracellularly located. This is primarily based on the presence of putative N-glycosylation sites (see figure 1.4) and the identification in the homologous nicotinic acetylcholine receptor of extracellular N and C-terminals (Barkas *et al*, 1987; Ratnam *et al*, 1986). The model also predicts that the region between M3 and M4 forms an intracellular loop consistent with putative phosphorylation sites in this region of ß (Grenningloh *et al*, 1990b) and α1 subunits (Malasio *et al*, 1991a). This model is also consistent with recent mapping of major antigenic determinants to the N-terminal region of α1 (Schröder *et al*, 1991).

In addition, functional domains have been implicated with the structural features. Particular attention has been paid to the domains that constitute the major functional properties, namely ligand binding and ion conductance. The abundance of uncharged polar amino acid residues in M2 sequence has favoured the idea that this provides the hydrophilic lining of the anion channel (Betz, 1990). The considerable homology between glycine and $GABA_A$ subunits in this region favoured the idea that this domain was important in anion conductance (Barnard *et al*, 1987; Betz, 1990). Also, the idea that M2 domains come together to form a channel structure (Betz, 1990) is consistent with the proposal that 5 α helices were required to form an anion channel of correct pore diameter (Bormann, 1990). However, it should be noted that the M2 sequence of the ß subunit, which should be involved in pentameric structure (see earlier; Langosch *et al*, 1988), does not share the particular sequence identity that exists between α subunits and $GABA_A$ subunits (Grenningloh *et al*, 1990a). From both biochemical (Giraudat *et al*, 1986) and

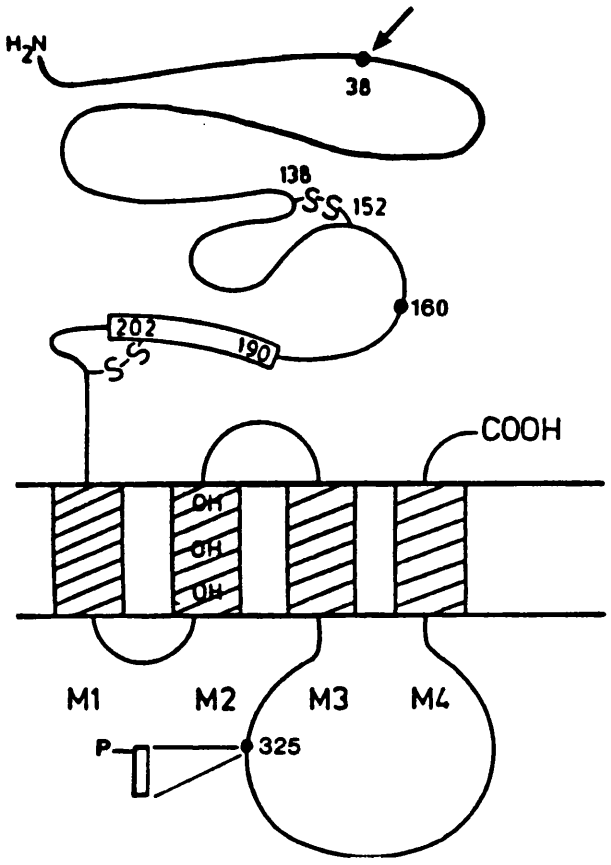


Figure 1.4: Transmembrane arrangement of the glycine receptor α1 subunit: The topology is predicted by hydropathy analysis. The arrow denotes a potential glycosylation site, putative disulphide bridges are denoted by -S-S-, and OH indicates the amphipathic character of the putative channel lining. Regions and residues thought to be important for agonist binding 190-202 and antagonist binding 160 taken from different alignments than described in Fig 1.3 are indicated. Also shown is the position of the eight amino acid insertion in the proposed cytoplasmic region that creates a possible phosphorylation site (P) (taken from Betz, 1991).

molecular genetic analysis (Leonard *et al*, 1988; Charnet *et al*, 1990; Revah *et al*, 1991) the corresponding M2 region of nicotinic acetylcholine receptors have been shown to be involved in ion conduction. Therefore, it is interesting to note that a common amino acid periodicity has been identified between M2 domain of nicotinic acetylcholine, $GABA_A$ and glycine receptor subunit. This evokes small or polar residues arranged towards the channel lumen to allow passage of ions (Betz, 1990).

The predicted large N-terminal region, particularly that of the α subunits, are speculated to harbour the glycine and strychnine binding sites (Betz, 1990; 1991). This region displays structural motifs that are conserved in nicotinic acetylcholine and $GABA_A$

receptors (Barnard *et al*, 1987; Betz, 1990). In this respect all subunits of this receptor subunit superfamily have two cysteine residues conserved in an equivalent position (182-197, see figure 1.3). These are thought to form a disulphide bridge. In the case of the glycine receptor subunits (see figures 1.3, 1.4) two additional cysteines (244-256) more distal to the N-terminal are conserved and speculated to be disulphide linked (Grenningloh *et al*, 1987). This cystine lies in a similar region to the vicinal cystine located in the α subunits of nicotinic acetylcholine receptors known to be important in ligand recognition (Changeux, 1990). The significance of the postulated cystines in glycine receptor function is presently unclear and further discussed in Chapter 3.

The above speculations form the basis for the diagrammatic representation of glycine receptor subunit structure shown in figure 1.4. Despite the construction of attractive functional models on the basis of sequence homologies, Maelicke (1989) offers a more restrained view when he points out that different levels of structural homology may not directly correlate to functional specialization, the possibility being that sequence homologies arise through structural rather than functional constraints that are common to ion channels.

1.6.c Functional expression of receptor isoforms

Heterologous expression in *Xenopus* oocytes of poly(A)$^+$ RNA prepared from spinal cord led to incorporation of glycine receptor channels in the oocyte membrane which had many similarities to those detected in neurones (Gundersen *et al*, 1984). This system has been used with cloned subunits of the inhibitory glycine receptor. Individual expression of rat or human α1, α2 or α3 isoforms led to glycine evoked currents which were sensitive to concentrations of strychnine that antagonised glycine receptors (Schmieden *et al*, 1989; Grenningloh *et al*, 1990a; Kuhse *et al*, 1990b; Malosio *et al*, 1991a; Kuhse *et al*, 1991). Such results indicated that the homooligomeric association of subunits could form functional channels.

A structural role for the ß subunit has been implied as it appears functionally inert with respect to ligand binding. Thus, the ß subunit only evokes weak responses to glycine and related agonists when introduced into the nucleus of oocytes, conditions that favour its over expression (Grenningloh *et al*, 1990a). In addition these responses had only a

slight sensitivity to strychnine even at about 50,000 times estimated K_D from previous ligand binding experiments. These results, coupled to biochemical data (see section 1.4.b) strongly argue that the strychnine and probably also the glycine recognition sites predominantly reside in the α-subunit.

The consensus characteristic of the channels induced by expression of α1, α2 and α3 isoforms is that they are stimulated by agonist in a strychnine sensitive manner. However they do exhibit differences in agonist potencies (Grenningloh *et al*, 1990b; Kuhse, *et al*, 1990a; Kuhse *et al*, 1990b). This could relate to the use of heterologous systems or expression of individual isoforms in absence of associated subunits. Alternatively, it might reflect differences in the amino acid sequence of putative ligand binding domains of different isoforms (Betz, 1991). This possibility is indicated by the differential sensitivity to strychnine and glycine of the currents expressed by α2 and α2* subunits which have sequences that differ by only one amino acid (Kuhse *et al*, 1990a). In addition, sequence differences in other putative functional domains might exert differential channel activities and have significance for different physiological roles that these isoforms play.

1.6.d Developmental expression and anatomical distribution of glycine receptor isoforms

On the basis of discrepancies in the quantitated glycine receptor immunoreactivity and [^{3}H]-strychnine binding during post-natal development, two forms of receptor designated strychnine-sensitive and insensitive were speculated (see section 1.5.e). Subsequent physical heterogeneity was identified in the mRNA species derived from adult and neonatal spinal cord, that code for glycine sensitive anion channels when expressed in oocytes. Thus, mRNA coding for glycine receptors as identified by sucrose gradients were predominated by a heavy form in adult and a light form in neonate. Interestingly, a corresponding light form coding for glycine receptors was identified in adult cerebral cortex (Akagi and Miledi, 1988). Heterogeneity was further vindicated when it was shown that antisense mRNA to cloned rat α1 blocked expression of glycine sensitive responses in oocytes injected with adult but not neonate mRNA (Akagi *et al*, 1989). However, unlike the biochemically defined isoforms, the responses evoked by adult and neonate mRNA appeared strychnine sensitive as they could be antagonised by 0.5μM of the

alkaloid (Akagi and Miledi, 1988).

Identification of α2* cDNA as an isoform capable of forming relative strychnine-insensitive glycine receptors argued that this corresponded to the developmentally regulated isoform identified biochemically (Kuhse *et al*, 1990a). Indeed, PCR amplification of α2* message in spinal cord was present at birth but disappeared postnatally; a temporal expression consistent with the strychnine insensitive receptor described during development (Kushe *et al*, 1990a). Subsequently rat α2A, a closely related variant of α2*, was shown to express glycine evoked currents with characteristic sensitivity to strychnine. As the cDNA sequences of the two isoforms vary at only 2 nucleotide positions, they would have been indistinguishable by PCR amplification. Thus, the expression at birth and subsequent decrease postnatally could relate to α2*, α2A or its alternatively spliced form α2B (Kuhse *et al*, 1991) or a combination of the three. Indeed, α2A and α2B probes hybridize to spinal cord slices from early postnatal ages (see Kuhse *et al*, 1991); this means that it is difficult to assess the significance during development of the isoforms with varying strychnine sensitivities. Yet, strychnine at 0.5μM blocked glycine evoked currents produced by expression of neonatal mRNA (Akagi and Miledi, 1988) and at 2μM blocks evoked inhibitory post synaptic currents in neonatal spinal cord slices (Edwards *et al*, 1989). These experiments might argue against significant contributions of α2* in neonate, as this expresses glycine induced current in oocytes that are antagonized by strychnine with an IC_{50} value of 7μM (Kuhse *et al*, 1990a).

The expression of different isoforms during development and in the adult have been investigated by in situ hybridization using probes specific to α1, α2, α3 and ß cDNA sequences (Malosio *et al*, 1991b). In spinal cord, α1 and ß expression at birth increases during a postnatal development. In agreement with PCR amplification (Kuhse *et al*, 1990a), α2 although highly expressed at birth falls to barely detectable levels by postnatal day 15 (Malosio *et al*, 1991b). Interestingly, this is the stage at which the strychnine insensitive subtype, defined biochemically, disappears (see section 1.5.e; Becker *et al*, 1988). As defined by northern blot analysis, α3 is under-expressed in CNS (Malosio *et al*, 1991b) and only hybridization to spinal cord was detected which was restricted to late postnatal development. This temporal expression is consistent with PCR amplifications (Kuhse *et al*, 1990b).

The isoform expression in other brain regions indicates that α1 expression can be detected in colliculus and hypothalamus in addition to spinal cord where its expression predominates (Malosio *et al*, 1991b). In addition to the neonatal expression of α2 in spinal cord this isoform can be detected in forebrain regions (Malosio *et al*, 1991b). Unlike spinal cord this expression is maintained postnatally, even in regions where [^{3}H]-strychnine or receptor subunit immunoreactivity have not previously been detected (Malosio *et al*, 1991b; Betz 1991). This pattern was confirmed by probing with sequences to α2A and α2B isoforms. These investigations indicated a differential expression of the 2 alternatively spliced isoforms although a α2A is the predominantly expressed form particularly during early development (Kuhse *et al*, 1991). In the case of α3 only weak expression was indicated in brain regions, cerebellum providing the strongest signal (Malosio *et al*, 1991b).

The ß isoform is highly expressed in forebrain regions throughout development and this pattern extends into the adult (Malosio *et al*, 1991b). This is consistent with investigation using PCR amplification (Kuhse *et al*, 1990a) and northern blot analysis of poly (A)$^+$ RNA (Grenningloh *et al*, 1990b). Indeed ß subunits are expressed in regions which have no associated α isoforms expression (Malosio *et al*, 1991b). This could indicate there are other isoforms which await identification. Alternatively, it has been suggested that it could associate with other subunits of this ion channel superfamily. It was also suggested that this subunit could associate with NMDA receptor subunits to constitute the glycine site identified on this cation channel (Malosio *et al*, 1991b; Betz 1991). This idea was based on the glycine site associated with the ß subunit upon expression in oocytes. However, mismatch in aspects of the regional distribution of ß isoform expression and NMDA channel localization (Malosio *et al*, 1991b) along with the distinct pharmacological profiles of the glycine site at the inhibitory glycine receptor and the NMDA channel argue against this possibility.

Isoform expression may provide an explanation for previously described mismatches between immunoreactivity and [^{3}H]-strychnine binding during development (see section 1.5.d) and in investigations into glycine receptor localization (see section 1.4.a). This expression also points to possible significance of the glycine transmitter system in synapse formation and the control of neurone pathways more extensively located in CNS than previously imagined (Betz, 1991).

1.7 Non-glycinergic action of convulsant alkaloid strychnine on nerve preparations

The previous sections have reviewed the use of strychnine for investigation of the processes underlying glycinergic transmission particularly in spinal cord and brainstem pathways. Barron and Guths (1987) have offered a more critical appraisal of the use of strychnine as a probe for the inhibitory glycinergic system. They describe a plethora of non-glycinergic actions of strychnine that have been reported. However, it has been argued that these effects do not compromise the use of the alkaloid to probe the inhibitory glycine receptor (Becker and Betz, 1987). This is based on the fact that much higher concentrations then those used to antagonise the inhibitory actions of glycine are required to manifest the non-glycinergic actions of strychnine. In addition, the interaction with the inhibitory receptor has a distinct pharmacological specificity (Betz and Becker, 1987). However, it should be noted in this context that recent identification of a relative strychnine insensitive receptor isoform diminishes the value of using concentration dependence as the sole criteria for specificity.

It is less clear whether or not the non-glycinergic actions of strychnine contribute to the convulsant properties of the alkaloid. This point has been debated (Faber and Klee, 1974) with reference to high level centres in brain, such as cerebral cortex. These form foci for strychnine seizures despite indications that glycine is not a transmitter (Krnjèvic and Kelly, 1969) and that strychnine enhances excitatory activity without reducing inhibition (Krnjèvic *et al*, 1966). Broadly speaking this can be attributed to effect on nerve membrane excitability or synaptic transmission, and non-glycinergic actions of strychnine that might underlie such phenomenon are considered below.

The ability of amino acids to depress neuronal activity was discriminated into the 'glycine-like' or 'GABA-like' on basis of whether they were strychnine sensitive or insensitive (Curtis *et al*, 1968a). However, this criteria was disputed when it was shown that depression of spinal interneurons by glycine and GABA was blocked by strychnine (Davidoff *et al*, 1969). It has been confirmed that strychnine can antagonise GABA evoked neuronal depression (Curtis *et al*, 1969) and the associated increase in chloride conductance (Yakushiji *et al*, 1987; Kandeda *et al*, 1989). This action of strychnine is competitive (Yakushiji *et al*, 1987) and although fairly potent would appear to require higher concentrations than those that characteristically antagonize glycine evoked neuronal

depression (Curtis *et al*, 1969, 1971b).

Strychnine has also been shown to inhibit synaptic responses and those evoked by ionophoretic application of various other transmitter substances. In cortex it was shown that strychnine, at relatively high concentrations, abolished or reduced the depressant effects of 5-hydroxytryptamine (5HT), acetylcholine (ACh) and noradrenaline (NA) on spontaneous or glutamate-evoked firing (Phillis and York 1967; Jordan and Phillis, 1972). This was contrasted in spinal cord where strychnine had no effect on the depression of spontaneous or DL homocysteic acid induced firing induced by NA (Biscoe and Curtis, 1966). Based on the disparate nature of the compounds whose actions were blocked it was postulated that strychnine was exerting its effects on membrane permeability changes that these transmitters induced (Phillis and York, 1967). An inhibitory action of strychnine on transmitter activated responses in *Aplysia* has been reported (Klee and Faber, 1971) and dose-response relation tentatively considered (Faber and Klee, 1974). It was argued that ACh and dopamine depolarizing responses were competitively inhibited by strychnine at membrane receptors for the applied drugs. In contrast, the ACh hyperpolarising response and 5HT depolarising response were non-competitively inhibited by strychnine compatible with an effect on mechanism underlying the changes in membrane permeability.

The idea that strychnine affects mechanisms underlying the changes in membrane permeability activated by transmitters would be compatible with the description that strychnine blocks the NMDA subclass of glutamate activated cation channels (Bertolino and Vincini, 1988). The concentration of alkaloid required are at least 100 times those that effect an antagonism at the inhibitory glycine receptor. This action is unrelated to the glycine site on this receptor, rather, the single channel analysis of the effect indicate strychnine is interacting through an open channel block.

Thus, non-glycinergic actions of strychnine might arise by a competition possibly with one of several endogenous transmitters at their receptor sites (see Barron and Guth, 1987). Alternatively, there is evidence that suggests it might block transmitter action by preventing the induced changes in membrane permeability by interacting at associated ion channels. The latter possibility may underlie the ability of strychnine, at relatively high concentrations, to alter excitability in a way that is independent of synaptic inputs (Washizu *et al*, 1961; Klee *et al.*, 1973). Such effects lead to changes in action potential

duration (Maruhashi *et al*, 1956; Washizu *et al*, 1961) and are associated with underlying membrane currents (Klee *et al*., 1973; Shapiro *et al*, 1974). At even higher concentrations this can lead to a complete conduction block by strychnine (Klee *et al*. 1973; Shapiro *et al*, 1974). In both vertebrate and invertebrate preparations, there is a reduction in both sodium and potassium currents, although other properties of ion channel function are modulated (Klee and Faber, 1973; Shapiro *et al*, 1974). A more detailed analysis of the effects of strychnine and its membrane impermeant derivative, N-methylstrychnine on frog and squid axons revealed possible mechanisms that underlie these actions (Shapiro, 1977a; 1977b; Cahalan and Almers, 1979). These investigations concluded that both sodium and potassium channels were blocked by strychnine entering an open channel from the cytoplasmic side of the membrane. Open channel block was consistent with frequency dependence of strychnine block of sodium channels (Cahalan, 1978). In addition, based on differences in potassium and sodium tail currents it was postulated that blocked channels could not close or inactivate unless they were first unblocked (Shapiro, 1977a; 1977b; Cahalan and Almers, 1979). The structural similarity between strychnine and quaternary ammonium ions was noted and this paralleled similarities in block of potassium channels by these compounds (Shapiro, 1977a). In the case of sodium channels, the structural similarities between strychnine and other cationic amphipathic drugs that blocked these channels in a similar way, was also noted (Shapiro, 1977b). A further aspect of the interaction of N-methylstrychnine and sodium channels was the almost complete immobilization of gating charge which was more pronounced than that associated with normal channel inactivation (Cahalan and Almers, 1979). This feature, is common to other polycyclic cationic amphipathic drugs, indicates that the drug molecule entraps the protein in a state that hinders subsequent conformational change (Cahalan and Almers, 1979). Strychnine has been shown to block subclasses of potassium channels that are activated by voltage or increases in internal calcium concentration (Cook, 1988).

Recent experiments designed to investigate the strychnine antagonism of GABA induced chloride conductances in disassociated rat dorsal ganglion described a novel action of the alkaloid on neuronal membrane conductance (Aibara *et al*, 1991). At concentrations supramaximal for antagonism of GABA-evoked chloride currents, strychnine induced an outward current. This was carried by potassium ions and was independent of voltage or external Ca^{++}. Also it was blocked by potassium channel blockers in order of potency Ba^{2+}>4-aminopyridine>tetraethylammonium. At the higher

concentrations of strychnine tested the peak conductance was suppressed, but was followed upon washout by a transient hump current. This result is consistent with strychnine activating a K^+ conductance which is subsequently blocked by the alkaloid, the transient hump associated with unblocking during washout.

In conclusion, the structural determinants of the strychnine molecule allow it to interact with a range of structures that mediate changes in membrane excitability. This extends the range of sites through which this drug may exert its convulsant actions beyond the inhibitory glycine receptor. However, the selectivity that strychnine exhibits for the glycine receptor indicates it is the primary site for the action of the alkaloid. This concept is only reinforced by considering the pivotal role that strychnine has played in experiments designed to elucidate the organization and structure of the inhibitory glycine receptor.

CHAPTER TWO

MATERIALS AND METHODS

2.1.a Equipment

Autoradiography cassette: Protex (Everything X-ray Ltd.)

Cell Harvester: 24 well (Brandel)

Centrifuges: High Spin 21 (M.S.E.)

Labspin (Gallenkamp)

Microfuge 12 (Beckman-RIIC Ltd.)

Ultracentrifuge-L8M (Beckman-RIIC Ltd.)

Chart recorder: 2210 (L.K.B. Instruments Ltd.)

Chromatography columns: (Pharmacia Ltd.)

Computers: Model TM 7003 (Tandon Corporation)

Tektronix 4052 (Tektronix)

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

Mini-Protean II Cell (Bio-Rad Laboratories)

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

Filtration manifold: 12 well (Millipore UK Ltd.)

Fraction collectors: 2211 SuperRac (L.K.B. Instruments Ltd.)

2112 RediRac (L.K.B. Instruments Ltd.)

Gel Slab Drier: 483 (Bio-Rad Laboratories)

Liquid Scintillation Spectrometer: Tri-carb model-D 3375 (Packard)

Peristaltic pumps: 2132 Microperpex (L.K.B. Instruments Ltd.)

P1 (Pharmacia Ltd.)

pH meter: Mode 109 (Corning-EEL)

Polytron tissue homogeniser (probe PTA 10S): Kinematica GmbH (Northern supply Ltd.)

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

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

650-10S (Perkin Elmer)

Torsion balance (Gallenkamp)

UV flow cell recorder: 2238 Uvicord SII (L.K.B. Instuments Ltd.)

Ultraviolet Lamp: UVGL-58 (CamLab Ltd.)

2.1.b Consumables

Dialysis tubing (Pharmacia Ltd.)

Glass fibre filters (Whatman GF/B): cut into sheets (30 x 5 cm)
discs (25 mm diameter)

Plastic culture plates (Falcon): 24 Multiwell Plate

Plastic petri dishes (Falcon): 3.5 cm diameter

Polypropylene eppendorfs (Sarstedt): 0.5, 1.5, and 2.2 ml

Polypropylene centrifuge tubes (Sarstedt): 5 ml

Polystyrene conical tubes (Falcon): 50 ml

Scintillation insert vials (Sterilin Ltd.)

Universal containers (Gibco Ltd.)

2.2.a General chemicals and reagents

Acetic acid (BDH Chemicals Ltd.); acrylamide (Bio-Rad Laboratories or BDH Electran); Affi-gel 10 (Bio-Rad Laboratories); albumin (bovine serum, fraction V; Sigma Chemical Co. Ltd.), ammonium sulphate (enzyme grade; Fisons plc); ammonium persulphate (Bio-Rad Laboratories); aprotoninin (Sigma Chemical Co. Ltd.); bacitracin (Sigma Chemical Co. Ltd.); blue dextran 2000 (Pharmacia Ltd.); bovine-γ-globulin (labile enzyme free, fraction II; Miles Laboratories Ltd.); bromophenol blue (BDH Chemicals Ltd.); 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulphonate (CHAPS; Sigma Chemical Co. Ltd.); Coomassie Brilliant Blue (Sigma Chemical Co. Ltd.); dimethyldichlorosilane (2% in 1,1,1-trichloroethane; BDH Chemicals Ltd.); dimethylformamide (BDH Chemicals Ltd.); Enhance (New England Nuclear); ethanol (absolute alcohol A.R.; James Burrough (F.A.D.) Ltd.); ethylenediaminetetracetic acid (disodium salt, EDTA; BDH Chemicals Ltd.); N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES; Sigma Chemical Co. Ltd.); iodomethane (Sigma Chemical Co. Ltd.); ß-lactoglobulin (Sigma Chemical Co. Ltd.); merthiolate (thimerosal, sodium ethylmercurithiosalicylate; Sigma Chemical Co. Ltd.); methanol (BDH Chemicals Ltd.); methyl green (BDH Chemicals Ltd.); molecular weight standards (Dalton Mark VII-L; Sigma Chemical Co. Ltd.); pepsin (Sigma Chemical Co. Ltd.); octylphenol ethylene oxide condensate (Nonidet P-40; BDH Chemicals Ltd.); phenylmethane sulphonyl fluoride (PMSF; Sigma Chemical Co. Ltd.); L-α-phosphatidylcholine (Sigma Chemical Co. Ltd.); Polyethylene glycol 1500 (PEG; BDH Chemicals Ltd.); polyethylenimine (Sigma Chemical Co. Ltd.); scintillation cocktails (Optiphase X; LKB; Ecoscint H; National Diagnostics); Sephadex G25M (Pharmacia Ltd.); Sephadex G50F (Pharmacia Ltd.); sodium dodecyl (lauryl) sulphate (BDH Electran); soybean trypsin inhibitor (Sigma

Chemical Co. Ltd.); Staphylococcus aureus cells (Gibco BRL or Sigma Chemical Co. Ltd.); sucrose (BDH Chemicals Ltd.); N,N,N',N'-tetramethylethylene diamine (TEMED; Bio-Rad Laboratories); Tris-(hydroxymethyl)-methylamine (Aristar; BDH Chemicals Ltd.); trypsinogen (Sigma Chemical Co. Ltd.); urea (BDH Chemicals Ltd.); vitamin B_{12} (BDH Chemicals Ltd.).

All other chemicals , were A.R. grade and purchased from BDH Chemicals Ltd or Sigma Chemical Co. Ltd.

2.2.b Protein Modifying Reagents

N-acetylimidazole (Sigma Chemical Co. Ltd.); 5-aminotetra zole (Sigma Chemical Co. Ltd.); n-bromosuccinimide (NBS; Aldrich Chemical Co. Ltd.); 2,3 butanedione (BD; diacetyl; Aldrich Chemical Co. Ltd.); camphorquinone-10-sulphate (CQS; Fluka); n-chloroacetyltyrosine (Aldrich Chemical Co. Ltd.); 1,2-cyclohexanedione (CHD; Sigma Chemical Co. Ltd.); diethylpyrocarbonate (DEP; BDH Chemicals Ltd.); dithiothreitol (DTT; Sigma Chemical Co. Ltd.); p-hydroxyacetophenone (Aldrich Chemical Co. Ltd.); hydroxylamine monohydrochloride (Sigma Chemical Co. Ltd.); Iodoacetamide (IAA; Sigma Chemical Co. Ltd.); phenylglyoxal monohydrate (PG; Aldrich Chemical Co. Ltd.); pyridoxal phosphate (PLP; Sigma Chemical Co. Ltd.); selenium dioxide (Sigma Chemical Co. Ltd.); sodium borohydride (BDH Chemicals Ltd.); tetranitromethane (TNM; BDH Chemicals Ltd.); trinitrobenzosulphonic acid (TNBS; picrylsulphonic acid; Aldrich Chemical Co. Ltd.).

2.2.b.1 Synthesis of diazonium tetrazole (Riordan and Vallee, 1974)

Sodium nitrite (1M, 10 ml) was slowly added with constant stirring to 5-aminotetra zole (0.42 M; 23 ml) dissolved in hydrochloric acid (1.6 M), the reaction was kept on an ice bath. After 6-8 mins, the pH of the incubation was adjusted to 5 with NaOH (3M), as the product, diazonium tetra zole, is unstable at higher pH. The final volume of the incubation was noted and based on a reaction yield of 75%, the product (0.09 moles) was diluted in chilled water to give a stock of diazonium tetra zole (estimated to be 62.5 mM). Controls in which the above protocol was followed without addition of 5-aminotetra zole were also processed. The stock should be stable for at least 1 hr on ice, and was used immediately for protein modification. Simultaneously, quantification of the stock was performed by reacting it with the model phenol n-

chloroacetyltyrosine. The stock diazonium tetratzole or control reaction (0.5 ml) were added to n-chloroacetyltyrosine (0.4 M; 0.5 ml) and incubated for 1 hour at 0-4°C. The reactant products were diluted into sodium hydroxide (0.1 M) and the absorbance at 480 nm was measured. Using the Beer-Lambert Law the concentration of diazonium tetratzole was estimated on the basis that the monazo derivative has a molar absorb.tivity of 8400.

2.2.b.2 p-Hydroxyphenylglyoxal (Fodor and Kovács, 1949)

Selenium dioxide (0.2 moles) was dissolved in dioxane* (120 ml) by heating at 60° C before addition of water (4ml) and p-hydroxyacetophenone (0.2 mole). The reaction was boiled (4hrs) and subjected to a double reflux to prevent the loss of water. The selenium was filtered and the yellow mother liquor was evaporated under vacuum. The residue remaining was boiled with 150 ml water under reflux conditions with constant stirring. Charcoal was added to the solution to decolorize it, before being removed by filtration. Following concentration to a small volume the product was crystallized out and finally recrystallized from the fourth amount of water. The melting point of the greenish white prisms was about 111°C which is coincidental with published value.

2.2.c Pharmacological agents

Unless otherwise stated compounds were freshly dissolved in phosphate buffered saline. Superscripts indicate other solvents used; distilled water (^), methanol (*), dimethylformamide (#) and sodium hydroxide (~).

*acridine (Sigma Chemical Co. Ltd.); ß-alanine (BDH Chemicals Ltd.); γ-amino-n-butyric acid (Sigma Chemical Co. Ltd.); ^9-aminoacridine (gift, D. Haylett, Dept. of Pharmacology, U.C.L.); ^4-aminopyridine (gift, D.Attwell, Dept. of Physiology, U.C.L.); ^#2-aminostrychnine (synthesized by Professor G.A.R. Johnston and Ms. H. Tran, University of New South Wales, Sydney); *amiodarone (gift, Sanofi UK. Ltd.); *anthracene (gift, teaching laboratory, Dept. of Chemistry, U.C.L.); ^apamin (gift, D. Haylett); *benzocaine (gift, Prof. D. Colquohn, Dept. of Pharmacology, U.C.L.); ^boldine, ^bromodesoxyisostrychnine (gifts, G.A.R. Johnston); *brucine (Koch Light Laboratories Ltd.); *brucine-N-oxide (Aldrich Chemical Co. Ltd.); ~cacotheline (Aldrich Chemical Co. Ltd.); *capsaicin (E.Merck Ltd.); *carbazole (Aldrich Chemical Co. Ltd.); #chlorothiazide (Sigma Chemical Co. Ltd.); *D-600 (methoxy-verapamil; Sigma Chemical Co. Ltd.);

^decamethonium (Allan & Hanburys Ltd.); ^dendrotoxin (gift, Professor D. Brown, Dept. of Phamacology, U.C.L.); #diaboline (gift, Prof. D Curtis, Department of Pharmacology, The Austrailian National University, Canberra.); *diazoxide (Sigma Chemical Co. Ltd.); *5,5-diphenylhydantoin (Sigma Chemical Co. Ltd.); ^disopyramide (Sigma Chemical Co. Ltd.); ^encainide (gift, Bristol-Myers Co.); ^flecainide (gift, Riker 3M); *gallamine (May & Baker); *gelsemine (gift, G.A.R. Johnston); ~genestrychninic acid (N-oxystrychninic acid; Aldrich Chemical Co. Ltd.); #giblencamide (gift D. Haylett); glycine (Sigma Chemical Co. Ltd.); ^hydrastine (gift, G.A.R. Johnston); *8-hydroxyquinoline (BDH Chemicals Ltd.); ^imipramine (Sigma Chemical Co. Ltd.); ^iso-THAZ (5,6,7,8-tetrahydro-4H-isooxazolo[3,4-d]azepin-3-ol; gift, Dr. P Krogsgaard-Larsen, Dept. of Chemistry, The Royal Danish School of Pharmacy, Copenhagen); ~isostrychnic acid (Aldrich Chemical Co. Ltd.); *laudanosine (gift, G.A.R. Johnston); *lemakalim (gift M. Collier, Dept. of Respiratory Medicine, St. Thomas's Hospitol); ^lidocaine (Sigma Chemical Co. Ltd.); *mephenesin (Sigma Chemical Co. Ltd.); N-methylstrychnine (synthesized see below); ^mexiletin (gift, Boehringer Ingelheim KG); *minoxidil (Sigma Chemical Co. Ltd.); ^MK-801 (gift, Dr. A.H. Dickerson, Dept. of Pharmacology, U.C.L.); *naphthalene (gift, teaching laboratory, Dept. of Chemistry, U.C.L.); *nifedipine (gift, D.Eisner, Dept of Physiology, U.C.L.); # 2-nitrostrychnine (Aldrich Chemical Co. Ltd.); ^pancuronium (gift, T. Biscoe, Dept. of Physiology, U.C.L.); *picrotoxin (Koch-Light Labs); ^procainamide (Sigma Chemical Co. Ltd.); ^procaine (Sigma Chemical Co. Ltd.); #propafenone (gift, Knoll); ^propranolol (Sigma Chemical Co. Ltd.); ^quinidine (gift, D. Haylett); ^quinine (gift, D. Haylett); *quinoline (BDH Chemicals Ltd.); ^QX-222 (gift, Prof. D. Colquohn); ^QX-314 (gift, Prof. D. Colquohn); *R5135 (gift, Roussel-Uclaf); #RP-52891 (gift Rhône-Poulenc); ^(see below) scorpion toxin (<u>leirius quinquestriatus hebraeus</u>; Sigma Chemical Co. Ltd.); *skatol (BDH Chemicals Ltd.); ^sparteine (gift D.Haylett); ^strychnine (hemisulphate and free base; Sigma Chemical Co. Ltd.); taurine (BDH Chemicals Ltd.); ^tetracaine (Sigma Chemical Co. Ltd.); ^tetraethylammonium (BDH Chemicals Ltd.); tetrodotoxin (gift, M. Duchen, Dept. of Physiology, U.C.L.); *thebaine (Aldrich); ^tocainide (gift, Astra); tolbutamide (gift D.Haylett); *1,3-o-tolyl-guanidine (D. Haylett); *tryptamine (BDH Chemicals Ltd.); ^tubocurarine (Burroughs Wellcome & Co.); ^veratridine (Sigma Chemical Co. Ltd.); ^vincamine (Aldrich Chemical Co. Ltd.); #vomicine (Aldrich Chemical Co. Ltd.); #Weiland-Gumlich aldehyde (gift, D.R. Curtis).

2.2.c.1 Synthesis of n-Methyl-strychnine (Shapiro, 1977a)

Strychnine (0.0149 moles) as a free base was reacted in iodomethane (0.4 moles, 25 ml) at room temperature (2 hrs). The n-methylstrychnine forms as a precipitate which was filtered and washed copiously with cold methanol (500 ml). The washed precipitate was dried in a dessicator and synthesis of the product verified by the elevation of the melting point (>300°C) over the value for free base strychnine (268°C).

2.2.c.2 Preparation of scorpion Venom

Distilled water (1.25 ml) was added to the sample of scorpion venom (5mg) to extract the toxin (1 hr, 4°C). The mucus that forms from the precipitation of glycoproteins was pelleted by centrifugation (12 000 g; 10 mins; 4°C). The supernatant was carefully removed and the pellet re-extracted with water (1.25 ml). After centrifugation the supernatant was removed, added to the first, to give a stock (2 mg/ml) which was stored (-70°C) in small aliquot (10 × 0.5 ml).

2.2.d Radiochemicals

n-Hexadecane [^{3}H], 2.07 µCi/mg (Amersham International plc); methylated protein mixture [^{14}C], 0.833 µCi/ml (Amersham International plc); strychnine [2,4,11-^{3}H], 23- 28 Ci/mmol (Amersham International plc) or strychnine (benzenering-^{3}H), 22.7-24.5 Ci/mmol (New England Nuclear).

2.2.e General buffers

Double distilled water was used for all aqueous solutions: pH was adjusted using pH meter which was calibrated before use by standard phosphate buffers of known pH at an appropriate temperature.

Standard A (pH 6.951 at 5°C); solution of potassium dihydrogen orthophosphate (0.025 M) and disodium hydrogen orthophosphate (0.025M).

Standard B (pH 7.497 at 5°C); solution of potassium dihydrogen orthophosphate

(0.013 M) and disodium hydrogen orthophosphate (0.053 M).

Phosphate buffered saline (PBS): a solution of sodium chloride (0.1 M) and disodium hydrogen orthophosphate (0.041 M) adjusted to pH 6, 7.4 or 8 with sodium dihydrogen orthophosphate (0.2 M).

PBS containing 0.1% (w/v) bovine serum albumin (PBS/BSA).

PBS/BSA containing 0.01% (w/v) merthiolate (PBS/BSA/M).

Phosphate buffered borate (PBB): a solution containing disodium tetraborate (0.025M) and disodium hydrogen orthophosphate (0.1M) adjusted to the required pH with sodium dihydrogen orthophosphate (0.5 M).

Tris-citrate: Tris-(hydroxymethyl)-methylamine (50mM or 100mM) adjusted to required pH, at the temperature it was used experimentally, with saturated citric acid.

HEPES-Sodium (HEPES/Na^+): N-2 Hydroxyethylpiperazine-N-ethanesulphonic acid (10mM) adjusted to pH 7.4 with sodium hydroxide (0.1M).

2.3 Preparation of 2-Aminostrychnine-agarose affinity column

The column was prepared essentially as described by Pfeiffer *et al.* (1982). Affigel 10 (40 ml) was gently spun (2 000 rpm; 1 min; Gallenkamp labspin) and the supernatant removed. The affigel was washed 3 times in an equal volume of dimethylformamide (DMF) before the addition of DMF (20 ml) containing 2-aminostrychnine (500 mg). The reactants were gently mixed by rotation. The test tube was wrapped in silver foil to ensure the exclusion of light. After 48 hours ethanolamine was added (final concentration of 10 mM) and the reactants incubated for a further 2 hours before centrifugation, as described. The supernatant was discarded and the 2-aminostrychnine-agarose was washed once in DMF (20 ml) followed by 3 washes in PBS/M (20 ml).

The 2-aminostrychnine-agarose was poured into a column placed in the cold room, and washed by pumping through PBS/M (20 column vols; 20 ml/hr). As before, silver foil

was used to protect the column from light.

An aliquot of the 2-aminostrychnine-agarose (100 µl bead volume) was incubated (overnight) with strychnine binding antisera (0.5ml; 1:1265; overnight) in PBS/BSA/M. The incubation was centrifuged and the level of [^{3}H]-strychnine binding remaining in the supernatant was estimated by radioimmunoassay (see section 2.10.c). The ability of the 2-aminostrychnine-agarose to remove the strychnine binding capacity of the antisera was used as criterion to show that the coupling reaction had been achieved.

Prior to use the column was sequentially washed in both directions by 5 volumes of urea (3M), KCl (1M), and PBS (containing 0.5% triton-X-100).

2.4 Silanization of test tubes

Glass test tubes and polypropylene eppendorfs were rinsed in dimethylchloroethane solution (approximately 2% in 1,1,1,-trichlorosilane in chloroform). The chloroform was evaporated off in a fumecupboard before thoroughly washing the tubes with tap water followed by distilled water. All tubes were oven dried before use.

2.5 Animals

Animals were bred in the joint animal house, University College London. Normal mice were of the C57Bl/6J strain. Spastic mutant (spa/spa) and unaffected littermate mice (spa/+ or +/+) were obtained by mating known heterozygote (spa/+) from a C57Bl/6J colony carrying the spastic gene. Male mice at 16 weeks or older were used in all experiments. Where appropriate normal, spastic and littermates were age matched.

Male or female Wistar rats older than 20 weeks were used for experiments that required larger individual tissue weights.

2.6 Dissection of animals

2.6.a Dissection to remove spinal cord tissue

Mice and rats were stunned before decapitation and the heads discarded unless

brain tissue was required. Laminectomy was performed and the spinal cord removed. Four mice spinal cords were placed in centrifuge tubes (MSE, 50 ml) on dry ice before storage (-70°C). Age matched normal, littermate and spastic mice and rat spinal cords were individually placed in foil and snap frozen in liquid nitrogen and stored at (-70°C).

2.6.b Fine dissection into brain regions

Brain tissue was obtained by opening the skull with a v-shaped cut and scooping out the brain with the olfactory lobes intact on to a petri dish held on ice. The medulla and pons (with cerebellum in situ) were isolated by a v-shaped coronal cut between the rostral boundaries of the anterior colliculus and the pons. The cerebellum was removed from the brainstem by opening the 4th ventricle with fine iridectomy scissors and severing the two white connecting tracts between the brainstem and the cerebellum. The olfactory lobes were trimmed and a coronal cut from the ventral surface down was made at the level of the optic chiasma, which divided the brain into the anterior and posterior portions.

From the anterior section, the rostral striatum was scooped out, then the septal area plus olfactory tubercles were removed. The remaining tissue constituted the cerebral cortex.

The cerebral cortex was removed from the posterior portion by opening the lateral ventricle and the caudal striatum was cut away, leaving the thalamus, hypothalamus, and amygdala, which were separated from each other by cuts with a sharp knife. The dissection of brain was into 7 regions: medulla and pons, cerebellum, thalamus, striatum, cortex, hippocampus, and remainder; which contained hypothalamus, amygdala, and the olfactory tubercles. Each region together with the spinal cord from the same rat were wrapped individually in silver foil, placed on dry ice prior to storage at -70°C.

2.6.c Dissection of non-neural tissue

Mice were dissected to supply non neural tissue. After laminectomy and removal of the spinal cord the gastroenemius muscle of the right hind limb was removed using fine dissecting scissors to ensure separation from the underlying muscle. The mouse was turned over and the abdominal and thoracic cavity were opened. Lungs, heart, spleen, one kidney, a cm^2 of liver lobe and 10 cm of ileum were removed, wrapped individually in

foil, placed on dry ice before storage at -70°C. Ileum was washed through with 3x5 ml PBS.

2.7 Preparation of crude membranes

All tissue types whether pooled or processed individually were subjected to the same protocol. Tissue weights were recorded and placed in polypropylene centrifuge tubes. Samples were homogenized in 20 volumes (tissue weight/volume) of ice cold Tris-citrate (50mM) with a polytron (speed 10; 10 secs). The polytron probe was washed with 4 ml Tris-citrate between samples and the wash buffer added to the homogenized tissue. This was then centrifuged at 27 000 x g for 30 mins at 4°C. The supernatant was discarded and the pellet was resuspended in 20 vols Tris-citrate by sonication (amplitude 25 microns; 10 secs) at 4°C, followed by centrifugation as before. This was repeated twice before the pellet was finally resuspended in 20 vols of Tris-citrate buffer. The crude membranes were stored at -70°C.

2.8 Affinity purification of glycine receptor on a 2-aminostrychnine-agarose column

2.8.a Solubilization and affinity purification

This was essentially as described by Pfeiffer *et al.* (1982). Mouse spinal cord membranes used as a source of glycine receptor were prepared as described (see section 2.7), except that the homogenization buffer contained: bacitracin (25 µg/ml), soybean trypsin inhibitor (SBTI; 25 µg/ml), phenylmethanesulphonylfluoride (PMSF; 17.5 µg/ml), ethylenediaminetetracetic acid (EDTA; 1mM), dithiothreitol (DTT, 5mM), and iodoacetamide (IAA, 2.5mM) as a cocktail to limit proteolysis. Membranes were centrifuged (27 000 g; 30 mins; 4°C) and solubilized by resuspending in PBS (20 vols, tissue weight/vol) containing 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulphonate (CHAPS; 1.5% w/v), phosphatidylcholine (PTC; 1.5 mg/ml) and the protease inhibitor cocktail described above. The membranes were incubated and continually agitated (20-21°C; 15 mins) before being cooled and incubated (4°C; 15 mins). Equal volumes of the solubilized membranes were aliquoted into polycarbonate centrifuge tubes and subjected to ultracentrifugation (100 000 g; 1 hr; 4°C). The supernatants were diluted with an equal volume of PBS (containing PTC; 1.5 mg/ml) and

the protease inhibitor cocktail. This reduced the concentration of CHAPS (1.5% to 0.75% w/v) before loading onto the column. When required the membrane pellet was resuspended in PBS (20 vols) containing the protease inhibitor cocktail.

The solubilized fraction was loaded onto the 2-aminostrychnine-agarose column that had previously pre-equilibrated with PBS (10 column vols; 10 ml/hr) containing CHAPS (0.75% w/v) PTC (1.5 mg/ml) and protease cocktail inhibitor. The pre-equilibration buffer (10 column vols; 10 ml/hr) was also used to wash the loaded column before eluting glycine receptors from the column using wash buffer (2 column vols, 2.5 ml/hr) containing glycine (200 mM).

4 ml fractions were collected from the column during the pre-equilibration, load and washing stages and 1.5 ml fractions during the elution. Aliquots designated as; membrane homogenate, membrane homogenate with added CHAPS, solubilized fraction prior to loading and resuspended pellet were taken at each stage of the purification and stored at 4°C prior to assay of [^{3}H]-strychnine binding.

2.8.b Wash of column fractions

A protein precipitation procedure was used to wash out glycine from the eluted fraction and for controls performed on other fractions, prior to [^{3}H]-strychnine binding and protein assay. Bovine-γ- globulin (200 µl, 10 mg/ml) and PEG (800 µl; 50% w/v) were added to aliquots (1 ml) of individual fractions. After vortexing and incubating on ice (10 mins) the precipitated protein was pelleted by centrifugation (10 000 g, 5 mins). The pellet was washed twice by resuspension and centrifugation using PEG (1ml, 20% w/v) before redissolving the precipitated proteins in CHAPS (0.75% w/v), PTC (1.5 mg/ml) and protease inhibitor cocktail. The eluted fractions that showed the highest [^{3}H]-strychnine binding activity (see section 2.10.b.) were pooled and used as a source of purified glycine receptor.

2.9 Chemical modification and ligand protection

2.9.a Treatment of crude membrane preparations with protein modifying reagents

Membranes were thawed and washed twice by centrifugation (27 000 g; 30 mins;

4°C) and resuspension in the buffers (40 vols) indicated in the text. Different buffers were used in an attempt to increase the specificity of the various protein modifying reagents. Fresh solutions of the reagents were made for each experiment, dissolved in reaction buffer or ethanol and then added at 1.7 vols to the membrane preparations to give the required reaction concentration. For each reagent, controls were performed in which membranes were exposed to the same buffer solution or ethanol, but without the reagent under investigation. Modifications were performed at 20-21°C with the exception of diazonium tetrazole (4°C). Incubations were subjected to constant mixing in opaque polypropylene tubes. The reactions were terminated after the required incubation time by the addition of PBS/BSA (160 vols tissue wt./vol). The membranes were then pelleted by centrifugation (27,000 g; 30 mins) and washed three times by resuspension and centrifugation in PBS (160 vols). The membranes were finally resuspended in PBS for measurement of [^{3}H]-strychnine binding. In the case of some reagents, 1,2 cyclohexandione (CHD) and 2,3 butanedione (BD), modification experiments were also performed in which the buffer used in the termination and washing of the membranes was PBB (phosphate buffered borate).

The ability of strychnine and glycine to protect against perturbation of [^{3}H]-strychnine binding was assessed by incubating membranes with strychnine (10^{-4}M) and glycine (10^{-2}M) prior to (15 mins; 20-21°C) and during the protein modification. These were compared against membranes that were treated with protein modifying reagents without either of the protecting ligands. In addition, unprotected, strychnine protected and glycine protected incubations were paralleled in membranes that were not subjected to treatment with protein modifying reagents.

2.9.b. Treatment of purified glycine receptors with the protein modifying reagent 2,3-butanedione

The eluted fractions from the affinity purification (see section 2.8.a) which showed specific [^{3}H]-strychnine binding were pooled and used as the source of purified glycine receptor. Glycine was removed by the protein precipitation procedure described (see section 2.8.b). The pooled fraction was divided and treated with BD (80 mM) or PBS to serve as control. After incubation (40 mins; 20-21°C) the reaction was terminated by the addition of PBS/BSA (4 incubation volumes) containing bovine-γ-globulin (0.125% w/v),

and PEG (3.6 incubation volumes at 50% w/v). The mixture was vortexed and subjected to the previously described protein precipitation (see section 2.8.b) incorporating four washes to ensure removal of unreacted reagent. The pellet was redissolved in PBS containing CHAPS (0.75% w/v) and PTC (1.5 mg/ml) and assayed for [^{3}H]-strychnine binding.

Purified glycine receptor preparations were incubated with strychnine (10^{-4}M), glycine (10^{-2}M) or no protecting ligand, prior to and during the treatment with the protein modifying reagent BD. The reactions were terminated and the preparations were washed as described above. Parallel incubations which were not treated with BD but subjected to ligand protection were included as controls and to show that protecting ligands were removed by the washing procedure.

2.9.c.1 Chemical modification of 2-AS-KLH antisera

Antisera dilutions (1:320) in PBS/BSA (200 µl) of the appropriate pH were treated with protein modifying reagents under conditions utilized for membranes. The reactions were terminated by the addition of chilled PBS/BSA (800 µl) containing bovine-γ-globulin (1.25 mg/ml). Immunoglobulins were then precipitated by addition of an equal volume ammonium sulphate (saturated) and pelleted by centrifugation (10,000g, 4 mins), as originally described by Farr (1958). The supernatant was aspirated off and the pellet washed by resuspension in ammonium sulphate (1 ml; 50% of saturated) followed by centrifugation and removal of the supernatant. This procedure was repeated three times before redissolving the antibodies in PBS/BSA/M for use in [^{3}H]-strychnine binding assays (see section 2.10.c).

2.9.c.2 Strychnine protection of 2-AS-KLH antisera during protein modification

Antisera dilutions (1:320) were incubated with strychnine (10^{-5}M) prior to (15 mins; 20-21°C) and during protein modification for comparison with antisera that were not strychnine protected. The concentration of strychnine used to protect was approximately 100 fold higher than the maximal binding capacity of this dilution of antisera. The protocol for chemical modification of antisera was followed (see section 2.9.c.1), however after washing, the redissolved antisera were dialysed (21°C, 36 hrs) against PBS/BSA/M (8 x 1.5 L), before a final dialysis against PBS/M (1 x 1L) containing

bovine-γ-globulin (0.2% w/v). Antisera that were strychnine protected but not subsequently treated with protein modifying reagents were included to illustrate that the unlabelled strychnine was completely removed prior to the [^{3}H]-strychnine binding assay.

2.10 [^{3}H]-strychnine binding assays

2.10.a [^{3}H]-strychnine binding to crude membrane homogenate

Triplicate samples of untreated or chemically modified membranes (50-100 µg protein/ml) were incubated (4°C; >2 hrs) with [^{3}H]-strychnine (6 nM). Incubations were performed in PBS unless otherwise stated. Parallel incubations performed in the presence of either unlabelled strychnine (10^{-4}M) or glycine (10^{-2}M) were used to define the level of non-specific binding. Assay blanks were measured by including incubations in which the membrane was replaced by assay buffer.

When [^{3}H]-strychnine binding to untreated and chemically modified membranes of the mutant mouse spastic or membranes other than spinal cord of normal mouse, was measured, more membrane protein was added to the assay (250 µg protein/ml). Previous experiments had shown this to be the upper limit for spinal cord membrane in filtration binding assays (Phelan, 1987).

2.10.a.1 Inhibition of [^{3}H]-strychnine binding by unlabelled drugs

Incubations were set up as described (see section 2.10.a.) but in the presence of increasing concentrations of unlabelled strychnine (10^{-10}-10^{-2}M), glycine (10^{-9}-10^{-2}M) or other unlabelled drugs of interest (10^{-9}M-10^{-3}M), by addition from stocks which were tenfold higher than the desired final concentration. These were made by making the appropriate serial dilutions of a primary stock into volumes of assay buffer, PBS or Tris/Citrate. Control binding in these experiments was defined by incubation in the presence of an appropriate dilution of the solvents used to make the primary stocks.

2.10.a.2 Saturation of [^{3}H]-strychnine binding to spinal cord membranes

Untreated or chemically modified mouse spinal membranes were equilibrated with increasing concentrations of [^{3}H]-strychnine using the conditions described previously (see

section 2.10.a). The stocks of [^{3}H]-strychnine were made up by two different methods depending on the concentration range required for the saturation experiment. Direct dilution of the manufacturer stock into assay buffer and subsequent serial dilutions to the required concentration, were made for assays that used 40 nM as the highest concentration of [^{3}H]-strychnine. When the experiment required μM concentrations isotopic dilutions were made: where a stock [^{3}H]-strychnine was mixed with an equal volume of decreasing concentrations of unlabelled strychnine; both were made up in assay buffer.

2.10.b [^{3}H]-strychnine binding to affinity purified glycine receptors

Aliquots of untreated and protein modified purified glycine receptor (50 ul; see section 2.8) were incubated in PBS (final vol 500 μl), containing CHAPS (0.75% w/v) and PTC (1.5 mg/ml), with [^{3}H]-strychnine (6 nM). The assay blanks and the ability of glycine and strychnine to inhibit total binding were defined as in the membranes (see section 2.10.a). After equilibration (4°C; >2 hrs) bovine-γ-globulin (100 μl; 10mg/ml) and PEG (400 μl; 50% w/v)were added, the tubes vortexed and left on ice (15 mins) before filtration (see section 2.11.a.2).

2.10.c [^{3}H]-strychnine binding to 2-AS-KLH antisera

Modified or untreated antibody preparations (100 μl; see section 2.9.c.1) were incubated in PBS/BSA/M (final volume 250 μl) containing bovine-γ-globulin (1 mg/ml), with [^{3}H]-strychnine (5 nM). Assay blanks were estimated by incubations in which the antibody addition was replaced by buffer. After incubation (overnight; 4°C), antibodies were precipitated by the addition of ammonium sulphate (saturated; 250 μl) and pelleted by centrifugation (12 000 g; 4 mins). The supernatant was removed and the pellet was washed once by resuspension and centrifugation in ammonium sulphate (50% of saturated; 250 μl). The final pellet was redissolved in PBS (250 μl) and an aliquot (50 μl) was removed for the measurement of bound radioactivity by liquid scintillation counting.

2.11 Termination of ligand binding assays (separation of free and bound [^{3}H]-strychnine)

2.11.a Filtration

Filtration under vacuum was performed on a 24 well Brandel harvester or a 12 well manifold by passing samples through glass fibre sheets (30 x 5 cm) or individual discs respectively. Unless otherwise stated the glass fibre filters were presoaked (1 hr) in polyethylenimine (PEI; 0.1% v/v) prior to filtration.

2.11.a.1 Membrane receptor binding assay

After prewashing the filters in ice chilled PBS, the samples were diluted with chilled PBS (3ml) and filtered. The assay tubes were rinsed with chilled PBS (4 ml) and the contents filtered. This washing procedure was repeated and meant that membranes were in contact with ligand free buffer for approximately 15 secs. The radioactivity bound in each sample was measured by pinching out the filter area underlying individuals wells and placing them in insert vials in readiness for liquid scintillation counting.

2.11.a.2 Solubilized receptor binding

Polyethylene glycol (PEG) precipitated samples (see section 2.10.b) were diluted with ice chilled PBS (containing 10% PEG) and passed through glass fibre filters which had been prewashed in chilled PBS (5 ml; containing PEG, 10% w/v). The sample tube was then rinsed with chilled PBS (3 ml; containing PEG, 10% w/v) and the contents filtered before finally washing the filter by directly adding chilled PBS (5 ml; containing PEG, 10% v/v) to the manifold well. The filters were vacuum dried and the individual discs placed in liquid scintillation vials.

2.11.b Centrifugation assays

This was performed in the cold room. Membrane receptor assays were set up in silanized eppendorfs unless otherwise stated. The samples were spun in a microfuge (12

000 g, 10 mins) that had been placed in the cold room for at least one hour. The supernatant was aspirated off using a flame polished pasteur pipette, connected to a vacuum line, and the remaining pellet given a superficial rinse with chilled PBS (1.5 ml).

2.12 Photoaffinity labelling mouse spinal cord membranes

2.12.a Photolabelling spinal cord membranes with [^{3}H]-strychnine

Untreated or BD modified membranes (1mg protein/ml) prepared as previously described (see secion 2.9.a) were incubated with [^{3}H]-strychnine (40 nM or 100 nM) in PBS (2-4 hrs; 4°C). The ability of unlabelled drugs to prevent the irreversible incorporation of [^{3}H]-strychnine was examined by incubations which contained strychnine (10^{-3} or 10^{-4}M), glycine (10^{-2}M), 9 aminoacridine (10^{-3}M) or quinine (10^{-3}M). Blank incubations in which membrane was replaced by assay buffer were also included. The incubations were decanted into either plastic culture dishes (3.5 cm diameter), if 1 ml in volume; or into individual wells (1.5 cm diameter) of a 24 well plate, if 0.25 ml in volume. The incubations were illuminated (30 mins) by short wave light (nominal peak 254 nm, 300 $\mu W/cm^2$ at a distance 15.2 cm) using an ultraviolet lamp.

Non-irradiated samples were run in parallel as controls. The incubations were pipetted into test tubes and the reaction vessels rinsed with a reaction volume (1 ml or 0.25 ml) of unlabelled strychnine (2 mM), which was then added to the incubation. After this chase (1 hr; 4°C) with unlabelled strychnine, the samples were filtered or solubilized for analysis by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and fluorography (see section 2.13).

2.12.b Filtration of photolabelled membranes

The samples were aliquoted in triplicate into assay tubes to estimate the level of [^{3}H]-strychnine irreversibly bound into spinal cord membranes. Presoaked glass fibre filters (PEI, 1 hr) were used to filter samples as described (see section 2.11.a.1) except the filters were subsequently washed 3 times after sampling. The amount of [^{3}H]-strychnine retained on glass fibre filters for non irradiated samples was subtracted from the level of [^{3}H]-strychnine retained for irradiated samples to give the irreversibly bound ligand. If the membranes showed irreversible incorporation of [^{3}H]-strychnine they were

further analysed by SDS-PAGE.

2.12.c Solubilization of photolabelled membranes

The photolabelled membranes were pelleted by centrifugation(10 000 g; 5 mins), the supernatant removed and the pellet washed once by resuspension in Tris/HCl (50 mM, pH6.8). After a second centrifugation, the supernatant was again removed and the membranes were taken up in SDS (10% w/v) to a final protein concentration of approximately 1 µg/µl and left to solubilize overnight at 4°C.

2.13 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Peptides were electrophoresed by the method described by Laemmli (1970); the gradient gel system was taken from Hames (1983).

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

Reservoir buffer: Tris (0.025 M), Glycine (0.192 M) were mixed to give buffer (pH 8.3).

Upper reservoir buffer: Reservoir buffer containing SDS (0.1%).

Stacking gel buffer: Tris (0.5M) adjusted to pH 6.8 with hydrochloric acid (HCl; 1 M) and filtered.

Master mix: Tris/HCl (0.05 M; pH 6.8) containing SDS (3% w/v), ß-mercaptoethanol (7.5% w/v), EDTA (15 mM), glycerol (45%; w/v), bromophenol blue (0.015% w/v).

Acrylamide: Stock (30% w/v) containing acrylamide (29.2% w/v) and N,N'-methylene-bis-acrylamide (0.8% w/v). Filtered and stored in the dark.

Stacking gel (20 ml/slab gel or 5ml/mini slab gel): Final concentrations; acrylamide (3% w/v), Tris/HCl (0.125 M; pH 6.8) and freshly made stock (1.5% w/v) of ammonium persulphate in water was diluted into the mix (1:200).

10% resolving gel: (45 ml/slab gel or 5ml/mini slab gel): Final concentrations; acrylamide (10% w/v), Tris-HCl pH 8.8 (0.375 M), SDS (from 10% w/v stock in water diluted to 0.1% w/v), ammonium persulphate (0.075%).

5% resolving gel (30 ml/slab gels): Final concentrations; acrylamide (5% w/v), Tris/HCl (0.375 M), SDS (0.1% w/v), ammonium persulphate (0.035% w/v) and sucrose (0.15% w/v; this contributes 2.5 ml to the final volume).

The stacking and the resolving gels were degassed (15 mins) and the polymerization catalyst N,N,N',-N'- tetramethylethylenediamine (TEMED) added immediately prior to use. The final TEMED concentration was 0.1% in the stacking gel mix, 0.08% in the 10% resolving gel, and 0.03% in the 5% and 20 % resolving gels.

2.13.b Preparation of gels

2.13.b.1 10% uniform concentration resolving gel

Freshly prepared resolving gel was pipetted between an electrophoretic plate sandwich , overlayed with SDS (0.1%) and left to polymerize (overnight, 20-21°C).

2.13.b.2 5-20% linear gradient resolving gel

Equal volumes (22.5 ml) of 5% and 20% freshly prepared resolving gel were placed in the two chambers of a gradient former. The 5% gel was initially placed in the chamber nearest the outlet to the electrophoretic plate sandwich, this chamber was stirred constantly throughout the gradient formation. The hole between the two chambers of the gradient former was opened and the resulting acrylamide mixture pumped into the bottom of the gel sandwich at 3 ml/min using a peristaltic pump. The top of the gel sandwich which corresponds to the 5% end of the linear gradient was overlayed with SDS (0.1%) and left to polymerize (overnight, 20-21°C).

2.13.b.3 Stacking gel

The SDS overlay was removed and the gel surface dried and rinsed with freshly prepared stacking gel. Stacking gel was then pipetted above the resolving gel and a spacer

comb carefully inserted into the gel to avoid trapping air bubbles under the loading well. The comb was removed after allowing the gel to polymerize (> 1 hr). The wells were rinsed with upper reservoir buffer and the gel sandwiches loaded onto the gel mounts. The mount used for the large gels served as a cooling core.

2.13.c Preparation of the samples for SDS-PAGE

2.13.c.1 Affinity purified glycine receptor

Spinal cord membranes, used as a source of glycine receptors, were pelleted and resuspended in SDS (10% w/v) to a protein concentration of approximately (4 µg/µl), followed by a dilution in master mix (1:2 v/v). Solubilized fractions (preload and active fraction) and controls of PBS containing CHAPS (0.75% w/v), PTC (1.5 mg/ml) and protease inhibitor cocktail, were mixed with master mix (1:2 v/v). These fractions were also subjected to a protein precipitation in an attempt to concentrate the protein component. Volumes of purified receptor, preload and control fractions (300 µl) were made up to volume (1ml) in H_2O. SDS (0.1 ml, 1%) was added to the samples which were left to stand (10 mins, 20-21°C). Trichloroacetic acid (0.1 ml, 72%) was added to the incubation and the protein was pelleted by centrifugation (12 000 g, 15 mins). The supernatant was removed and the pellet resuspended in an ether/ethanol mix (3:1). After further centrifugation the pellet was resuspended in SDS (4% w/v, 10 µl) containing sodium hydroxide (NaOH 16 mM) and phenol red indicator (0.1% w/v). The pH of the mixture was adjusted to < 7 by additions of NaOH (2 µl), the final volume was recorded before adding master mix (1:2 v/v).

2.13.c.2 Photolabelled spinal cord membranes

Samples in SDS (10% w/v) solubilized overnight as described (see section 2.12.c) were mixed with master mix (1:2 v/v).

2.13.c.3 Molecular weight markers

Dalton Mark VII-L in Tris/HCl (1 mg/ml) was diluted in master mix (1:2 v/v).

[^{14}C]-methylated protein mix was diluted in Tris/HCl (1:4 v/v) before master mix

(1:2 v/v).

The samples in master mix were boiled (3 mins) to ensure they were denatured, a process facilitated by ß-mercaptoethanol. The samples were cooled in readiness before loading.

2.13.d Loading and gel electrophoresis of samples

The samples were applied to the stacking gel, for large gels a maximum of 100 µg protein in a maximum volume of 60 µl; for mini-gels a maximum 50 µg protein in a maximum volume of 30 µl. Upper and lower reservoir buffers were added and the samples run at constant current through the stacking (15 mA) and resolving (30 mA) gel. When the bromophenol dye ran towards the end of the of the resolving gel the current was turned off. The large gels were run at constant temperature (15°C).

2.13.e Detection of peptides

At all stages the gels were incubated by gentle mixing.

2.13.e.1 Detection of unlabelled peptides by silver staining

Large gels (200 ml/wash volume) and mini gels (100 ml/wash volume) were fixed by two consecutive washes (2 x 15 mins) in a water/methanol/acetic acid mix (40%/50%/10% v/v/v) followed by a further wash (15 mins) in a water/methanol/acetic acid mix (88%/5%/7%). The gels were washed (10 mins) in distilled water and reduced by incubation (30 mins) in dithiothreitol (5 µg/µl) before rinsing in distilled water. The gels were stained by incubating (30 mins) in a silver nitrate solution (0.1% w/v). A rinse in distilled water was followed by 2 superficial rinses in developer containing formaldehyde (0.0005% v/v) and sodium carbonate (3% w/v) before the gels were incubated in developer. When bands appeared in the gels, the reaction was stopped by addition of citric acid (0.115M, final concentration). The gels were given 2 washes in distilled water and stored in sodium carbonate (0.03% w/v) for photography before being dried down.

2.13.e.2 Detection of radioactive peptides by autoradiography

10% gels were fixed by incubation (1 hr) in a water/methanol/acetic acid mix (50%/40%/10% v/v/v) and could be stored in acetic acid (7% v/v) prior to further processing. Alternatively, gradient gels were fixed by incubation (3 hrs) in water/methanol/acetic acid mix (50%/40%/10% v/v/v) containing glycerol (3% w/v). After fixing both types of gels were incubated (1 hr) in Enhance, followed by an incubation in cold water (1 hr).

The gels were dried under heat (60°C) and vacuum until they lay flush on the filter paper that was used as backing support. Gradient gels were overlayed with cellophane membrane to prevent cracks forming during or after the drying process. The gels were placed in X-ray cassettes and exposed to Kodak X-OMAT AR film and stored at -70°C for up to 4 months prior to development of the films. [^{14}C]-molecular weight markers were defined by re-exposing the gels for one week as the longer exposures, required to define [^{3}H]-labelled peptides, allowed detection of signal from proteolytic products which make interpretation of marker lanes difficult.

The films were developed under safelight, by incubating (4 mins) in X-ray developer (25% v/v). The films were placed (30 secs) in a stop bath (4% v/v) before being fixed by incubation (5 mins) in X-ray fixative. The films were then thoroughly washed in running water and air dried. The developed autoradiographs were laid over duplicating film, exposed to a short flash UV light (nominal peak 254 nm; 1-5 secs) and the transferred image processed automatically at the X-ray department at University College London. The reproductions used in this thesis are reversals of contact prints.

2.14 Estimation of protein content

Protein levels were estimated using the dye-binding method of Bradford (1976).

2.14.a Preparation of Coomassie brilliant blue

Coomassie brilliant blue reagent G-250 (150 mg) was dissolved in ethanol (7.125% v/v, final concentration) and mixed gently (overnight). Phosphoric acid (10.2% v/v, final concentration) was slowly added to the ethanol and the mixture was brought to volume

(2 l) by the addition of distilled water. The reagent was left to cool, filtered and stored in a dark bottle at room temperature.

2.14.b Macro assay

Care was taken to add protein at a final concentration that lay within the linear range of the assay. Samples (10µg-100µg protein) were added to quadruplicate test tubes containing distilled water (100 µl). The fourth tube of each quadruplicate contained BSA (25 µg) or bovine-γ-globulin (50 µg), as standard proteins. Coomassie brilliant blue solution (5 ml) was added to all tubes and the incubations were vortexed. The absorbance at 595 nm was read in a spectrophotometer, the complex formed between dye and protein is stable for 1 hour.

The tubes containing the standard protein addition served as external standards used in the calculation of sample protein content. In addition, a comparison of the absorbance at 595 nm of appropriate blank incubations, containing a sample volume (100 µl) of distilled water or buffer, were performed to assess potential interference from additives in the experimental protein samples (*e.g.* ions, detergents or protease inhibitors).

2.14.c Micro assays

A micro assay was used to detect low protein quantity due to low sample volume or sample protein concentration. Quadruplicate samples (1-10 µg) were added to test tubes containing distilled water (50 µl). The fourth tube of each quadruplicate contained in addition BSA (5µg) or bovine-γ-globulin (10 µg). The relevant incubations were included in each assay and the absorbance measured as described.

If protein was still undetectable, samples and equal volumes of blanks were subjected to the protein precipitation procedure described (see section 2.13.c.1). The protein content was estimated by addition of Commassie brilliant blue (1 ml) directly to the pelleted protein.

2.15 Liquid scintillation counting

Radioactive filters or liquid samples (50 µl) in 5 ml insert vials were prepared for estimation of tritium by addition of scintillation cocktail (3 ml). In an assay terminated by centrifugation (see section 2.11.b) the membrane pellet was dissolved in scintillant cocktail (1 ml) and the sample thoroughly vortexed. The assay tube was directly used to estimate the level of tritium associated with the membrane pellet. Aliquots of [^{3}H]-ligand (20 µl) were prepared for liquid scintillation counting to check the concentration of the assay stocks. Prior to assay counting the photodetection system was normalized by counting an unquenched [^{14}C]-standard. The samples were counted and the background value estimated by the first vial which contained only scintillant cocktail, this was automatically subtracted from sample counts. Assays were usually counted for 10 mins or until 10,000 counts had been detected. The counts/min were converted to disintegrations/mins by estimating the counting efficiency in individual samples. This was achieved by obtaining a spectral index produced in samples by an external standard source of γ-radiation (^{133}Ba). The spectral index had previously been related to counting effifciency by counting a series of [^{3}H]-hexadecane standards quenched by adding increasing volumes of chloroform. This quench correction was held in the memory of the counter.

2.16 Analysis of binding data

2.16.a Principles of ligand binding experiments

A ligand binding experiment in which the law of mass action is valid can be represented by:

$$R + F + I \rightleftharpoons RF + RI$$

R= concentration of free binding sites
F= concentration of free radioactive ligand
I= concentration of unlabelled drug
RF and RI= concentration of receptor bound with ligand
and drug respectively

Although the free ligand concentration in experiments is often taken to be the same as the added ligand concentration, it is more precisely represented by $L = L_T - RL$ (where L_T = total ligand). If this value and the non specific binding are adequately defined, measurement of the experimental variable RF can be used to define parameters of:

(1) Saturation experiments in which L_T is increased and RF determined at equilibrium (I=0)

(2) Inhibition experiments in which RF is determined as the concentration of I is increased L_T is held constant. The case where I and F are the same drug, represents a saturation experiment in which the specific activity of the radioligand is lowered by the addition of unlabelled drug (isotopic dilution) and can consequently be analyzed as an inhibition or saturation experiment.

A saturation experiment allows the estimation of the equilibrium dissociation constant (K_D) and the total receptor concentration (B_{max}) as defined mathematically:

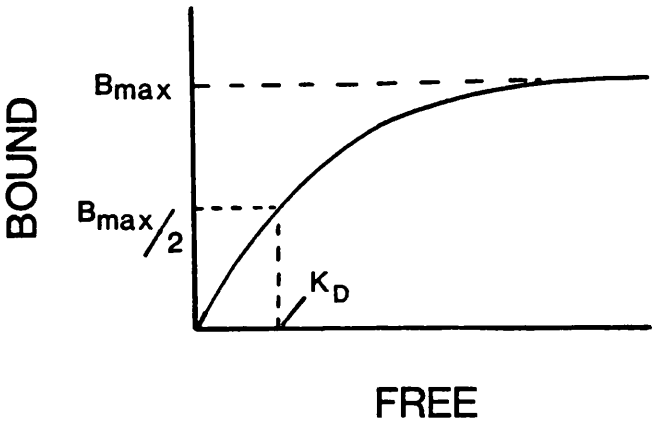
$$K_D = R.F/RF,$$

$$B_{max} = R+RF,$$

These equations can be substituted and solved for RF (bound ligand; B) to give a fuller description which is mathematically equivalent to the Michaelis-Menton equation of enzyme kinetics and defines a rectangular hyperbola.

$$B = B_{max}.F/K_D+F \qquad \text{Equation 1}$$

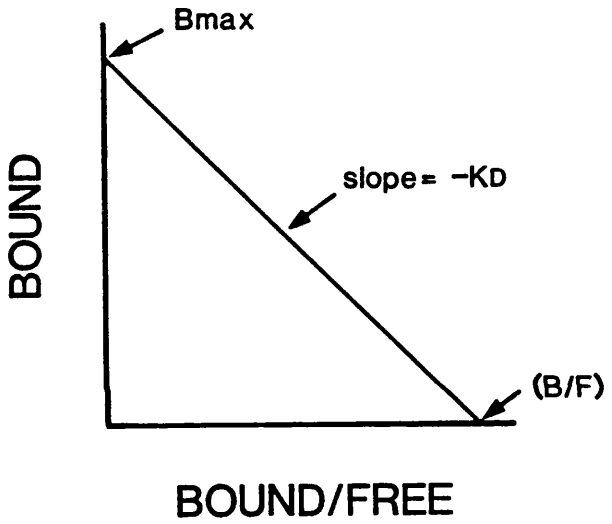
Experimentally this can be defined by subtracting non-specific binding from total binding to give specific binding from which the binding parameters can be obtained.



Two important linear forms of the equation 1 that have been used in the analysis of the binding data are discussed below.

$$B = B_{max} - K_D B/F; \quad \text{Equation 2}$$

Equation 2 is analogous to those initially derived by Eadie (1942) and Hofstee (1952) for enzyme kinetic analysis. As shown below the Eadie/Hofstee plot transforms the saturation binding isotherm into a linear form which can be used to estimate the B_{max} and K_D.



This represents the most simple case where only one class of binding site exists in the preparation. When more than one class of binding site exists, the concentration of bound ligand at each concentration of free ligand is the sum of that bound at each of the different sites.

$$B = B^1 + ...B^m = B_{max}{}^1.F/K_D{}^1 + F + ...B_{max}{}^m.F/K_D{}^m + F$$

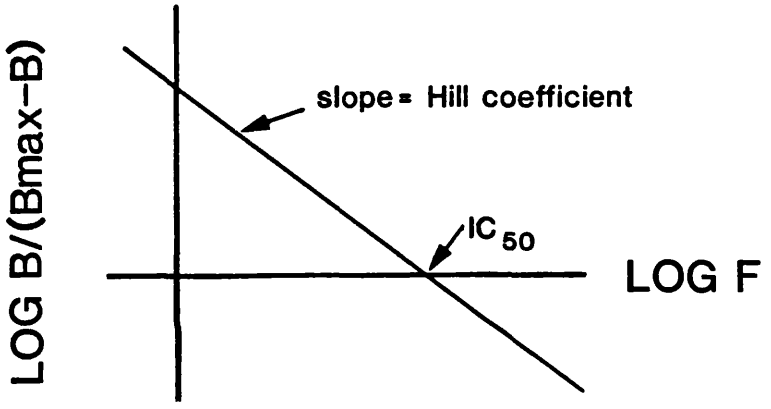
The components that define the parameters of multiple binding sites are not usually well resolved by the saturation experiment or the linear transformations. Although the non-linear Eadie/Hofstee Plots are indicative of deviation from simplest situation of a single class of non-interacting binding sites. The resolution of the individual parameters

of multiple binding sites is most precisely acheived by using specialized computer programs (Unnerstall, 1990) to generate a non-linear least squares fit of the data to a saturation isotherm.

The second transformation of equation 1 was initially derived by Hill (1910) and has been used to define co-operativity and specificity of the binding sites.

$$\log B/B_{max}\text{-}B = n(\log F - \log K_D); \quad \text{Equation 3}$$

This logarithmic transformation of Equation 1 can be plotted out in the form of Hill slopes.



The Hill coefficient is the slope at $B = B_{max}/2$. The value of the Hill coefficient is used to indicate the nature of the interaction between ligand and receptor.

>1 positive co-operativity or facilitation of binding

=1 no-co-operativity

<1 either negative co-operativity or multiple classes of binding sites

The Hill plot was also important in defining specificity of binding sites. When unlabelled drug is added to the binding assay, it can compete with the radioligand for the binding to reduce B in equation 1, which than becomes

$$B = B_{max}.F/F + K_D\ (1 + /K_I); \quad \text{Equation 4}$$

where K_I= equilibrium dissociation constant for the unlabelled ligand receptor complex

(the inhibition constant).

Specificity experiments used the concentration of I that produced 50% inhibition (IC_{50}) to describe the potency of unlabelled ligand for the binding site.

At I= IC_{50} $\quad B_{max}.F/F.K_D(1 + IC_{50}/K_I)= 1/2.B_{max}.F/K_D + F$

Therefore $IC_{50}= K_I(1 + F/K_D)$; Equation 5,

The logarithmic transformation of equation 4 gives a form of equation 3 where the free ligand (F) becomes the concentration of the unlabelled inhibitor (I).

$$logB/B_{max}-B= logI -logK_I(1+F/K_D)$$

A Hill plot of log I against log $B/B_{max}-B$ intercepts the ordinate at $I= K_I (1 + F/K_D)= IC50$ (see equation 5).

2.16.b Analysis of binding data

Experimental data were analyzed using a linear regression package (Graphpad) or the packages EBDA (Mcpherson, 1983) and SCAFIT (Munson and Rodbard, 1980).

EBDA gives linear least square estimates of binding parameters from Hofstee and Hill plots and subjects the estimates to an iterative curve fitting program to produce final estimates and standard errors associated with these estimates. Graphpad gives linear least square fits of Hill Plots to give IC_{50} values, Hill coefficents and the 95% confidance limits on these estimates.

Although all binding sites that obey the law of mass action will have parameters which can be defined by the procedures discussed, neither linear least squared fitting programs are able adequately to resolve results into multiple binding sites. SCAFIT gives non-linear least square curve fit to experimental data using initial estimates of K_D and B_{max} from analysis of curve segments in EBDA. SCAFIT allows fits to the experimental data for single or multiple binding sites. The appropriateness of each fit is indicated by the F-test criteria on residual variance and by estimating the randomness of fit using a runs test. This package can also be used to test the appropriateness of the definition of non-specific

by treating it as a fitted parameter.

2.17 Statistical analysis of data

Where appropriate, data was compared using Student's t test (unpaired and paired). The Pearson coefficient was calculated to test for the significance of correlation between two data sets.

CHAPTER 3

Characterization of ligand recognition by strychnine binding proteins

3.1 Introduction

3.1.a Ligand interactions at the inhibitory glycine receptor

The general introduction to this thesis highlights the extensive use of the convulsant alkaloid strychnine as a probe of the inhibitory glycine receptor. Although it is clearly established that strychnine antagonizes the actions of glycine at this ligand gated anion channel there are fundamental differences in the models that have been developed to describe the nature of the interaction between strychnine and glycine.

In 1974 Young and Snyder used the [^{3}H]-strychnine binding assay they had developed (Young and Snyder, 1973) to investigate the interaction between glycine and strychnine at the inhibitory glycine receptor. It appeared that the inhibition of [^{3}H]-strychnine binding by glycine produced Hill plots with a slope of 1.7, suggesting that the inhibition of strychnine binding by glycine showed positive cooperativity. In addition Young and Snyder (1974a) detected non-competitive kinetics when the saturation of [^{3}H]-strychnine binding was performed in the presence of unlabelled glycine. Finally they showed that treatment of spinal cord membranes with the protein modifying reagents diazonium tetrazole and acetic anhydride differentially effected the interaction of glycine with the inhibitory glycine receptor. As the effects of these protein modifying reagents could be exclusively prevented by ligand protection with the agonist glycine they argued that strychnine and glycine were binding to distinct sites on the receptor protein. Indeed this idea was compatible with the non-competitive nature of the interaction between glycine and strychnine. These findings were summarized in a model which proposed that glycine and strychnine bound at distinct sites which were coupled by an allosteric interaction which showed positive co-operativity.

A more recent study which used [^{3}H]-strychnine binding and protein modification has similarly concluded that strychnine and glycine have distinct binding sites on the receptor protein (Marvizón *et al.*, 1986a). However in contrast to previous data (Young

and Snyder, 1974a) it was shown that saturation of [^{3}H]-strychnine binding in the presence of unlabelled glycine described a competitive interaction between the two ligands. In addition it appeared that the proportion of total [^{3}H]-strychnine binding inhibited even at high concentrations of glycine was much less than inhibited by unlabelled strychnine. For this reason glycine was described as a partial competitive inhibitor of strychnine. This type of interaction was used to explain the shallow slope of the Hill plots, derived from the inhibition of [^{3}H]-strychnine binding by unlabelled glycine, which were significantly less than 1. This data was modelled mathematically and can be qualitatively described by a concomitant binding of strychnine and glycine to form a ternary complex in which glycine can never completely abolish the affinity of the receptor for strychnine. The ability to effect the degree of partial inhibition by glycine through changes in the ionic composition of the incubation buffer (Marvizón *et al.*, 1986b), were argued to arise by modulation of the allosteric interaction that couples the glycine and strychnine binding sites.

A competitive interaction between glycine and strychnine was found in experiments performed in this laboratory (Fry and Phelan, 1986; Phelan, 1987). However, unlabelled glycine was able to inhibit a similar proportion of total [^{3}H]-strychnine binding as unlabelled strychnine. Furthermore rather than detecting slopes >1 (Young and Snyder, 1974a) or <1 (Schaffer *et al.*, 1981; Marvizón *et al.*, 1986a; Ruíz-Gómez *et al.*, 1989) from the inhibition of [^{3}H]-strychnine binding by glycine the data from this laboratory was in agreement with independent workers who have shown Hill slopes equal to one (White, 1985; Braestrup *et al.*, 1986; Becker *et al.*, 1986).

The three apparent conflicting descriptions of the interaction between strychnine and glycine at inhibitory receptor have arisen from very similar experimental strategies which have considered the parameters of [^{3}H]-strychnine binding to rat or mouse spinal cord membranes. The initial studies described in this chapter have attempted to address these discrepancies experimentally and discuss why they arise.

3.1.b Chemical modification of proteins: general principles

Topography of the strychnine and glycine binding sites on the inhibitory glycine receptor was examined by treating spinal cord membranes with protein modifying reagents

and measuring the effect on subsequent [^{3}H]-strychnine binding. This technique which considers the effect of protein modification on function has been extensively used to probe enzyme structure/function relations (see Cohen, 1970; Glazer, 1976; Eyzaguire, 1987).

The ability chemically to modify proteins depends on the physicochemical properties of the amino acid side chains. Of the 20 amino acids residues found in proteins, some have a reactive nature which makes them prone to classical derivitization (see Table 1).

Functional Group	Reactions
Amino of lysine	Acylation; alkylation; arylation; reaction with carbonyls.
Guanidinium of arginine	Condensation with dicarbonyls.
Carboxyl of glutamic and aspartic acid	Esterification; amide formation with carbodilmides; reduction.
Thiol of cysteine	Oxidation; arylation; alkylation; ß-elimination;heavy metal derivatives
Imidazole of histidine	Alkylation; diazonium coupling; iodination; oxidation; photoxidation.
Thioether of methionine	Oxidation; alkylation.
Phenol of tyrosine	Acylation; alkylation; iodination; nitration; oxidation; diazonium coupling.
Indole of tryptophan	Alkylation; formylation; oxidation; ozonolysis.
Hydroxyl of serine and threonine	Esterification; phosphorylation; alkylation.

Table 3.1: Reactions for modification of functional groups in proteins (Riordan, 1979).

Classically chemical modification has been used to investigate protein composition. However the development of protein modifying reagents that utilize milder reaction conditions has extended the use of this technique to investigations of protein function. The reactivity is limited to the amino acids with polar side chains listed in Table 3.1. Therefore not all types of amino side chains possess the required reactivity, although those that do might be expected to be those involved in the dynamic features of protein function, including ligand receptor interactions. Nonetheless, there are limitations in the

use of chemical modification to probe protein function (Cohen, 1970), the major one being specificity. Common reactivities of distinct amino acids means that no modifying reagent will have an absolute specificity for a single residue. However, the reaction between an amino acid and reagent will have optimum conditions. The most obvious of these is pH, which relates to the reactive index being greatest when the residue is uncharged. Thus by varying the reaction conditions, selectivity of a reagent for a specific residue can be increased but not guaranteed. Further consideration is required as even though the selectivity towards one type of amino acid can be ensured reactivity of individual residues will vary and be dependent on the microenviroment in which the residue is located (Eyzaguirre, 1987). This can either lead to a hyper- reactivity which facilitates specific labelling (Eyzaguirre, 1987) or a inaccessibility of reagent to residue due to steric factors (Cohen, 1970).

Finally the effect of a reagent on protein function cannot be presumed to occur via a modification of residue directly involved in protein function as conformational changes may accompany protein modification. A common strategy used in conjunction with chemical modification, in an attempt to circumvent such limitation is ligand or substrate protection. This compares the degree of chemical modification on assayed biological function when the reaction is performed in the presence or absence of ligand or substrate that interacts with the site of protein that is functionally important (Eyzaguirre, 1987).

3.1.c Protein modification of [^{3}H]-strychnine binding as a probe of the strychnine/ glycine interaction at the inhibitory glycine receptor.

Protein modifying reagents have been widely used in attempts to characterize the interaction between strychnine and glycine at the inhibitory glycine receptor protein. These experiments considered the effect of protein modifying reagents on the strychnine binding site by direct measurement of [^{3}H]-strychnine binding. In the absence of a suitable agonist ligand, an effect at the glycine binding site could only be measured indirectly by comparing the ability of glycine to inhibit [^{3}H]-strychnine binding to modified and unmodified preparations. The specificity of the effects of protein modifying reagents for the ligand binding sites has been addressed using ligand protection procedures (Young and Snyder, 1974a; Marvizón *et al.*, 1986a; Ruíz-Gómez *et al.*, 1989; Ruíz-Gómez *et al.*, 1990).

The initial report that diazonium tetrazole and acetic anhydride differentially modified the interaction of glycine with the receptor, gave further weight to the suggestion that strychnine and glycine were binding to distinct sites on the receptor (Young and Snyder, 1974a). This ability differentially to affect the interaction of glycine with its receptor has been confirmed in experiments that used fluorescein isothiocyanate (FI, Ruíz-Gómez *et al.*,1989). A recent publication has argued for the idea of distinct strychnine and glycine binding sites based on the exclusive ability of strychnine protection to prevent the reduction in [^{3}H]-strychnine binding to spinal cord membranes or reconstituted receptor preparations treated with the protein modifying reagents diazotised sulphanilate (DSA) or phenylglyoxal (Ruíz-Gómez *et al.*, 1990). However, in another study DSA has been shown to decrease the acceleration of the rate of [^{3}H]-strychnine dissociation that was induced by unlabelled glycine. This was apparent despite only a small decrease in the level of [^{3}H]-strychnine binding to DSA treated membranes and was argued to result from a modification by DSA of residues involved in the allosteric interaction between distinct glycine and strychnine binding sites (Maksay, 1990). The effects of chemical modification on the parameters of [^{3}H]-strychnine binding has led to the present consensus view that there are distinct binding sites for strychnine and glycine.

In addition to detailing the possible gross topographies of the strychnine and glycine binding sites the specificity of the reagents has led to speculations concerning the residues involved in the molecular recognition of the two ligands. Young and Snyder (1974a) proposed that the selectivity of diazonium tetrazole and acetic anhydride pointed to a role for histidyl or amino functions in the interaction between strychnine and glycine. They also speculated that the reduction in [^{3}H]-strychnine binding to membranes treated with tetranitromethane (TNM) or dinitrofluorobenzene argued for a direct role of aromatic residues in strychnine binding, although this possibility was not fully addressed by ligand protection.

The differential reactivity of FI for the glycine binding site was taken to infer a direct role of lysine residues in the molecular recognition of the agonist at the inhibitory glycine receptor (Ruíz-Gómez *et al.*, 1989). The lysine residues at position 236, 239, 246 and 253 (numbering according to fig 1.3) of the rat α 1 subunit were speculated to be those involved as intrinsic florescence of FI was specifically located in a peptide sequence, cleaved from the ligand binding subunit, whose size was characterized based

on possible cleavage sites in the primary sequence.

DSA and PG differentially modified the strychnine binding site and were argued to exert this effect selectively at tyrosine and arginine residues (Ruíz-Gómez *et al.*, 1990). In addition from the kinetics of the inactivation of [^{3}H]-strychnine it was argued that a single residue was being modified to produce the effect. Based on the peptide cleavage pattern of ligand binding subunit photolabelled with [^{3}H]-strychnine it was argued that the tyrosine and arginine residues that function in the molecular recognition of strychnine lie in a region of the sequence that include the lysine residues discussed above.

In contrast to the above study DSA at a similar concentration was shown to only effect the interaction between strychnine and glycine. This result was discussed in terms of a modification of a tyrosine residue or residues that function in the allosteric interaction that couples the strychnine and glycine binding sites (Maksay, 1990).

The experiments described in this chapter were designed to investigate the topography of the strychnine and glycine binding sites at the inhibitory glycine receptor by similar strategies to those outlined above. The selectivity of the reagents used will be addressed under the relevant section of the discussion.

3.1.d Chemical modification of strychnine binding antisera

The antisera raised against the 2-aminostrychnine-keyhole limpet haemocyanin conjugate (2-AS-KLH antisera) have a pharmacological specificity for strychnine and related alkaloids that is similar to the strychnine binding site of the inhibitory glycine receptor (Phelan *et al.*, 1989). For this reason the antisera have been used as tools to probe for endogenous strychnine-like immunoreactivity and in attempts to produce anti idiotypic antibodies which may serve as markers for the inhibitory glycine receptor (Phelan, 1987). The final approach used in this chapter was to consider the effect of treatment of the 2-AS-KLH-antisera with protein modifying reagents on subsequent ability to bind [^{3}H]-strychnine. A comparison of the reactivity of protein modifying reagents for the strychnine binding site in spinal cord membranes and in 2-AS-KLH antisera allowed further investigation of the hypothesis that there is a congruence in the detail of molecular

recognition of the alkaloid strychnine at two unrelated proteins, a ligand gated ion channel and an immunoglobulin.

3.2 Results

3.2.a Inhibition of [^{3}H]-strychnine binding by increasing concentrations of unlabelled strychnine and glycine: comparison of assays terminated by filtration through untreated and polyethylenimine (0.1%) treated glass fibre filters.

Hill plots of the inhibition of [^{3}H]-strychnine binding by unlabelled glycine have been used (see section 3.1.a) in previous attempts to define the interaction between glycine and strychnine. However, the results of such analyses generate have led to divergent interpretations of the nature this interaction and could arise through the use of different assay conditions and procedures. The present experiments examined the possible effect of [^{3}H]-strychnine binding to the glass fibre filters, used to terminate the binding assays, on the parameters of the inhibition curve and the derived Hill plot.

Identical assays were set up as described (see section 2.10.a) in Tris/citrate buffer (50mM, pH 7.1, 4°C) and terminated by filtration through untreated or polyethylenimine treated glass fibre filters (0.1% v/v). The binding to glass fibre filters alone was estimated by parallel incubations from which the spinal cord membranes were omitted (fig 3.1.a and 3.1.b).

[^{3}H]-strychnine bound to the glass fibre filter alone. However, this interaction is greater in untreated glass fibre filters. The polyethylenimine pretreatment is an effective precaution against filter binding reducing it by approximately 80%. A comparison of the binding of [^{3}H]-strychnine to untreated glassfibre filters in the presence of increasing concentration of unlabelled strychnine indicates that unlabelled strychnine inhibits [^{3}H]-strychnine binding to untreated glass fibre filters in a concentration dependent manner. The inhibition of [^{3}H]-strychnine binding to spinal cord membranes described by the assay terminated by filtration through untreated and pre-treated filters show that the amounts of ligand binding converge to a similar level at high concentrations of unlabelled ligand (fig 3.1.a). This was apparent despite a very marked increase in the level of [^{3}H]-strychnine binding to spinal cord in the absence of unlabelled ligand for incubations terminated by filtration through untreated filters. This can be explained by the [^{3}H]-

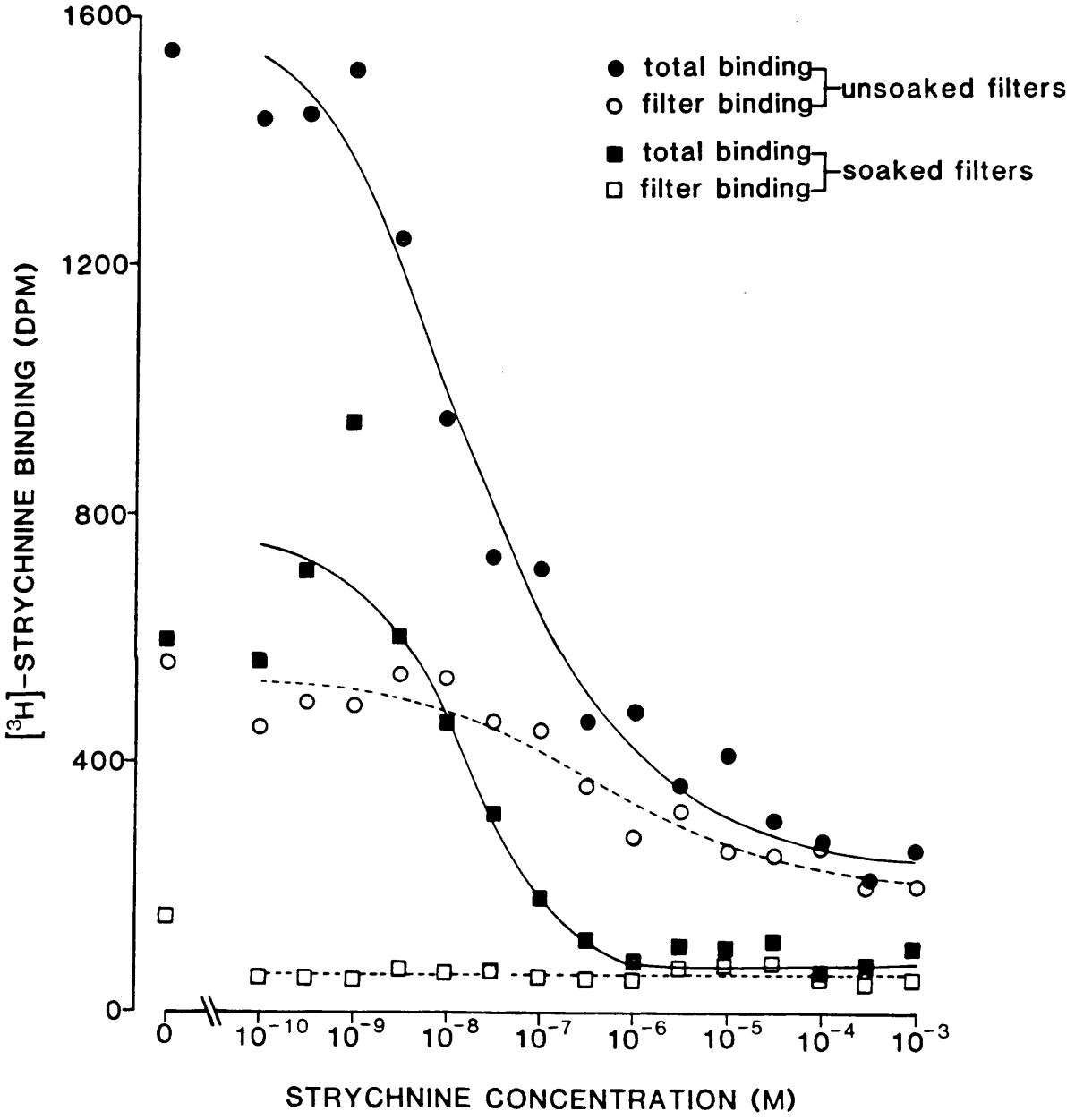


Figure 3.1.a: Inhibition of total [^{3}H]-strychnine binding by increasing concentrations of unlabelled strychnine. Comparison of incubations terminated by filtration through untreated or polyethylenimine (0.1%) presoaked glass fibre filters. Binding to filters alone was assessed by incubations set up without spinal cord membranes. Each value represents the mean of triplicate estimates; the experiment was repeated with similar results.

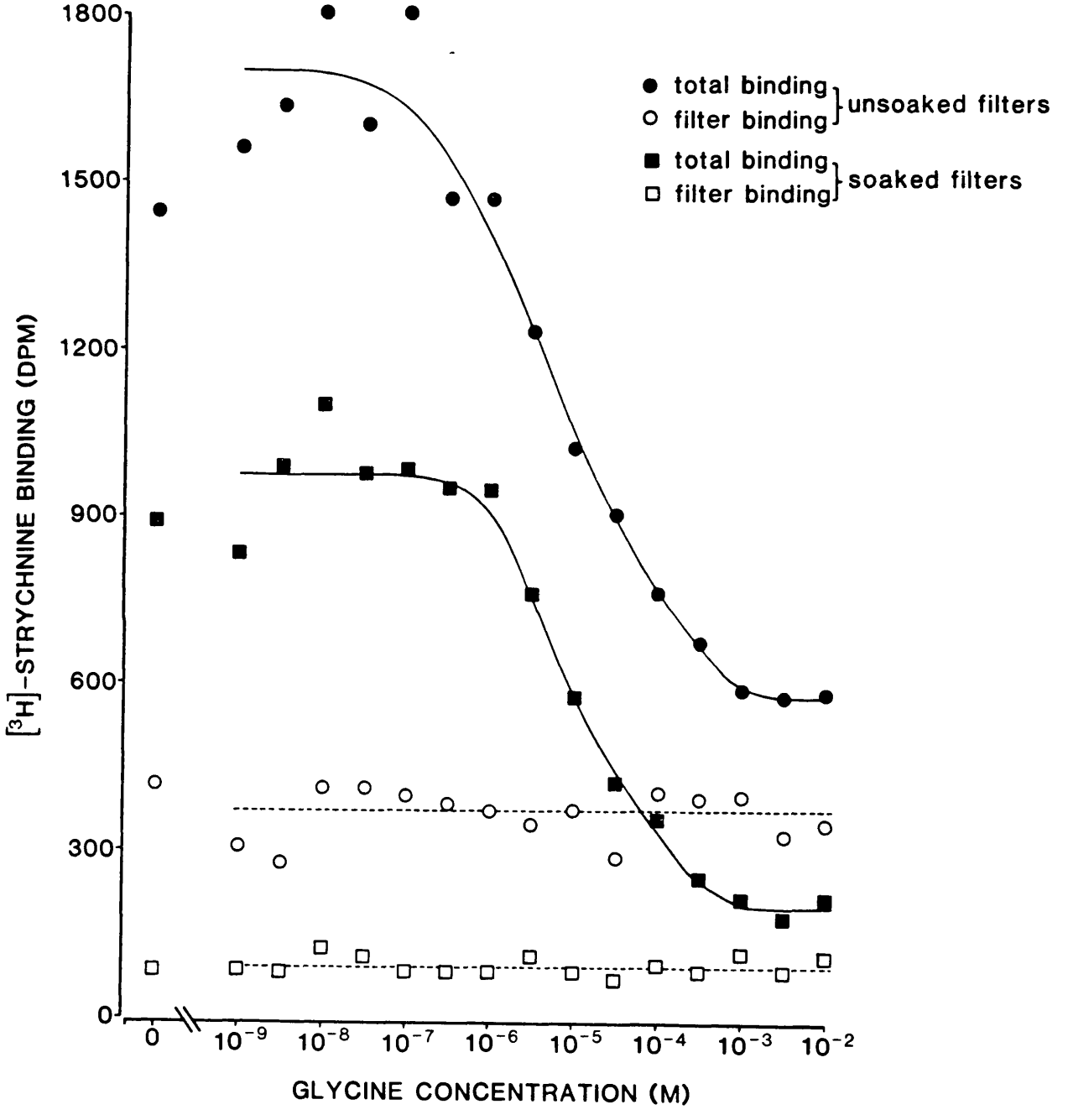


Figure 3.1.b: Inhibition of total [^{3}H]-strychnine binding by increasing concentrations of glycine. Comparison of incubations terminated by filtration through untreated or polyethylenimine (0.1%) presoaked glass fibre filters. Binding to filters alone was assessed by incubations set up without spinal cord membranes. Each value represents the mean of triplicate estimates; the experiment was repeated with similar results.

strychnine binding to untreated glass fibre filters and its inhibition by unlabelled strychnine.

In contrast glycine is unable to inhibit [^{3}H]-strychnine binding to untreated glass fibre filters (fig 3.1.b). This accounts for the increase in the [^{3}H]-strychnine binding to mouse spinal cord membranes even at high concentration of unlabelled glycine for assays terminated using untreated glass fibre filters (fig 3.1.b). This difference of approximately 400 dpm means that glycine appears to act as a partial inhibitor of [^{3}H]-strychnine binding.

The inhibition of [^{3}H]-strychnine to spinal cord membranes by increasing concentration of unlabelled strychnine and glycine (fig 3.1.a and 3.1.b) were corrected for non-specific binding defined by incubations which contained 10^{-4}M strychnine, as routinely used by other workers (Young and Snyder, 1974a; Marvizón *et al.*, 1986a). The corrected data was transformed to Hill plots and estimates of the Hill slope and IC_{50} were obtained using linear least square fit programs (see Table 3.2).

The results show inhibition of [^{3}H]-strychnine by increasing concentration of glycine using untreated glass fibre filters gives estimates for the Hill coefficient which are significantly less than 1. This contrasts with the estimates for the Hill coefficient for the inhibition of [^{3}H]-strychnine binding by increasing unlabelled strychnine or glycine separated using polyethylenimine presoaked filters, which are not significantly different to one. The Hill coefficient estimated for the inhibition of [^{3}H]-strychnine binding by increasing unlabelled strychnine separated using untreated filters has an intermediate value of 0.6.

Filter	Unlabelled ligand	IC_{50} (M)	Hill slope
Untreated	Strychnine	$1.25x10^{-8}$ ($8.57x10^{-9}$-$1.92x10^{-7}$)	0.60 (0.47-0.73)
Polyethylenimine Treated	Strychnine	$6.29x10^{-8}$ ($1.85x10^{-8}$-$2.10x10^{-7}$)	1.07 (0.82-1.32)
Untreated	Glycine	$8.93x10^{-5}$ ($2.79x10^{-5}$-$2.85x10^{-4}$)	0.31 (0.23-0.39)
Polyethylenimine Treated	Glycine	$8.45x10^{-5}$ ($4.47x10^{-5}$-$1.76x10^{-4}$)	0.86 (0.62-1.19)

Table 3.2: Hill slope and IC_{50} values with 95 % confidence limits in brackets from data Illustrated in fig 3.1.a and 3.1.b corrected for nonspecific binding and estimates made using linear least square fit program. This experiment was repeated and gave similar results.

3.2.b Optimising reaction conditions for treatment mouse spinal cord membranes with protein modifying reagents.

The conditions for chemical modification were optimized by incubating well washed spinal cord membranes in the appropriate buffer (see text) with reagent for increasing reaction times. After washing (see section 2.9.a) the levels of total [^{3}H]-strychnine binding were estimated and a comparison of the ability of strychnine and glycine to displace total binding made by addition of unlabelled strychnine (10^{-4}M) or glycine (10^{-2}M). These values were used as the strychnine- and glycine-specific [^{3}H]-strychnine binding respectively. The chemically modified membranes were compared to untreated controls carried through the same procedure but in which the reagent volume was replaced with a solvent control. The amount of total [^{3}H]-strychnine binding displaced by strychnine (10^{-4}M) and glycine (10^{-2}M) was essentially the same and accounted for at least 85% of total bound in untreated membranes. These experiments were performed to define conditions under which 50 percent of [^{3}H]-strychnine binding sites were inactivated for use in further studies.

3.2.b.1 Lysine selective reagents.

Pyridoxal phosphate (12 mM) + sodium borohydride (30mM) in PBS pH 7.4

Chemical modification of mouse spinal cord membranes followed by reduction

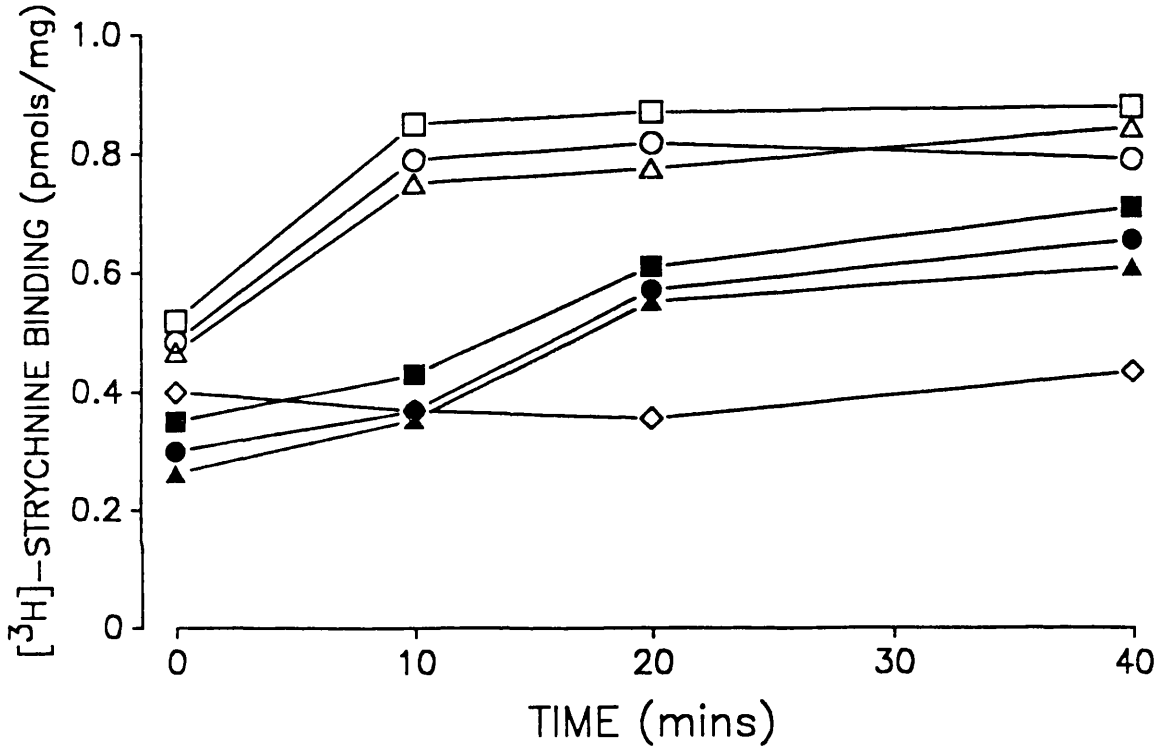


Figure 3.2: The effect of PLP treatment of spinal cord membranes on [^{3}H]-strychnine binding: Mouse spinal cord membranes in PBS (pH 7.4) were reacted with PLP (12mM) for increasing time before addition of sodium borohydride (20mM) and a further 20 min incubation (closed symbols). Control membranes not treated with PLP but subjected to the borohydride incubation are also shown (open symbols). Triplicate estimates of total (square), strychnine specific binding (circle) and glycine specific (triangle), [^{3}H]-strychnine binding to treated membranes are shown. Strychnine specific binding to untreated membranes (◇) represents 85% of total binding and was almost identical to glycine specific binding to untreated spinal cord membranes.

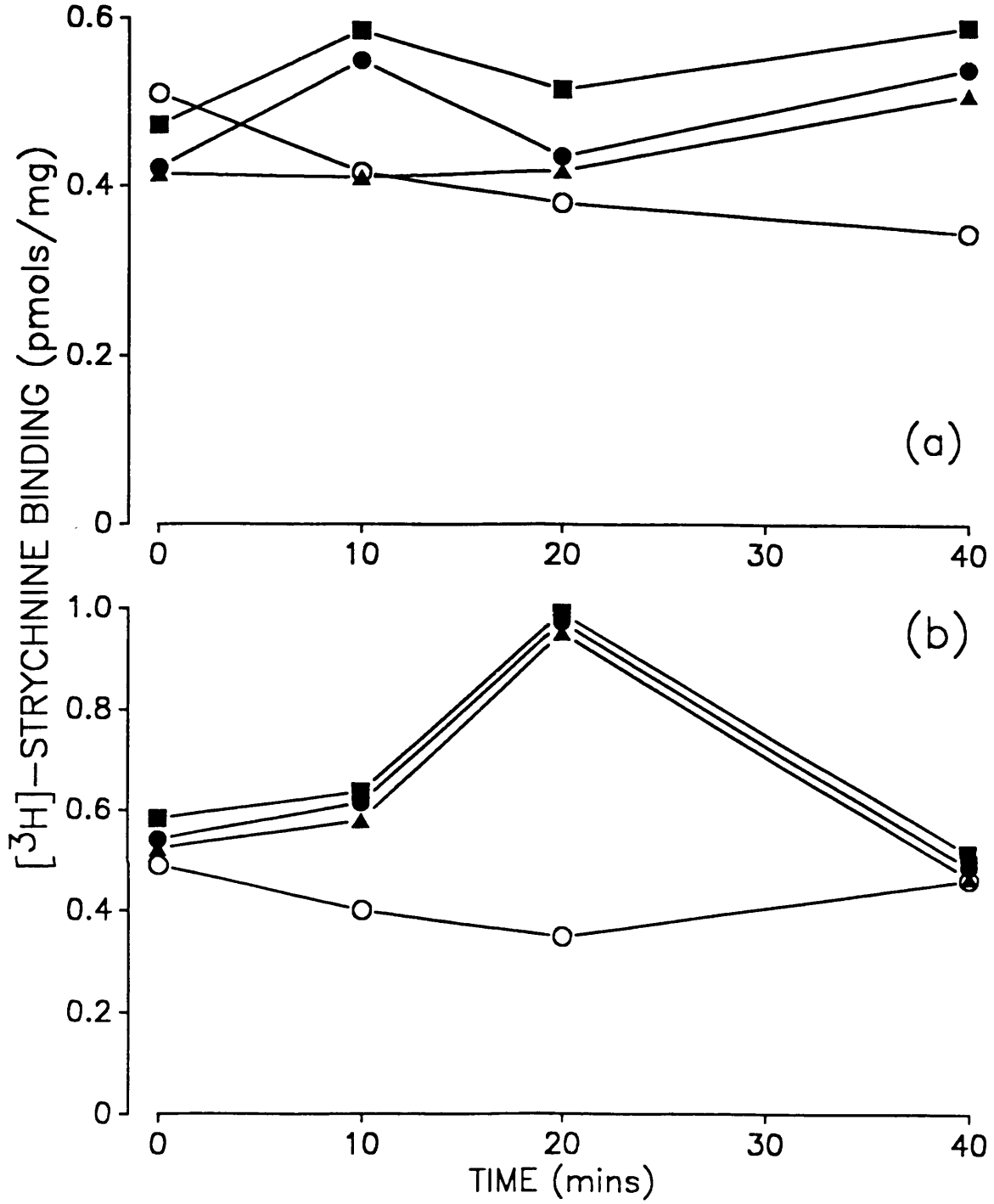


Figure 3.3: The effect of TNBS treatment of spinal cord membranes on [^{3}H]-strychnine binding: Mouse spinal cord membranes were reacted with TNBS (20mM) for increasing time in PBS at pH 8 (fig a) and pH 7.4 (fig b). After chemical treatment the membranes were washed and triplicate estimates of total (■), strychnine-specific (●), and glycine-specific (▲) [^{3}H]-strychnine binding to treated membranes were made by assaying in PBS pH 7.4. The strychnine specific [^{3}H]-strychnine binding (○) to untreated membrane represents 85% total binding and was almost identical to the glycine-specific binding.

with sodium borohydride, a procedure required to stabilize the Schiff base formed between lysine residues and pyridoxal phosphate, leads to an increase in [^{3}H]-strychnine binding (fig 3.2). However, as indicated by [^{3}H]-strychnine binding to membranes which are treated with borohydride alone, this increase can be attributed to effects of the reducing agent. It was also clear that the [^{3}H]-strychnine binding defined by unlabelled glycine and strychnine are very similar and account for much of the total binding. As a direct effect of PLP modification seems to be masked by a indirect effect of the reducing agent, this modification procedure was not investigated further.

2,4,6-Trinitrobenzenesulphonic acid (TNBS; 20 mM) in PBS pH 7.4 and 8

Spinal cord membranes were chemically modified with TNBS at pH 7.4 (fig 3.3.a) and pH 8 (fig 3.3.b), the latter condition favours the modification of lysine residues. Both treatments (fig 3.3.a and 3.3.b) tended to elevate the subsequent [^{3}H]-strychnine binding to mouse spinal cord membranes but were not consistent at different time points.In further experiments this reagent was reacted with membranes at pH 8 for 40 min.

3.2.b.2 Arginine selective reagents

1,2-cyclohexandione (CHD; 20 mM) in PBS (pH 7.4) and PBB (pH 7.4 and 9).

Spinal cord membranes were modified with CHD using phosphate buffered saline (PBS, pH7.4, fig 3.4.a) and phosphate buffered borate (PBB pH 7.4 and 9; fig 3.4.b and 3.4.c respectively). It would appear that chemical modification of spinal cord membrane with CHD had no clear effect on subsequent [^{3}H]-strychnine binding. There is little change in the [^{3}H]-strychnine binding to membranes subjected to CHD treatment for increasing reaction times. Specific [^{3}H]-strychnine binding as defined by excess unlabelled glycine and strychnine are very similar.

2,3-butanedione (BD; 20 mM in PBS pH 7.4 and PBB pH 7.4)

Spinal cord membranes were modified with BD using PBS (pH 7.4) or PBB (pH 7.4; fig 3.5.a and 3.5.b). Chemical modification with this reagent under both sets of conditions caused a time-dependent increase in the total [^{3}H]-strychnine binding. It can also be seen that, under both reaction conditions, the increase in total binding was

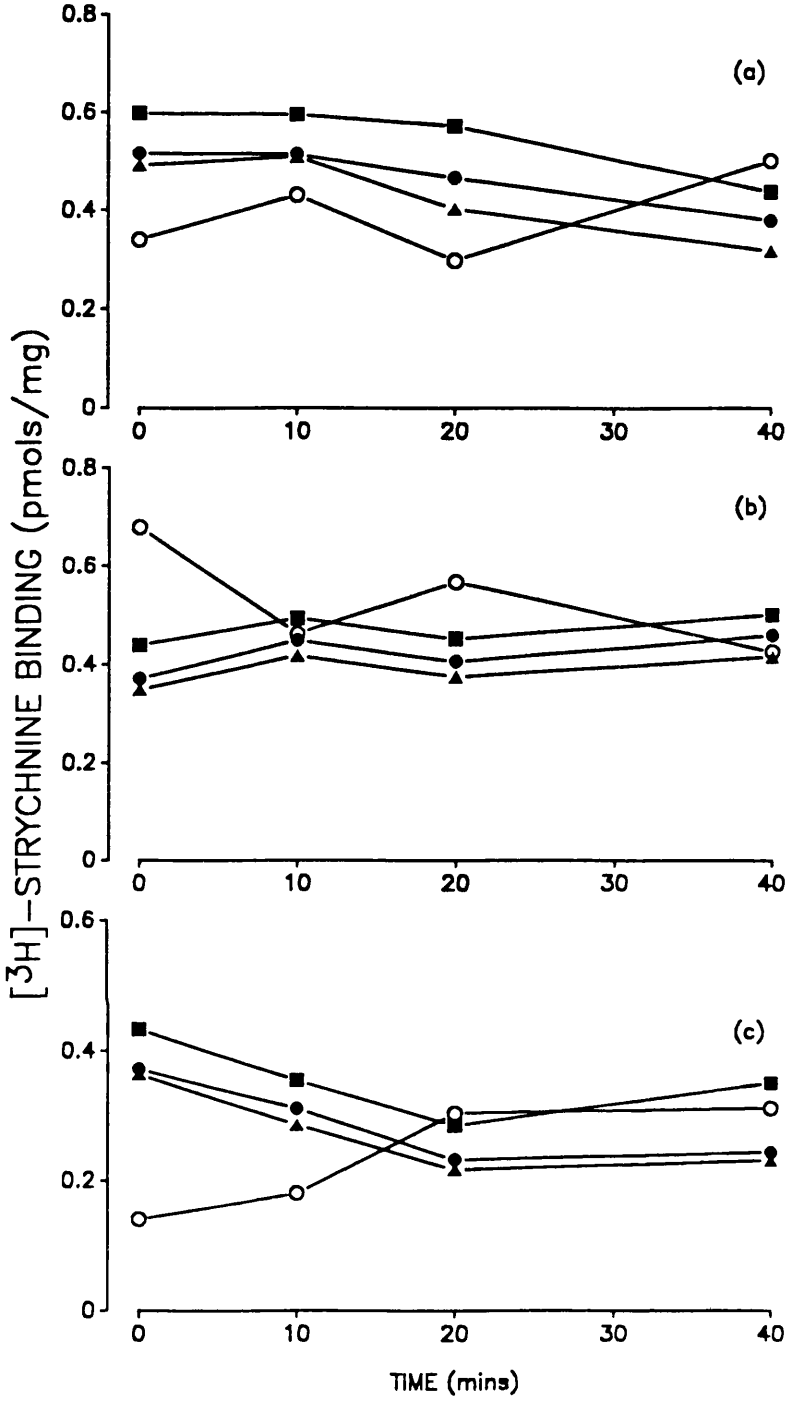


Figure 3.4: The effect of CHD treatment of spinal cord membranes on [^{3}H]-strychnine binding: Mouse spinal cord membranes were incubated with CHD (20mM) for increasing time in PBS (pH 7.4; fig a) and PBB (pH 7.4 & 9; fig b & c respectively). After treatment the membranes were washed and triplicate estimates of total (■), strychnine-specific (●), and glycine-specific (▲) [^{3}H]-strychnine binding to treated membranes were made. The binding assays were performed in PBS pH 7.4 (fig a) or PBB (fig b and c). The strychnine-specific [^{3}H]-strychnine binding (○) to untreated membrane represents 85% total binding and was almost identical to the glycine-specific binding to untreated membranes.

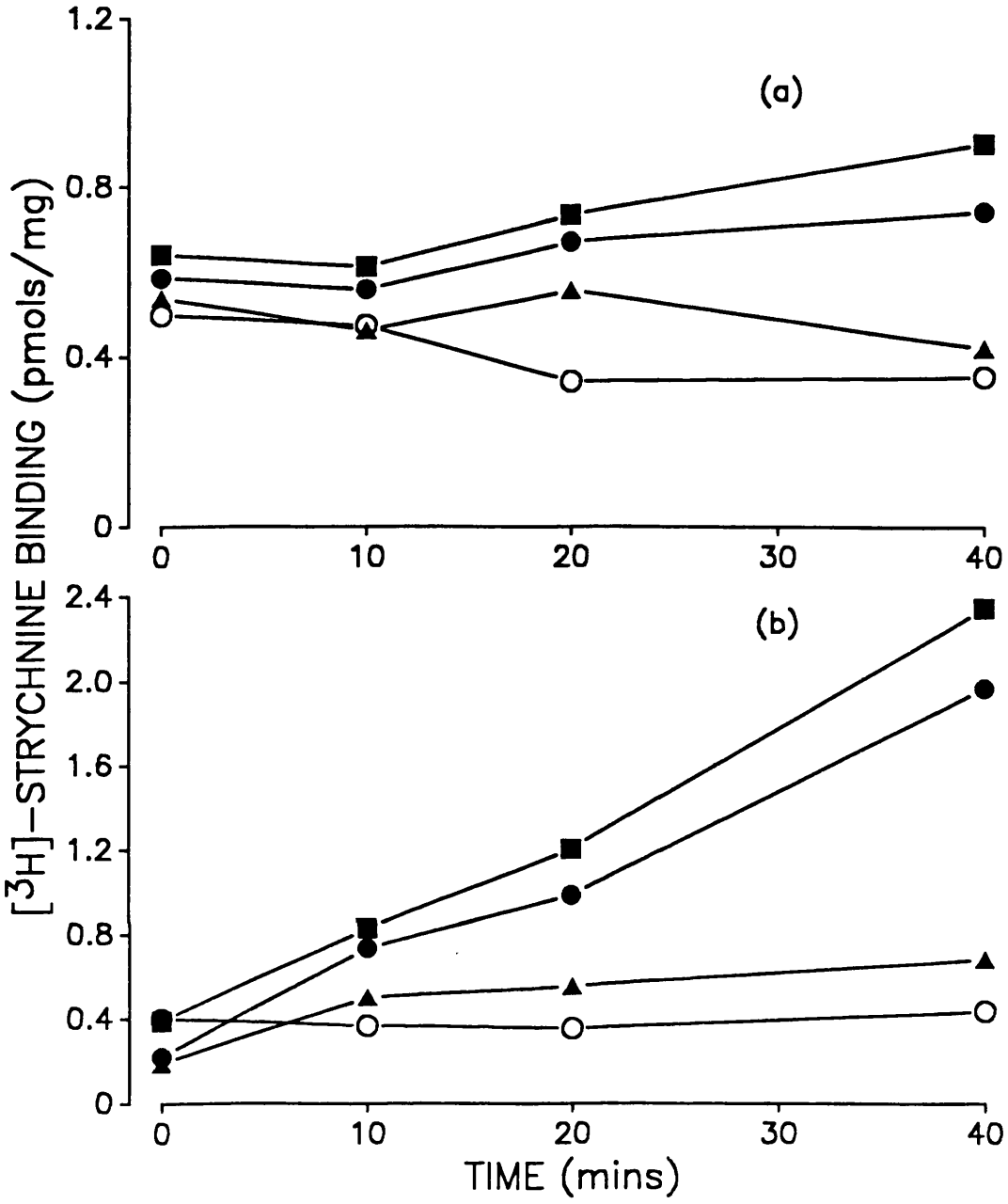


Figure 3.5: The effect of BD treatment of spinal cord membranes on [^{3}H]-strychnine binding: Mouse spinal cord membranes were incubated with BD (20mM) for increasing time at pH 7.4 in PBB (fig a) or PBS (fig b). After chemical treatment the membranes were washed and triplicate estimates of total (■), strychnine-specific (●) and glycine-specific (▲) binding to treated membranes, were made by assaying in PBS pH 7.4. The strychnine-specific [^{3}H]-strychnine binding to untreated membranes (○) represents 85% of total binding and was almost identical to the glycine-specific binding.

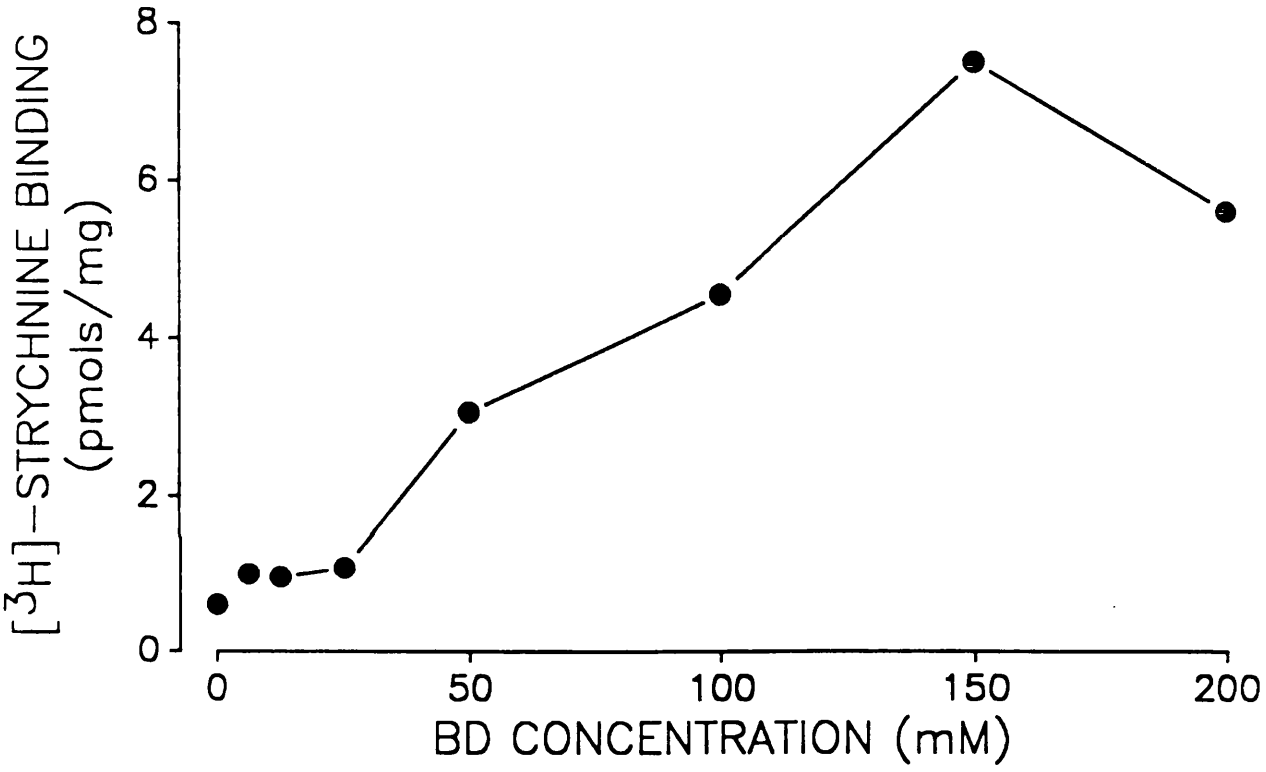


Figure 3.6: The effect of treating spinal cord membranes with increasing concentrations of BD on [^{3}H]-strychnine binding: Mouse spinal cord membranes were treated with increasing concentration of BD in PBS pH 7.4 for 40 min. After treatment the membranes were washed and triplicate estimates were made of strychnine specific [^{3}H]-strychnine binding (●) by assaying in PBS pH 7.4.

associated with a decrease in the specific [^{3}H]-strychnine binding defined by unlabelled glycine. This represented less than 30% of total binding in the most extreme case, membranes modified with BD for 40 min. This contrasted with specific binding defined by strychnine which represented at least 85% of total [^{3}H]-strychnine binding for all points.

A comparison of BD modification performed in PBS and PBB (fig 3.5.a and fig 3.5.b) indicated that the reagent caused a more rapid increase in [^{3}H]-strychnine binding when performed in PBS. The ability of BD to enhance [^{3}H]-strychnine had not reached a maximum under either of the conditions used. A further optimization experiment was attempted to define reaction conditions for use in later experiments.

Well washed spinal cord membranes in PBS were incubated with increasing concentration of BD (0-200 mM) and the [^{3}H]-strychnine binding estimated after treatment for 10, 20, and 40 min. At each BD concentration there was a time dependent increase in the total [^{3}H]-strychnine binding (data not shown). As before, this increase in total [^{3}H]-strychnine binding to treated spinal cord membranes was characterized by low specific [^{3}H]-strychnine binding defined by unlabelled glycine (data not shown). Spinal cord membranes incubated with increasing concentration of BD for 40 min showed a maximum increase in strychnine-specific [^{3}H]-strychnine binding at 160 mM (fig 3.6). In subsequent experiments, membranes were treated with 80 mM BD for 40 min in PBS (pH 7.4), the conditions under which the increase in [^{3}H]-strychnine binding is approximately 50 percent of the maximum (fig 3.6).

3.2.b.3 Tryptophan selective reagent

N-bromosuccinimide (NBS; 1 mM in PBS at pH 7.4 and 6)

Treatment with NBS in PBS at pH 7.4 (fig 3.7.a) and 6 (fig 3.7.b) caused a decrease in subsequent total [^{3}H]-strychnine binding to modified membranes. When membranes were treated at pH 7.4 the effect was rapid but increased with reaction time. In membranes treated with the same concentration of NBS but at pH6 maximal effect of the treatment occurred very rapidly as indicated by the [^{3}H]-strychnine binding at 0 min (fig 3.7.b; see Discussion). Treatment of spinal cord membranes at both pH 6 and 7.4 caused specific [^{3}H]-strychnine binding defined by unlabelled glycine to fall to values as

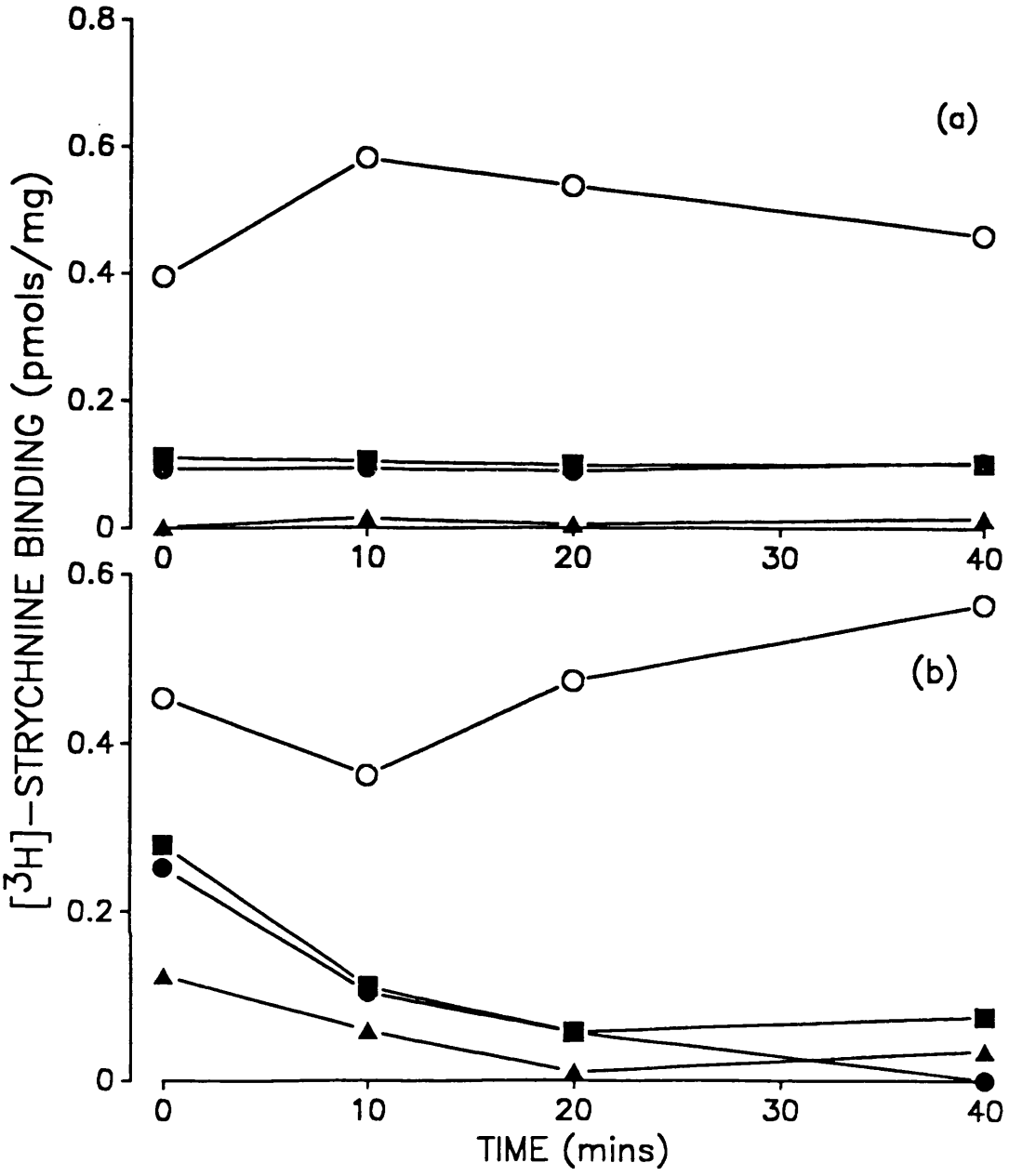


Figure 3.7: The effect of NBS treatment of spinal cord membranes on [^{3}H]-strychnine binding: Mouse spinal cord membranes were incubated with NBS (1mM)for increasing time in PBS pH 6 (fig a) and pH 7.4 (fig b). After treatment the membranes were washed and triplicate estimates of total (■), strychnine-specific (●), and glycine-specific (▲) [^{3}H]-strychnine binding to treated membranes were made by assaying in pH 7.4. The strychnine-specific [^{3}H]-strychnine binding (○) to untreated membranes represents 85% of total binding and was almost identical to the glycine-specific binding in untreated membranes.

low as 10% of total binding; while at all time points this value was lower than 50% of total binding. However, the strychnine-specific [^{3}H]-strychnine binding was at least 85% of total binding.

3.2.b.4 Tyrosine selective reagent

Tetranitromethane (TNM; 5mM in PBS pH 7.4 and 8)

Chemical modification of spinal cord membranes with TNM in PBS at pH 7.4 or pH 8, at which the reagent has greatest selectivity for tyrosine residues (see Discussion), caused a fall in subsequent [^{3}H]-strychnine binding. When the reaction was performed at pH 7.4 (fig 3.8.a) the decrease in [^{3}H]-strychnine is greater as the time of chemical modification was increased. As the length of time that spinal cord membranes were treated with TNM was increased the specific [^{3}H]-strychnine binding defined by unlabelled strychnine and glycine falls. In the case of strychnine-specific binding this was still 70% total for membranes modified with TNM at pH 7.4 for 40 min but glycine-specific binding fell to 23% of total binding at this time point.

The treatment of spinal cord membranes with TNM at pH 8 had a qualitatively similar effect (fig 3.8.b). However, the decrease in [^{3}H]-strychnine binding was maximal for membranes modified under these conditions for 20 min. Although there was a fall in the glycine-specific [^{3}H]-strychnine binding, to 57% of total for membranes modified for 20 min under these conditions. However, at the 40 min time point the [^{3}H]-strychnine binding defined by both strychnine and glycine was only 70% of total binding. This compared to strychnine specific binding which accounts for 85% of total binding in membranes treated with TNM for 0, 10, and 20 min. In later experiments membranes were reacted with 5mM TNM at pH 8 for 15 mins.

3.2.b.5 Histidine selective reagent

Diethylpyrocarbonate (DEP; 20 mM in PBS at pH 7.4 and 8)

At pH 7.4 (fig 3.9.a) this reagent caused a time-dependent decrease in total [^{3}H]-strychnine binding. Reaction of membranes at pH 7.4 also caused a time dependent fall in the specific [^{3}H]-strychnine binding defined by unlabelled glycine. At time 0 this

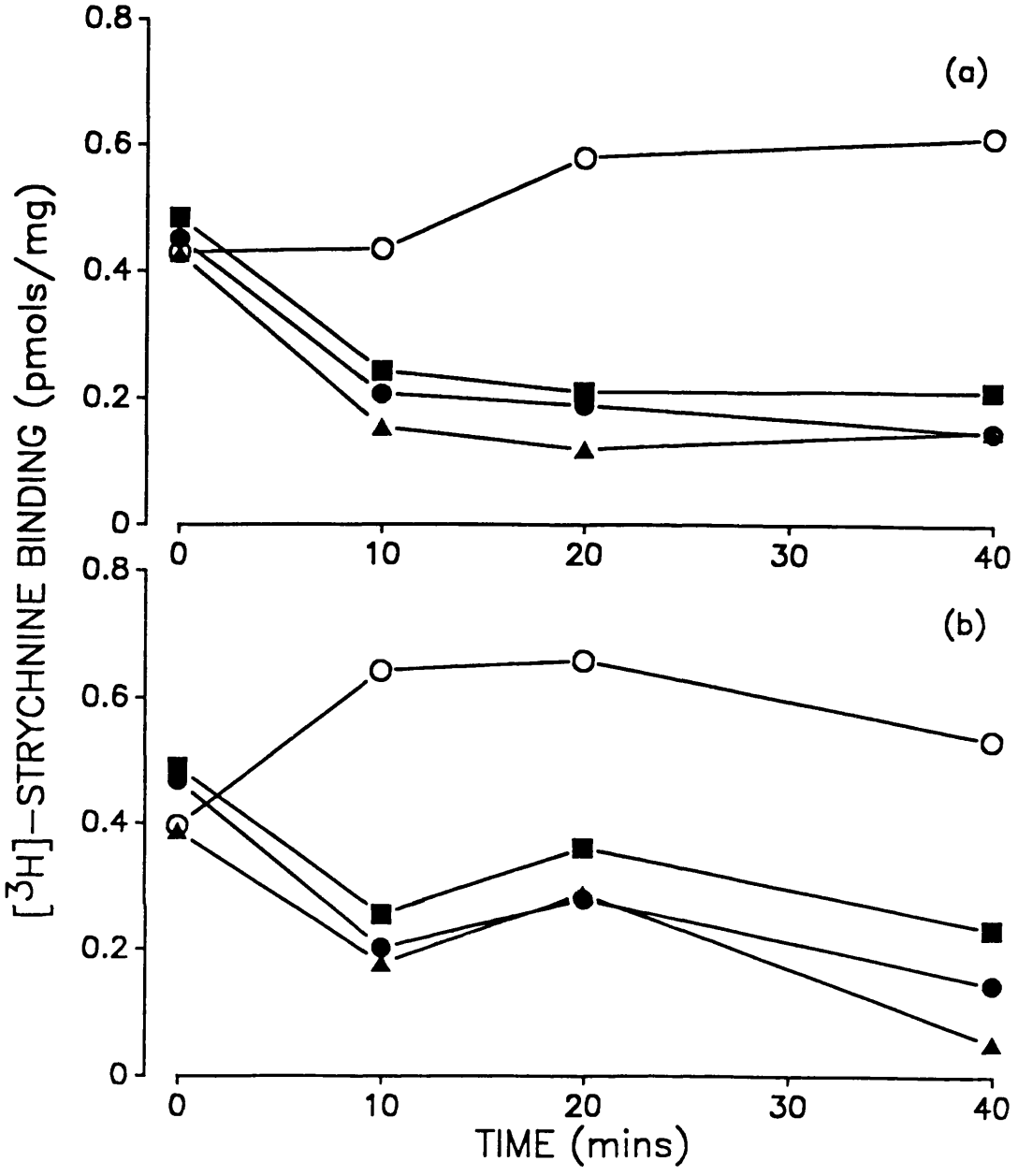


Figure 3.8: The effect of TNM treatment of spinal cord membranes on [^{3}H]-strychnine binding: Mouse spinal cord membranes were incubated with TNM (5mM) for increasing time in PBS pH 8 (fig a) and pH 7.4 (fig b). After treatment the membranes were washed and triplicate estimates of total (■), strychnine-specific (●), and glycine-specific (▲) [^{3}H]-strychnine binding were made by assaying in PBS pH 7.4. The strychnine-specific [^{3}H]-strychnine binding (○) to untreated membranes represents 85% total binding and was almost identical to the glycine-specific binding to untreated membranes.

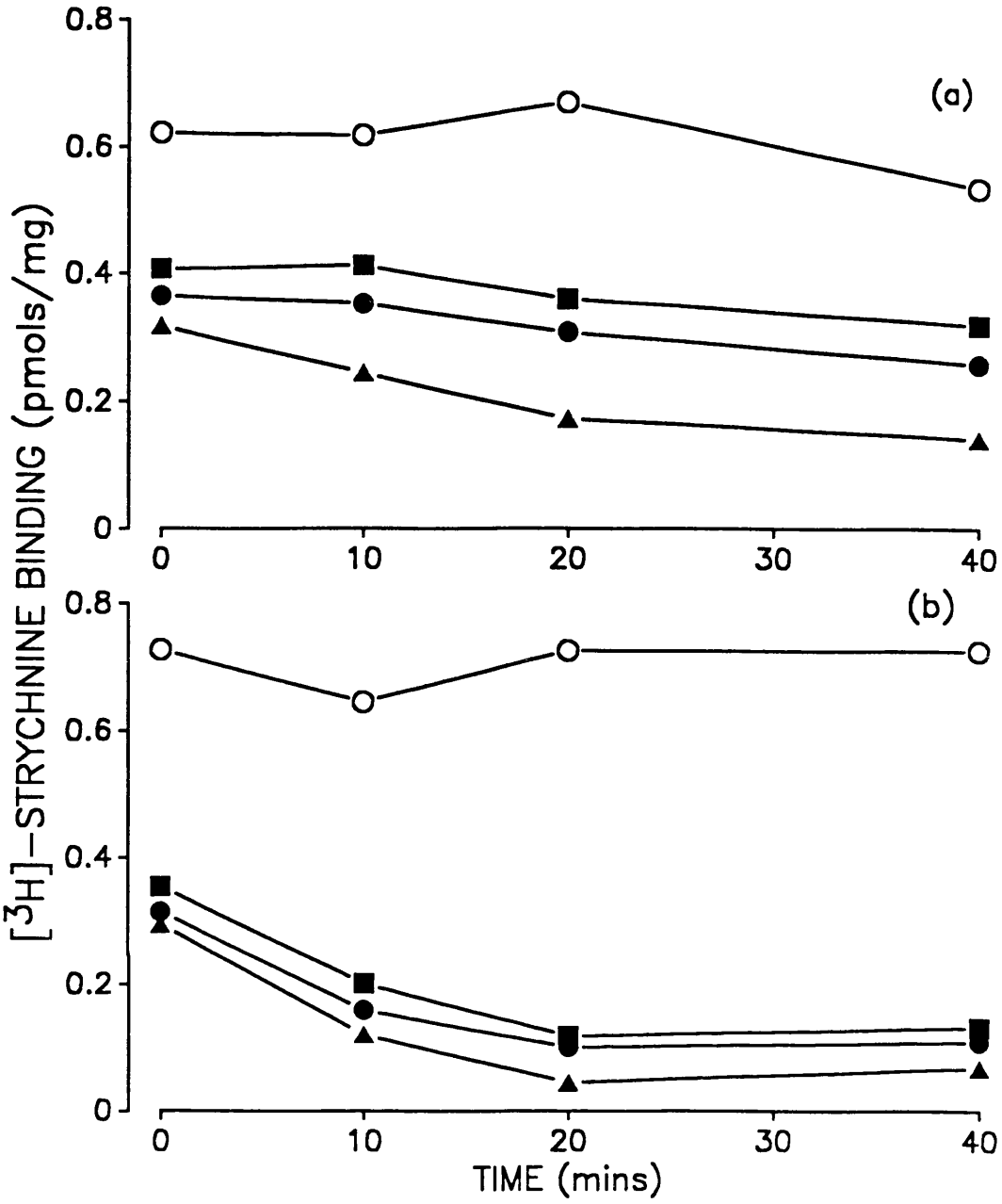


Figure 3.9: The effect of DEP treatment of spinal cord membranes on [^{3}H]-strychnine binding: Mouse spinal cord membranes were incubated with DEP (20mM) for increasing time in PBS pH 6 (fig a) and pH 7.4 (fig b). After treatment the membranes were washed and triplicate estimates of total (■), strychnine-specific (●), and glycine-specific (▲), [^{3}H]-strychnine binding were made. The strychnine-specific binding to untreated membranes (○) represents 85% of total binding and was almost identical to the glycine-specific binding.

represents 85% of total strychnine binding but this fell to 50% at 40 minute time point. In contrast specific binding defined by unlabelled strychnine remains at least 85% of total binding after all treatment times.

Again modification with the same concentration of DEP but at pH 6 causes a time dependent decrease in subsequent [^{3}H]-strychnine binding to spinal cord membranes (fig 3.9.b). The decrease in total binding is rapid and continues with increasing modification. The decrease in strychnine defined specific binding in treated membrane represents approximately 50% the value in untreated membrane for the maximum modification time period of 40 mins. This contrasts with the DEP modified membranes at pH 7.4 where the level of strychnine defined specific binding was only 15% the value of untreated membranes. There seems to be a smaller effect on subsequent [^{3}H]-strychnine binding for membranes treated with DEP at pH 6, the condition at which this reagent is most selective for histidine residues.

Although there appeared to be less an effect on subsequent [^{3}H]-strychnine binding when the membranes were modified at pH 6, this condition caused the level of specific binding defined by unlabelled glycine to decrease with increasing reaction time, so that at 40 min it represented only 50% of total binding (fig 3.9.b). This contrasted with the level of specific [^{3}H]-strychnine binding to treated membranes defined by unlabelled strychnine which remained at least 85% of total for all time points tested (fig 3.9.b) and for untreated control membranes (data not shown).

3.2.b.6 Reagents selective for cysteine and cystine residues

Disulphide bonds are susceptible to chemical modification by reducing agents, for this reason the effect of treatment of spinal cord membranes with dithiothrietol (DTT) on subsequent [^{3}H]-strychnine binding was tested. Three different concentrations (0.2, 1.0, 10 mM) of DTT were tested for their effects using PBS pH 8 as reaction buffer (fig 3.10.a, 3.10.b, and 3.10.c). Although there was an elevation in [^{3}H]-strychnine binding to mouse spinal cord membranes treated with DTT (0.2M) for 20 and 40 minutes when compared to the control it is slight and does not appear in membranes treated with a higher concentration of reagent (fig 3.10.b and 3.10.c). Membranes treated with DTT have almost identical specific [^{3}H]-strychnine binding defined by unlabelled strychnine and glycine at each of the reagent concentrations. In all cases this represents at least 80% total

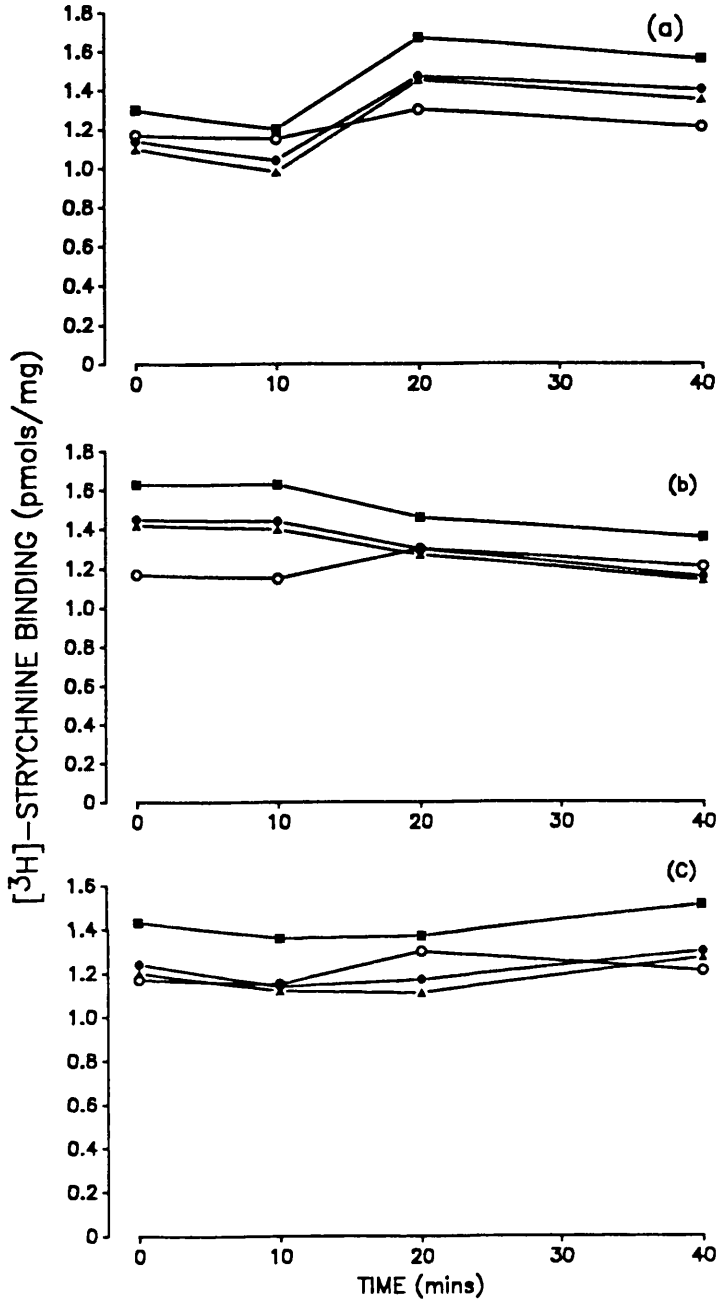


Figure 3.10: The effect of reduction of spinal cord membranes with DTT on [^{3}H]-strychnine binding: Mouse spinal cord membranes were incubated in PBS (pH8) with DTT at 0.2mM (fig a), 1mM (fig b) and 10mM (fig c), for increasing times. After treatment the membranes were washed and triplicate estimates of total (■), strychnine-specific (●), and glycine-specific (▲), [^{3}H]-strychnine binding were made by assaying in PBS pH 7.4. The strychnine-specific [^{3}H]-strychnine binding (○) to untreated membranes represents 85% of total binding and was almost identical to the glycine-specific binding.

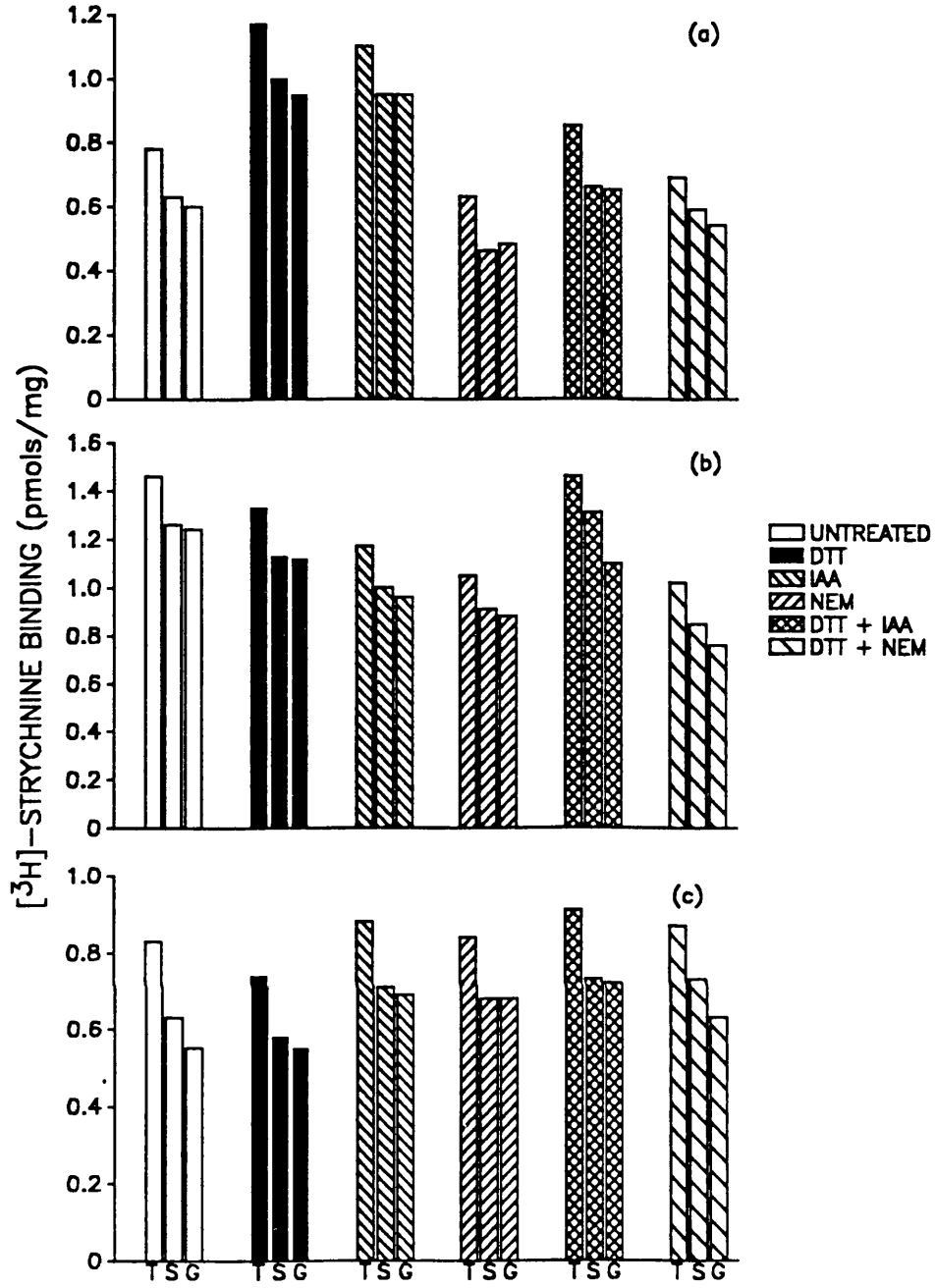


Figure 3.11: The effect of reduction and alkylation of spinal cord membranes on subsequent [^{3}H]-strychnine binding: Mouse spinal cord membranes in PBS (pH 7) were untreated or treated with DTT 10mM (fig a), 1mM (fig b) or 0.2mM (fig c) for 20 minutes. These procedures were followed by the addition of buffer or one of two alkylating reagents NEM or IAA; 50mM (fig a), 5mM (fig b) or 1mM (fig c). After further incubation (20 min) the membranes were washed and triplicate estimates of total (T), strychnine-specific (S) or glycine-specific (G) [^{3}H]-strychnine binding were made by assaying in PBS pH 7.4.

binding and compares favourably with the % total [^{3}H]-strychnine binding that is specific in untreated membranes.Lack of an effect of DTT could be attributed to re-oxidization of the reduced disulphide during the extensive washing procedures used to remove unreacted reagent (see section 2.9.a). Therefore, experiments were performed in which the membranes in PBS (pH 7.4) were treated with DTT (0.2; 1.0; 10m M) for 20 minutes followed by the addition of a 5 fold excess of the alkylating reagent N-ethylmaleimide (1, 10, 50 mM) or iodoacetamide (1, 10, 50 mM; see figure 3.11.a, 3.11.b and 3.11.c respectively) before terminating the reaction. This procedure should favour the alkylation of free sulphydryls and ensure they do not reoxidize and reform disulphide bonds.

Compared to untreated membranes reduction (DTT) and alkylation (NEM and IAA) protocol does not seem to effect any clear change in total [^{3}H]-strychnine binding or the level of specific binding defined by unlabelled strychnine or glycine (fig 3.11.a, 3.11.b and 3.11.c). The exceptions could be the slight decrease in the total binding estimated for membranes treated with DTT (1 mM) and alkylated by NEM (5 mM; fig 3.11.b).

This experiment also considered as controls the effect of alkylation of membrane on the subsequent [^{3}H]-strychnine binding. Although treatment of membranes with 100 mM IAA slightly increased subsequent [^{3}H]-strychnine binding when compared to untreated controls the same concentration of NEM (fig 3.11.c) effected a slight decrease in total [^{3}H]-strychnine binding. This effect of two different alkylating agents and lack of an effect on [^{3}H]-strychnine binding to spinal cord membranes with lower concentration of NEM or IAA (fig 3.11.a and 3.11.b) did not implicate functional role for cysteine residues in the interaction between strychnine and glycine at the inhibitory glycine receptor.

3.2.c Optimizing protection protocol

3.2.c.1 Removal of protecting ligands

Protection experiments were designed to look at the ability of unlabelled strychnine and glycine to prevent the effects of chemical modification on the subsequent [^{3}H]-strychnine binding to mouse spinal cord membranes. Protection was attempted using concentration of unlabelled strychnine (10^{-4}M) or glycine (10^{-2}M) which produced full

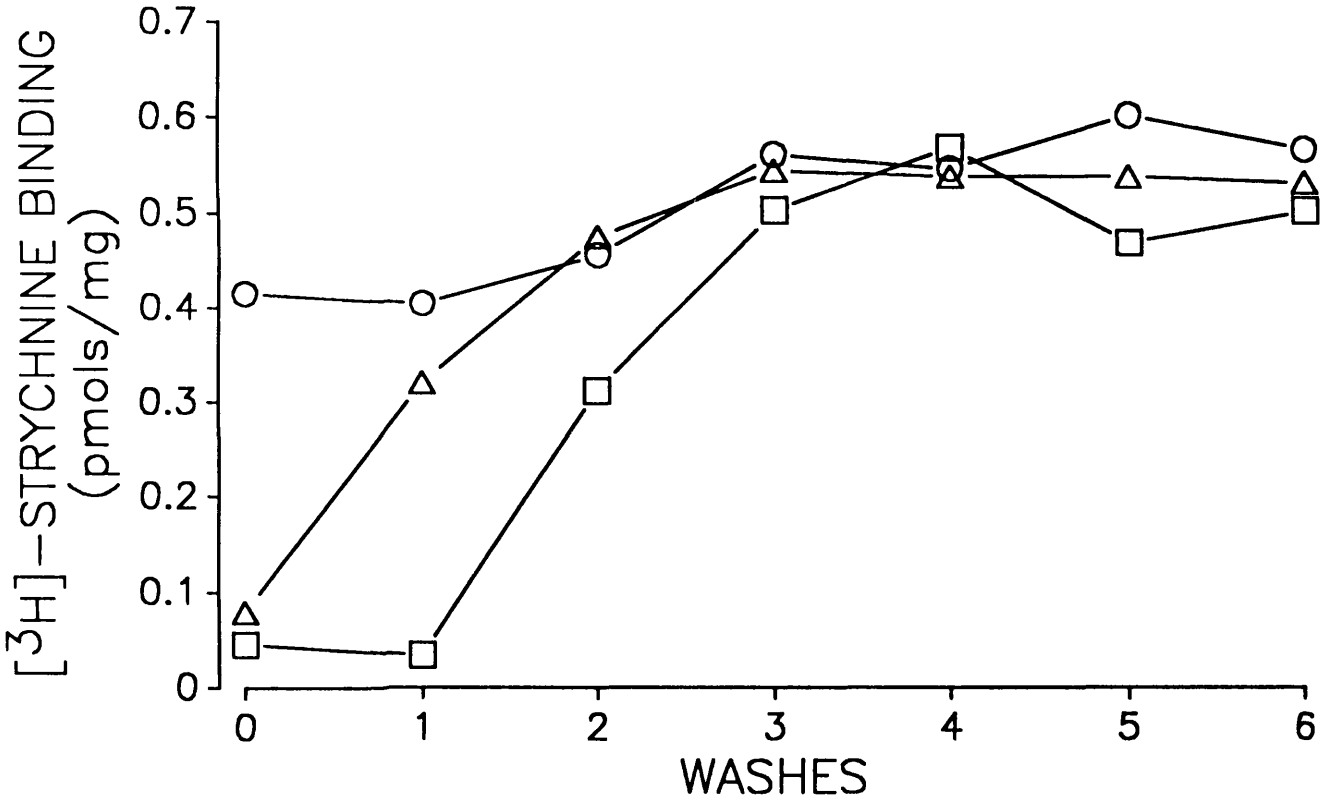


Figure 3.12: Optimization of washing procedure required to remove protecting ligands from spinal cord membranes: Membranes were incubated with no ligand (○), strychnine (10^{-4}M; □) or glycine (10^{-2}M; △). Samples of membrane were removed and triplicate estimates of specific [^{3}H]-strychnine binding were made. The remaining membranes underwent a series of washes and triplicate estimates of the specific [^{3}H]-strychnine binding were made after each wash.

occupancy of the receptor protein recognition sites for these ligands; that is the concentration used to define non-specific binding. An important criterion of these experiments was the complete removal of the protecting ligands, prior to the subsequent measurement of [^{3}H]-strychnine binding to chemically modified spinal cord membranes.

Well washed spinal cord membranes in PBS pH 7.4 (40 vols tissue weight/vol) were incubated with strychnine (10^{-4}M), glycine (10^{-2}M) or no ligand (buffer control) for 15 mins at 20/21°C. Samples of these membranes were taken to indicate the level of [^{3}H]-strychnine binding after no washes. PBS/BSA (160 vols tissue weight/vol) was added to each of remaining membrane suspensions from which an aliquot was taken spun and resuspended in PBS (40 vols tissue weight/volume) and assayed to give estimates of [^{3}H]-strychnine binding after one wash. The remaining aliquot was spun, the supernatant discarded and the pellet resuspended in PBS (160 vols tissue weight/volume) from which an aliquot was used to estimate the [^{3}H]-strychnine binding after 2 washes. This was repeated until estimates of [^{3}H]-strychnine binding were made in membranes subjected to up to 6 washes.

The results from this experiment (fig 3.12) show that [^{3}H]-strychnine binding in membranes preincubated with unlabelled strychnine (10^{-4}M) or glycine (10^{-2}M) return to that of controls after 2 or 3 washes for membranes preincubated with glycine. However 3 to 4 washes are required for membranes preincubated with strychnine. Four washes in PBS (160 vols tissue weight/vol) were used to remove unlabelled strychnine and glycine from spinal cord membranes used in the ligand protection experiments. The same procedure was shown to be sufficient to remove protecting ligand when PBB was used as wash buffer (data not shown).

3.2.c.2 pH profile of strychnine and glycine binding

The selectivity of chemical reagents is dependent on pH. Experiments were therefore designed to ensure that treatment of spinal cord membranes was performed where possible under conditions in which the reagent has greatest selectivity for a single type of amino acid residue. The ability of ligands to interact with recognition site will also be affected by pH either at the receptor protein or on the chemical properties of the ligand. This could lead to a pH dependence of the ability of the ligand to afford protection against the effect of chemical modification. For this reason, [^{3}H]-strychnine binding to

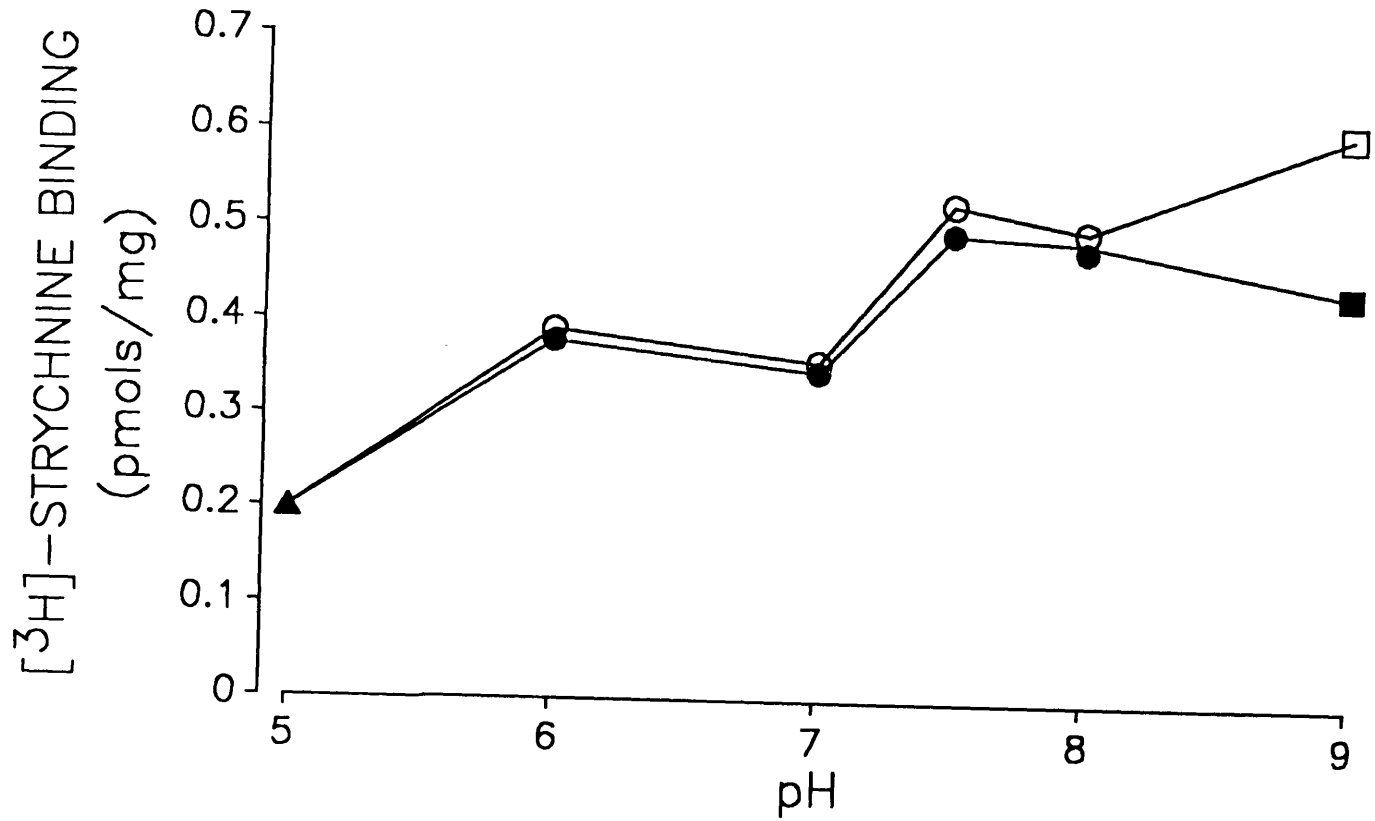


Figure 3.13: Effect of pH on the specific [^{3}H]-strychnine binding to spinal cord membranes: Strychnine-specific (open) and glycine-specific (closed) [^{3}H]-strychnine binding to mouse spinal cord membranes incubated at different pH. Buffers used are illustrated: 0.1M disodium phosphate/citrate buffer (triangle), PBS (circle), 0.025M borate/HCl (square).

well washed spinal cord membranes was estimated in a series of incubations set up over a range of pH values. Specific [^{3}H]-strychnine binding at each pH was defined by unlabelled strychnine and glycine as described. [^{3}H]-strychnine binding tended to increase as pH varies from 5 to 9 (fig 3.13). However the specific [^{3}H]-strychnine binding defined by unlabelled strychnine and glycine was almost identical at the pH values tested with the exception of pH 9 where there was a fall in specific binding defined by glycine so that it was only 50% total binding. At all other points specific binding represented at least 85% of total binding. Although total binding changed with pH the potency of strychnine and glycine, as indicated by the specific [^{3}H]-strychnine binding, seemed unaffected between pH 6-8 the range over which the protection experiments were performed.

3.2.d An assessment of the ability of glycine or strychnine to protect against the effect of chemical modification on [^{3}H]-strychnine binding to mouse spinal cord

The ability of glycine and unlabelled strychnine to protect against the effects of five protein modifying reagents on subsequent ability of treated mouse spinal cord membranes to bind [^{3}H]-strychnine was investigated. An effect of four reagents NBS, TNM, DEP and BD had been shown in the optimization experiments (see section 3.6) and encouraged their use in further study. The fifth reagent investigated was diazonium tetrazole (DT) because it had been used by other workers (Young and Snyder, 1974a; Marvizón *et al.*, 1986a) in investigation of ligand interactions at the glycine receptor.

Spinal cord membranes were protected with strychnine and glycine before being chemically modified (see section 2.9.a) and subjected to the washing procedure optimized above (see section 3.2.a.1). All experiments were performed in parallel with untreated membranes and the [^{3}H]-strychnine binding was assayed as described. The specific [^{3}H]-strychnine binding defined by unlabelled strychnine and glycine in untreated membranes was very similar and accounted for at least 85% of total binding. The [^{3}H]-strychnine binding in unprotected, strychnine and glycine protected membranes which were not treated with protein modifying reagents were not significantly different ($p>0.05$) indicating both the complete removal of protecting ligands and a lack of effects of these protection protocols on [^{3}H]-strychnine binding (fig 3.14; 3.15; 3.16; 3.18 and 3.19).

Statistical comparison were made using the paired t-test between untreated and

treated membranes. The ability to effect the complete removal of protecting ligands also allowed valid comparison of treated membranes subjected to strychnine or glycine protection with treated membranes that were unprotected. Two criteria were thus used to define a protection: lack of significance when compared to untreated control but significant increase in binding when compared to treated membranes that were unprotected.

3.2.d.1 N-bromosuccinimide (fig 3.14)

Chemical treatment with NBS at pH 6 should ensure a relative selectivity for tryptophan residues (see Discussion). Reaction with NBS (1mM, pH 6 for 10 mins) caused a decrease in [^{3}H]-strychnine binding as shown by a significant decrease in total, strychnine and glycine-specific [^{3}H]-strychnine binding. In addition there was a decrease in the ability of glycine to displace total binding in the treated membranes which is not the case for unlabelled strychnine, as defined by strychnine-specific binding.

Strychnine and glycine protect against the loss of [^{3}H]-strychnine binding as defined by insignificant change in total [^{3}H]-strychnine and strychnine specific [^{3}H]-strychnine binding when compared to corresponding untreated controls. Although there was an increase in total and strychnine specific [^{3}H]-strychnine binding in strychnine and glycine protected treated membranes when compared to unprotected treated membranes, this is only significant ($p<0.05$) in the glycine protected membranes. These results suggest that both strychnine and glycine can protect against decrease in total and strychnine-specific binding caused by the NBS treatment although strychnine protection satisfies only one of the statistical comparisons.

In contrast, neither strychnine or glycine were able to protect against the loss of the ability of glycine to displace total [^{3}H]-strychnine binding. This was illustrated by the significant decrease in specific binding defined by glycine in unprotected, strychnine protected and glycine protected treated membranes when compared to the corresponding untreated controls. Thus, the glycine-specific [^{3}H]-strychnine binding was similar in unprotected, strychnine and glycine protected treated membranes.

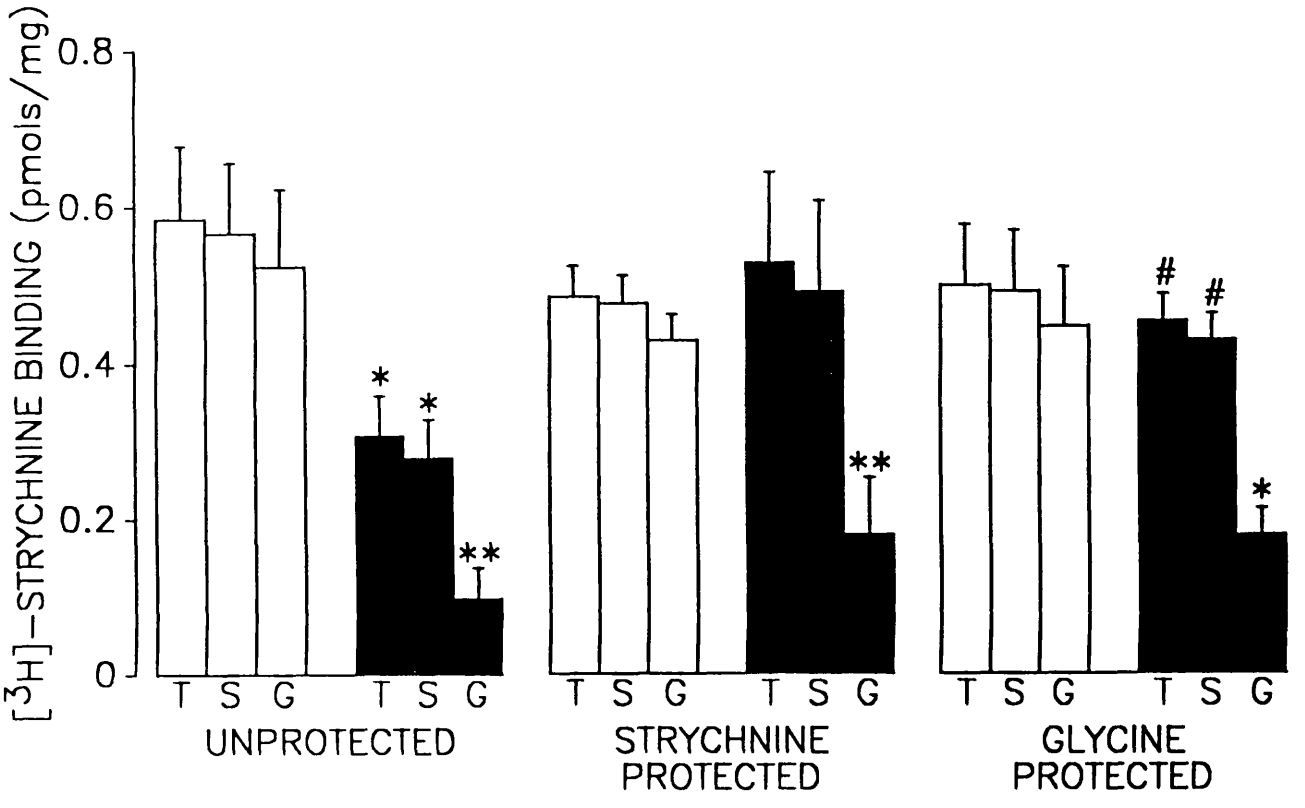


Fig 3.14: Effect of NBS treatment of mouse spinal cord membranes on subsequent [^{3}H]-strychnine binding: an assessment of strychnine and glycine protection. Untreated (open bars) or NBS treated (1mM, pH 6, 10 min, 21°C; solid bars) membranes were incubated with no ligand (unprotected), 10^{-4}M strychnine (strychnine protection) or 10^{-2}M glycine (glycine protection) prior to and during the modification procedure. The membranes were washed to remove protecting ligands and triplicate estimates of total (T), strychnine-specific (S) and glycine-specific (G) [^{3}H]-strychnine binding were made by assaying membranes in PBS pH 7.4. Values are the means of 5 treatments and the vertical bars indicate standard errors; significant differences from corresponding untreated control *$p<0.05$, **$p<0.01$ and from corresponding unprotected value #$p<0.05$(Students paired t-test).

3.2.d.2 Tetranitromethane (fig 3.15)

Chemical modification of spinal cord membranes with TNM was performed in PBS pH 8 to increase selectivity for tyrosine residues. Treatment with this reagent (15mM, pH 8, 15 mins, 21°C) produced a significant decrease in the total and the strychnine- and glycine-specific [^{3}H]-strychnine binding to unprotected treated spinal cord membranes when compared to untreated controls. The decrease in specific [^{3}H]-strychnine binding defined by glycine relative to specific [^{3}H]-strychnine binding defined by strychnine indicated that the ability of glycine to displace was perturbed in unprotected treated membranes.

The decrease in total and strychnine-specific binding can be prevented by both strychnine and glycine protection. This was indicated by the insignificant difference in the total and strychnine specific [^{3}H]-strychnine binding in strychnine and glycine protected treated membranes when compared to corresponding untreated controls. A protection by both ligands was paralleled by a significant increase in the total and strychnine specific [^{3}H]-strychnine binding in glycine and strychnine protected treated membranes when compared to unprotected treated membranes. The shared ability of both protecting ligands to prevent the decrease in total and strychnine specific [^{3}H]-strychnine binding was very similar to the effect described for chemical modification with NBS.

As described for reagent NBS, strychnine does not protect against the loss of the ability of glycine to displace total binding, as specific [^{3}H]-strychnine binding defined by glycine in TNM treated membrane was significantly different to corresponding untreated control membrane. This was the same effect that the TNM treatment produced in the unprotected membranes.

The glycine-specific [^{3}H]-strychnine binding in glycine protected TNM treated membranes indicated that glycine might partially protect against the loss of the ability to displace total [^{3}H]-strychnine binding that has been described in unprotected membranes. This parameter was increased significantly ($p<0.01$) in glycine protected treated membranes when compared to the level of glycine specific [^{3}H]-strychnine binding in unprotected treated membranes. In contrast there was a significant difference ($p<0.05$) in glycine specific [^{3}H]-strychnine binding in glycine protected treated membranes when compared to the corresponding untreated controls. This suggested that the ability of

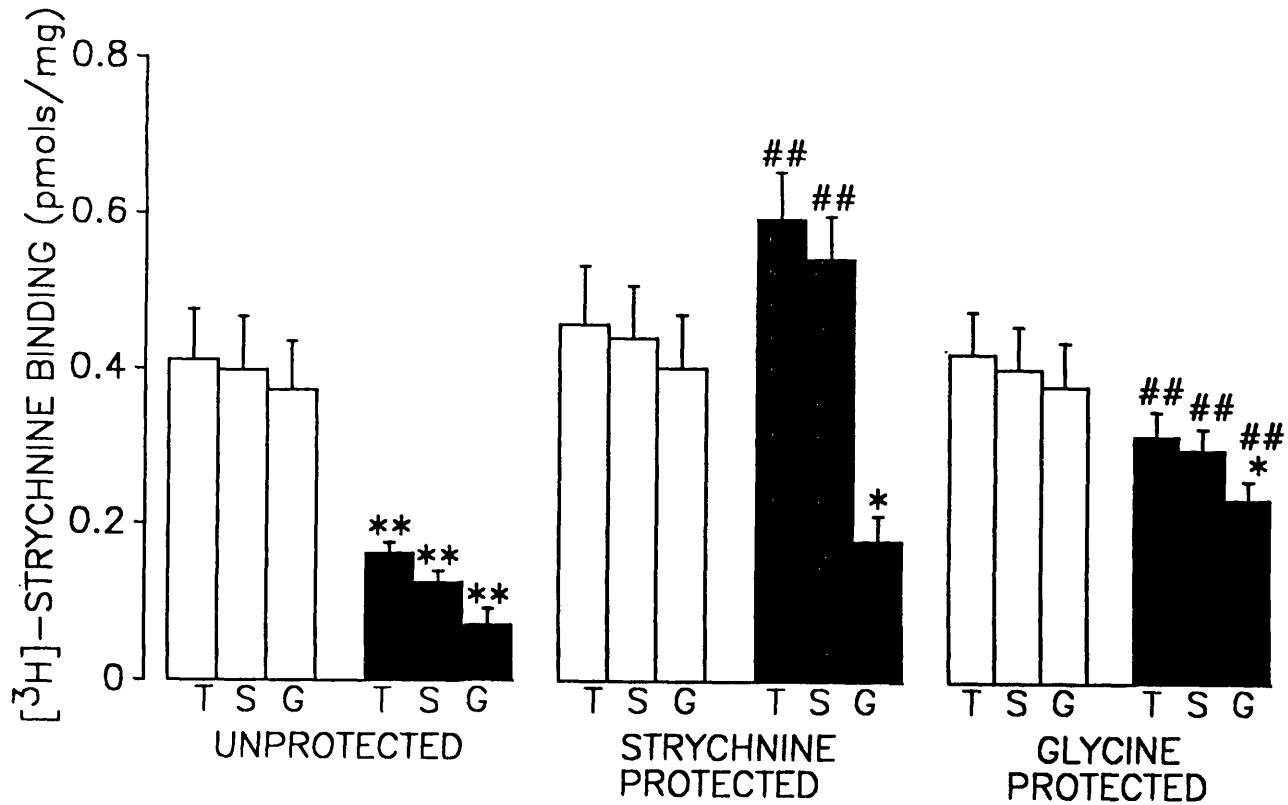


Fig 3.15: Effect of TNM treatment of mouse spinal cord membranes on subsequent [^{3}H]-strychnine binding: an assessment of strychnine and glycine protection. Untreated (open bars) or TNM treated (5mM, pH8, 15 min, 21°C; solid bars) membranes were incubated with no ligand (unprotected), 10^{-4}M strychnine (strychnine protection) or 10^{-2}M glycine (glycine protection) prior to and during the modification procedure. The membranes were washed to remove protecting ligands and triplicate estimates of total (T), strychnine-specific (S) and glycine-specific (G) [^{3}H]-strychnine binding were made by assaying membranes in PBS pH 7.4. Values are the means of 5 treatments and the vertical bars indicate standard errors; significant differences from corresponding untreated control *$p<0.05$, **$p<0.01$ and from corresponding unprotected value ##$p<0.01$ (Students paired t-test).

glycine to protect against the loss of glycine displacement of [^{3}H]-strychnine binding was not complete.

3..2.d.3 Diethylpyrocarbonate (fig 3.16)

Protein modification with DEP was performed at pH 6 to increase selectivity of this reagent for histidine residues. Treatment with DEP (20mM, pH 6, 20 min, 21°C) caused a significant ($p<0.01$) decrease in the total and strychnine- and glycine-specific [^{3}H]-strychnine binding in unprotected membranes when compared to untreated controls. As for NBS and TNM this decreased binding was accompanied by a decrease in the ability of glycine to displace total [^{3}H]-strychnine, as defined by glycine-specific [^{3}H]-strychnine. This contrasts with a lack of effect on the relative strychnine specific-[^{3}H]-strychnine binding which indicated that the ability of strychnine to displace total binding was unaffected.

In common with reagents TNM and NBS, the decrease in total and strychnine specific [^{3}H]-strychnine binding effected by DEP treatment of spinal cord membranes was prevented by strychnine and glycine protection. Thus, total and strychnine-specific [^{3}H]-strychnine binding in strychnine and glycine protected treated membranes was not significantly different to corresponding untreated controls. In addition the total and strychnine-specific [^{3}H]-strychnine binding was significantly increased ($p<0.05$) when compared to unprotected treated membranes. This was further evidence that glycine and strychnine protected against loss of total and strychnine-specific binding seen in treated membranes.

Membranes treated with NBS, TNM and DEP showed a decrease in the ability of glycine to displace total [^{3}H]-strychnine binding. In the two previously described treatment ligand protection against this was only clear in the TNM treated membranes with glycine. However in spinal cord membranes treated with DEP both strychnine and glycine afforded some protection against the decrease in ability of glycine to displace [^{3}H]-strychnine binding in unprotected membranes. The glycine-specific [^{3}H]-strychnine binding in both strychnine and glycine protected membranes was not significantly different ($p>0.05$) from corresponding untreated controls. There was also an increase in this parameter in strychnine and glycine protected membranes when compared to unprotected treated membranes. This increase was significant ($p<0.05$) in the strychnine protected membranes

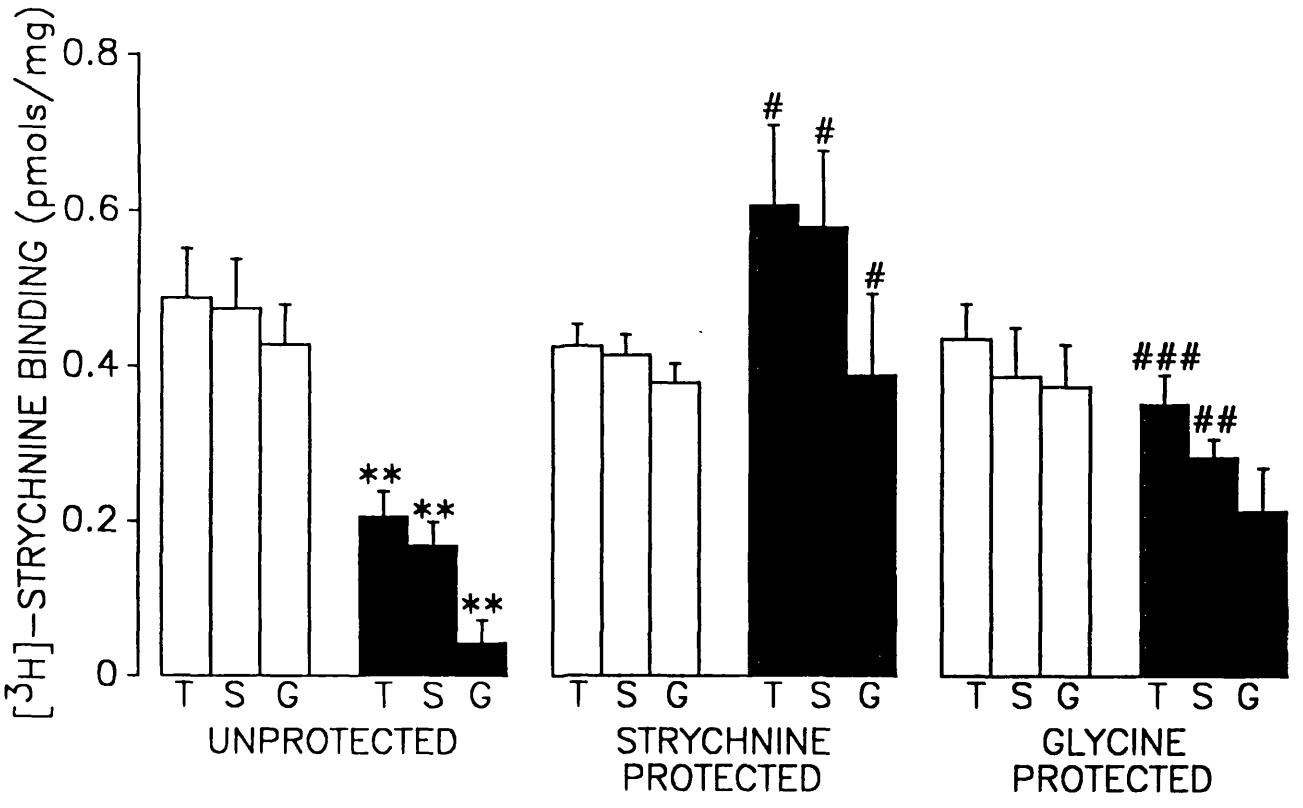


Fig 3.16: Effect of DEP treatment of mouse spinal cord membranes on subsequent [^{3}H]-strychnine binding: an assessment of strychnine and glycine protection. Untreated (open bars) or DEP treated (20mM, pH6, 20 min, 21°C; solid bars) membranes were incubated with no ligand (unprotected), 10^{-4}M strychnine (strychnine protection) or 10^{-2}M glycine (glycine protection) prior to and during the modification procedure. The membranes were washed to remove protecting ligands and triplicate estimates of total (T), strychnine-specific (S) and glycine-specific (G) [^{3}H]-strychnine binding were made by assaying membranes in PBS pH 7.4. Values are the means of 5 treatments and the vertical bars indicate standard errors; significant differences from corresponding untreated control **p<0.01, and from corresponding unprotected value #p<0.05 , ##p<0.01, ###p<0.001 (Students paired t-test).

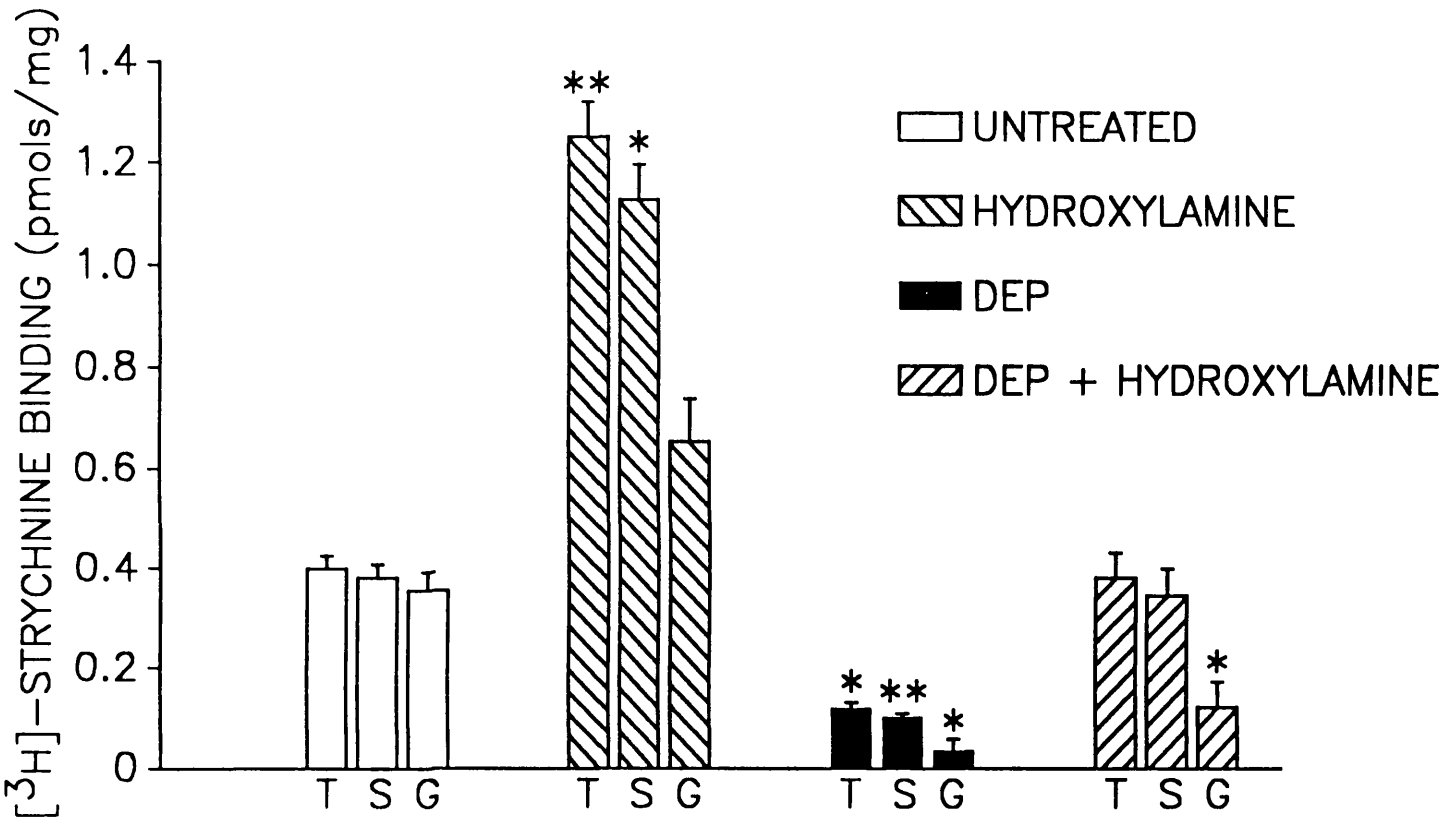


Fig 3.17: Effect of incubating DEP treated membranes with hydroxylamine on [^{3}H]-strychnine binding: Untreated or DEP treated (20 mM, pH 6, 20 min, 21°C) spinal cord membranes were washed and resuspended in PBS pH7 or hydroxylamine (0.5M) neutralized with HCl and incubated for 1 hour, 21°C. The membranes were washed and triplicate estimates of total (T), strychnine-specific (S) and glycine-specific (G), [^{3}H]-strychnine binding were made by assaying membranes in PBS at pH 7.4. Values are the mean of three treatments and the vertical bars indicate standard errors. Significant differences from corresponding untreated control #p<0.05, ##p<0.005 (Student paired t-test).

and only just insignificant in the glycine protected membranes ($p>0.05$).

Although the treatment of spinal cord membranes with DEP at pH 6 ensures a selectivity towards histidine residues of proteins the effect may be mediated through a reaction with lysine residues. This possibility can be investigated by testing for a reversal of the effect of chemical modification by reacting the modified preparations with neutral hydroxylamine which reverse the adduct formed between DEP and histidyl residues but not lysyl residues.

Mouse spinal cord membranes were treated with DEP and the reaction was terminated and the membrane washed as described. After the final wash treated and untreated membranes were either resuspended in hydroxylamine (0.5 M; 40 vols;tissue weight/vol; neutralized with HCl) or PBS and incubated for 1 hr at 21°C. The membrane were then taken through the same wash protocol used to remove DEP and finally resuspended in PBS for estimate of total and specific [^{3}H]-strychnine binding.

Treatment of spinal cord membranes with hydroxylamine alone caused an increase in total and specific [^{3}H]-strychnine binding (fig 3.17). Also the ability of glycine to displace total [^{3}H]-strychnine binding was reduced although the ability of unlabelled strychnine to displace total [^{3}H]-strychnine remained unaffected (fig 3.17). Effects of DEP alone were as described above (see figure 3.16). It appeared that hydroxylamine treatment was able to reverse the effect of DEP on total [^{3}H]-strychnine binding but not on the ability of glycine to displace total binding. However the increased total and strychnine specific [^{3}H]-strychnine binding, in DEP treated spinal cord membranes subsequently incubated in hydroxylamine, could be due to a direct effect of hydroxylamine on [^{3}H]-strychnine binding. The direct effect of hydroxylamine on the [^{3}H]-strychnine binding to untreated membranes precluded clear conclusion concerning the ability of hydroxylamine to reverse the effect of DEP on [^{3}H]-strychnine binding to spinal cord membrane.

3.2.d.4 Diazonium tetrazole (fig 3.18)

Spinal cord membranes were treated with diazonium tetrazole (approximately 2mM, 4°C, 1 hr) in PBS pH 7.4. DT is unstable and had to be synthesized as required, its production was determined by its reaction with n-acetylchlorotyrosine with which it forms a coloured product (see section 2.2.b.1). The experiment illustrated (fig 3.18) was

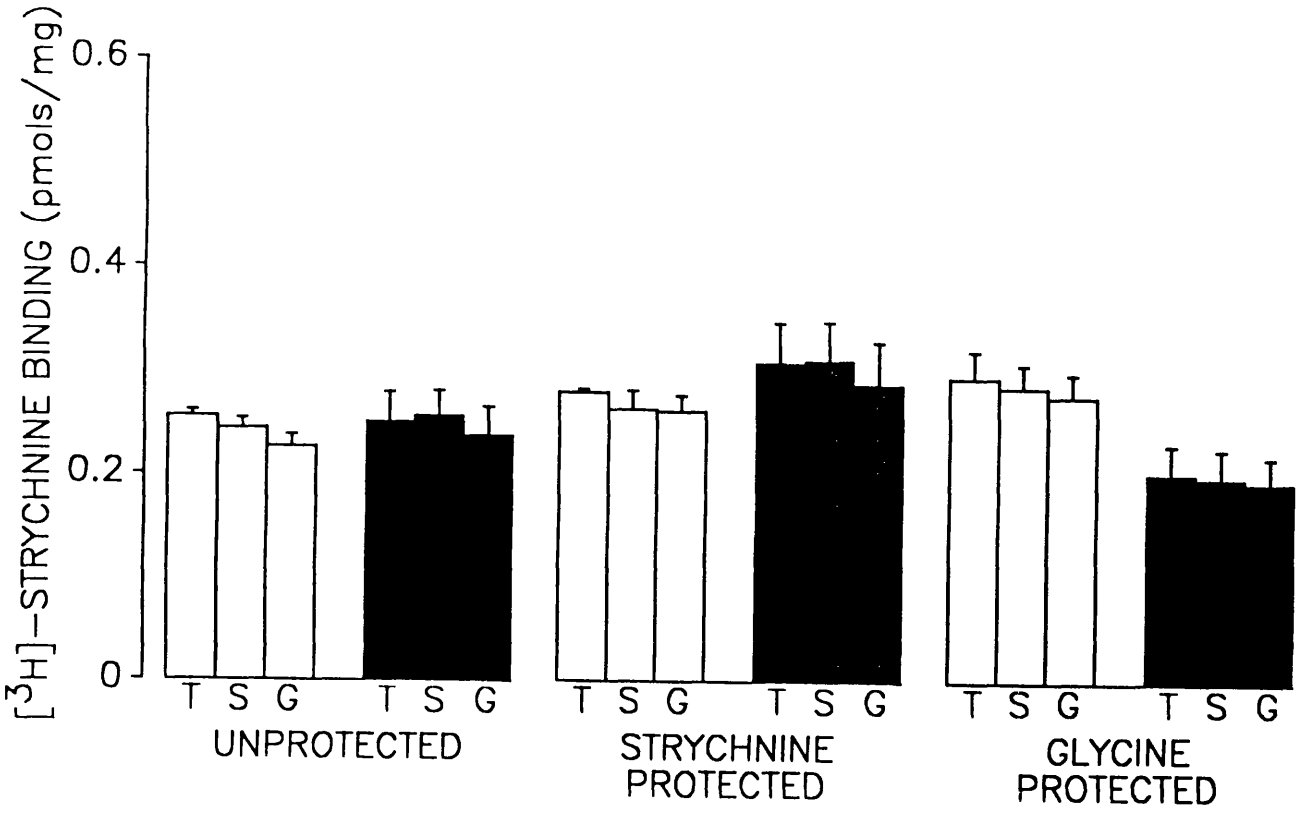


Fig 3.18: Effect of DT treatment of mouse spinal cord membranes on subsequent [^{3}H]-strychnine binding: an assessment of strychnine and glycine protection. Untreated (open bars) or DT treated (1.7 mM, pH7.4, 1 hr, 4°C; solid bars) membranes were incubated with no ligand (unprotected), 10^{-4}M strychnine (strychnine protection) or 10^{-2}M glycine (glycine protection) prior to and during the modification procedure. The membranes were washed to remove protecting ligands and triplicate estimates of total (T), strychnine-specific (S) and glycine-specific (G) [^{3}H]-strychnine binding were made by assaying membranes in PBS pH 7.4. Values for untreated membranes are the means of 2 treatments, values for the treated membranes are the mean of 4 treatments; the vertical bars indicate standard errors. Significant difference from corresponding unprotected value #p<0.05 , ##p<0.01, ###p<0.001 (Students unpaired t-test).

performed using synthesized DT that was concomitantly characterized spectrophotometrically by reaction with n-acetylchlorotyrosine. The results for untreated and treated membranes are the means of two and four observation respectively performed in triplicate. The data was compared using an unpaired t-test.

When compared to corresponding controls treatment of spinal cord membranes with DT at concentration estimated to be 1.7 mM had no effect on the total or specific [^{3}H]-strychnine binding whether defined by unlabelled strychnine or glycine. Although there was a decrease in subsequent [^{3}H]-strychnine binding to treated membranes that were glycine protected this was not statistically significant ($p>0.05$).

3.2.d.5 2,3-Butanedione (fig 3.19)

The reagent BD has a selective reactivity towards arginine residues. Chemical modification of unprotected mouse spinal cord membranes with BD (80 mM, 40 min, 21°C) caused an approximate tenfold increase in subsequent [^{3}H]-strychnine binding when compared to corresponding controls. This increase in the total [^{3}H]-strychnine binding to BD-treated spinal cord membranes was also seen in strychnine and glycine protected membranes. In the glycine protected membranes the increase in subsequent total [^{3}H]-strychnine binding was similar to unprotected membranes. However, in membranes protected with strychnine the increase in subsequent total [^{3}H]-strychnine binding was potentiated. This potentiation induced by incubating the membranes with strychnine prior to and during the modification was not statistically significant for data shown but has been reinvestigated and shown to be significant ($p<0.01$; see fig 6.5.a).

In unprotected, strychnine and glycine protected membranes the increase in total [^{3}H]-strychnine binding induced by BD was differentially sensitive to inhibition by unlabelled strychnine and glycine. This was indicated by differences in the strychnine- and glycine-specific [^{3}H]-strychnine binding. Similarly neither strychnine or glycine protection of BD treated spinal cord membranes caused the increased total [^{3}H]-strychnine binding to become glycine displaceable. This was in contrast to untreated membranes in which total [^{3}H]-strychnine binding was displaced equally well by the concentrations of unlabelled strychnine or glycine used to define specific [^{3}H]-strychnine binding.

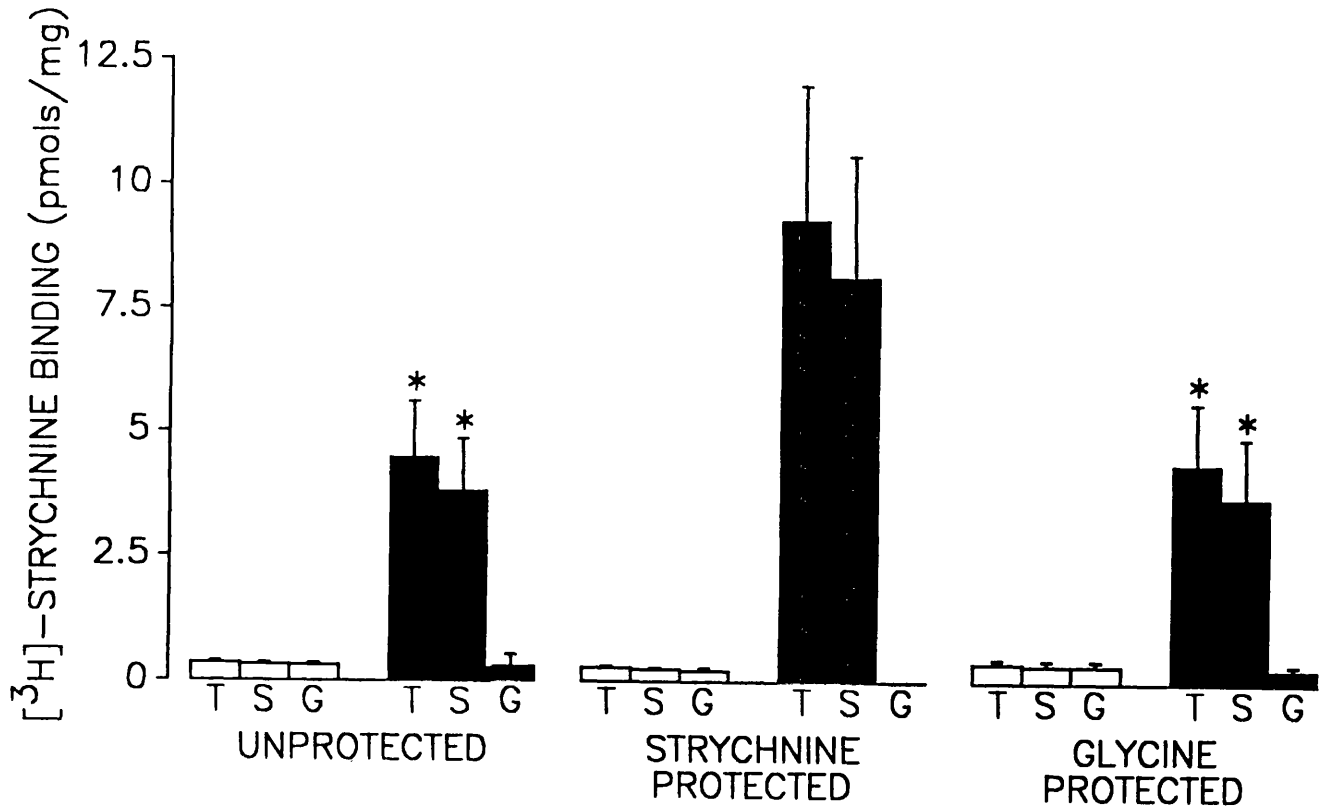


Fig 3.19: Effect of BD treatment of mouse spinal cord membranes on subsequent [^{3}H]-strychnine binding: an assessment of strychnine and glycine protection: Untreated (open bars) or BD treated (80mM, pH7.4, 40 min, 21°C; solid bars) membranes were incubated with no ligand (unprotected), 10^{-4}M strychnine (strychnine protection) or 10^{-2}M glycine (glycine protection) prior to and during the modification procedure. The membranes were washed to remove protecting ligands and triplicate estimates of total (T), strychnine-specific (S) and glycine-specific (G) [^{3}H]-strychnine binding were made by assaying membranes in PBS pH 7.4. Values are the means of 4 treatments and the vertical bars indicate standard errors; significant differences from corresponding untreated control *$p<0.05$ (students paired t-test).

3.2.d.6 Comparison of the effects of different arginine reagents on subsequent estimates of [^{3}H]-strychnine binding to treated mouse spinal cord membranes

The unexpected effect of the arginine selective reagent BD on subsequent [^{3}H]-strychnine binding to treated mouse spinal cord membranes prompted an investigation of other arginine selective reagents. A comparison of the effect of chemical treatment on subsequent [^{3}H]-strychnine binding to treated mouse spinal cord membranes was made for five arginine selective reagents BD, phenylglyoxal (PG), p-hydroxyphenylglyoxal (pPG), CHD and camphorquinone-10-sulphate (CQS). The first four of these reagents are classified as membrane permeable in contrast to CQS which is membrane impermeable.

Well washed mouse spinal cord membranes were treated under the conditions indicated in Table 3.3 which should ensure selectivity for arginine residues. Protein modification and ligand protection were performed as previously described before measurement of [^{3}H]-strychnine binding. Untreated membranes were subjected to the same procedures without the addition of protein modifying reagent and total [^{3}H]-strychnine binding was represented as percent untreated control. As for previous experiments specific [^{3}H]-strychnine binding was defined by binding assay incubations that contained unlabelled strychnine (10^{-4}M) or glycine (10^{-2}M). The % total [^{3}H]-strychnine binding displaced by strychnine and glycine as shown (Table 3.3) is used to indicate the specificity of total binding. For all untreated control membranes at least 85% total [^{3}H]-strychnine binding was displaced by unlabelled strychnine and glycine.

The effect of the arginine selective reagent BD on subsequent [^{3}H]-strychnine binding to mouse spinal cord membranes was as characterized previously. There was 10 fold increase in total and strychnine-specific binding and an inability of glycine to displace the increased binding. In addition, strychnine protection of spinal cord membranes appeared to potentiate the subsequent increase in total and strychnine specific [^{3}H]-strychnine binding. In contrast, BD treatment of glycine protected membranes had almost identical effects as those seen in unprotected membranes.

The pattern of total, strychnine-specific and glycine-specific [^{3}H]-strychnine binding to unprotected, strychnine and glycine protected spinal cord membranes treated with PG and pPG was qualitatively similar to that described for BD. Both reagents caused an increase in total and strychnine-displaceable [^{3}H]-strychnine to treated spinal cord

Modification conditions	Unprotected			Strychnine protected			Glycine protected		
	Total binding % untreated control	% Total Strychnine displaced	% Total Glycine displaced	Total binding % untreated control	% Total Strychnine displaced	% Total Glycine displaced	Total binding % untreated control	% Total Strychnine displaced	% Total Glycine displaced
2,3-butanedione (BD) 80 mM PBS pH 7.4	1471	87	6	4814	88	0	1424	85	7
Phenylglyoxal (PG) 60mM PBS pH 7.4	1054	86	5	1848	87	13	1039	85	10
p-hydroxyphenylglyoxal (pPG) 60 mM PBS pH 7.4	196	92	14	660	91	30	192	84	0
1,2-Cyclohexandione (CHD) 80 mM PBB pH 8	98	81	58	170	87	46	173	90	61
Camphorquinone-10-sulphate (CQS) 20 mM PBB pH 8	59	99	74	140	98	90	121	98	94

Table 3.3 Comparison of the effects of different arginine selective reagents on subsequent [^{3}H]-strychnine binding to mouse spinal cord membranes: Mouse spinal cord membranes were untreated or treated with various arginine selective reagents for 40 min under the conditions shown above. Membranes were preincubated prior to and during the modification procedure with no ligand (unprotected), 10^{-4}M strychnine (strychnine protected) or 10^{-2}M glycine (glycine protected). The membranes were washed and triplicate estimates made of the level of total [^{3}H]-strychnine binding to untreated and treated membranes. The level of binding to treated membranes is represented as % binding in untreated controls. The % of total binding to treated displaced by unlabelled ligands was estimated by including assay incubations that contained unlabelled strychnine (10^{-4}M) or glycine (10^{-2}M). In the untreated, unprotected, strychnine and glycine protected membrane at least 85% of total binding was displaced by unlabelled strychnine or glycine. Values are the mean of two experiments determined in triplicate.

membranes. As indicated by the low values for % total binding inhibited by glycine, this increased [^{3}H]-strychnine binding was not displaced by unlabelled glycine. In addition, the increase in total [^{3}H]-strychnine binding was potentiated in treated membranes that were strychnine protected. However, a quantitative comparison of the effects produced by membranes treated with p-hydroxyphenylglyoxal and phenylglyoxal under apparently identical conditions indicated that phenylglyoxal was the more potent compound.

Modification with the membrane permeable arginine selective reagent 1,2 cyclohexanedione had no detectable effect on total [^{3}H]-strychnine binding to unprotected membranes. However, when compared to corresponding untreated controls, treatment of strychnine or glycine protected spinal cord membranes with CHD did exert some effect on subsequent [^{3}H]-strychnine binding. Total [^{3}H]-strychnine binding is slightly elevated in strychnine protected membranes, although this effect was less marked then described for the three other membrane permeable arginine specific reagents. In addition when compared with unprotected treated membranes the [^{3}H]-strychnine binding was increased in glycine protected treated membranes. This was not seen for the other three membrane permeable reagents. In unprotected, strychnine and glycine protected membranes treated with CHD, the percent total binding displaced by unlabelled strychnine was at least 80%. This was in good agreement with the corresponding untreated control membranes (data not shown). In contrast, the percent of total binding displaced by unlabelled glycine in unprotected and ligand protected membranes was lower than 80%, the percent total that unlabelled glycine displaced in corresponding untreated controls. Thus CHD treatment caused a differentiation in the ability of glycine and strychnine to displace total [^{3}H]-strychnine binding in treated membranes which was not prevented by either strychnine or glycine protection. It was interesting that this effect was not apparent in the earlier optimization experiments which used a lower concentration of CHD to modify spinal cord membranes (see section 3.6.b).

The final reagent tested was the membrane impermeable arginine selective reagent CQS. This reagent caused a decrease in total [^{3}H]-strychnine binding in unprotected membranes. The % total binding displaced by unlabelled strychnine or glycine was 99% and 74% respectively. As at least 85% of the total binding was displaced by these ligands in corresponding untreated membranes this might suggest a slight effect of this treatment on the ability of glycine to displace total [^{3}H]-strychnine binding. Experiments in which the membranes were protected with strychnine or glycine indicated that both ligands can

prevent the decrease in total [^{3}H]-strychnine binding. Also the slight difference in percent total binding displaced by glycine in unprotected CQS treated spinal cord membranes is prevented by strychnine and glycine protection.

3.2.e Chemical modification of anti 2-AS-KLH sera

The effects of chemical modification of strychnine binding antisera on its subsequent ability to bind [^{3}H]-strychnine were investigated. The antisera raised against the hapten 2-aminostrychnine have been shown to have to have a pharmacological specificity for strychnine and structurally related alkaloids similar to the strychnine recognition site in mouse spinal cord membranes. However [^{3}H]-strychnine binding to the antisera is not inhibited by glycine or other agents thought to act through the glycine recognition site at the inhibitory glycine receptor.

As for the previously described experiments on mouse spinal cord membranes, the specificity of the effects of chemical modification on the ligand recognition was investigated using protection experiments.

Antisera diluted 1/320 in a final volume of PBS (200 µl) at the appropriate pH were treated and reacted with modifying reagent (see section 2.9.c.1). Strychnine protection was attempted by preincubating the antisera with unlabelled strychnine (10^{-5}M) prior to and during chemical treatment with protein modifying reagents. The concentration of unlabelled strychnine represents a 1000 fold excess over the estimated number of strychnine binding sites present in a serum dilution of 1/320 (rabbit 1, Phelan *et al.*; 1989).

3.2.e.1 Optimising the removal of unlabelled strychnine

The protection experiments require the removal of unlabelled protecting ligand to ensure valid estimates of [^{3}H]-strychnine binding to modified antisera can be made. An experiment was performed to compare the [^{3}H]-strychnine in untreated antisera preincubated with or without strychnine (10^{-5}M) after increasing number of washes by ammonium sulphate precipitation prior to assay (fig 3.20). However after 10 washes the estimated [^{3}H]-strychnine binding in the antisera preincubated with strychnine did not

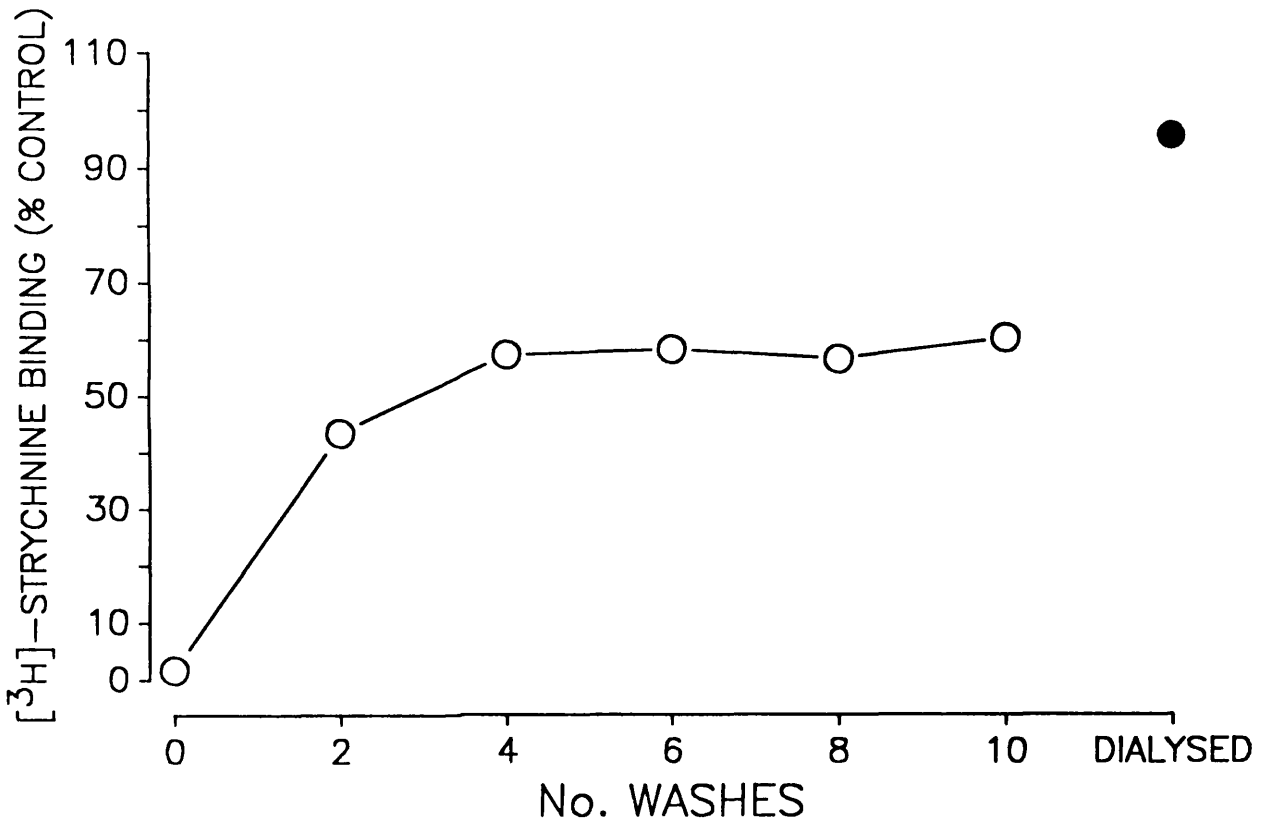


Fig 3.20: Optimizing the removal of unlabelled strychnine from 2-AS-KLH antisera prior to measurement of [^{3}H]-strychnine binding: Antisera (1/320) were incubated with no ligand or with unlabelled strychnine (10^{-5}M). The incubations were washed by ammonium sulphate precipitation. Quadruplicate estimates of [^{3}H]-strychnine binding were made to antisera subjected to increasing number of washes (○). A batch of antisera that were washed 4 times were also subjected to extensive dialysis (●) before estimating the [^{3}H]-strychnine binding. The binding to 2-AS-KLH antisera preincubated with strychnine was expressed as % of binding to antisera preincubated with no ligand but subjected to the same precipitation and dialysis procedures.

return to the amounts estimated in antisera taken through the same procedure without preincubation with strychnine (see fig 3.20). This suggested that the concentrations of unlabelled strychnine used for ligand protection were not removed by this procedure. Complete removal of the unlabelled strychnine could be achieved by washing the protected antisera four times by ammonium sulphate followed by an extended dialysis (see section 2.9.c.2). This ensured that [^{3}H]-strychnine binding in antisera preincubated with strychnine was similar to that taken through the same procedure to which unlabelled strychnine was not added during the incubation period.

3.2.e.2 The effect of chemical modification on subsequent [^{3}H]-strychnine binding to 2-AS-KLH antisera.

Experiments were designed so that a comparison of the effects of chemical modification of antisera on subsequent ability to bind [^{3}H]-strychnine and the ability of strychnine protection to prevent these changes could be made within individual experiments. Thus both untreated and treated antisera were subjected to preincubation with strychnine for comparison with antisera not subjected to strychnine protection. Treated antisera were incubated under identical conditions but the addition of reagent was controlled for by appropriate volume of solvent control. The reactions were terminated and the antisera dialysed as described above (see section 3.9.a) and assayed for [^{3}H]-strychnine binding (see section 2.10.c).

The effects of five protein modifying reagents under conditions used for treatment of mouse spinal cord membranes (see Table 3.4) were investigated for effects on [^{3}H]-strychnine binding to antiserum. These experiments indicated that NBS, TNM, DEP, TNBS and BD were all able to effect a significant decrease in the [^{3}H]-strychnine binding in unprotected antiserum when compared to untreated controls (see Table 3.4). An ability to prevent these decreases in [^{3}H]-strychnine binding to treated antiserum by strychnine protection was investigated and used to intimate that the effect of chemical modification was mediated at the strychnine binding site of the antibody. A complete prevention of the effects of chemical modification of the antiserum as indicated by an increase in [^{3}H]-strychnine binding levels to those of untreated controls was never seen. However, a significant protection against the decrease in [^{3}H]-strychnine binding to antiserum caused by treatment with NBS, TNM and BD was shown. This was indicated by a significant increase in the [^{3}H]-strychnine binding to antisera that were protected during modification

Reagent	Reaction conditions	% Untreated control	
		Unprotected	Strychnine protected
n-bromosuccinimide (NBS)	1mM, pH6, 10 min, 21°C	2.3 (±0.8)#; n=2	17.6 (±0.7)*; n=2
tetranitromethane (TNM)	5mM, pH8, 15 min, 21°C	24.3 (±1.4)#; n=3	56.8 (±2.7)*; n=3
diethylpyrocarbonate (DEP)	20mM, pH6, 20 min, 21°C	71.3 (±7.4)#; n=3	77.1 (±1.5)*; n=3
2,4,6,-trinitrobenzene -sulphonic acid (TNBS)	20mM, pH8, 20 min, 21°C	49.2 (±3.8)#; n=3	51.3 (±1.7)*; n=3
2,3-butanedione (BD)	80mM, pH7.4, 40 min, 21°C	46.9 (±9.8)#; n=3	79.2 (±5.4)*; n=3

TABLE 3.4 [^{3}H]-strychnine binding to chemically modified 2-aminostrychnine keyhole limpet haemocyanin antisera: antisera (1/320) were untreated or treated with protein modifying reagent under conditions cited. Batches of untreated and treated antisera were preincubated prior to and during the modification period with no ligand or 10^{-5}M unlabelled strychnine (strychnine protected). Antisera were washed and subjected to dialysis prior to making quadruplicate estimates of the level of [^{3}H]-strychnine binding to unprotected and strychnine protected treated antisera is expressed as % the level of binding to untreated control subjected to identical incubation conditions. Values are means and standard errors of the number of experiments indicated are shown in brackets. Significant differences from untreated control, # $p < 0.05$; from unprotected antisera * $p<0.05$. (Student paired t-test).

with these reagent (see Table 3.4).

In contrast although antisera showed a significant decrease in [^{3}H]-strychnine binding when treated with TNBS and DEP this effect was not considered specific to the recognition site of the antibody as strychnine did not afford any protection against the decreased binding.

3.3 Discussion

3.3.a The interaction between strychnine and glycine binding sites at the inhibitory glycine receptor

In the experiments described, [^{3}H]-strychnine binding to well washed mouse spinal cord membranes was used to further understanding of the interaction between the glycine and strychnine recognition sites of the inhibitory glycine receptor. This was prompted by a study from this laboratory which described the inhibition of [^{3}H]-strychnine binding by increasing concentration of unlabelled glycine as a fully competitive interaction that produced a Hill Plot with a slope equal to one (Fry and Phelan, 1986; Phelan, 1987). This contradicted the results of two earlier studies that were designed to consider the strychnine/glycine interaction (Young and Snyder, 1974a; Marvizón *et al.*, 1986a).

Such disparities could have arisen through differences in the assay conditions used. This was highlighted by an experiment in which the inhibition of [^{3}H]-strychnine binding by increasing unlabelled strychnine and glycine was compared between assay set up under identical conditions but terminated through glass fibre filters that were untreated or presoaked (0.1% polyethylenimine) prior to filtration. In the experiment terminated using unsoaked glass fibre filters, glycine appears only to partially inhibit [^{3}H]-strychnine binding even at very high concentrations. In addition when this data was analyzed as a Hill plot it gave values for the slope that were significantly less than one. In comparison in the assays terminated using PEI treated glass fibre filters, glycine acts as a full inhibitor of [^{3}H]-strychnine binding and the Hill coefficient derived from Hill slope are equal to one. Thus the inhibition of [^{3}H]-strychnine binding by unlabelled glycine in assay filtered through unsoaked filters appear to produce data that is compatible with the partial inhibition model previously proposed. However, this data appears to have arisen through

an artifactual binding to glass fibre filters used to separate free and bound ligand. This is also suggested by results from another laboratory in which the glass fibre filters are also treated and whose studies on the inhibition of [^{3}H]-strychnine binding by unlabelled glycine are also described by Hill plots with slopes not significantly different from one (Becker *et al.*, 1986). It is likely that pretreatment of glass fibre filters with polycations; polylysine (Becker *et al.*, 1986) or polyethylenimine blocked negative charges associated with glass fibre filters with which strychnine in its protonated form would interact. Although strychnine was able to inhibit [^{3}H]-strychnine binding to untreated glass fibre filters glycine did not share this property. As the interaction between [^{3}H]-strychnine and the glass fibre filters was likely to occur through a charge effect; ionic strength of the assay buffer could modulate the level [^{3}H]-strychnine binding to glass fibre filters. Thus, the phenomenum which previously described that the degree the partial inhibition, of [^{3}H]-strychnine binding by glycine, to be dependent on the ionic strength of the assay buffer (Marvizón *et al.*, 1986b) could be explained by artifactual binding to untreated glass fibre filters rather than modulation of the ligand recognition properties of the inhibitory glycine receptor. Also, the inability of glycine to inhibit [^{3}H]-strychnine binding to untreated glass fibre filters probably explains the differences seen in Hill plots derived from inhibition of [^{3}H]-strychnine binding by unlabelled glycine that were terminated through untreated filters. Hill slopes equal to one were described for the inhibition of [^{3}H]-strychnine by glycine and structurally related amino acid ß-alanine; however, these experiments used unlabelled glycine to define the level of non-specific binding (Braestrup *et al.*, 1986). In contrast experiments in which the inhibition curves are described by Hill plots significantly less than one (Marvizón *et al.*, 1986a; Schaffer and Anderson, 1981) used unlabelled strychnine to define non-specific binding. Thus defining non specific binding with unlabelled glycine may offer another means of avoiding the misinterpretation of filter binding as glycine receptor binding. In contrast, Marvizón and Skolnik (1988) made an effort to control for this by using a concentration of unlabelled strychnine (10^{-6}M) that inhibits [^{3}H]-strychnine to receptors but not glass fibre filters.

Differences in assay conditions might also explain discrepancies in data from this laboratory and another which have shown that inhibition of [^{3}H]-strychnine binding by increasing concentration of unlabelled glycine produces Hill plots with a slope greater than 1 (Young and Snyder, 1974a). This suggested that there was a positive co-operativity between the glycine and strychnine recognition sites. When the pH of the incubation

buffer was raised from pH 7.1 to pH 7.4 the estimated Hill slopes were much closer to one but whether or not it was significantly greater than one was not indicated (Young and Snyder, 1974a). Interestingly, the experiments from this laboratory and others (Becker *et al.*, 1986) which have described Hill slopes, for the inhibition of [^{3}H]-strychnine by glycine equal to one use assay incubated at pH 7.4. However there are experiments in which the inhibition of [^{3}H]-strychnine binding by unlabelled glycine was measured at pH 7.1 yet described Hill plots with slopes not significantly different from one (White, 1985). Thus, reasons other than differences in pH of the incubation medium could underlie discrepancies in the Hill slopes derived from experiments that consider the inhibition of [^{3}H]-strychnine binding to spinal cord membranes by unlabelled glycine. In the experiments which describe a positive co-operativity between the glycine and strychnine recognition sites the incubations were performed under conditions in which receptor number was between 20-70% of the estimated K_D (Young and Snyder, 1974a). It has been shown that in binding assays at which receptor number was greater than 10% of the estimated K_D the binding isotherms may exhibit positive cooperativity. This deviation is due to the membrane preparation binding significant proportions of free ligand (Bennet and Yamamura, 1985).

In contrast to the experiments which described a positive co-operativity for the interaction between strychnine and glycine, the [^{3}H]-strychnine binding assays performed in our laboratory have receptors at a concentration which were about 2.5% of the estimated K_D. Thus 0.05-0.07 mg membrane protein was routinely used for the study of [^{3}H]-strychnine binding to mouse spinal cord; a preparation which has a B_{max} of approximately 1000 fmol/mg protein and a K_D of 6nM (Phelan,1987). Thus in addition to previously discussed differences in incubation buffer, species of animal (Phelan, 1987) and pH (see above) the detection of positive co-operativity between strychnine and glycine binding sites could relate to the amount of membrane protein used in the relative binding assays.

Thus the data from this laboratory are compatible with the interaction between glycine and strychnine, at the inhibitory glycine receptor, being fully competitive in nature (Fry and Phelan, 1986; Phelan 1987). It appeared from the foregoing discussion that different experimental conditions can explain the discrepancy between this description and those previously used to explain the interaction between glycine and strychnine. However

interaction which are fully competitive usually arise through an interaction between two ligands or substrates at a common binding site. The conclusion that glycine and strychnine interact in this manner would be incompatible with experimental evidence that has suggested that there are distinct binding sites for agonist and antagonist on the inhibitory glycine receptor protein. (Young and Snyder, 1974a; Marvizón *et al.*, 1986a; Ruíz-Gómez *et al.*, 1989; Ruíz-Gómez *et al.*, 1990).

3.3.b The gross relationship of the strychnine and glycine binding sites at the inhibitory glycine receptor as revealed by protein modification of [^{3}H]-strychnine binding to spinal cord membranes

In common with previous experiments (Young and Snyder, 1974a, Marvizón *et al.*, 1986a; Ruíz-Gómez *et al.*, 1989; 1990) a comparison of the parameters of [^{3}H]-strychnine binding to a crude receptor preparation that were untreated or chemically treated with protein modifying reagents was made in an attempt to readdress the nature of the interaction between strychnine and glycine binding sites. A comparison of the effects of chemical modification on total [^{3}H]-strychnine binding and the ability of unlabelled strychnine and glycine to inhibit binding to treated membranes allows an investigation of the topography of the glycine and strychnine binding site. However the limited specificity of these protein modifying reagents and multiplicity of reactivities within a single protein mean that residue(s) other than those directly involved in ligand binding might be modified. For this reason the specificity of the reagent for the binding site was addressed using protection protocols. These experiments involved incubation with concentration of glycine or strychnine that fully occupied the binding sites in the receptor preparation during protein modification procedure. It was pertinent that these procedures had no direct effect on the subsequent measurement of parameters of [^{3}H]-strychnine binding to spinal cord membranes. This was shown by experiments which used untreated membranes subjected to protection protocols (fig 3.14, 3.15, 3.16, 3.18 and 3.19). This contrasted with the previous experiments for the $GABA_A$ receptor (Maksay and Ticku, 1984a).

The protein modification experiments reported in this study allowed an investigation of the gross topography of the strychnine and glycine binding sites. In addition, by considering the reactivity of the reagents tested the nature of residues involved in ligand binding could be addressed.

In terms of addressing the gross nature of the strychnine and glycine binding sites, at the inhibitory glycine receptor, three reagents NBS, TNM and DEP proved revealing. These three reagents were all shown to cause a decrease in total [^{3}H]-strychnine binding to treated spinal cord membranes when compared to untreated controls. Although total [^{3}H]-strychnine binding was reduced the proportion of this total binding inhibited by unlabelled strychnine was similar to controls. This implied that a population of remaining binding sites unlabelled strychnine can bind and inhibit [^{3}H]-strychnine binding. In contrast the proportion of total [^{3}H]-strychnine binding inhibited by unlabelled glycine was much reduced in the membranes treated with NBS (fig 3.14), TNM (fig 3.15) and DEP (fig 3.16). Thus these reagents effect a modification which either prevent glycine binding to the receptor or upon binding prevented it inhibiting [^{3}H]-strychnine binding.

Although modification with TNM, NBS and DEP effected the interaction of both strychnine and glycine with the inhibitory glycine receptor it was not clear whether the effects of the reagents on the strychnine and glycine interaction with the receptor protein are mediated through common or distinct reactivities. In spinal cord membranes that were protected with glycine and strychnine during protein modification with TNM, NBS or DEP, the decrease in total [^{3}H]-strychnine binding reported in the unprotected membranes was prevented. In the protected membranes the total [^{3}H]-strychnine binding was not significantly from corresponding untreated controls. In addition, the total [^{3}H]-strychnine binding to treated membranes was increased when compared to unprotected membranes, this increase was statistically significant for TNM (fig 3.15) and DEP (fig 3.16) treated membranes. As these reagents effect no change in the strychnine-specific [^{3}H]-strychnine binding the pattern of protection described for total binding was paralleled for the strychnine-specific [^{3}H]-strychnine binding.

The fact that both ligands are able to protect against the decrease in total and strychnine specific [^{3}H]-strychnine binding, caused by chemical modification of spinal cord membranes with NBS, TNM and DEP, supports the possibility that strychnine and glycine interact at a common binding site. This description would be compatible with this laboratories kinetic data (Fry and Phelan, 1986; Phelan, 1987). However, it requires further discussion in view of the pattern of the ligand protection for the parameter of [^{3}H]-strychnine binding defined by unlabelled glycine. As described, in spinal cord membranes treated with NBS (fig 3.14), TNM (fig 3.15) and DEP (fig 3.16), there was a decrease in the proportion of total [^{3}H]-strychnine binding inhibited by glycine. If the effects of these

reagents on this parameter were mediated solely through a modification at a common binding site, one would expect both strychnine and glycine protection to successfully prevent this decrease. Neither strychnine or glycine protection prevent the decrease in the proportion of total [^{3}H]-strychnine binding inhibited by unlabelled glycine, caused by NBS treatment, in spite of the shared ability of the two ligands to prevent the decrease in total [^{3}H]-strychnine binding. An explanation for this apparent divergence could be that NBS effects a modification at a common binding site but has additional effects on the receptor protein that are not directly involved in ligand binding but are important in the interaction between glycine and strychnine.

Since glycine exhibits a differential ability to protect against the effects of protein modification, on total and strychnine specific [^{3}H]-strychnine binding when compared to glycine specific binding, this is taken as indirect evidence that protection was not due to a direct reaction between protecting ligand and reagent.

TNM causes a similar effect to that described above, on the ability of glycine to inhibit total [^{3}H]-strychnine binding, when used to chemically modify membranes. In this instance glycine protection partially prevented the decrease in the ability of glycine to inhibit total [^{3}H]-strychnine binding, however strychnine afforded no protection. It has already been suggested the shared ability of strychnine and glycine protection to prevent the effect of this reagent on total [^{3}H]-strychnine binding suggested a common binding site. As discussed for the reagent NBS, the fact that both ligands do not protect against the decrease in [^{3}H]-strychnine binding defined by unlabelled glycine might be explained by a modification of TNM outside the common binding site. It was interesting to note that in the membranes treated with TNM glycine was able partially to protect against the decreased potency of glycine in inhibiting total [^{3}H]-strychnine binding. This might argue that the glycine and strychnine binding site although common, are not identical.

It was only in the case of membranes treated with DEP that both glycine and strychnine protection protocols were able to prevent the decrease in the proportion of total [^{3}H]-strychnine binding inhibited by glycine. This was paralleled by the common ability of strychnine and glycine protection to prevent the decrease in total [^{3}H]-strychnine to DEP treated spinal cord membranes. The most straightforward interpretation of these data is that modification of the inhibitory glycine receptor by DEP was mediated at the common binding site and strongly suggestive of a DEP sensitive residue at this common

binding site.

Thus the experiments provided evidence that strychnine and glycine might share a common binding site at the inhibitory glycine receptor. However it appeared that the interaction between the two ligands could be perturbed by modifications that could not be accounted for by reactions with the ligand binding sites. By considering some of the general principles that are thought to underlie the interaction of competitive antagonist and agonist with proteins a model has been developed which includes various facets of the presented data and offers an interpretation to explain the interaction between strychnine and glycine at the inhibitory glycine receptor.

Competitive antagonists are considered to bind to receptor and the occupation of the recognition site is associated with minor conformational change relative to the non-liganded receptor. In comparison upon binding an agonist the receptor undergoes more drastic conformational change. Thermodynamically this is represented as the agonist molecule falling to an energetically lower state when bound. This energy is transferred to the receptor protein structure and expressed as a change in receptor conformation (Bowman and Rand, 1980) which in the case of the glycine receptor would presumably open the anion channel. In this scheme, although agonist and antagonist bind to a common site residues outside this site might be important in the interaction between two ligands if they are involved in conformational changes in the receptor protein in response to ligation.

Conformational differences between receptor bound by agonist or antagonist may underlie the differential effect of TNM and NBS on the ability of glycine to inhibit total [^{3}H]-strychnine binding to treated membranes. Thus by effecting a modification of residues required for conformational changes on agonist binding might have hindered the inhibition of [^{3}H]-strychnine binding by glycine. Glycine protection would induce receptor into an agonist conformation during the treatment with modifying reagent in addition to protecting the ligand binding site by physically occupying it. The induced conformational change could have masked residues that are modified in the unprotected preparations and explain the partial protection afforded by glycine against the decrease in the ability of glycine to inhibit [^{3}H]-strychnine in TNM treated membranes.

The proposed model has two requirements; firstly the inhibitory glycine receptor

adopts different conformational states on binding glycine or strychnine; secondly that the receptors interaction with agonist induces a more marked conformational change.

The conformational flexibility of the inhibitory glycine receptor is most clearly inferred from electrophysiological studies which indicate that the protein can exist in multiple states which are thought to relate to distinct conformations (Hamill *et al.*, 1983; Twyman and Macdonald, 1991) . It should be noted that these experiments also highlight that the conformational states of the glycine receptor are more complex than proposed in the model outlined above, the kinetic schemes evoking the existence of multiple agonist states.

The concept that there is a more marked change in conformation associated with agonist binding is inferred from the higher affinity that antagonists exhibit for receptors relative to agonists. Indirect experimental evidence for this comes from studies using the fluorescent probe 1-(5-di-methylaminonaphthalene-1-sulphonamido)propane-3-trimethyl-ammonium iodide at the *Torpedo* electric organ nicotinic acetylcholine receptor (Cohen and Changeaux, 1973). These studies indicated that spectral shifts were apparent from the probe when agonist but not antagonist were added. This was taken to indicate that agonists induced change in receptor conformation in contrast to antagonists. However, it should be noted that at the nicotinic receptor, there has been a recent report that the competitive antagonist induced the more marked effect on receptor conformation (McCarthy and Stroud, 1989). The study used tritium hydrogen exchange which reports on the global changes in receptor conformation. Therefore, the conformational changes associated with receptor ligation remains an open question requiring further experimental investigation.

Thus this laboratory's experimental data can be explained by a model in which the interaction between strychnine and glycine occurs through a common recognition site which is conformationally distinct when bound with agonist or antagonist. This accounts for the described effect of chemical modification and previous kinetic description of the strychnine and glycine interaction (Fry and Phelan, 1986; Phelan, 1987). However it would appear that the above model is at odds with previous descriptions (Young and Snyder, 1974a; Marvizón *et al.*, 1986a) which argue on the basis of the differential susceptibility to chemical modification for physically distinct strychnine and glycine

binding sites.

It is interesting to note that the reagent diazonium tetrazole which had previously been shown differentially to effect the interaction between glycine and the inhibitory glycine receptor (Young and Snyder, 1974a; Marvizón *et al.*;1986a) was shown to have no effect on the parameters of [^{3}H]-strychnine binding to modified spinal cord membranes (see fig 3.18). This reagent has to be synthesized as required but inappropriate synthesis can not explain the lack of an effect, as the production of diazonium tetrazole was quantified by reacting it with the model phenol n-acetylchlorotyrosine.

In the previous studies that have evoked a distinct nature of the two binding sites, reagents were reported to differentiate between the strychnine and glycine binding site (Young and Snyder, 1974a; Marvizón *et al.*, 1986a; Ruíz-Gómez *et al.*, 1989; 1990). The reagents diazonium tetrazole and acetic anhydride showed a preferential reactivity towards disruption of the interaction of glycine with the inhibitory glycine receptor (Young and Snyder, 1974a; Marvizón *et al.*, 1986a). However they also caused a decrease in the [^{3}H]-strychnine binding which argues that they have effects at the strychnine binding site. In addition, although it was reported that only strychnine protection prevented the reduction of [^{3}H]-strychnine binding spinal cord membranes treated with PG, glycine also appeared to afford a partial protection against the effect of PG. Although there appears to be a differential susceptibility to some protein modifying reagents, for the interaction of ligands with the inhibitory glycine receptor, this has rarely been shown singularly to effect the agonist or antagonist interaction. This could relate to non- specific modifications but may also prove indicative of an overlap or common nature of the strychnine and glycine binding sites at the inhibitory glycine receptor.

Recently a chemical modification with the reagent flurescein isothiocyanate has been shown specifically to effect the interaction of glycine with the interaction of glycine with the inhibitory glycine receptor (Ruíz-Gómez *et al.*, 1989) thus providing strong argument for physically distinct strychnine and glycine binding sites. However as discussed previously modification of residues outside the common binding site could specifically effect the interaction between glycine and the receptor by perturbing the conformational changes induced by agonist binding.

It is interesting to note that recent experiments have indicated that the rate of dissociation of [^{3}H]-strychnine binding to spinal cord membranes in the presence of unlabelled glycine was reduced by chemical modification, although the [^{3}H]-strychnine was only slightly reduced (Maksay, 1990). This effect was attributed to modification of the allosteric interaction between distinct strychnine and glycine binding sites at the inhibitory glycine receptor. As the allosteric interaction could be accounted as a shift in conformation the effect of chemical modification on the interaction between glycine and [^{3}H]-strychnine is equally well interpreted as a modification of the receptor protein outside a common binding site. In conclusion much of the previous data that was taken to indicate the distinct nature of the two binding sites can be adequately accommodated by evoking an interaction between overlapping binding sites that are conformationally distinct.

The possibility that glycine and strychnine share a common binding site at the inhibitory glycine receptor has been proposed on the basis that there is a molecular congruence between the structure glycine and structure (Aprison *et al.*, 1987). This has been offered to explain how the apparently dissimilar structure of strychnine is able to antagonize the action of glycine.

3.3.c Speculations on the molecular structure of the strychnine and glycine binding sites on the inhibitory receptor

By considering the selectivity of the reagents, used in this study, for amino acids residues, the scope of the discussion can be expanded to consider the amino acid residues involved in ligand interactions at the inhibitory glycine receptor. It should be stressed that the reactivity that exist even between residues of one amino acid type means that the extrapolation of the effects of different reagents on [^{3}H]-strychnine binding to modified spinal cord membranes from a literature, based on the reactivity of the reagents towards purified proteins or model compounds, can only serve to guide interpretation of which residue might be involved.

On the basis of chemical modification with various reagents it has been speculated that lysine residues are important in the interaction between in the interaction between glycine and inhibitory receptor (Young and Snyder, 1974a; Marvizón *et al.*, 1986a; Ruíz-Gómez *et al.*, 1989). In this study we were unable to produce evidence for the role of

lysine residues using two reagents which have a selective reactivity for lysine residues. Chemical modification with pyridoxal phosphate followed by a subsequent reduction with sodium tetraborohydride can be used to specifically modify lysine residues (Uyeda, 1969). Indeed under identical reaction conditions used in this study PLP modification has been used to implicate a role for lysine residues at the GABA binding site of the $GABA_A$ receptor (Tunnicliff, 1979; Maksay and Ticku, 1984a). However what appeared to be a direct effect of borohydride treatment in [^{3}H]-strychnine binding to spinal cord membranes (fig 3.2) prevented any conclusions about possible role of lysine residues in ligand recognition. It is interesting to note that a direct effect of borohydride could be mediated by a reduction; indeed borohydride at a higher concentration has been used to specifically reduce one of six disulphide bonds of trypsinogen (Light *et al.*, 1969). However the possible role of disulphides in ligand recognition at the inhibitory glycine receptor was not obvious as will be discussed.

The second lysine selective reagent used in this study TNBS has proved a convenient method for estimating the amino group in proteins in spite of the compromised specificity which arises through disulphide cleavage under certain conditions (Fields, 1972). Treatment of mouse spinal cord membranes with this reagent caused no obvious effect on the subsequent [^{3}H]-strychnine binding. The lack of effect of TNBS modification on total [^{3}H]-strychnine binding or the ability of glycine to displace is surprising in view of previous reports that modification of spinal cord membranes with modifying reagents selective for amino groups caused little effect on total [^{3}H]-strychnine binding yet selectively inhibited the proportion of total binding displaced by glycine (Young and Snyder, 1974a; Marvizón *et al.*, 1986a; Ruíz-Gómez *et al.*, 1989). For the earlier reports (Young and Snyder, 1974a; Marvizón *et al.*, 1986a) which used diazonium tetrazole and acetic anhydride, relatively unselective reagents, the effect could have been due to modification of non-lysyl residues (Andres and Atassi, 1973). However, the more recent study used FI, under conditions which ensure selective reactivity, have stressed that a lysine residue is involved. For this reason it is assumed that TNBS under conditions used had little reactivity for the lysyl residue that was proposed to be modified by FI (Ruíz-Gómez *et al.*, 1989). The different reactivity of residue to different reagents could be caused by the effect of the protein environment lysyl residues (Glazer, 1976). However, It is interesting that the experiments were unable to reproduce the effect of diazonium terazole that had been previously reported (Young and Snyder, 1974a; Marvizón *et al.*,

1986a). For this reason it is speculated that the differences in the buffers in which the modifications were performed may also effect the reactivity. In this respect in this study the buffer contained 100 mM sodium chloride and this anion and cation that have previously been shown to modulate ligand recognition at the inhibitory glycine receptor (see section 1.4.a).

In addition to inferences being made on the molecular basis of ligand recognition at the inhibitory glycine receptor from the experimental work, speculations have been made based on comparison of the primary sequence of subunits derived from molecular cloning different classes of ligand gated ion channels (Grenningloh *et al.*, 1987). These comparisons highlight conservation of structural domains including the N-terminal region of in which the proposed ligand binding sites are thought to reside (see 1.6.b). In the case of the nicotinic acetylcholine receptor α subunit, whose sequence are thought to harbour the ligand binding site (Deneiris *et al.* 1991), two cysteine pairs have been found conserved. These two cysteine have been shown to be cystine linked in the α subunit from the Torpedo electric organ tissue (Kao and Karlin, 1986; Kellaris and Ware, 1989). Although both these cystine have been speculated to have a role in ligand recognition (McCormick and Atassi,1984), reduction of the vicinal cystine, which of the two cystine is located most distally from the N-terminal, has been shown to effect acetylcholine receptor function (Karlin, 1969) and located in close proximity to the subsite that binds the quaternary ammonium of the agonist acetylcholine (Karlin, 1969).

By homology it has been speculated that the cysteine pair at position 244-256 in rat α-1 glycine receptor (see Fig 1.3) are similarly disulphide bonded and functionally important. Although unlike the nicotinic acetylcholine receptor the cysteine pair in the glycine receptor is conserved in the ß-subunit. DTT treatment did not perturb the parameters of [^{3}H]-strychnine binding to treated binding to treated spinal cord membranes. Chemical treatment with this reagent specifically cleaves disulphide bridges in proteins. As this reaction leads to the production of an energetically favourable moiety this disulphide reduction can be achieved at relatively low reactant concentration (Konigsberg, 1972). In addition, membranes which were alkylated with NEM or IAA after DTT treatment had total and specific [^{3}H]-strychnine binding consistent with untreated membranes. This indicates that an effect of DTT treatment was not lost by reoxidation of disulpide during the washing procedure. The inability to detect an effect of DTT treatment

on [^{3}H]-strychnine binding may relate to the to a difference in the structure of the proposed cystine in the glycine which in contrast to the acetylcholine receptor is not vicinal. The amino acids spaced between the adjacent cysteines in the glycine receptor favouring stability (Thornton, 1981). Another possibility is that although lying in close proximity to the ligand binding site disulphide bridge was not directly involved in ligand recognition.In this respect it has been shown that chemical treatment of the reconstituted glycine receptor preparation with protein modifying reagent, PG and DSA, have a less deleterious effect on subsequent [^{3}H]-strychnine binding when the reactions are performed in the presence of DTT (Ruíz-Gómez *et al.*, 1990). This was taken to indicate a role for disulphides in modulation of receptor conformation rather than being directly involved in ligand recognition.

Consideration of the selective reactivity of the three reagents NBS, TNM and DEP under the conditions used should supplement the ideas on the molecular detail that underlie ligand recognition at the inhibitory glycine receptor.

NBS has been used to modify tryptophan residues in proteins through the oxidation of the indole ring (Spande *et al.*; 1970). However under conditions of excess reagent and low pH this reagent has been successfully used to cleave peptides at tryptophan, tyrosine and histidine residues (Spande *et al.*, 1970) and also effect bromination or oxidation of methionine, tyrosine, histidine and lysine residues (Imoto and Yamada, 1989). Nevertheless it has been argued that pH values closer to neutrality favour the differential oxidation of tryptophan residues without cleavage of the protein (Okada *et al.*, 1963). The oxidation of sulphydryls could still compromise the specificity of NBS as this modification can occur at pH 6. However the effect of NBS on [^{3}H]-strychnine binding to treated mouse spinal cord membranes are unlikely to occur through such a reaction because treatment with the selective sulphydryl reagents IAA or NEM have no effect on subsequent [^{3}H]-strychnine binding. Thus under the conditions used in these experiments the effect of NBS on parameters of [^{3}H]-strychnine binding to spinal cord membranes were likely mediated through a selective reactivity to tryptophan residues. It should be noted that the ability of NBS stopped at time 0 with an excess buffer containing BSA (see fig 3.7.a and 3.7.b) suggested that it was difficult to quench the reactivity of this reagent. This would increase the likely heterogeneity of the residues that react with NBS (Ohnishi *et al.*, 1980).

The protection experiments with NBS suggested that the reagent was not only directly modifying the common ligand binding site for strychnine and glycine but also effecting residues involved in the interaction between the two ligands. At present one can only speculate which amino acid residues might be important in the effects but it does agree with previous speculation that tryptophan residues might be involved in strychnine binding (Young and Snyder, 1974a). In view of the selectivity of NBS it is interesting to note that the tryptophan residues are precisely conserved in the inhibitory glycine receptor α subunits and the ß subunit that have thus far been sequenced (see fig 1.3). This precise conservation could be taken to suggest a structural or functional significance for these residues.

As for NBS it was concluded that TNM might be modifying residue at a common binding site for strychnine and glycine, in addition to effecting the interaction between the ligands. This reagent has a high selectivity for tyrosine residues (Riordan and Vallee, 1972b). At pH 8, the condition used in the protection experiments TNM also reacts with sulphydryls however as discussed for NBS, the effects of TNM on [^{3}H]-strychnine to TNM treated membranes was not likely to occur by such a reaction. However, under conditions in which the reagents is at a high molar excess over protein TNM can effect polymerizations (Vincent *et al.*, 1970) or oxidation of other residues most notably tryptophans (Sokolovski *et al.*, 1970). The modification of spinal cord membranes with TNM was performed with a lower reagent concentration than used in experiments to investigate polymerization (Vincent *et al.*, 1970) or reactivity towards tryptophanyl compounds (Sokolovski *et al.*, 1970) but it is difficult relate this reagent concentration to protein in crude membrane preparation. The fact that a second tyrosine selective reagent n-acetylimidazole (Riordan and Vallee, 1972c) effected similar changes as TNM in parameters of [^{3}H]-strychnine binding to treated spinal cord membranes (J. P. Fry personal communication; data not shown) argues in favour of the possibility that the effect of TNM was mediated through a reaction with tyrosine residues.

On the basis that TNM could modify the parameters of [^{3}H]-strychnine binding to spinal cord membranes (Young and Snyder, 1974a) it has been speculated that tyrosine residues are involved in strychnine binding at the inhibitory glycine receptor. Unlike this study, the effect of TNM seen by Young and Snyder (1974a) was not extended to consider the ability of ligand protection to prevent the changes [^{3}H]-strychnine binding.

However, it has been proposed that reactivity of a single tyrosine residue exclusive to the strychnine binding site was responsible for the decrease in [^{3}H]-strychnine binding to spinal cord membranes treated with diazotized sulphanilate (Ruíz-Gómez *et al.*, 1990).

Therefore there is convergent evidence that the aromatic residues tyrosine and tryptophan are directly involved in strychnine and glycine binding to the inhibitory glycine receptor. This idea is compatible with the proposal that aromatic residues at the ligand binding site are responsible for an energy transfer process which leads to the covalent incorporation of strychnine into the inhibitory glycine receptor upon exposure to ultraviolet light (Graham *et al.*, 1983).

Chemical modification with the tyrosine selective reagent DSA has also implicated a role for tyrosine residues in the interaction between glycine and strychnine (Maksay, 1990). Thus the implication of tyrosine residues at the ligand binding site and in the interaction between strychnine and glycine finds good agreement with the reported effect of TNM on [^{3}H]-strychnine binding in this study.

Unlike the tryptophan residues which are conserved in the sequenced α and ß subunits of the inhibitory glycine receptor, the tyrosine residues are not always conserved when comparisons between subunits are made (see fig 1.3). Expression of different subunits reveals that they can form functional channels which often vary in sensitivities to agonist and antagonist (Grenningloh *et al.*, 1990a; Grenningloh *et al.*, 1990b; Kuhse *et al.*, 1990a; Kuhse *et al.*, 1990b). A comparison of the tyrosine content of the N-terminus, region that harbours ligand binding sites, could implicate where important residues might lie. In this respect, note that 4 tyrosines located in the α 1 subunit which has expected affinities for agonist and antagonist are not conserved in the ß subunit, the so called non-ligand binding subunit which nevertheless has about 100 fold lower affinity for glycine and no detectable sensitivity to strychnine. It is interesting to note that the loss of tyrosine in the α-subunit at position which corresponds to 243 (see Fig 1.3) in the α-subunit is consistent with the proposal that this residue is important in ligand binding (Grenningloh *et al.*, 1987; Ruíz-Gómez *et al.*, 1990). However the possibility that the other tyrosine residues implicated here might be important can not be excluded. Indeed other residues are likely to play a role at the ligand binding site of the inhibitory glycine receptor because in the α2* subunit all the tyrosines are conserved in the N-terminal when

compared to α1. Yet α2* has been shown to represent a ligand binding subunit which has a lower affinity for glycine and strychnine than α1 (Kuhse *et al.*, 1990a).

The existence of aromatic residues at the ligand binding sites may emerge as a general phenomenum at ion channels gated by the ligands as they have been suggested to be important at $GABA_A$ (Maksay and Ticku, 1984), nicotinic acetylcholine (Dennis *et al.*, 1988) and glycine receptors (see discussion). At the nicotinic acetylcholine receptor it appears that several aromatic residues located some distance apart in the primary sequence are directly involved in the interaction of competitive antagonist and the receptor binding site (Galzi *et al.*, 1990). A clustering of aromatic residues could provide negative subsite to which cationic quaternary ammonium of acetylcholine binds. This possibility has been highlighted by binding acetylcholine to a synthetic heterocyclic compound consisting of aromatic rings, through cation-π interaction (Dougherty and Stauffer, 1990). There is emerging evidence that distant domains of the primary sequence are involved in forming the binding sites of the glycine receptor and it therefore that possible clustering of aromatic residues are involved in binding strychnine and glycine, it is interesting to note that in this respect both ligands carry a cationic charge at physiological pH.

Diethylpyrocarbonate (DEP) reacts with histidine residues to form the monosubstituted N-carboxyhistidyl derivative. Under certain conditions, particularly those of reagent excess, the monosubstituted N-carboxylhistidyl derivative can react further and undergo disubstitution (Miles, 1977). The selectivity of DEP for histidine residues is greatest when the reaction is performed at pH 6 in phosphate buffers (Miles, 1977). Although these conditions were used a specificity for histidine residues can not be guaranteed as a reaction with tyrosine and lysine residues can occur particularly under conditions of reagent excess. The reagent concentration used in this study was 20 mM and in spite of a selective modification of a functional histidine in ribonuclease with 40 mM DEP reactivity beyond histidine residues at the inhibitory glycine can not be excluded.

However, the effect of DEP treatment on protein function is often ascribed to a histidine modification if the effect can be reversed by treatment with hydroxylamine (Melchoir and Fahrney, 1970). Indeed a reversal of the effects of DEP treatment has been used to specify the role of histidine residues in benzodiazepine binding at the $GABA_A$/benzodiazepine receptor (Duggan and Stephenson, 1987). Although this criteria

is not absolute as tyrosine adducts are reversed and disubstituted histidine adducts are not, the effect of hydroxylamine on DEP treated spinal cord membranes was investigated. Unfortunately a direct effect of hydroxylamine precluded any conclusion about the residue type that might have been involved in the DEP modification.

The protection experiments with DEP treated membranes supported the idea that modification of a DEP sensitive residue at a common binding site was responsible for the major effect that this reagent exerted on the parameters of [^{3}H]-strychnine binding. It has been shown that DEP treatment of spinal cord membranes can effect a two fold reduction in the affinity for [^{3}H]-strychnine binding and this implicated a role for histidine residues in binding or the interaction between strychnine and glycine.(Ruíz-Gómez *et al.*, 1990). However the ability of ligand protection to prevent these changes were not addressed. The effect of DEP in the experiments described in this study (fig 3.16) were more marked than previously reported (Ruíz-Gómez *et al.*, 1990) and probably relate to the higher concentration of reagent used. It is interesting to speculate that the aromatic nature of histidine may relate to this possible role in ligand recognition at the inhibitory glycine receptor.

A comparison of the of distribution of histidine residues in the N-terminal of different subunit isoforms reveals some interesting possibilities (see fig 1.3). Sequence alignments indicate there are two histidines in the α1 at position 152 and 247 (see Fig 1.3) that are exchanged for an asparagine and tyrosine respectively in the ß subunit (Grenningloh *et al.*, 1990b). The histidine at position 247 lies in between the proposed cystine bond that has been postulated to function or lie in close proximity to the ligand binding site. However at present one can only speculate as to whether the exchange in this histidine residue is in any way related to the different glycine and strychnine sensitivities between α1 and ß subunit when expressed as homologimers. The histidine at 201 in the α1 subunit is conserved between rat α1, α2* and α3 despite this the α2* subunit has been shown to have a low affinity binding site for strychnine and glycine (Kuhse *et al.*, 1990a).

However, a second histidine at position 152 in the rat α1 subunit that is exchanged for asparagine in the ß, α2* and α3 subunit. It is interesting to point out that the three subunits that have this exchange have reduced sensitivity to the agonist glycine (Grenningloh *et al.*, 1990b, Kuhse *et al.*, 1990a; Kuhse *et al.*, 1990b) which could indicate

a role for the histidine in the interaction between glycine and the inhibitory glycine receptor. It should be stressed that discussion based on the sequence homology can only speculate on possible significance of a single residue in receptor structure and function. The value of these speculation is only brought to fruition if they are then experimentally investigated as for the α2* subunit of the inhibitory receptor (Kuhse *et al.*, 1990a). Although such a strategy can give compelling evidence on the molecular basis of receptor functionally, the definitive answers lie in the resolution of the 3 dimensional structure of wild type and mutant receptor proteins.

3.3.d The effect of chemical modification with arginine selective reagents on subsequent [^{3}H]-strychnine binding to spinal cord membranes

With a similar aim as has been discussed, the effects of the arginine selective reagent BD (Riordan, 1979) on subsequent [^{3}H]-strychnine binding to treated spinal cord membranes, were investigated. Rather surprisingly chemical modification with BD caused an approximate ten fold increase in the [^{3}H]-strychnine binding. The increased binding was characterized by the fact that it could be inhibited by unlabelled strychnine but not glycine.

A specificity of this reagent for arginine residues has often been implied on the grounds that the reactivity has a dependence on borate ions (Riordan, 1979). However when BD modification were performed using PBB the increase in [^{3}H]-strychnine binding as a function of time appeared to be less than experiments that used PBS. The borate dependence of the BD reaction is often empirical and the non-dependence of the BD reactivity on borate in these experiments may reflect an inappropriate borate concentration. Indeed, an apparent decrease in the effect of BD treatment to increase [^{3}H]-strychnine binding to spinal cord membranes, when the modification was performed in borate buffer may reflect an interaction between BD and borate thus reducing the BD concentration available for reaction (Riordan, 1979). In addition it has been shown before that arginine residues can be irrevresably modified through a borate insensitive reaction (Ehrlich and Colman, 1977).

The concentration of BD used to modify membranes was optimized at 80 mM (see fig 3.7). This rather high concentration of reagent may reflect the environment and the

reactivity of the residue(s) that are modified. However at higher reagent concentrations the specificity of arginine residues may be compromised, to react with lysine residues (Riordan, 1979). In this respect it should be noted that in this study and others (Ruíz-Gómez *et al.*, 1989) have not been able to cause an increase in [^{3}H]-strychnine binding to spinal cord membranes treated with lysine selective reagents.

The specificity of BD for arginine residues can also be compromised by its photosensitization (Grippon and Hofmann, 1981) which can lead to modification of histidine, tyrosine and tryptophan residues. However, this was unlikely to account for the effect reported here. Firstly modification was performed in opaque reaction vessel to preclude activation by light and even when the reaction was performed in complete darkness the effect was identical (result not shown). Secondly, modification of spinal cord membranes with tyrosine, tryptophan and histidine selective reagents does not cause an increased in [^{3}H]-strychnine binding as seen with BD-treated membranes.

Neither strychnine or glycine protection prevented the increase in [^{3}H]-strychnine binding or were able to maintain an inhibition of the [^{3}H]-strychnine binding by unlabelled glycine in spinal cord membranes treated with BD. This argues that the residue(s) that BD treatment effect are probably not associated with the strychnine or glycine binding site at the inhibitory glycine receptor. However, the inability of glycine to inhibit [^{3}H]-strychnine and the potentiation of the effect of BD in membranes that were strychnine protected during the modification led to the speculation that the modification was entrapping the inhibitory glycine receptor in an antagonist conformation. This stimulated the work that is described in the next two chapters and will not be further discussed here.

In an attempt more clearly to define the nature of the residue(s) that were modified by BD with a resultant increase in [^{3}H]-strychnine binding a range of arginine selective reagents were studied. Of the reagents tested all with the exception of CQS were classified as membrane permeable (Findlay, 1987). BD, pPG and PG had a common reactivity to the residue(s) which when modified led to an increase in [^{3}H]-strychnine binding which was potentiated when the membranes were protected with strychnine during the chemical treatment. Although the fourth membrane permeable reagent, CHD, does not have a BD like effect on [^{3}H]-strychnine binding to treated membranes, the common reactivity of the other membrane permeable reagent indicate that the residues responsible for the increased

[^{3}H]-strychnine binding are in an environment which is buried or restricted access to protein modifying reagents. Interestingly a differential reactivity of a functional arginine residue towards PG and CHD in Band 3 protein has been explained on basis that the planar PG molecule can gain access to a residue which the non-planar CHD molecule cannot reach (Falke and Chan, 1986). In this respect, both BD and pPG are also planar molecules.

The increase in [^{3}H]-strychnine binding to membrane treated with PG contrast with similar experiments that use a lower concentration of PG and have shown a decrease in subsequent [^{3}H]-strychnine binding (Ruíz-Gómez, 1990). This difference could be attributed to reagent concentration but even at comparably low concentration of the reagent BD a decrease in [^{3}H]-strychnine binding was not seen (see fig 3.6).

Treatment of spinal cord membranes with the membrane impermeable arginine selective reagent CQS did not effect an increase in subsequent [^{3}H]-strychnine binding, which is in agreement with the idea that the residues involved in the manifestation of the increase [^{3}H]-strychnine were in buried lipophilic sites. This reagent reduced [^{3}H]-strychnine binding. Previously modification of spinal cord membranes with the arginine selective reagent PG was shown to decrease [^{3}H]-strychnine binding (Ruíz-Gómez, 1990), perhaps CQS modifies the same residue. Although there was a suggestion that both strychnine and glycine protection might prevent the decreased [^{3}H]-strychnine binding to membranes modified with PG (Ruíz-Gómez *et al.*, 1990), it was clear that both ligands fully protected against the effect of CQS which argues in favour of modification at a common binding site for strychnine and glycine.

3.3.e Reactivity of 2-AS-KLH antisera towards chemical modification:a comparison of perturbation of [^{3}H]-strychnine binding to antisera and spinal cord membranes treated with protein modifying reagents

A major difficulty in interpreting the effect of chemical modification and ligand protection on the parameters of [^{3}H]-strychnine binding was that the receptor has different conformational states that are associated with binding the agonist, glycine or antagonist, strychnine. In an attempt to extend the investigation of the details that underlie molecular recognition of strychnine and circumvent problem of difference in protein conformation

the effects of chemical modification of 2-AS-KLH antisera on subsequent [^{3}H]-strychnine binding was investigated. Structure activity study with strychnine and related alkaloids suggested there was a congruence in strychnine binding to the inhibitory glycine receptor of spinal cord membranes and 2-AS-KLH antisera (Phelan *et al.*, 1989). However the antisera does not interact with glycine and thus was specifically in a strychnine binding conformation.

The antisera were treated with the protein modifying reagents under incubation conditions used to modify the spinal cord membranes in order to facilitate a selective reactivity of NBS, TNM, DEP, BD and TNBS for tryptophan, tyrosine, histidine, arginine and lysine residues respectively. The possibility that modification of amino acid outside the antibody binding site might be responsible for reported effect of [^{3}H]-strychnine binding was addressed using a strychnine protection protocol. For all five reagents at least some of the effect appeared to be mediated by such modifications as strychnine protection was never able completely prevent the decrease in [^{3}H]-strychnine binding to treated antisera. However, NBS, TNM and BD appeared to exert part of their effect by modifying residues directly involved in strychnine binding as strychnine protection was able to afford some protection against the decrease [^{3}H]-strychnine binding reported for unprotected antisera.

In the case of NBS and TNM there appears to be a reactivity towards the reagents at the strychnine binding site of both the inhibitory glycine receptor and the strychnine binding antisera. Whether this relates to a common interaction between aromatic residues and the alkaloid molecule at the two binding sites can only speculated, however with the previously reported structure activity relations (Phelan *et al.*, 1989), it may point to a convergence in the detail of molecular recognition of strychnine at the two binding sites. The strychnine protection afforded against the decrease in [^{3}H]-strychnine binding to antisera treated with BD suggests an arginine residue may be important at the antisera binding site. The unusual effect of the BD treatment on subsequent [^{3}H]-strychnine binding to spinal cord membranes mean it is difficult to compare this with BD modification of the antisera. In spite of this a direct role of arginine residues in the strychnine binding to the inhibitory glycine receptor has been discussed in relation to the reagent CQS (see section 3.3.d) and PG (Ruíz-Gómez *et al.*, 1990). The existence of a common residue, arginine could further implicate a congruence at the strychnine binding

site of the inhibitory glycine receptor and 2-AS-KLH antisera.

As has been discussed the effect of DEP treatment of spinal cord membranes on the parameters of [^{3}H]-strychnine binding can be explained by a modification of residue(s) at a common binding site for strychnine and glycine. However the reactivity of DEP was not extended to the strychnine binding site of the antisera. The non congruence of DEP reactivity, contrasted by NBS, TNM and possibly BD, suggest some difference in the residues involved in the molecular recognition of strychnine at the two binding site. This has previously been implied on the basis of structure activity studies, most notably with the alkaloid, diaboline (Phelan *et al.*, 1989). The apparent non-reactivity of DEP for the strychnine binding sites of the antisera compared to the inhibitory glycine receptor might reflect the loss of a DEP sensitive residue, found at the common binding site for strychnine and glycine at the receptor, in the strychnine binding site of the antisera.

The interaction of ligand with the antibody is specified by the structural arrangements of the complimentary determining regions of the antibody which are located in the variable domain the light and heavy chains; these residues are brought together to form the binding site by folding of the protein which has the characteristic immunoglobulin fold (Padlan, 1977; Rees, 1987; Davies *et al.*, 1988). Whether maturation of the immune response (Berek and Milstein, 1987) to the 2-AS-KLH conjugate produces a population of antibodies whose strychnine binding sites reproduce protein ligand interactions that underlie molecular recognition of this ligand and inhibitory glycine receptor can only be speculated from data presented.

Similarities in the molecular recognition of ligands at membrane receptors and antibodies have been discussed for acetylcholine (Dougherty and Stauffer, 1990) and the ß-adrenergic antagonist alprenol (Nahimas *et al.*, 1988). In this respect the antibody binding site which is comprised of amino acids from distant regions of the same or separate polypeptide chains that are brought together by the protein fold. The possibility that the ligand binding site of ligand gated ion channels proteins are made up of discontinuous sequences is an emerging theme (Blount and Merlie, 1989; Galzi *et al.*,1990; Pederson and Cohen, 1990; Conti-Tronconi *et al.*, 1990) which likely hold at the inhibitory glycine receptor (Kuhse *et al.*, 1990a). This might encourage attempts to use pharmacologically specific antisera as an approach to define amino acid that are directly

involved in ligand recognition at ligand gated channel proteins.

CHAPTER FOUR

Biochemical characterization of [^{3}H]-strychnine binding to untreated and 2,3-butanedione treated rodent spinal cord membranes

4.1 Introduction

In the previous chapter, the ligand recognition properties of the inhibitory glycine receptor of mouse spinal cord were investigated by examining the effect of protein modification of spinal cord membranes on the subsequent parameters of [^{3}H]-strychnine binding. In general, the reagents tested were without effect or caused a decrease in [^{3}H]-strychnine binding. The notable exceptions to this were a class of membrane permeable arginine selective reagents which caused a large increase in [^{3}H]-strychnine binding. Unlike binding to untreated membranes, the increased [^{3}H]-strychnine binding to BD-treated membranes was not inhibited by unlabelled glycine. This effect was best characterized for the reagent 2,3-butanedione (BD). Although arginine residues have been implicated in direct recognition of strychnine at the inhibitory glycine receptor (Ruíz-Gómez *et al.*, 1990), modification of such residues was unlikely to underlie the effect of BD as neither strychnine or glycine protection prevented the increase in [^{3}H]-strychnine binding. Indeed, the presence of strychnine during BD-treatment enhanced the increase in subsequent [^{3}H]-strychnine binding, suggesting a conformation dependent modification of residues outside the ligand binding site.

In the discussion of Chapter 3, it was suggested that the conformation dependence of BD modification could relate to the receptor being entrapped in an antagonist conformation and this might also explain the inability of glycine to inhibit the induced binding. Yet, it is equally plausible that BD treatment modifies the strychnine binding property of a totally unrelated protein. The experiments described in this chapter were designed to characterize features of the BD-induced increase in [^{3}H]-strychnine to spinal cord membranes and address its relationship to the previously characterized binding site for strychnine which exists on the inhibitory glycine receptor protein (see Introduction to thesis). A pharmacological characterization of the BD-induced strychnine binding site is presented in Chapter 6.

4.2 Results

4.2.a Distribution of [^{3}H]-strychnine binding to untreated and BD-treated membranes from different regions of the rat central nervous system (CNS) and non-neuronal tissue

As an initial attempt further to characterize the increase in [^{3}H]-strychnine binding to spinal cord membranes a comparison of the binding to untreated and treated membranes from different regions of the CNS were made. Mouse membranes were used in the initial experiments which described the effect of BD treatment on binding. However, rat membranes were used for comparison of the regional distribution to ensure sufficient tissue could be obtained to allow estimates of binding to each region from an individual animal. Membranes prepared from CNS tissue dissected to give eight regions, were untreated or treated with BD and subsequently assayed for [^{3}H]-strychnine binding (see fig 4.1).

As expected from previous studies (see Discussion) the specific [^{3}H]-strychnine binding to untreated membranes was highest in the spinal cord and brain stem (medulla and pons). Although specific [^{3}H]-strychnine binding was detected in membranes prepared from remainder (hypothalamus and amygdala), thalamus, hippocampus, and also striatum it was only 10-25% of that measured in spinal cord or the medulla and pons. In the membranes prepared from the cerebellum or cortex specific [^{3}H]-strychnine binding could not reliably be measured and was considered non-detectable.

In the BD-treated membranes, the specific [^{3}H]-strychnine binding was highest in the spinal cord and brainstem and although increased when compared to untreated, the amount bound was lower in membranes from the other CNS regions. Indeed a comparison of the amount of strychnine bound to untreated and BD-treated membranes indicated that the two sets of data show a statistically significant correlation ($r=0.759$; $p>0.05$). However, the correlation apparent when comparing untreated and BD treated membranes from all regions was not always seen when [^{3}H]-strychnine binding from untreated and BD-treated membranes from individual regions of six different animals were compared.

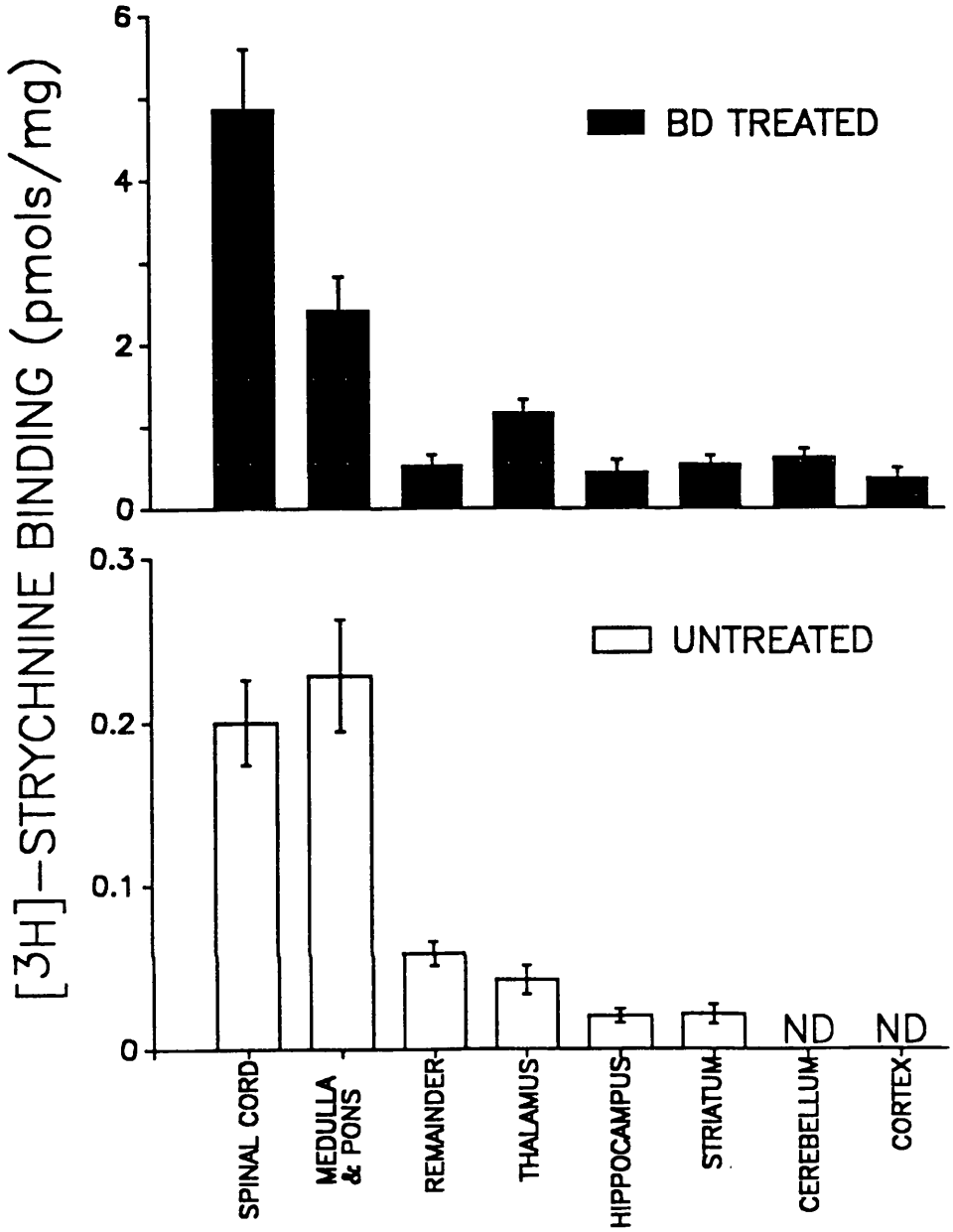


Figure 4.1: Regional distribution of specific [^{3}H]-strychnine binding to untreated and BD-treated rat central nervous tissue: Membrane prepared from different regions of the central nervous system of the rat were untreated or incubated with BD (80 mM, 40 min, pH 7.4, 20/21°C), washed and assayed for [^{3}H]-strychnine binding (6 nM). The values represent specific [^{3}H]-strychnine binding defined by unlabelled strychnine (10^{-4}M) and are mean and standard error of triplicate estimates for 6 individual animals. The correlation in the amount bound between untreated and BD treated was given by r= 0.759, $p<0.05$. Binding was depicted as non-detectable (ND) if total binding was not at least twice non-specific.

When untreated membranes prepared from non-neuronal tissue of mouse were assayed the specific [^{3}H]-strychnine binding was not twice the value of the non-specific defined by unlabelled strychnine and therefore considered beyond the limit of assay sensitivity. In contrast some specific binding, although low when compared to spinal cord, was detected in the membranes of non-neuronal tissue treated with BD. The binding in pmol/mg for different tissues are indicated and represent the mean ± standard error of four batches of membrane in which triplicate estimates of the [^{3}H]-strychnine bound were made: spinal cord 4.98±1.00; skeletal muscle 0.09±0.01; lung 0.15±0.03; ileum 0.07±0.01; heart ND<0.04; liver ND<0.04; spleen ND<0.05; kidney ND<0.05. ND, indicating that specific binding was not twice value of estimated non-specific binding for that tissue.

4.2.b The effect of BD treatment on subsequent [^{3}H]-strychnine binding to spinal cord membranes the mutant mouse *spastic*

An extension of the approach which correlates the amount of [^{3}H]-strychnine binding between different treatments was attempted by comparing [^{3}H]-strychnine bound to untreated and BD-treated spinal cord membranes from normal, unaffected littermate and spastic mice. The spinal cord membranes from the mutant mouse *spastic* have been shown to be deficient in high affinity strychnine binding sites (White, W.F. 1985; Becker *et al.*, 1986). In agreement, the amount of [^{3}H]-strychnine binding to untreated spastic mouse spinal cord membranes was only 20-25% that measured in age matched normal and littermate mouse spinal cord membranes assayed under identical conditions (fig 4.2). In contrast, there was no apparent difference between the binding to untreated normal and littermate spinal cord membranes.

Treatment with BD caused the subsequent [^{3}H]-strychnine binding to normal, littermate and *spastic* spinal cord membranes to increase. This was characterized by the inability of glycine to inhibit the increased binding (data not shown) in each of the treated preparations. Despite the decrease in [^{3}H]-strychnine binding to untreated spastic membranes when compared to littermate, the absolute binding to corresponding BD-treated membranes was not different. However, the increased amount of [^{3}H]-strychnine to BD-treated normal membrane was significantly greater than in treated littermate and *spastic* spinal cord membranes. Interestingly this contrasted with the untreated membranes where there was no apparent difference in the binding between normal and littermate.

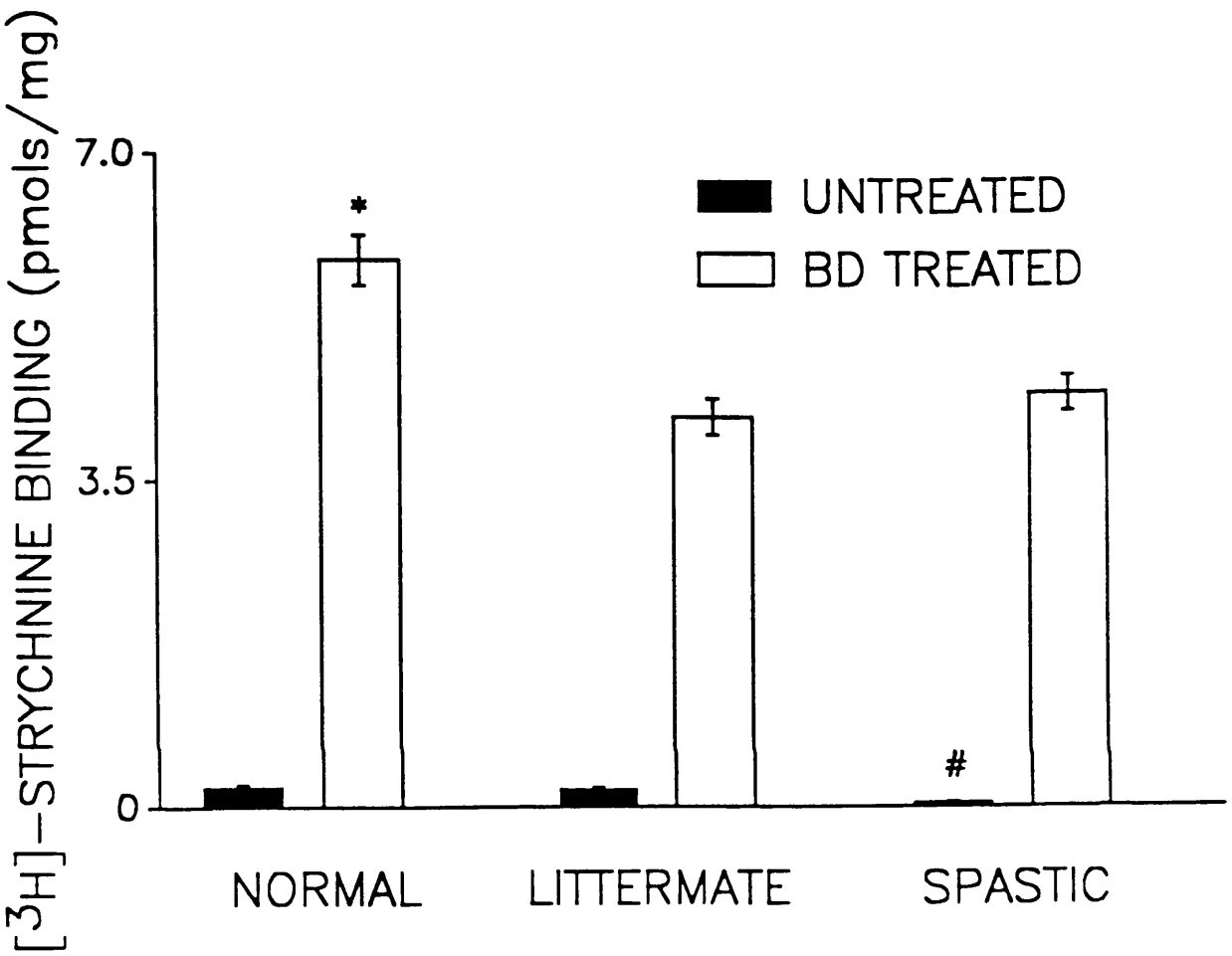


Figure 4.2: A comparison between [^{3}H]-strychnine binding to untreated and BD-treated spinal cord membranes from normal, littermate and spastic mouse: Normal, unaffected littermate and spastic spinal cord membranes prepared from age-matched mice were untreated or incubated with BD (80 mM, 40 min, pH 7.4, 21°C) washed and assayed for [^{3}H]-strychnine binding (6 nM). Specific [^{3}H]-strychnine binding was defined by unlabelled strychnine (10^{-4}M). Values are means and standard errors from four experiments in which triplicate estimates were made. # significantly different from untreated normal and littermate; $p < 0.01$: * significantly different from treated littermate and *spastic* (Students paired t-test).

4.2.c The effect of BD treatment on [^{3}H]-strychnine binding to purified glycine receptors

To directly address whether the increased [^{3}H]-strychnine binding to CNS membranes treated with BD was associated with glycine receptor complex required the purification of the receptor protein and a comparison of [^{3}H]-strychnine binding to untreated and BD-treated preparations.

4.2.c.1 Solubilization and affinity purification of the inhibitory glycine receptor.

Mouse spinal cord membranes were solubilized in the zwitterionic detergent 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulphonate (CHAPS; 1.5% w/v) in the presence of the exogenous lipid phosphatidyl choline and the [^{3}H]-strychnine binding sites affinity purified on a 2-aminostrychnine-agarose column (see section 2.8).

The results shown (Table 4.1) summarize the detail of a purification procedure in which the initial material was obtained from 40 mouse spinal cord membranes. The binding was determined in each fraction by assaying with [^{3}H]-strychnine (6nM). The purification shown (Table 4.1 and Fig 4.3) used the most starting material but was also representative of the four preparations performed for the work in this thesis. The addition of CHAPS to the membrane homogenate caused an apparent decrease in the number of detectable binding sites (Table 4.1). Although this could be due to an inhibitory effect of CHAPS on ligand binding, the mild nature of this detergent favours the interpretation that solubilization of these sites underlies this slight decrease. This was compatible with an apparent 4-fold increase in protein concentration of the homogenate with added CHAPS. Solubilization of the protein would increase potential protein Commassie dye interactions responsible for light absorbance, giving an apparent increase in the amount of detectable protein.

CHAPS, under the conditions used, solubilized 17% of the [^{3}H]-strychnine binding sites present in membrane homogenate. This was consistent with the fact that 87% of the sites found in the source were measured in the pellet which remained after ultracentrifugation of the membrane homogenate used to separate the solubilized fraction. CHAPS was constantly found to remove only 20% of the [^{3}H]-strychnine binding from membrane homogenate, a value which was lower than the value that has been described

Fraction	Volume (ml)	[^{3}H]-strychnine binding (pmols)	Yield (%)	Protein (mg/ml)	pmol bound/mg protein
Membrane Homogenate	26.0	69.4	_	0.69	3.75
Membrane Homogenate (containing CHAPS 1.5%)	26.0	46.6	100 (67.7)MH	2.43	0.74
Resuspended Pellet (containing CHAPS 1.5%)	26.0	40.6	87.0	0.780	2.94
Solubilized Fraction (Load fraction)	52.0 (R); 45.0 (L)	8.1 (R) 6.9 (L)	17.4	0.533	0.29
Active Fraction	5.1	2.6	3.7	N.D.*	_

Table 4.1: Purification of the glycine receptor. Activities at each stage of the purification procedure which used 40 mouse spinal cords as starting material (see section 2.10). [^{3}H]-strychnine binding in fractions was assayed with 6 nM ligand. R; activity recovered in fraction: L; activity loaded onto 2-AS-Agarose column: MH; value expressed as % activity assayed in membrane homogenate that contained no added CHAPS: N.D.*; non-detectable despite assaying fraction that was subjected to protein precipitation procedures to concentrate sample.

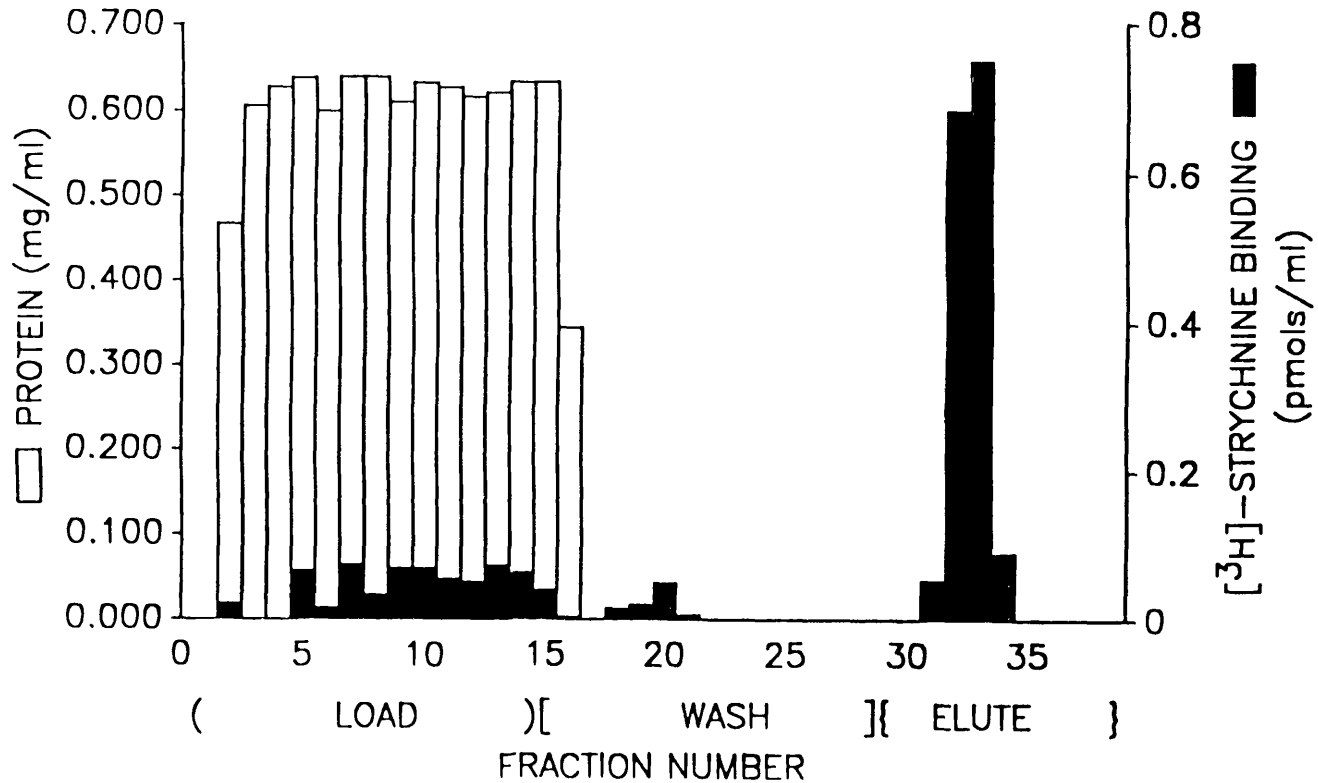


Figure 4.3: Affinity chromatography of the glycine receptor on 2-aminostrychnine-agarose column: 6.9 pmol of [^{3}H]-strychnine binding sites solubilized in CHAPS were loaded (2.5ml/hr) onto a 2-aminostrychnine-agarose column. The column was washed (10 ml/hr) before eluting with wash buffer containing 200mM glycine (2.5 ml/hr). Fractions were collected throughout the load (4 ml), wash (4ml) and elution (1.5 ml). [^{3}H]-strychnine binding to an aliquot of each fraction was estimated after PEG precipitation to remove unlabelled glycine from eluted fractions. 35% of binding loaded onto the column was recovered in eluted fractions. Proteins was estimated in each fraction using wash buffer containing 200 mM glycine as the appropriate zero controls.

for previous solubilizations of mammalian spinal cord membranes with Triton-X-100 or sodium cholate (Pfeiffer *et al.*, 1982; Graham *et al.*, 1985).

The solubilized fraction was loaded onto a 2-aminostrychnine-agarose column which was then washed and eluted using wash buffer containing 200 mM glycine (see fig 4.3). This procedure was successful in fractionating [^{3}H]-strychnine binding activity of solubilized fraction from the protein component. Indeed, the peak [^{3}H]-strychnine binding associated with the eluted fractions contained undetectable amounts of protein, even if the fractions were subjected to a protein concentrating procedure prior to Commassie assay (see section 2.13.c.1). As the extent of protein purification requires the expression of ligand binding activity/unit of protein it was not possible to quantitate for procedures described. This procedure ensured that 34% of binding sites loaded onto the column were recovered in the active fraction; this represented a marked improvement on the procedures that use triton-X-100 or sodium cholate. The final yield for the purification illustrated in Table 4.1 was 2.6 pmols of assayed [^{3}H]-strychnine binding sites. The percent yield was comparable to previous procedures (Pfeiffer *et al.*, 1982; Graham *et al.*, 1985); the low amount of recovered protein relates to the quantity in the starting material, 40 mouse spinal cords.

4.2.c.2 SDS polyacrylamide gel electrophoresis of fractions from glycine receptor purification.

Previous procedures have shown that 3 peptides are found associated with the glycine receptor affinity purified from the mammalian CNS (Pfeiffer *et al.*, 1982; Graham *et al.*, 1985; Becker *et al.*, 1986; García-Calvo *et al.*, 1989). Therefore the peptides associated with active fraction and from different stages of the purification protocol were analyzed for their peptide content. This was done by resolving the peptides solubilized in SDS by gel electrophoresis through a 10% polyacrylamide before the peptides were visualized by silver staining (see section 2.13).

Polyacrylamide gel electrophoresis was performed on fractions from the four receptor purifications undertaken, but the gel shown (plate 4.1) was from the purification that has been described in detail (see table 4.1 and fig 4.3). The gel was chosen because it was loaded with active fraction that had the highest number of [^{3}H]-strychnine binding

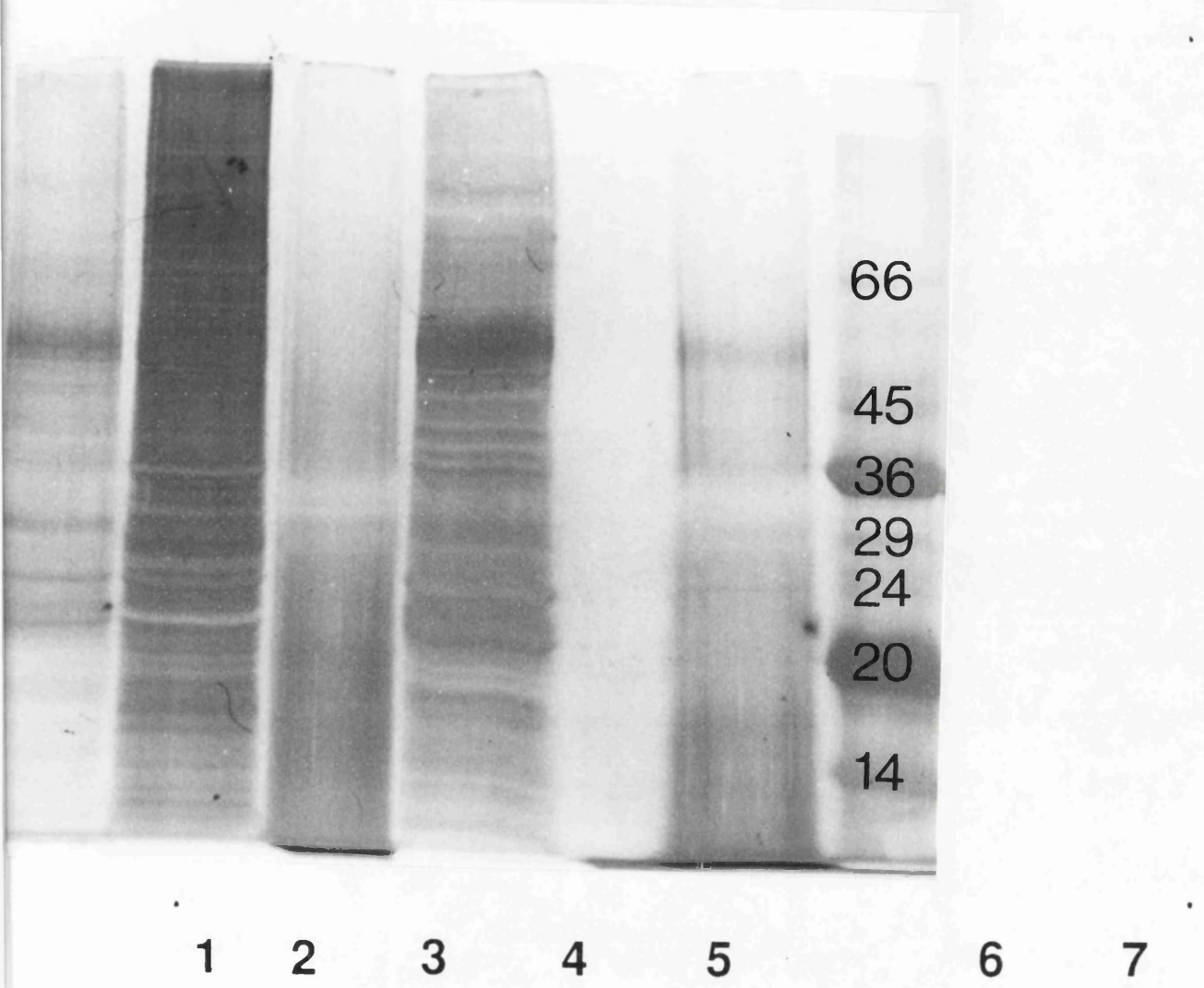


Plate 4.1: SDS-polyacrylamide gel electrophoresis on 10 % gels of fractions from different stages of the glycine receptor purification: samples were prepared by diluting (1.2 v/v) in master mix to give load volumes and boiled at 95°C for 3 min. All load volumes were 20 μl with the exception of the active fraction which was 25 μl. The samples diluted with master mix were: Lane 1, 7 μl of wash buffer; Lane 2, 7 μl of solubilized fraction (0.533 μg protein/μl; Lane 3, 7 μl membrane homogenate (containing 40 μg protein) which had been solubilized in 10% SDS overnight; Lane 4, 300 μl wash buffer TCA precipitated and taken up into load volume; Lane 5, 300 μl solubilized fraction TCA precipitated to concentrate to a load volume; Lane 6, 300 μl eluted fraction containing 0.2 pmole [^{3}H]-strychnine binding sites TCA precipitated to concentrate load volume; lane 7, molecular weight markers (15 μg protein). After electrophoresis the gel was fixed and silver stained.

sites (0.2 pmols). Priority was given to use of the active fraction in experiments which considered the effect of BD treatment on subsequent [^{3}H]-strychnine binding to the glycine receptor and this limited the amount of purified receptor available for analysis by gel electrophoresis.

As expected there were numerous peptides associated with the crude membrane homogenate and it was also clear that CHAPS solubilized a large range of the protein component of membrane homogenate. By comparing the peptide pattern associated with solubilized fraction directly loaded onto the gel (Lane 2) with that subjected to trichloroacetic acid (TCA) precipitation (Lane 5) it appeared that the procedure effectively precipitated proteins which indicated its usefulness in reducing sample volume to concentrate protein loaded. Despite the concentration of the sample, only light staining appeared in the lane loaded with the active fraction. This fraction contained [^{3}H]-strychnine binding and undetectable amounts of protein when assayed by Commasssie blue (Bradford, 1976); however, this was not associated with the 3 subunits which make up the receptor complex. In the lane loaded with active fraction some faint staining visible between the 24 and 29 kd markers appeared to be contaminants from the adjacent marker lane. Interestingly there was a fairly broad band that stained at approximately 50 kd. This could correspond to a previously described peptide of the glycine receptor complex, which harbours the ligand binding sites. However, comparison with the solubilized fraction precipitated by TCA indicated (Lane 5) a heavy band at a similar position and it is unlikely that the subunit associated with the receptor would be a major component of the membrane fraction. Thus, the staining could represent an artefact. Indeed it was not associated with the 2 other peptides that characterize the purified glycine receptor.

The inability clearly to show a typical pattern of peptide staining associated with fraction that contained purified glycine receptor was attributed to the sensitivity of the silver staining protocol. The active fraction loaded (plate 4.1, lane 6) contained 0.2 pmole of [^{3}H]-strychnine binding sites, assuming 100% of the sites were precipitated by TCA and effectively resolubilized. The receptor molecular weight assumed to be made up of a pentameric arrangement of 3, 48 kD and 2, 58 kd subunits associated with one 93 kd subunit has a molecular weight of 353,000. On the assumption that each receptor complex has one [^{3}H]-strychnine binding site there would be 70 ng of protein loaded in 0.2 pmoles of binding sites. This is at the detection limit of the silver stain method (Haga *et al.*,

1990).

4.2.c.3 The effect of BD treatment on the affinity purified glycine receptor on subsequent level of [^{3}H]-strychnine binding.

To test for the effect of chemical modification of purified glycine receptor with the arginine selective reagent, the fractions eluted from 2-aminostrychnine-agarose column which showed peak [^{3}H]-strychnine binding activity were washed (see section 2.8.b) to remove the unlabelled glycine. The purified receptors were then thoroughly mixed and divided into fractions of equal volume. In one experiment the fractions were either untreated or incubated with BD (see section 2.9.b) prior to assay of total, strychnine and glycine specific [^{3}H]-strychnine binding (see section 2.10.b). In a second experiment equal volumes of purified receptors were treated as above, however they were incubated with no ligand, strychnine (10^{-4}M) or glycine (10^{-2}M) prior to and during the periods of BD-treatment to assess the effects of ligand protection. There was minimal delay between elution, identification by [^{3}H]-strychnine binding and chemical modification of purified receptor (16-20 hrs) in an attempt to prevent receptor inactivation.

As for the receptor in spinal cord membrane, both glycine and strychnine were able to inhibit equal proportions of the total [^{3}H]-strychnine binding to purified glycine receptor (fig 4.4). However, the amount of specific [^{3}H]-strychnine binding was only 60-65% of total bound and lower than the binding to receptor in spinal cord membranes where specific binding in filtration assays was at least 85% of total (see Chapter 3). This reflected a non-specific absorbtion of [^{3}H]-strychnine to the constituents used in the PEG precipitation. In contrast to results with spinal cord membranes described in the previous Chapter (see fig 3.19), treatment of purified receptor with the reagent BD did not cause subsequent 10 fold increase in [^{3}H]-strychnine binding when compared to the untreated preparation. In fact there was a slight decrease in the amount of total [^{3}H]-strychnine binding. BD treatment of purified glycine receptor was also found to decrease the amount of specific binding defined by unlabelled glycine. In contrast the proportion of total binding inhibited by strychnine in the BD treated preparation was comparable with the untreated control.

In a second experiment which used glycine receptors from a second solubilization and purification, an assessment of strychnine and glycine protection on the effect of BD

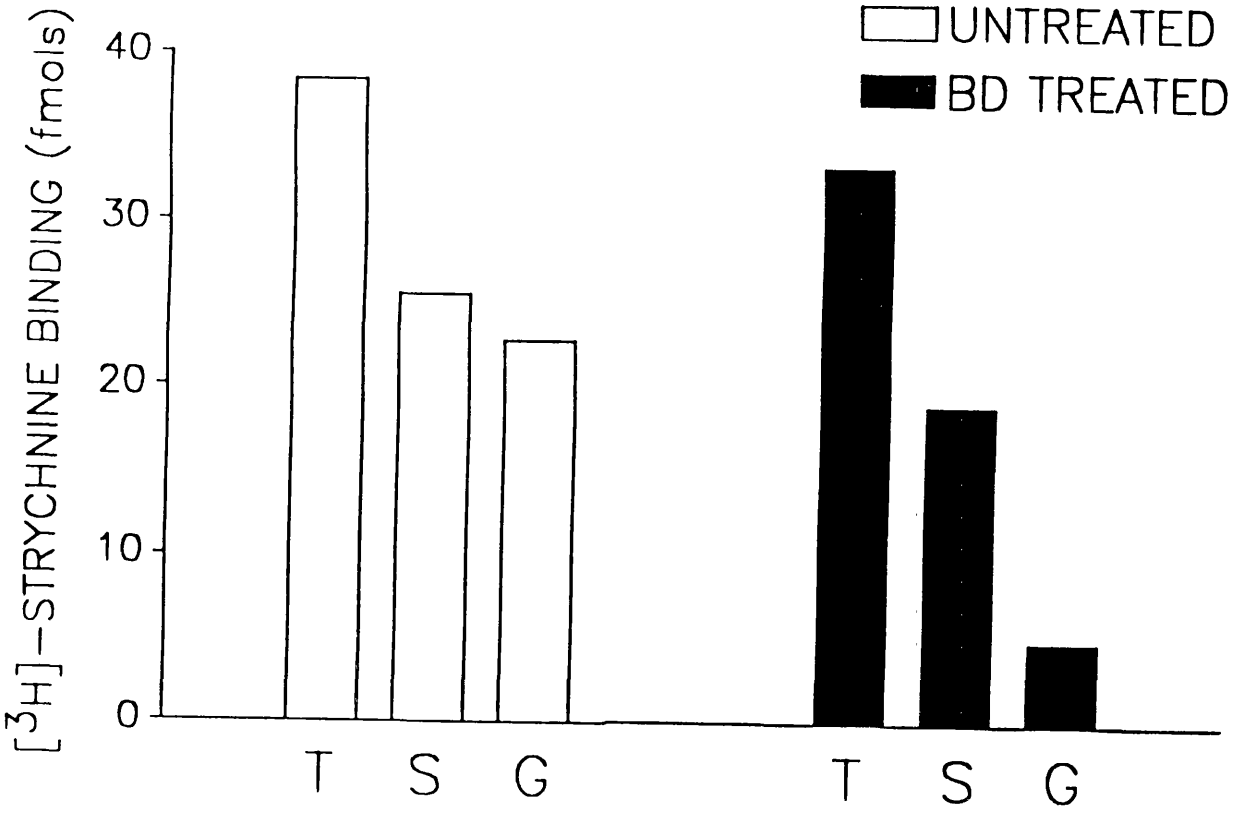


Figure 4.4: Effect of BD treatment of purifed glycine receptor on subsequent [^{3}H]-strychnine binding: Active fractions containing approximately 1 pmols of assayed strychnine binding sites were PEG precipitated and washed to remove the glycine used to elute from 2-aminostrychnine agarose column. Pooled fractions resolubilized in PBS (containing CHAPS 0.75% w/v: phosphatidyl choline; 1.5 mg/ml) were divided into two equal volumes which were untreated or incubated with BD (80 mM, 40 mins, 20/21°C). Reaction was terminated by addition of 4 reaction volumes of chilled PBS (containing 0.1% BSA; bovine-γ-globulin, 1.2mg/ml) and washed 4 times by PEG precipitation to remove unreacted reagent. [^{3}H]-strychnine (6 nM) was incubated with fraction to enable triplicate estimates of total binding (T). Incubations containing unlabelled strychnine (10^{-4}M) or glycine (10^{-2}M) defined strychnine-specific (S) and glycine-specific (G) binding. Assays were terminated by PEG precipitation and filtered.

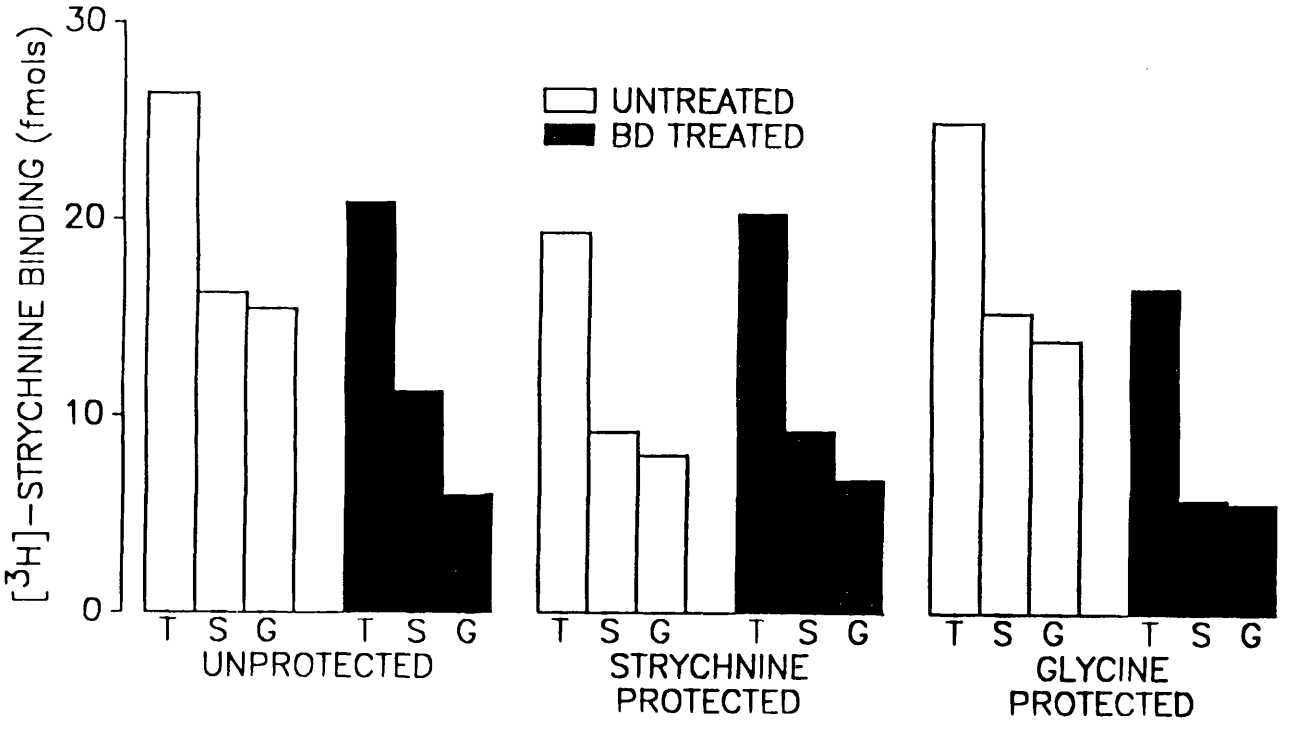


Figure 4.5: Effect of BD treatment of purified glycine receptor on subsequent [^{3}H]-strychnine binding: an assessment of strychnine and glycine protection: Active fractions containing 2.4 fmols of assayed strychnine binding sites were PEG precipitated and washed to remove the glycine used to elute from 2-aminostrychnine-agarose column. Pooled fraction resolubilized in PBS (containing CHAPS, 0.75% w/v; PTC 1.5 mg/ml) were divided into 6 equal volumes. Fractions preincubated with no ligand (unprotected), strychnine (10^{-4}M; strychnine protected) or glycine (10^{-2}M; glycine protected) were untreated or incubated with BD (80 mM, 40 mins, 20/21°C). Reaction was terminated by addition of 4 volumes of chilled PBS (containing BSA, 0.1% w/v; bovine-γ-globulin, 1.2, mg/ml) and washed 4 times by PEG precipitation to remove protecting ligands and untreated reagent. [^{3}H]-strychnine (6 nM) was incubated with each sample to enable triplicate estimates of total binding (T). Incubations containing unlabelled strychnine (10^{-4}M) or glycine (10^{-2}M) defined strychnine-specific (S) and glycine-specific (G) binding. Assays were terminated by PEG precipitation and filtered.

treatment was made (see fig 4.5). The equivalent amount of [^{3}H]-strychnine bound to unprotected and glycine protected preparations indicated that glycine was removed by PEG washes. However, in the untreated strychnine-protected purified receptors, the decreased amount of total [^{3}H]-strychnine when compared to the corresponding unprotected preparation indicated either that the unlabelled strychnine was not completely removed or that the protection protocol had ancillary effects on subsequent [^{3}H]-strychnine binding.

When compared to untreated, BD treatment of unprotected purified receptor caused a decrease in total [^{3}H]-strychnine binding and differentially effected the ability of glycine to inhibit total binding. This was consistent with the effects of BD described in the previous experiment (see fig 4.4). In contrast, in the strychnine protected BD-treated receptor preparation the amount of total, strychnine and glycine specific [^{3}H]-strychnine binding were almost identical to untreated strychnine protected receptors. This could indicate that strychnine protection of the preparations during BD treatment prevented the effects on binding described in the unprotected receptors. However, this interpretation was complicated by the apparent effect of strychnine protection on total [^{3}H]-strychnine binding to untreated receptors, when untreated and unprotected receptors are considered as the controls.

Finally it appeared that when purified glycine receptors were protected with glycine the effects of the BD treatment on subsequent [^{3}H]-strychnine binding were not prevented and may have been enhanced. Thus,the decreased total [^{3}H]-strychnine binding to BD-treated preparations was greater than in unprotected preparation. In addition, it appeared that BD treatment of glycine protected receptor decreased the proportion of total binding inhibited by both strychnine and glycine. This compared with unprotected BD-treated receptor in which only the ability of glycine to inhibit total binding was affected.

4.2.d Photolabelling the [^{3}H]-strychnine binding sites in untreated and BD treated mouse spinal cord membranes.

[^{3}H]-strychnine has been successfully employed as a photoaffinity label for the inhibitory glycine receptor in spinal cord membranes (Graham *et al.*, 1981; Graham *et al.*, 1983). For this reason, a photolabelling procedure was developed and used in an attempt

to identify the peptide(s) associated with increased [^{3}H]-strychnine binding to BD-treated spinal cord membranes.

4.2.d.1 Effect of UV irradiation on reversible [^{3}H]-strychnine binding to untreated and BD treated spinal cord membranes

The stability of [^{3}H]-strychnine binding sites could be compromised by subjecting them to ultraviolet (UV) irradiation. In case of BD it has been shown that the reagent is photosensitive (Grippon and Hofmann, 1981). Consequently, the reaction product formed on modification of amino acid residues might also be sensitive to UV irradiation. For this reason the effect of UV light on subsequent [^{3}H]-strychnine binding was assessed (fig 4.6). Membranes were either untreated or incubated with BD under standard conditions. After washing, the membranes were decanted into petri dishes and either non-illuminated or exposed to increasing periods of irradiation (nominal peak 254 nm, 30 $\mu W/cm^2$) at 4°C. Subsequently, reversible [^{3}H]-strychnine binding to the membranes was measured by filtration assays.

In the untreated membranes there was a time dependent decrease in [^{3}H]-strychnine binding which at 30 mins indicated that 40% of the sites were inactivated by UV illumination. This agreed with previously reported effects of UV irradiation of spinal cord membranes on the reversible [^{3}H]-strychnine binding (Graham *et al.*, 1983). In contrast there was a minimal effect on reversible [^{3}H]-strychnine binding to BD treated membranes indicating that the increased binding was stable to extended periods of UV illumination.

4.2.d.2 Effect of membrane concentration and irradiation time on the amount of [^{3}H]-strychnine irreversibly incorporated into spinal cord membranes.

A series of experiments was performed to optimize the photolabelling procedure for untreated and BD treated spinal cord membranes. The membranes were equilibrated with [^{3}H]-strychnine before being subjected to UV irradiation. Experiments utilized [^{3}H]-strychnine at a concentration which saturated the binding site in untreated membranes (30-40 nM). The membranes were then chased with unlabelled strychnine and the amount of [^{3}H]-strychnine irreversibly incorporated was estimated by filtering and thoroughly washing the membranes. The specificity of photolabelling was compared by estimating

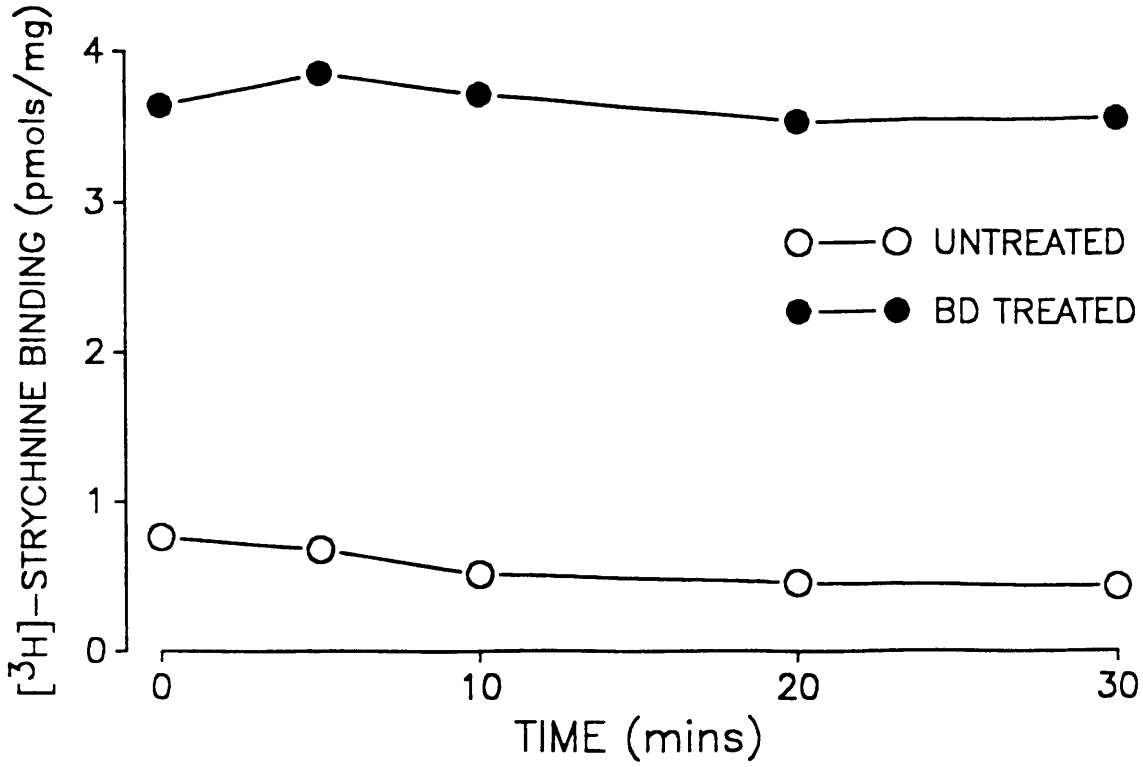


Figure 4.6: Effect of UV irradiation of untreated and BD-treated mouse spinal cord membranes on subsequent reversible [^{3}H]-strychnine binding: Untreated and BD-treated mouse spinal cord membrane at 4°C (40 vols tissue wt./vol) were irradiated for increasing times (nominal peak 254 nm, 30 μW/cm^2 at 15 cm). Triplicate samples of irradiated membranes were subsequently incubated with [^{3}H]-strychnine (6 nM) and strychnine-specific binding estimated using filtration assays.

the amount of ligand irreversibly incorporated into membranes that contained unlabelled strychnine and glycine during UV irradiation. Further controls included non-irradiated membranes and incubations that were irradiated but contained no membrane, essentially estimates of filter binding.

The preliminary experiment used an untreated preparation and considered the effects of changes in spinal cord membrane concentration on the amount of irreversibly incorporated [^{3}H]-strychnine (Table 4.2). Irradiation for 10 mins caused a linear, membrane concentration-dependent increase in the amount of [^{3}H]-strychnine irreversibly incorporated into untreated membrane. In subsequent experiments untreated and BD-treated membranes at 20 vols were subjected to irradiation in the presence of [^{3}H]-strychnine.

Tissue Concentration (protein mg/ml)	**Specific [^{3}H]-strychnine binding irreversibly bound (fmol/ml)**
320 vols (0.048 mg/ml)	10
160 vols (0.112 mg/ml)	22
80 vols (0.208 mg/ml)	50
40 vols (0.432 mg/ml)	88
20 vols (0.992 mg/ml)	213

Table 4.2 Effect of membrane concentration on [^{3}H]-strychnine irreversibly bound to spinal cord membranes irradiated with UV light: untreated spinal cord membranes prepared at 20 vols were serially diluted to 320 vols in PBS (pH 7.4). Membranes preincubated with [^{3}H]-strychnine (40 nM) were irradiated with UV light from lamp source (nominal peak 254 nM, 30 $\mu W/cm^2$ at 15.2 cm) for 10 mins at 4°C. The incubations were chased with unlabelled strychnine (1mM) for 1 hr before filtering triplicate aliquots and washing with 4 x 5ml PBS. Non-specific incorporation was estimated by incubations that contained unlabelled strychnine (10^{-4}M) during the irradiation.

A second experiment considered the effect of increasing irradiation time on the amount of [^{3}H]-strychnine irreversibly incorporated into untreated and BD treated membranes (Fig 4.7). There was a time-dependent increase in the irreversibly bound

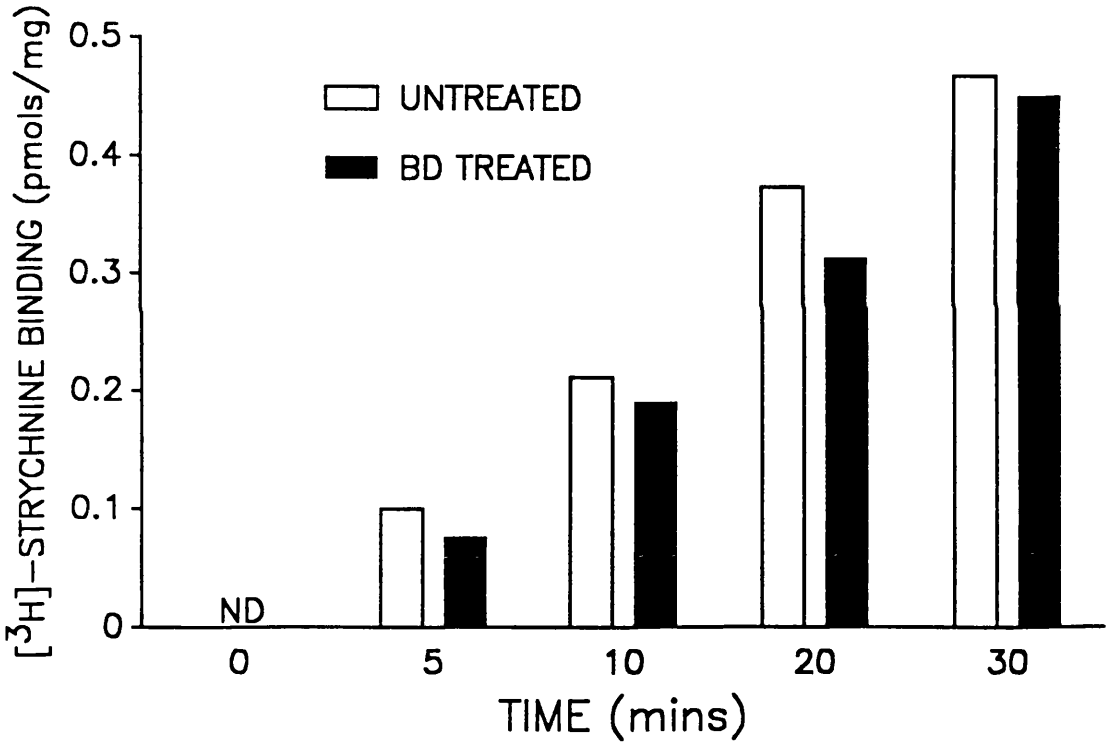


Figure 4.7: Effect of increasing irradiation time on the irreversible incorporation of [^{3}H]-strychnine into untreated and BD-treated mouse spinal cord membranes: Untreated and BD-treated membranes (20 vols tissue wt/vol) were incubated with [^{3}H]-strychnine for 2hr at 4°C. Incubations that contained unlabelled strychnine (10^{-4}M) defined non-specific binding. Non-irradiated membranes were compared with membranes subjected to increasing periods of irradiation (nominal peak 254 nm, 30 μW/cm^2 at 15 cm). Membranes were chased with unlabelled strychnine (1mM) and filtered to estimate the [^{3}H]-strychnine irreversibly bound. Values are mean specific irreversible binding from one experiment performed in duplicate. ND; (non detectable irreversible binding).

strychnine in both untreated and BD treated membranes. The amount bound at each time point, for the two treatments, although similar, was consistently lower in BD- treated membranes.

As the major aim of this approach was to develop a photolabelling procedure which could be used to detect peptides associated with the increased [^{3}H]-strychnine bound in BD-treated spinal cord membranes. 30 mins was chosen as the irradiation time. This was considered important as efficiency of photolabelling in BD-treated membranes was much lower than untreated controls (table 4.3). Identical incubations were either irradiated and filtered to estimate the irreversibly incorporated ligands; or directly filtered to estimate [^{3}H]-strychnine irreversibly bound.

		[^{3}H]-strychnine bound dpm	
		Untreated	**BD Treated**
Reversibly Bound	**Total**	10721	206582
	Non-specific	1414	44445
	Specific	**9307**	**162137**
Irreversibly Bound	**Total**	2637	3672
	Non-specific	1042	1899
	Specific	**1595**	**1773**
	Efficiency (%)	17	1

Table 4.3 Efficiency of photolabelling untreated and BD treated membranes with [^{3}H]-strychnine. Untreated and BD treated membranes (20 vols; tissue wt./vol) were incubated with ligand (30nM) for 2hrs at 4°C. Non specific binding was estimated by incubations that contained unlabelled strychnine (10^{-4}M). Non-irradiated membranes were filtered to estimate reversible [^{3}H]-strychnine binding. Irradiated membranes (30 min at 4°C were chased with unlabelled strychnine and filtered to estimate irreversibly bound [^{3}H]-strychnine. Efficiency of photolabelling was expressed as % specific binding reversibly bound that was irreversibly bound. Results are mean of one experiment performed in duplicate.

The results (table 4.3) indicated that BD treatment caused an approximate 16-fold increase in the reversible, specific [^{3}H]-strychnine binding. Despite this, the total ligand irreversibly bound to BD treated membranes when compared to untreated membranes, was

similar. Thus, the increased amount of strychnine reversibly bound to BD-treated membranes was not reflected when the incubations were irradiated and this was indicated by the calculated efficiency of photolabelling.

4.2.d.3 Peptides labelled by photolabelling untreated and BD-treated spinal cord membranes with [^{3}H]-strychnine as resolved on 10% acrylamide gels

In an attempt to define the peptides which irreversibly incorporated [^{3}H]-strychnine, untreated and BD-treated membranes were incubated with [^{3}H]-strychnine, photolabelled under defined conditions, solubilized in SDS and subjected to PAGE followed by fluorography (plate 4.2). In both the untreated and BD-treated membranes the ability to prevent irreversible incorporation by addition of unlabelled strychnine (10^{-4}M) or glycine (10^{-2}M) during photolabelling protocol was examined.

In untreated membranes, photolabelling was associated with the irreversible incorporation of [^{3}H]-strychnine into one clearly defined peptide with a molecular weight of 48 kd. Unlabelled strychnine and glycine were able to prevent photolabelling of this peptide. These results were consistent with previous reports that [^{3}H]-strychnine is specifically incorporated into the 48 kd subunit (α) of the inhibitory glycine receptor (Graham *et al.*, 1981; Graham *et al.*, 1983; García-Calvo et al., 1989). The fluorogram shown resulted from exposure of the acrylamide gel to photographic film for 3 months. When this was extended to 5-6 months photolabelling of a peptide of approximately 58 kd was also detected (result not shown). This was consistent with previous reports that [^{3}H]-strychnine was also irreversibly incorporated into the 58 kd (ß) subunit but at a lower level than α subunit (Graham *et al.*, 1985; García-Calvo *et al.*,1989).

In the BD treated membranes the only detectable band was at an identical molecular weight as the peptide labelled in untreated membranes which suggests that [^{3}H]-strychnine can specifically interact with the inhibitory glycine receptor. There was an approximate 10 fold increase in the reversible [^{3}H]-strychnine bound to treated membrane used for photolabelling when compared to untreated controls. However, it appeared that the amount irreversibly incorporated into the 48kd subunit of treated membranes was lower than in untreated membranes. Although the system was not set up for quantitative fluorography this interpretation was consistent with the results which showed that [^{3}H]-

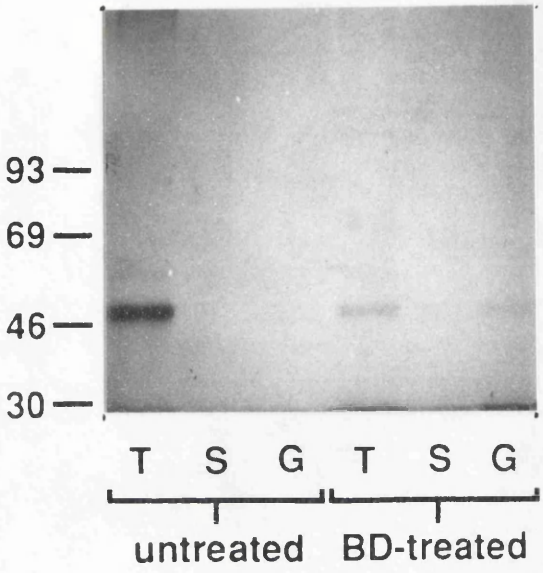


Plate 4.2: Fluorography SDS-polyacrylamide gel electrophoresis of untreated and BD-treated membrane photolabelled with [^{3}H]-strychnine: Untreated and BD treated membranes (20 vols, 1 mg membrane protein/ml) incubated with [^{3}H]-strychnine (40 nM) alone (T) or in the presence of unlabelled strychnine (10^{-4}M; S) or glycine (10^{-2}M; G) were irradiated for 30 mins at 4°C. Membranes were chased with unlabelled strychnine (1 mM) for 30 min, pelleted by centrifugation and washed in Tris.HCl pH 6.8. Washed membranes (8.5 μg protein/μl) solubilized overnight in 10% SDS were mixed with master mix (1:2 v/v), loaded (20 μl) and subjected to PAGE through a 10% resolving gel. Fixed gels were incubated in Enhance, dried and exposed to photographic film for 3-5 months at -70 °C. Radioactivity (dpm) associated with each loaded sample (20 μl) was: Untreated total 6100; strychnine-specific 1800; glycine-specific 1560; BD treated total 5300; strychnine-specific 3200; glycine-specific 6000. Gel was representative of four fluorographs of photolabelled membrane from 2 experiments.

strychnine bound to the BD-treated purified glycine receptor was decreased when compared to untreated preparations (fig 4.4 and 4.5).

As in the untreated membranes strychnine was able to prevent photolabelling at the 48 kd subunit in the BD treated membranes. Glycine, although able to prevent the irreversible incorporation of [^{3}H]-strychnine in untreated membranes was less effective in the BD-treated membranes. Thus, faint labelling of the 48kd subunit was apparent in the lane loaded with solubilized BD-treated membrane which had been photolabelled in the presence of glycine. Again, this was consistent with the experiments described in the previous section which showed the effective reduction in the ability of glycine to inhibit [^{3}H]-strychnine binding to BD-treated purified glycine receptor (see fig 4.4 and 4.5).

4.2.e An attempt to solubilize the [^{3}H]-strychnine binding to untreated and BD treated spinal cord membranes

The ability of the three detergents; Triton-X 100, CHAPS, and sodium cholate to solubilize [^{3}H]-strychnine binding from untreated and BD treated membranes was compared. As the long term goal of this approach was to develop a procedure which would enable the purification of the protein(s) harbouring the [^{3}H]-strychnine binding to BD-treated membranes, rat spinal cord was used, because it offered a potential source that provided more starting material.

Untreated and BD treated membranes (10 vols, 2mg protein/ml) in PBS were incubated with detergent Triton-X 100 (1.5%, w/v), CHAPS (1.5%, w/v) or sodium cholate (1%, w/v) in the presence of exogenous lipid PTC (1.5 mg/ml) and protease inhibitor cocktail as described previously (see section 2.8). The membranes were ultracentrifuged (100,000 g for 1 hr at 4°C), the supernatant removed and remaining pellet resuspended in a incubation volume of PBS. Aliquots of membrane and detergent, solubilized fractions and resuspended pellet were assayed (6 nM) for [^{3}H]-strychnine binding, non-specific binding was defined by unlabelled strychnine (10^{-4}M) or glycine (10^{-2}M). In addition the amount bound to source membrane incubated in PBS (buffer control), at equivalent tissue weight/buffer volume, was made.

There was a characteristic increase in [^{3}H]-strychnine bound to spinal cord membrane treated with BD, which glycine was unable to inhibit (see fig 4.8). The [^{3}H]-

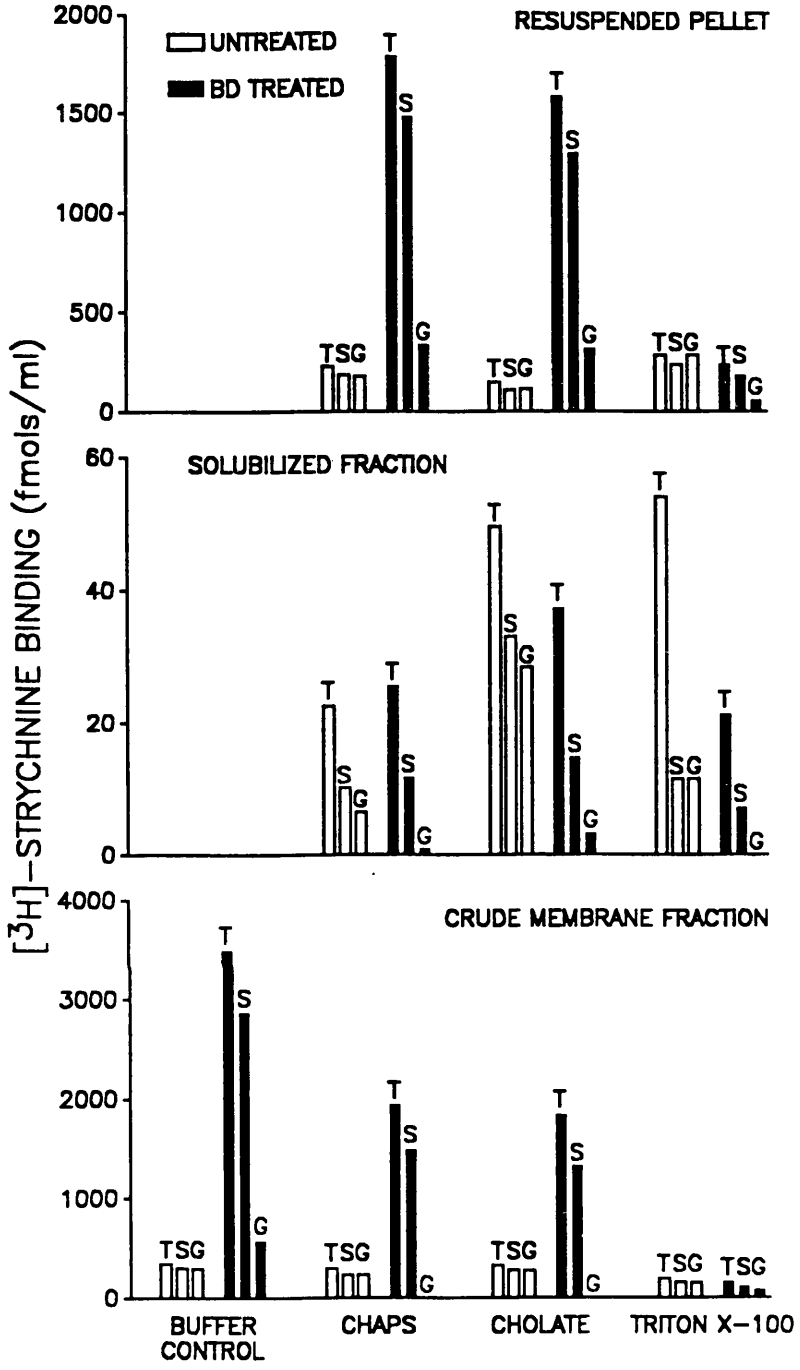


Figure 4.8: Solubilization of [^{3}H]-strychnine binding sites from untreated and BD-treated rat spinal cord membranes: Untreated and BD-treated membranes in PBS (10 vols tissue wt./vol) were incubated with CHAPS (1.5% w/v), sodium cholate (1% w/v) or Triton-X-100 (1.5% w/v) in presence of PTC (1.5 mg/ml) and a protease inhibitor cocktail. Buffer control contained no added detergent. Aliquots of detergent incubations were centrifuged (1 hr 100,000 g at 4°C), the supernatant (solubilized fraction) removed and the remaining pellet resuspended. [^{3}H]-strychnine (6 nM) was incubated with aliquots to enable triplicate estimates of total binding (T). Incubations which contained unlabelled strychnine (10^{-4}M) or glycine (10^{-2}M) were used to define strychnine (S) and glycine (G) specific binding. Assays were terminated by filtration, in solubilized fractions this was proceeded by a PEG precipitation. The experiment has been repeated once with similar results.

strychnine binding to untreated membranes assayed in the presence of detergent was similar to the amount bound to untreated membranes assayed without added detergent. In contrast, the binding to BD-treated membranes was sensitive to the presence of detergent. Although binding to BD treated membranes in the presence of detergents, CHAPS and sodium cholate was similar, it only represented approximately 55% the amount bound to treated membranes in which there was no added detergent. The third detergent tested Triton-X 100 caused a 95% reduction in the total [^{3}H]-strychnine bound when compared to BD-treated membranes assayed in the absence of added detergent. Although Triton-X 100 did effect some decrease in [^{3}H]-strychnine bound to untreated membranes, this was less drastic than the effect on binding to BD treated membrane.

The [^{3}H]-strychnine bound in the solubilized fraction indicated the three detergents were able to solubilize binding from untreated or BD treated membrane. 7, 15 and 16% of the binding associated with the untreated buffer control, were detected in the fractions solubilized by CHAPS, sodium cholate and Triton-X 100 respectively. The solubilized total binding from untreated membranes was equally sensitive to inhibition by unlabelled glycine or strychnine, although in the Triton solubilized fraction there appeared to be a marked fall in the proportion of the total binding inhibited by either displacer. The lower proportion of binding sites detected in the CHAPS solubilized fraction was compatible with the principle that this is a mild detergent. The proportion of the strychnine binding sites solubilized by sodium cholate and Triton-X 100 was lower than previously reported using the same concentration of these detergents (Pfeiffer *et al.*, 1982; Graham *et al.*, 1985) although this difference was likely related to the lower ionic strength of solubilization buffer used in the study reported here.

The [^{3}H]-strychnine binding in fractions solubilized from BD-treated membranes compared with those solubilized from untreated was similar when CHAPS was used or lower when sodium cholate and Triton-X 100 were used. This was despite the increase in [^{3}H]-strychnine binding to crude membranes treated with BD. Interpreting this result in relation to the [^{3}H]-strychnine bound to solubilized fractions of untreated membranes was complicated. Firstly the [^{3}H]-strychnine binding induced by BD-treatment was shown to be sensitive to inhibition by detergents, particularly Triton-X-100. This may account for the inability to detect an increase in the [^{3}H]-strychnine binding associated with fractions solubilized from BD-treated membranes. Secondly, although the total [^{3}H]-

strychnine binding in fractions solubilized from BD-treated membranes were characterized by their differential sensitivity to inhibition by strychnine and glycine, this could relate to solubilization of BD modified glycine receptor rather than the BD-induced strychnine binding site.

The pellet remaining after removal of the solubilized fraction was resuspended in PBS and in untreated membrane the [^{3}H]-strychnine binding was consistent with partial solubilization of [^{3}H]-strychnine binding by the three detergents. When strychnine-specific binding in the resuspended pellet and the solubilized fractions were summed they accounted for 65% (CHAPS), 40% (sodium cholate), and 45% (Triton) of the binding detected in buffer controls. A large proportion of the [^{3}H]-strychnine binding sites in the BD treated membranes assayed in the absence of detergent were no longer detectable in the pellets resuspended in PBS, which indicates either an inactivation of the sites or removal by solubilization. The removal of sites by solubilization was not reflected in the [^{3}H]-strychnine binding assayed in the solubilized fractions for any of the detergents. An inhibition of [^{3}H]-strychnine binding to the BD-induced site by the detergent could underlie the failure to detect binding to the solubilized BD-induced binding site. However, the reduced [^{3}H]-strychnine binding remaining in the pellets could indicate that sites were inactivated by the detergents or the binding prevented by detergent that remained trapped in the pellet. In this respect the reduction in binding to BD-treated membranes assayed in the presence of Triton-X 100 was not reversed when the Triton treated membranes were pelleted and resuspended in PBS.

4.3 Discussion

4.3.a Correlation of [^{3}H]-strychnine bound to untreated and treated membrane preparations

Initial experiments described in this Chapter adopted an indirect approach to address the question as to whether the increase in [^{3}H]-strychnine binding to BD- treated membranes occurred at the inhibitory glycine receptor. This used the amount of [^{3}H]-strychnine bound to untreated preparations as direct assay of the glycine receptor protein and considered how fluctuations in this parameter were reflected in the amount detected in BD-treated preparations.

In the first experiment [^{3}H]-strychnine binding to membranes from different regions of CNS indicated there was a heterogenous caudo-rostral distribution. This was in agreement with previous descriptions of [^{3}H]-strychnine binding to CNS membranes (Young and Snyder, 1973; Le Fort *et al.*, 1978) and tissue sections (Zarbin *et al.*, 1981; Frostholm and Rotter, 1985; Probst *et al.*, 1986; White *et al.*, 1990). Although minor discrepancies exist (see Section 1.4.a) gross caudal rostral distribution has been to reflect changes in distribution of receptor protein by immunocytochemistry using monoclonal antibodies raised against the inhibitory glycine receptor (Araki *et al.*, 1988; Basbaum, 1988; van den Pol and Gorcs, 1988). This simply reflects the predominant role of glycine as a inhibitory transmitter in the lower neuroaxis.

When membranes from eight regions of the CNS were treated with BD they all showed an increase in [^{3}H]-strychnine which showed a caudo-rostral distribution. However, it would be premature to state that the correlation between the amount of [^{3}H]-strychnine bound to untreated and BD-treated membranes indicates that the induced binding occurs at the inhibitory glycine receptor. Indeed, the correlation between the amount of [^{3}H]-strychnine bound to untreated and BD-treated membranes when all data was used to make the comparison was not always apparent when the data from individual regions of the CNS was compared. In addition, BD treatment allowed detection of [^{3}H]-strychnine binding to regions which had undetectable amounts of binding when untreated. However, sensitivity of membrane binding assay might prevent detection of low receptor concentration. Despite a correlation in the [^{3}H]-strychnine bound to untreated and BD-treated membranes there are discrepancies that argue against the inhibitory glycine receptor being the causal link.

This interpretation finds agreement with the experiment in which a comparison was made of the effect of BD treatment on subsequent [^{3}H]-strychnine binding to spinal cord membranes from normal, littermate and *spastic* mouse. The mutant mouse *spastic* suffers from a motor disorder of autosomal recessive inheritance which has been characterized to have an associated decrease in the amount of [^{3}H]-strychnine binding sites in CNS (White and Heller, 1982). The decrease in binding relates to a decrease in number of inhibitory glycine receptors rather than a mutation of the normal receptor protein (White, 1985; Becker *et al.*, 1986; Phelan, 1987). As a consequence there is a reduction in glycinergic synaptic inhibition which underlies the phenotypic expression of the mutant

(Biscoe and Duchen, 1986).

When the [^{3}H]-strychnine binding was measured in untreated spinal cord membranes of the mutant there was only 20% of the binding in the untreated normal or unaffected littermate mouse spinal cord membranes. Despite this significant difference in binding between the untreated *spastic* and the littermate mouse spinal cord membranes the increase in [^{3}H]-strychnine binding induced by BD treatment was not different in the two preparations. It is important to note that the concentration of BD used to treat membranes was sub-maximal. Therefore, similar amounts of [^{3}H]-strychnine bound to BD-treated littermate and *spastic* spinal cord membranes despite 75% fewer glycine receptor binding sites in untreated *spastic* membranes argues against the increase in binding in BD-treated membranes being at the inhibitory glycine receptor. The significant increase in the [^{3}H]-strychnine bound to BD-treated normal mouse membranes when compared to treated littermate and *spastic* was not considered a specific phenomenon. Most likely it related to genetic drift that occurred in the C57Bl (normal) colony which served as the background for the *spastic* colony some years ago.

4.3.b. Biochemical characterization of [^{3}H]-strychnine binding to untreated and BD treated preparations

Experiments run concurrently with those described above considered the direct effect of BD treatment on the binding of [^{3}H]-strychnine to the inhibitory glycine receptor. This initially involved the solubilization and affinity purification of [^{3}H]-strychnine binding from mouse spinal cord membranes. Experiments that described the effect of strychnine protection on the BD-induced [^{3}H]-strychnine binding in spinal cord membranes (see section 3.2.d.5) implicated a conformational-dependent modification which influenced the strategy used to affinity purify the inhibitory glycine receptor. The procedure that was developed endeavoured to purify the receptor while causing minimal disturbance to the protein-protein interactions that were suspected to underlie the conformational dependence of the modification. For this reason the spinal cord membranes were solubilized in the presence of exogenous lipid which is often necessary to preserve full functionality (Haga *et al.*, 1990) and avoided the use of high salt which can be routinely used to increase efficiency of solubilization (Pfeiffer and Betz, 1981) but might compromise protein function. In addition, CHAPS was chosen as a detergent because it

is considered non-denaturing (Hjemland, 1986) and has previously been shown to be important in the preservation of the repertoire of ligand binding to different sites on the membrane bound $GABA_A$/Benzodiazepine receptor when solubilized (Sigel and Barnard, 1984).

As indicated in section 4.2.c.1 the conditions used to solubilize the [^{3}H]-strychnine binding sites from spinal cord membranes compromised yield in solubilized fraction when compared to previous purifications (Pfeiffer *et al.*, 1982; Graham *et al.*, 1985). However, this was somewhat compensated by the number of sites that survived the affinity column and were recovered in the eluted fraction. In fact, the final yield of affinity purified sites in terms percent starting material compared fairly well with other procedures. A major problem of the purification described was the inability to detect protein in the concentrated active fraction by Coomassie assay or resolve peptides that have previously been characterized as constituents of the affinity purified glycine receptors. The association of [^{3}H]-strychnine binding with non-detectable amounts of protein indicated that the fraction was fairly pure but this could not be quantified. As percent yield of the purification was comparable to previous procedures the limiting factor in these purifications was the amount of receptor in the starting material and reflects the limitation of using mouse as the source tissue in the receptor purification.

There was no apparent increase in [^{3}H]-strychnine binding to purified receptors treated with BD under conditions which cause a 10 fold increase in binding to spinal cord membranes. Although detergent can inhibit the increased [^{3}H]-strychnine binding induced in BD-treated membranes it was unlikely that CHAPS, the detergent used to solubilize the membranes, would have completely masked a potential increase in [^{3}H]-strychnine binding to BD-modified receptor. Thus, direct use of purified receptor indicated that the increase in [^{3}H]-strychnine binding to BD-treated spinal cord membranes was not due to modification and binding at the inhibitory glycine receptor. A possible interference of binding might have been circumvented by removing detergent without perturbing protein function by reconstituting the receptor into liposomes prior to protein modification (Ruíz-Gómez *et al.*, 1989, 1990) but this was not attempted.

A further aspect of the experiments that considered the effects of BD treatment of purified glycine receptor on subsequent [^{3}H]-strychnine binding was the study of the direct

effects on the receptor protein. The treatment caused a decrease in total [^{3}H]-strychnine binding to the purified receptor and an associated decrease in the glycine- specific binding. As the strychnine-specific binding to the remaining sites appeared less effected, the BD-treatment appears differentially to effect the interaction of glycine with the receptor. Therefore it appears that strychnine specific [^{3}H]-strychnine binding to the inhibitory receptor largely survives the BD treatment and should be present in the BD treated spinal cord membranes. However, the receptor present in the treated membranes would have modified characteristic as the ability of glycine to inhibit binding to the receptor is perturbed. Consequently in BD-treated membranes neither the [^{3}H]-strychnine binding to the modified inhibitory glycine receptor or the BD-induced site are sensitive to glycine.

The interpretation that [^{3}H]-strychnine binding to the inhibitory glycine receptor prevailed with modified properties in the BD treated membranes was evident in experiments which attempted to use [^{3}H]-strychnine as a photoaffinity label for strychnine binding sites in untreated or BD treated membranes. Irradiation of membrane bound and purified glycine receptor incubated with [^{3}H]-strychnine has previously been shown to photolabel the 48kd subunit of the glycine receptor which has been speculated to harbour the strychnine binding site (see section 1.6.b). Based on published results, a procedure was developed and biased to maximize a potential incorporation into BD-treated membranes. Despite the increase in reversible [^{3}H]-strychnine binding to BD-treated membranes and the insensitivity of this site to UV irradiation the amount of ligand irreversibly incorporated into untreated and BD-treated membranes was very similar. This was reflected by extremely low efficiency of photoincorporation into BD-treated membranes. However, when photolabelled membranes were solubilized, subjected to PAGE and prepared for fluorography, it was possible to detect a single labelled peptide of 48 kd in untreated and BD-treated membranes indicating irreversible incorporation of ligand only occurred at the inhibitory glycine receptor in both preparations. However, as shown by the pattern of labelling the interaction with [^{3}H]-strychnine and the glycine receptor is modified by the BD treatment. Firstly, the intensity of labelling to BD-treated membrane was consistently lower which is taken to indicate an inhibition of irreversibly incorporated [^{3}H]-strychnine. Secondly, unlike the untreated membranes where incubations in the presence of unlabelled strychnine and glycine prevented photolabelling, the ability of glycine to prevent photolabelling to the BD-treated membranes appeared retarded.

Thus, the photolabelling experiments confirm the effect of BD treatment on the inhibitory receptor and provide further information that indicates that the increased [^{3}H]-strychnine binding associated with BD treatment of spinal cord membranes was not located at the inhibitory glycine receptor. In addition a photolabelled peptide specifically associated with increased [^{3}H]-strychnine binding was not detected. This may relate to different ligand recognition properties of strychnine binding to the inhibitory glycine receptor and the BD-induced binding site. Although membranes were irradiated at a wavelength (254 nm) at which strychnine absorbs strongly (Mackerer *et al.*, 1977), it has been proposed that photoincorporation of the molecule into the receptor depends on a energy transfer process from amino acids that lie at or close to the strychnine binding site (Graham *et al.*, 1983). The energy transfer from residues are particularly associated with aromatic residues (Goeldner and Hirth, 1983) and a lack of such residues might underlie the inability to photolabel the BD-induced site.

In the absence of quantitative fluorography the significance of the decrease in intensity of photolabelled peptide in the BD-treated membranes can only be speculated. However, it was consistent in 4 gels in spite of the relative similar amounts of radioactivity associated with each of the loaded fractions. Such a discrepancy was also associated with membrane photolabelled in the presence of glycine. It might be speculated that the induced site was labelled but at a level which was below the limit of sensitivity of the system used. Certainly, if this was dependent on a direct activation of the strychnine molecule rather than an energy transfer process it would be less efficient (Goeldner and Hirsch, 1983) than labelling at the inhibitory glycine receptor. A protein structure that might explain this discrepancy is described and discussed in Chapter 6.

4.3.c The effect of BD modification on ligand binding properties of the inhibitory glycine receptor:implications for ligand recognition

Although the increased [^{3}H]-strychnine binding induced in spinal cord membranes treated with BD was not associated with the inhibitory glycine receptor, this reagent clearly has an effect on the ligand recognition properties of the receptor protein. The BD treatment caused a decrease in total [^{3}H]-strychnine binding and the ability of unlabelled glycine to inhibit the total binding to the treated preparation. This was apparent whether reversible binding to the purified receptor or [^{3}H]-strychnine irreversibly bound to

membranes was used to assay ligand receptor interactions. The specificity of BD is not unequivocal (see Section 3.3.d). However, its selectivity means the effect is discussed in terms of a modification of arginine residues at the receptor protein.

As in previously discussed experiments (see Chapter 3) the effect of BD on ligand/receptor interaction was further investigated by ligand protection. These studies were preliminary in nature and clear interpretation of the results was compromised by an inability completely to remove strychnine, present as a protecting ligand, prior to estimates of reversible [^{3}H]-strychnine binding. Nevertheless, it appeared that strychnine afforded some protection against BD which might be interpreted as a modification of arginine residues directly involved in molecular recognition of strychnine. In contrast glycine protection appeared to accentuate the ability of BD treatment to reduce parameters of strychnine binding. It was interesting to note the reduction in inhibition of total binding by glycine was not prevented by either strychnine or glycine protection. This might indicate that the modified residues lie outside the binding site but are important in the conformational flexibility of the receptor protein. Interestingly, where an effect on ligand/receptor interaction by arginine selective reagent PG was discussed previously (Ruíz-Gómez *et al.*, 1990), it was in terms of residue(s) directly involved in strychnine recognition. The additional modification of residues important in receptor conformation could relate to a differential reactivity of reagents to BD and PG for relevant residues under the experimental conditions used. Alternatively, solubilized receptors as used in this study may be more susceptible to modifications that effect conformational flexibility whereas membrane bound or reconstituted receptors, modified by PG may be more robust.

The suggestion that arginine residues might be important in the molecular recognition of strychnine at the inhibitory glycine receptor was interesting in view of the identification of a BD modifiable residue(s) at the strychnine binding site of 2-AS-KLH antisera (see Section 3.3.e). It is interesting to speculate that the presence of an arginine residue at the binding site for the cationic hapten strychnine appears to contradict the idea of charge complimentary (Davies *et al.*, 1988). Indeed, there are descriptions that BD modifiable residues involved in hapten binding are absent in antibodies raised against uncharged or cationic haptens (Grossberg and Pressman, 1968). The selection for an arginine at strychnine binding site of the 2-AS-KLH antisera indicates the importance of this residue in the specific recognition of the alkaloid as reflected in the antisera and the

receptor binding. Details underlying such specificity are not understood although as pointed out previously (Ruíz-Gómez *et al.*, 1990) it could involve an interaction with carbonyl on ring III of strychnine (see fig 6.1) which has previously been implicated as a determinant of strychnine binding, particularly at 2-AS-KLH antisera (Phelan *et al.*, 1989).

By implicating differences in the homologous sequences of the α and ß subunits of the rat glycine receptor an attempt to rationalize the effect of the arginine selective reagent on the ligand binding properties can be made. This is based on the premise that the N-terminal sequence of the α subunit harbours the ligand binding site of the receptor complex. Interestingly of 3 arginines (242, 260 and 265; see Fig 1.3) located within this region of the α subunit thought to be important in the ligand recognition, 2 are not conserved in the ß subunit. These residues at position 242 and 260 are obvious candidate whose modification might effect and change the ligand recognition properties of the protein. Although this agrees with previous speculations (Grenningloh *et al.*, 1987; Ruíz-Gómez *et al.*, 1990) modification of other residues whether or not they are conserved in the α and ß subunit cannot be excluded.

In addition to a possible modification of residues directly involved in strychnine recognition it appeared that BD treatment modified protein interactions that effected the ability of glycine to inhibit strychnine binding which cannot be accounted for by residues directly involved in ligand recognition. These modifications may be specific to residues involved in conformational changes in receptor when bound with glycine or strychnine. However it is also possible that the modified residue(s) are more important in the more general function of structural integrity of the receptor. In the latter case, this could involve residues located on receptor domains or subunits that are not involved in ligand binding. In this respect arginine residues are particularly suited to the formation of extended patterns of hydrogen bonding (Riordan, 1979) or the formation of inter molecular salt bridges (Schulz and Schirmer, 1978). Thus, modification of one of the many arginine residues in the deduced sequence could have implications for structural integrity. Therefore speculation as to which residues are modified to cause the effects on the ability of glycine to displace [^{3}H]-strychnine bound to the receptor awaits more precise information concerning the residue involved.

4.3.d Implications of the attempts to solubilize the BD induced [^{3}H]-strychnine binding

The experiments described clearly rule out the possibility that the BD induced [^{3}H]-strychnine binding is a result of modification of binding to the inhibitory glycine receptor. In addition an attempt to identify peptides that are associated with the induced binding by photolabelling by procedures as described in this Chapter also proved unsuccessful. As an approach to identifying the site associated with the increased binding, the solubilization of the membranes was attempted with a view to eventually purifying a putative binding protein.

The preliminary experiments considered the ability of three detergents Triton-X 100 (Pfeiffer *et al.*, 1982), sodium cholate (Graham *et al.*, 1985; García-Calvo *et al.*, 1989) and CHAPS (see section 4.2.c) that have successfully been employed to solubilize the [^{3}H]-strychnine binding from mammalian spinal cord membranes. Indeed these detergents solubilized [^{3}H]-strychnine from the untreated membranes which was sensitive to both strychnine and glycine indicating an interaction with the inhibitory glycine receptor.

However, an inhibition or inactivation of the BD-induced [^{3}H]-strychnine binding to membranes was apparent for all 3 detergents particularly Triton-X 100. Thus, the BD-induced [^{3}H]-strychnine binding was markedly sensitive to detergent when compared to binding to untreated membranes. In fact, the [^{3}H]-strychnine binding detected in fractions solubilized from BD-treated membranes was compatible with binding to the inhibitory glycine receptor with modified characteristics. This indicated that BD-induced site was undetectable in its solubilized form. An inactivation of the BD-induced binding by detergent was further implicated by the [^{3}H]-strychnine binding detected in the resuspended pellet that remained after removal of the solubilized fraction. It was again apparent that the deleterious effect of the detergent was most obvious in membranes subjected to Triton-X 100 solubilization. Clearly the BD-induced [^{3}H]-strychnine binding site displays different requirements to the inhibitory glycine receptor to maintain strychnine binding activity in a solubilized form. This requires further experimental investigation.

Nevertheless, the susceptibility of induced [^{3}H]-strychnine binding in BD-treated membranes, to detergent, might further indicate the nature of the site of this binding. Unlike the BD-induced binding, [^{3}H]-strychnine bound to the inhibitory glycine receptor appeared relatively insensitive to the presence of detergent. This may relate to the location of the strychnine binding site to the large extracelluar N-terminal domain of the ligand binding subunit of the receptor (see section 1.6.c). An apparent insensitivity towards detergents of ligands (Sigel and Barnard, 1984) believed to bind to the homologous region of the $GABA_A$ receptor subunits may indicate this is a general feature of ligands that bind to extracelluar receptor domains. In contrast, ligand binding activity associated with the transmembrane regions of integral membrane receptors is considered vulnerable to solubilization because these regions have a high affinity for detergent and are consequently susceptible to disruption of protein integrity. Any effect of detergent will be compounded by detergents which disrupt weak protein-protein interactions and strip away endogenous lipids (Haga *et al.*, 1990). This is thought to underlie the sensitivity toward detergent of the binding activity of ligand-gated receptor channel blockers and ligands that interact with G-protein linked receptor proteins. In both cases the binding sites are thought to be located in transmembrane domains of the receptor proteins (Barnard *et al.*, 1987; Strader *et al.*, 1989). By inference the inhibition of the BD induced [^{3}H]-strychnine binding, most apparent for Triton-X 100, might indicate that the induced binding site is membrane buried. This finds agreement with the previously discussed observation that the increase in [^{3}H]-strychnine binding to spinal cord membranes require reaction with a membrane permeable arginine-selective reagent (see section 3.3.d) and is consistent with the pharmacological characteristics of the BD-induced [^{3}H]-strychnine site as described in Chapter 5 and 6.

CHAPTER FIVE

An investigation of the parameters of equilibrium binding to BD treated spinal cord membranes.

5.1 Introduction

For an interaction between a ligand and its binding site that obeys the law of mass action, the amount of bound ligand at equilibrium is defined as:

$$\text{Bound} = B_{max}.F/K_D + F$$

B_{max} = No. of binding sites.
K_D = affinity of site for ligand.
F = concentration of free ligand.

For this reason the estimation of bound ligand in response to variation of free ligand can be used as an experimental protocol for estimating K_D and B_{max}. These experiments involve saturation of specific binding sites either by an increase in concentration of labelled ligand or increasing the concentration of unlabelled ligand in the presence of a single concentration of labelled ligand (De Blasi *et al.*, 1989). The K_D and B_{max} serve as useful points of reference when considering the nature of the interactions between a ligand and its binding sites. Analysis of the equilibrium binding of [^{3}H]-strychnine to BD-treated membranes to estimate B_{max} and K_D therefore should provide insight into the nature of the binding induced in BD treated membranes. However, it was clearly shown in the previous chapter that BD treatment of spinal cord membrane has two distinct effects on subsequent [^{3}H]-strychnine binding. Firstly, BD treatment modified [^{3}H]-strychnine binding to the inhibitory glycine receptor that is detected in untreated membrane. This modification primarily affected the ability of glycine to inhibit total [^{3}H]-strychnine binding to the receptor, although there was also a decrease in the total [^{3}H]-strychnine bound to the purified receptor preparation. More obviously, BD treatment also affected the induction of a [^{3}H]-strychnine binding site that was not apparent in the untreated membrane. As a consequence, [^{3}H]-strychnine binding to the BD-treated membranes consists of a component to the modified inhibitory glycine receptor and the

induced strychnine binding site.

The primary aim of these studies was to define parameters of [^{3}H]-strychnine binding to BD treated membranes. Therefore it was appropriate to optimize the assay conditions to ensure the parameters B_{max} and K_D for this site were adequately defined. It was also important to resolve the [^{3}H]-strychnine binding to the modified glycine receptor in the BD-treated membranes in order to ensure that the induced site is adequately defined. In parallel experiments, equilibrium binding of [^{3}H]-strychnine to untreated and BD-treated membranes was addressed. The initial motivation behind this aspect of the investigation were attempts to resolve a [^{3}H]-strychnine binding site in untreated membranes, additional to that at the glycine receptor, whose modification might underlie the BD-induced binding.

From the above, it is clear that any analysis of binding to untreated and BD-treated membranes needs to consider that [^{3}H]-strychnine binding may be more complex than an interaction described by a ligand binding to a single class of homogenous sites. Indeed, the speculation that evokes two binding sites would mean that at equilibrium the bound ligand could be defined as:

$$\text{Bound} = B_{max}^{G}.F/F+K_D^{G} + B_{max}^{BD}.F/F+K_D^{BD}$$

B_{max}^{G} = No. of binding sites associated with glycine receptor

B_{max}^{BD} = No. of binding sites associated with BD induced site

K_D^{G} = affinity of strychnine for glycine receptor

K_D^{BD} = affinity of strychnine for BD induced site

F = concentration of free strychnine

Where ligand binds to a single class of homogeneous sites linearized forms of the binding data as in Scatchard or Hofstee plots are sufficient to define the parameters B_{max} and K_D (see section 2.16). Deviations from linearity can indicate the existence of more complex ligand binding interactions and although multiple components can be fitted to the curvilinear derivitizations, where multiple sites exist, the method provides poorly defined values for K_D and B_{max}. This approach is limited by the fact that the lines used to fit curvilinear data and define the components of multiple binding sites, are not independent.

The preferred method of analysis is to use computer assisted non-linear curve fitting techniques (Unnerstall, 1990). These procedures provide accurate assessments of binding parameters because they delineate the contribution of binding to one site from another. In general, the procedures are developed on the rationale of iterative curve fitting. Using initial estimates, usually derived from the cruder linearizing of data, the fitting programs estimates binding contributed by each site before comparing how well calculated values fit the experimental data. This process is performed iteratively until the "goodness of fit" between the calculated values and experimental data is optimized.

For saturation of [^{3}H]-strychnine binding or inhibition of [^{3}H]-strychnine binding by unlabelled strychnine initial analysis was performed using EBDA (McPherson, 1983). This program serves a dual purpose as it is convenient for entering crude binding data and also provides the initial estimates of K_D and B_{max} required for further analysis by non-linear iterative curve fitting. In EBDA the data is analyzed by a linear transformation from which estimates of the kinetic parameters of multiple sites can be made by linear interpolation. EBDA finally creates files which can be further analyzed by Scafit (Munson and Rodbard, 1980). Scafit is the non-linear curve fitting routine of the ligand binding analysis program Ligand (Munson and Rodbard, 1980).

In addition to re-iterating initial estimates for single or multiple binding sites to give final estimates of the parameters K_D and B_{max}, Scafit has additional facilities that are important for ligand binding analysis. In particular Scafit enables a statistical comparison to be made between the data by use of the F-test which compares the residual variance of the fit of data to models of increasing complexity. It also provides statistical analysis of the distribution of points to the fit by Runs test, where $p>0.05$ signifies random distribution and accordingly, a good fit to the experimental data. Thus, the F-test and Runs test are used as criteria to establish whether a model of multiple binding sites rather than a one site model provides a better fit to the experimental data. As Scafit allows non-specific binding to be treated as a fitted parameter it is convenient to compare such fits to those obtained when non-specific binding has not been treated as a fitted parameter. If the two fits so obtained are not significantly different, then the empirical estimate of non-specific binding can be taken as an adequate definition of this parameter. The parameter for the fitted non-specific (N) is defined by the ratio of bound/free at infinite free concentration.

Data from experiments that were designed to define the parameters of strychnine binding to untreated and BD treated membranes are presented and analyzed in accordance with the principles laid out above. The Scafit analysis for each experiment is summarized in tabular form. The tables illustrate final estimates of K_D, based on its reciprocal value K_A given by the Scafit analysis. Also shown are the calculated B_{max} at each site. Scafit also provides crude estimates of the standard errors of these calculated parameters which are shown. Significance of the F-value and Runs test are indicated and so is the ratio (N) if non-specific binding was a fitted parameter.

5.2 Results and discussion

5.2.a Saturation of [^{3}H]-strychnine binding (0.1-33 nM) to untreated and BD-treated spinal cord membranes

In the first of the series of experiments designed to determine the parameters of equilibrium [^{3}H]-strychnine binding, untreated and BD-treated mouse spinal cord membranes were incubated at 4°C with increasing concentrations of labelled strychnine between 0.1-33 nM. Non-specific binding was defined by unlabelled strychnine (10^{-4}M) and the incubations were terminated after 2 hrs by filtration.

In the untreated membrane, the [^{3}H]-strychnine binding saturated with increasing concentration of ligand (fig 5.1.a). Thus, it appeared that the increased binding to BD-treated spinal cord membranes was caused by the induction of a strychnine binding site that was of lower affinity and higher capacity than the site at the inhibitory glycine receptor. This interpretation was confirmed when data was linearized and analyzed as a Eadie-Hofstee plot (fig 5.1.b and 5.1.d). The [^{3}H]-strychnine binding to untreated membranes was characterized by a K_D of approximately 10nM and a B_{max} of approximately 1 pmol/mg protein. In BD treated membranes, the estimated K_D was approximately 10 fold higher and B_{max} 20 fold higher than in untreated membranes. However, the clustering of points in the Eadie-Hofstee plot for BD-treated membrane would lead to poor estimates. Despite the increased binding capacity of BD-treated membranes at each ligand concentration, less than 5% of the labelled ligand was bound when the assays were terminated after 2 hrs.

Further analysis of the data using Scafit attempted to resolve two [^{3}H]-strychnine

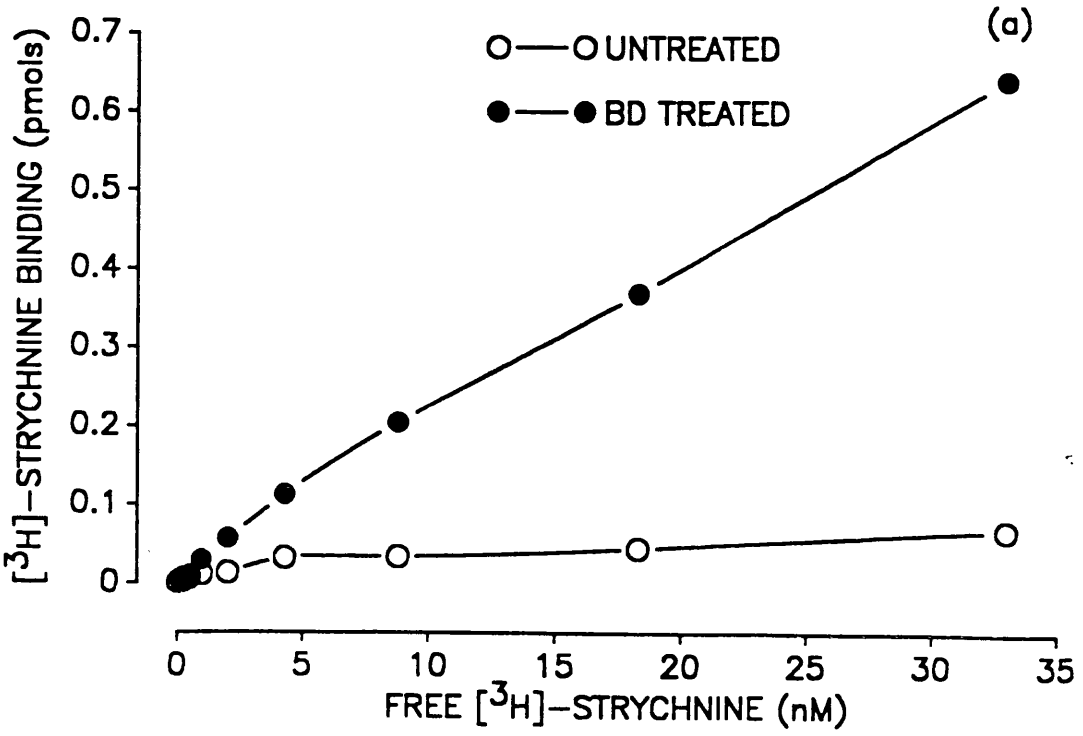
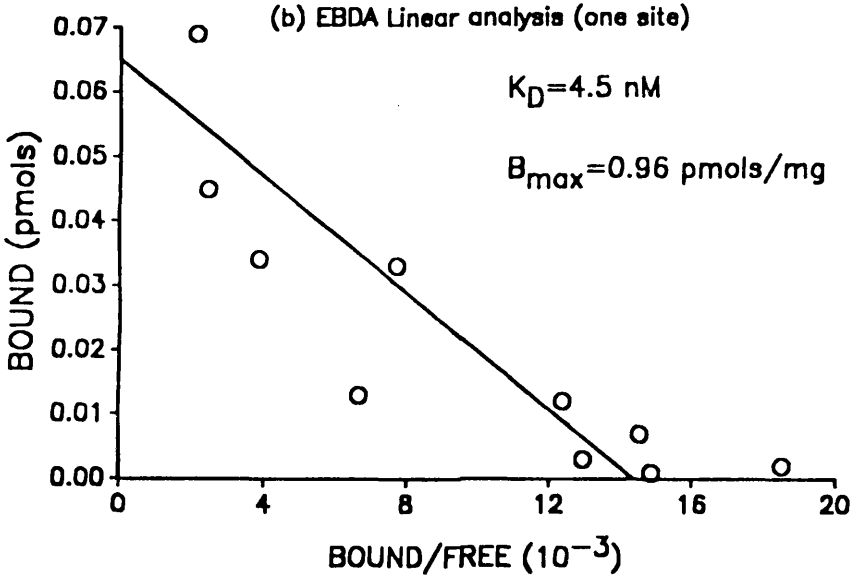
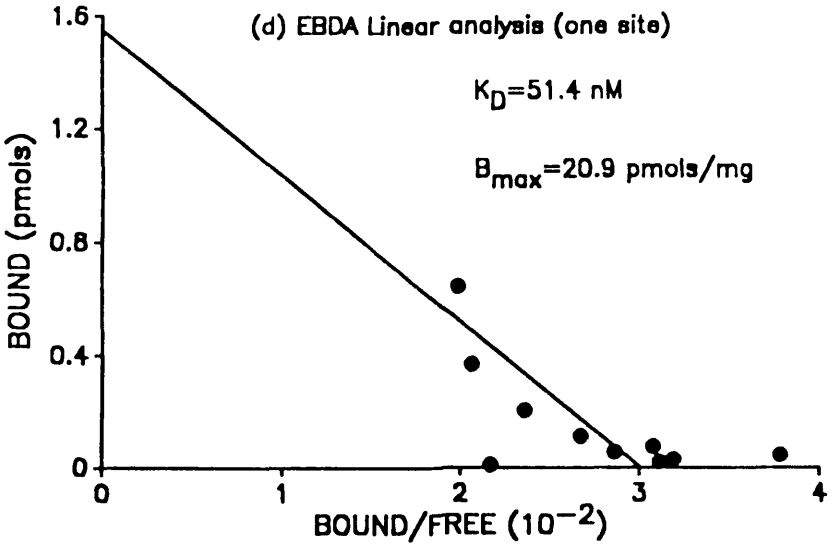


Figure 5.1: Estimates of equilibrium binding parameters for [^{3}H]-strychnine binding: (a) [^{3}H]-strychnine specifically bound to untreated (68 μg protein) and BD-treated (74 μg protein) membranes incubated with increased concentrations of ligand (0.01-35 nM). Non-specific binding was defined by incubations that contained unlabelled strychnine (10^{-4}M) and the assays incubated for 2 hr at 4°C were terminated by filtration. Each point represents the mean of triplicate estimates. The same data is shown as Eadie-Hofstee plots in (b) and (d), where the estimates of K_D and B_{max} were derived from linear least square fits of this data by EBDA. Tables (c) and (e) summarize SCAFIT analysis of this data by non-linear least square iterative curve fit procedures. Strychnine binding that predominated in untreated membranes was designated site^1 and that in BD treated membranes, site^2. The possibility that additional sites exist was investigated by fitting the data to more complex models and comparing goodness of fit by F-tests and the Runs test.



MODEL	[K_A ± S.E.] (mol^{-1}) K_D^1 (nM)	B_{max}^1 ± S.E. (pmols/mg)	[K_A ± S.E.] (mol^{-1}) K_D^1 (μM)	B_{max}^2 ± S.E. (pmols/mg)	N ± S.E.	Runs Test	F value	
one site	[2.5E8 ± 5.1E7] 4.02	0.882 ± 0.117	–	–	–	p> 0.05	3.97 (p=0.09)	-0.58 (p=1)
one site fitted NS	[4.7E8 ± 1.7E8] 2.11	0.475 ± 0.150	–	–	1.1E-3 ± 0.5E-3	p> 0.05	–	-2.1 (p=1)
two site	ILL CONDITIONED							
two site fitted NS	[5.4E8 ± 4.0E8] 1.89	0.496 ± 0.035	[2.0E5 ± 2.6E13] 4.88	34.6 ± 22,000	0	p> 0.05		–

(c) SCAFIT non-linear analysis



MODEL	[K_A ± S.E.] (mol^{-1}) K_D^1 (nM)	B_{max}^1 ± S.E. (pmols/mg)	[K_A ± S.E.] (mol^{-1}) K_D^2 (µM)	B_{max}^2 ± S.E. (pmols/mg)	N ± S.E.	Runs Test	F value		
one site			[2.0E7 ± 7.1E6] 0.05	19.2 ± 6.3		p>0.05	-6.64 (p=1)	0.76 (p=0.5)	-1.61 (p=0.5)
one site fitted NS			[1.7E8 ± 7.3E21] 0.06	6.4E-5 ± 21.8	0.06 ± 0.72	p<0.01	_	142 (p=0)	-0.92 (p=1)
two site	[1.5E8 ± 2.8E8] 6.8	1.31 ± 3.5	[7.5E4 ± 1.4E7] 13.2	3076 ± 580,000		p>0.05		_	-4.87 (p=1)
two site fitted NS	[1.7E8 ± 1.5E10] 5.7	1.19 ± 162.2	[1.1E6 ± 8.4E13] 0.9	0	0.89 ± 43.4	p>0.05			_

(e) SCAFIT non-linear analysis

binding sites in untreated and BD treated membranes. In untreated membranes this corresponded to an attempt to fit a low affinity site of high capacity, characteristic of BD treated membranes, in addition to the high affinity binding to the inhibitory glycine receptor. In contrast, in BD treated membranes the objective was to resolve high affinity binding to the inhibitory glycine receptor. The high affinity binding was designated site[1] and low affinity site[2]. Also, [^{3}H]-strychnine binding was presumed to be dominated by the high affinity site in untreated membrane and low affinity site in BD treated membrane and the bias of the one site fits reflect this assumption.

In the untreated membrane a comparison of fits to models of increasing complexity (fig 5.1.c) indicated the data tested fitted a one site model which was defined by a K_D of 4 nM and a B_{max} of 0.884 pmol/mg. The fit was not improved by treating non-specific as a fitted parameter. Neither was the data better fitted to either of the 2 site models.

When binding to BD treated membranes was analyzed the data best fitted a one site model but as expected the K_D of 50 nM and B_{max} of 19.2 pmol/mg indicated a site of lower affinity and higher capacity than described in the untreated membranes. An additional high affinity site with similar parameter estimates to the binding at the glycine receptor characterized in untreated membranes, could be fitted to data for BD-treated membranes. However, the resultant 2 site models did not fit the data as well as the one site model.

Qualititatively, these results indicate that the site induced by BD treatment was of lower affinity and higher capacity than binding in untreated membranes. However, in the BD-treated membranes the estimate of K_D and B_{max} are poorly defined, a direct result of the fact that in treated membrane the highest concentration of [^{3}H]-strychnine used was insufficient to saturate binding. In order to measure a site accurately it should be at least 70-80 % saturated (Bürgisser, 1984) which translates into a ligand concentration 8-10 times the K_D (Unnerstall, 1990). Also it is considered inappropriate to measure binding to a site with a K_D >10 nM using filtration as the method of separating free and bound ligand. This empirical rule is assumed on the basis that low affinity sites have a rapid rate of disassociation. Therefore, the excessive contact with ligand free buffer that arise during the filtration protocol promote disassociation and artifactually decreased estimates of bound ligand at equilibrium. This effect can be circumvented by separating free and bound ligand by centrifugation (Bennett and Yamamura, 1985).

The best definition of the K_D and B_{max} for [^{3}H]-strychnine binding to the BD-induced site would be derived from a model that resolves a second site in treated membranes. Thus ligand bound to the inhibitory glycine receptor could be accounted for when deriving estimates of K_D and B_{max} for the induced strychnine binding site. Using the experimental conditions described this was not achieved. However, as the number of experimental points used to define binding can limit the ability to resolve 2 sites (Bürgisser, 1984) limiting the experiments to 10 points might compromise attempts to define a second site. Such a problem might have been compounded by the inappropriate range over which experimental concentrations was extended.

In untreated membranes the saturation of [^{3}H]-strychnine binding under experimental conditions was best defined by a one site model. The detection of a single site and the estimates of the parameters K_D and B_{max} are in good agreement with previous reports that have used similar experimental conditions to investigate high affinity [^{3}H]-strychnine binding to the inhibitory glycine receptor in normal rodent spinal cord (Braestrup *et al.*, 1986; Marvizón *et al.*, 1986a, Becker *et al.*, 1986; Phelan, 1987). However, as indicated for BD-treated membranes, these conditions may have prevented the adequate definition of low affinity site in addition to binding to the inhibitory glycine receptor in untreated membranes.

5.2.b Saturation of [^{3}H]-strychnine binding to untreated and BD treated membranes over an extended range of ligand concentrations

In view of the points discussed in the previous section, saturation of [^{3}H]-strychnine binding was investigated in untreated membranes by incubating with 18 ligand concentrations in range 0.1-318 nM and in BD treated membranes by 32 ligand concentrations in range 0.1-1018 nM. For concentrations between 0.1-40 nM the ligand concentrations were achieved by adding a dilution of manufacturers stock in assay buffer. Ligand concentrations above this were achieved by isotopic dilution of 40 nM of manufacturers stock with increasing concentrations of unlabelled strychnine. Non-specific binding was estimated by incubations that contained unlabelled strychnine (10^{-4}M) and assay blanks were estimated by identical incubations that had no added membrane. The incubations were terminated after 2 hours at 4°C by centrifugation (see section 2.11.b).

Where the membranes were incubated with ligand directly diluted from stock strychnine bound was estimated directly from dpm bound. However when the free ligand was derived from isotopic dilution the bound strychnine was calculated (Bowery *et al.*, 1983) as indicated:

$$B=b(1 + C/H)$$

B=total ligand specifically bound
b=radioactive ligand specifically bound
C=molar concentration of non-radioactive ligand
H=molar concentration of radioactive ligand

For the purpose of EBDA and subsequent SCAFIT analysis the calculated bound ligand were converted to dpm which would correspond to the dpm bound if free ligand had not been derived from an isotopic dilution.

The strychnine specifically bound to untreated and BD-treated membranes when incubated with increasing concentrations of ligand are shown in fig 5.2.a. Some [^{3}H]-strychnine bound to incubation tubes in a strychnine displaceable manner (fig 5.3) therefore this component of total binding was added to the amount of strychnine defined as non-specifically bound to membrane to give the value used ultimately to define specific strychnine binding.

There was a small increase in the amount of strychnine defined as specifically bound to untreated membranes in comparison to the large increase in the BD-treated membranes (fig 5.2.a). This data transformed to an Eadie-Hofstee plot has a distinct non-linear distribution (fig 5.2.b). Although EBDA provided estimates of K_D (94 nM) and B_{max} (30.7 pmols/mg), these were considerably higher than those previously described for [^{3}H]-strychnine binding to untreated membrane (see section 5.2.a). It was also clear from visual inspection that non-linearity arose from two distinct components. Further, SCAFIT analysis of the data indicated that the one site model with similar estimates of K_D and B_{max} provided an inadequate fit to the data. This was indicated by the Runs test for the one site model ($p<0.01$) and F values ($p=0$) when more complex models were compared to the one site model.

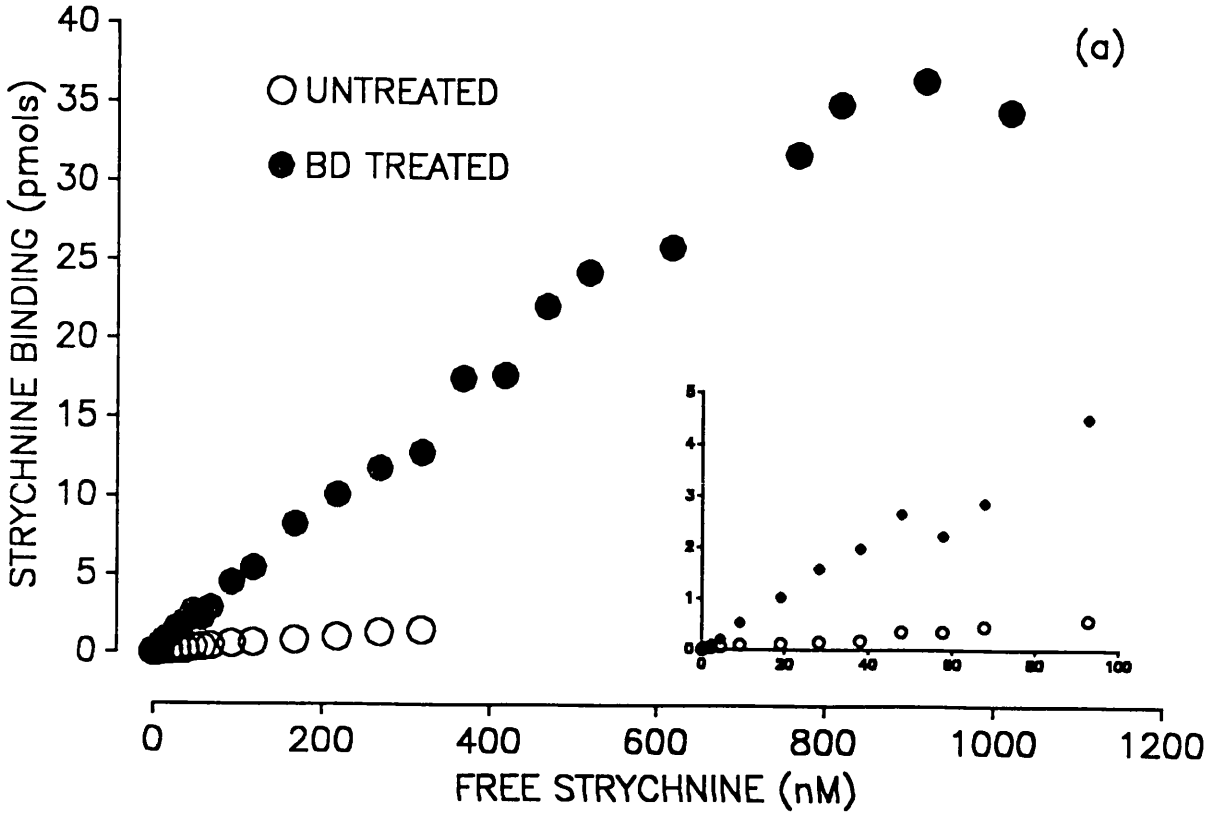
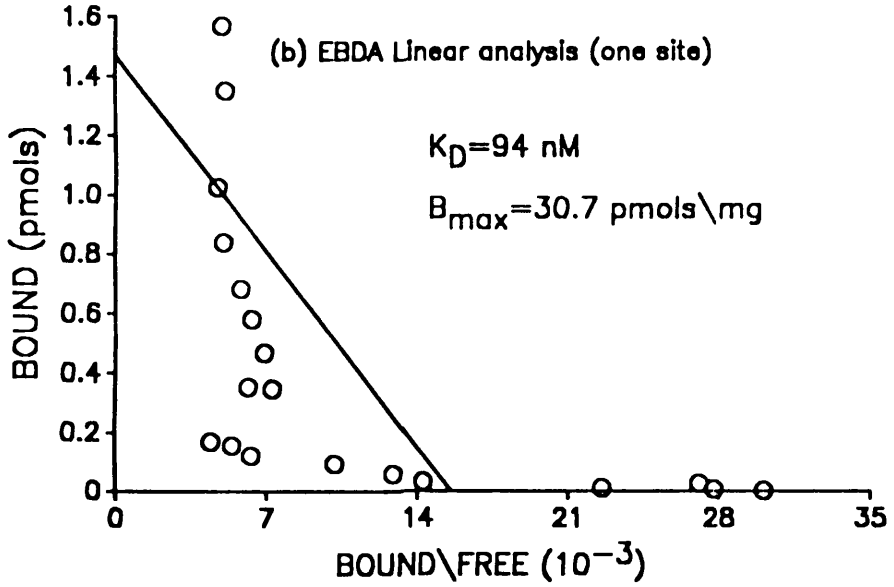
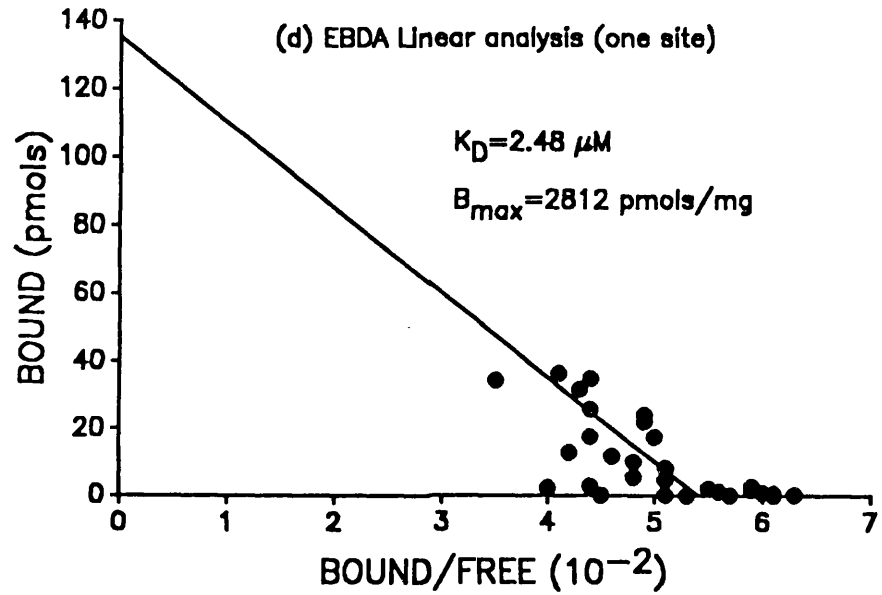


Figure 5.2: Estimates of equilibrium binding parameters for strychnine binding to untreated and BD-treated mouse spinal cord membranes: (a) Amount of strychnine specifically bound to untreated and BD-treated (48μg protein) membranes incubated with increasing concentration of strychnine. Untreated membranes were incubated in range 0.1-318 nM and treated membranes in the range 0.1-1018 nM. Inset data for strychnine bound to membranes incubated between 0.1-93 nM. Ligand concentrations between 0.1-40 nM were achieved by diluting the ligand in assay buffer and concentrations >40 nM were defined by isotopic dilution. Incubations that contained excess (10^{-4}M) unlabelled strychnine were used to estimate non-specific binding. Strychnine displaceable component of total ligand bound to test tube added to the amount non-specifically bound to membranes was the value used to define specific strychnine binding. Assays were incubated for 2 hrs at 4°C were terminated by centrifugation. Each point represents the mean of triplicate estimates. Eadie-Hofstee plots of specific [^{3}H]-strychnine binding are shown in (b) and (d) where estimates of K_D and B_{max} were derived from linear least square fits of this data by EBDA. Tables (c) and (e) summariise the SCAFIT analysis of the data. For detail of parameters see fig 5.1.



MODEL	[K_A ± S.E.] (mol^{-1}) K_D^1 (nM)	B_{max}^1 ± S.E. (pmols/mg)	[K_A ± S.E.] (mol^{-1}) K_D^2 (µM)	B_{max}^2 ± S.E. (pmols/mg)	N ± S.E.	Runs Test	F value		
one site	[1.6E7 ± 5.3E6] 63.8	23.7 ± 5.4				p<0.01	128 (p=0)	62.4 (p=0)	62.4 (p=0)
one site fitted NS	[5.2E8 ± 1.6E6] 1.9	1.0 ± 0.229			0.005 ± 0.0004	p>0.05	_	0.55 (p=0.47)	0.55 (p=0.47)
two site	[6.1E8 ± 2.3E8] 1.6	0.881 ± 0.627	[3.2E5 ± 6.4E5] 3.1	333.3 ± 627		p>0.05		_	_
two site fitted NS	[6.1E8 ± 2.3E8] 1.6	0.881 ± 0.627	[3.2E5 ± 6.4E5] 3.1	333.3 ± 627	0	p>0.05			_

(c) SCAFIT non-linear analysis



MODEL	[K_A ± S.E.] (mol^{-1}) K_D^1 (nM)	B_{max}^1 ± S.E. (pmols/mg)	[K_A ± S.E.] (mol^{-1}) K_D^2 (μM)	B_{max}^2 ± S.E. (pmols/mg)	N ± S.E.	Runs Test	F value		
one site			[4.2E5 ± 8.6E4] 2.37	2700 ± 510		p>0.05	0.03 (p=0.864)	1.45 (p=0.254)	1.45 (p=0.254)
one site fitted NS			[4.5E5 ± 1.0E6] 2.2	2354 ± 9145	0.003 ± 0.08	p>0.05	–	2.87 (p=0.103)	2.87 (p=0.103)
two site	[3.2E7 ± 8.5E7] 3.0	4.5 ± 14.2	[2.7E5 ± 1.8E5] 3.6	3812.5 ± 2131.2		p>0.05		–	–
two site fitted NS	[3.1E7 ± 7.9E7] 3.2	4.9 ± 15.1	[2.6E5 ± 1.8E5] 3.7	3916.6 ± 2292.0	0	p>0.05			–

(e) SCAFIT non-linear analysis

Although both 2 site models provided better fits of the data than the simple one site model , the best fit was to a one site model where non-specific binding was estimated by SCAFIT. The model also gave estimates of K_D (1.9 nM) and B_{max} (1.0 pmol/mg) which were in good agreement with previous estimates (see section 5.2.a). This was the favoured interpretation, because the F values for one site fit with non-specific estimated by SCAFIT when compared to both 2 site models gave p values >0.05. This interpretation indicated that the empirical estimate of non-specific binding derived from incubations that contained an excess of unlabelled strychnine (10^{-4}M) inadequately defined this parameter and caused the marked non-linearity of the Eadie-Hofstee plot. Non-specific binding sites are characterized by a constant percentage of free ligand being bound (Unnerstall, 1990). Therefore the cluster of points that run parallel to the Y-axis in Fig 5.2.b that have constant bound/free values, define the non-specific binding site resolved by SCAFIT.

In the BD-treated membranes the increase in strychnine bound as the concentration of free strychnine in the incubation was increased did not saturate (see fig 5.2.b). When the data for BD-treated membranes was linearized the points were clustered together and as a consequence the Eadie-Hofstee plot was poorly defined. The estimate of K_D (2.48 µM) and B_{max} (2812 pmol/mg) derived by EBDA using a linear least square fit were much greater than estimates defined by the previous experiment (see section 5.2.a) which used a more limited range of ligand concentrations.

SCAFIT analysis defined parameters for a two site model which had characteristics of strychnine binding to the glycine receptor (K_D, nM; B_{max} 1 pmols/mg) in addition to the BD-induced site (K_D, µM; B_{max} >1000pmols/mg). However, the best fit was to a simple one site model as indicated by the F-values when this model was compared with fits of data to more complex models. Also, unlike the untreated membranes the empirical estimate provided an adequate definition of non-specific binding as the model that includes non-specific as an estimated parameter does not provide a better fit of the data.

Even though the experiment described above used assay conditions that favoured the definition of a low affinity site the range of ligand concentrations used were not extensive enough to saturate the BD-induced site. The estimates of K_D and B_{max} defined a site of low affinity and high capacity when compared to [^{3}H]-strychnine binding to the glycine receptor. However, inability to saturate the site means that these estimates are poorly defined. It was also not possible adequately to resolve a high affinity site, which

characterizes strychnine binding to the glycine receptor, from the data for strychnine binding to BD-treated membranes. It is possible that the inability to saturate and better define the strychnine binding to BD-treated membranes may hinder the attempts to resolve 2 sites. However, a ratio of >1000 from best fit estimates of B_{max} for strychnine binding to BD-treated compared to untreated suggests that the binding to the glycine receptor in treated membranes represents only a small proportion of strychnine bound therefore may not be resolvable.

In the untreated membrane, strychnine binding was assayed using the same conditions in BD treated membranes but over a less extensive range of ligand concentrations. SCAFIT analysis of the data was only able to resolve a non-specific binding site in addition to the high affinity strychnine binding to the inhibitory glycine receptor. However, the attempts to resolve a low affinity strychnine binding site could have been restricted by the fact that the ligand concentrations used to measure binding to untreated membranes were not over an extensive enough range to define such a site. If the induction of strychnine binding in treated membranes involved the modification of properties of an existing protein, it could involve an increase in the affinity of the site upon BD treatment. Therefore, to define such a site in its unmodified state might require an even more extensive range of ligand concentrations than needed to define the site in BD treated membranes.

In addition, although SCAFIT resolved the binding to a non-specific site, ill defined non-specific binding may also have hindered attempts to resolve 2 sites in the data for strychnine binding to untreated membranes. A major limitation with the experimental protocol was that isotopic dilution made it difficult accurately to determine nM specific-strychnine binding because of the low number of counts that were bound. In addition the best fits by SCAFIT (see fig 5.2.c) indicated the K_D for low affinity binding was at least 1 μM, hence the 10^{-4}M of unlabelled strychnine used may not provide sufficient excess to define non-specific binding especially in untreated membranes where it is possible that the low affinity site may have an even lower K_D. Finally, as indicated (see fig 5.3), there was a strychnine-displaceable component of total binding to the assay tubes. Although this was corrected for, if it was inappropriately defined it might have contributed to the ill definition of non-specific binding.

From the foregoing discussion, it was clear that experimental limitations might

prevent adequate definition of the parameters that define equilibrium binding to the BD-induced site and may also underlie inability to resolve the strychnine binding isotherm for untreated and BD-treated spinal cord membranes into two components. For this reason the assay conditions were further investigated.

5.2.c Optimizing the assay conditions used to define the parameters of equilibrium strychnine binding to untreated and BD-treated membranes

The possibility that inappropriate experimental conditions might prevent the adequate analysis and estimation of the parameters of equilibrium strychnine binding prompted an investigation of aspects of the assay conditions used to measure binding to untreated and BD-treated membrane. These experiments examined a method of preventing strychnine binding to the assay tube, incubation time required to achieve equilibration and compared assays terminated by filtration and centrifugation. The aim was to optimize the assay conditions used before attempting further equilibrium binding experiments.

5.2.c.1 Silanization of assay tubes to reduce tube binding

In the previous experiment (fig 5.3) the strychnine displaceable [^{3}H]-strychnine binding to the assay tubes was described. Although this was corrected for when estimating strychnine specifically bound to membrane a more thorough approach would attempt to prevent strychnine-displaceable strychnine binding to the assay tubes. The retention of biologically active compounds by laboratory hardware is often reduced by derivatizing the polar moieties that underlie these interactions (Williams and Wilson, 1981).

For this reason, the assay volumes of PBS were incubated with increasing concentration of [^{3}H]-strychnine in silanized and untreated eppendorf tubes and subjected to the centrifugation procedure used to terminate the binding assays. Incubations that contained unlabelled strychnine (10^{-4}M) were included to estimate the strychnine displaceable [^{3}H]-strychnine binding to the assay tube.

There was an increase in [^{3}H]-strychnine bound to both untreated and silanized tubes as the concentration of [^{3}H]-strychnine added to the incubation was increased (fig 5.4). However, the amount bound to the untreated tubes was at least 3 times higher than to the silanized tubes. In addition, at each ligand concentration at least 65% of total

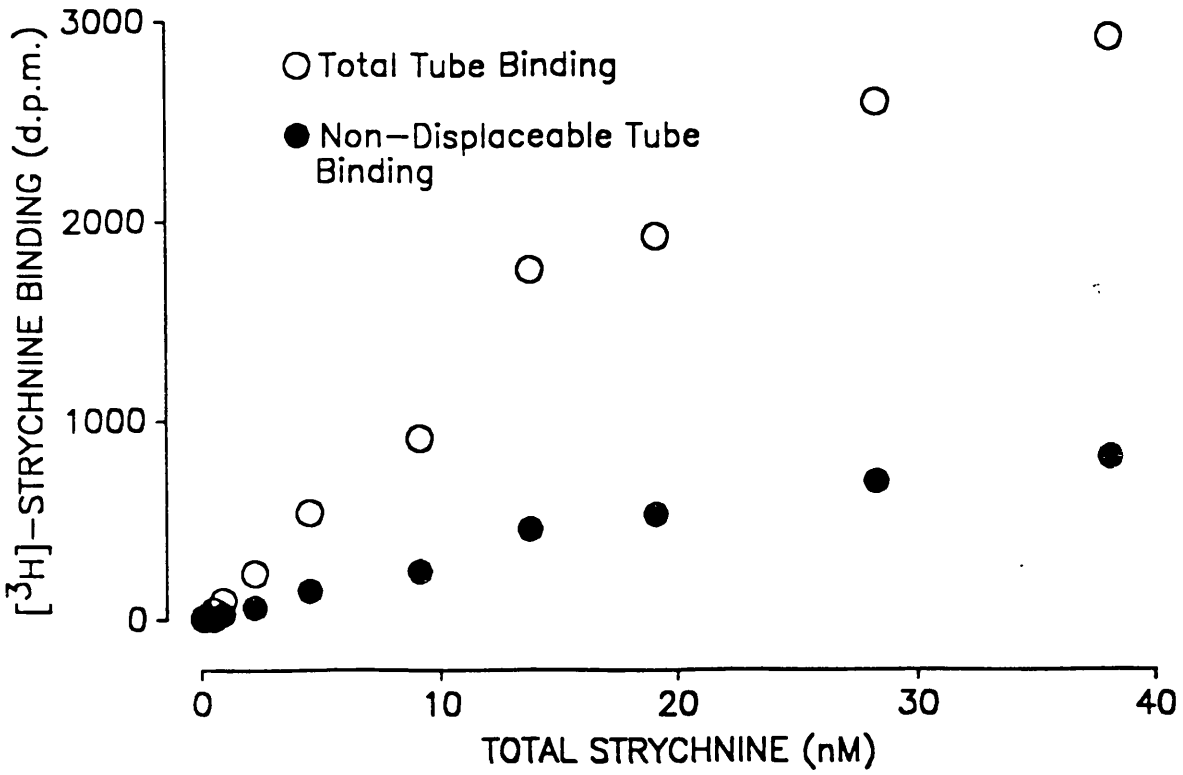


Figure 5.3: Effect of increasing strychnine concentration on the amount bound to assay tubes: Data from experiments described in fig 5.2.a are shown to illustrate strychnine displaceable binding to tubes. Assay volumes which contained no membrane were incubated with concentrations of [^{3}H]-strychnine between 0.1-40nM for 2 hrs at 4°C and terminated by centrifugation to estimate total binding to tubes. Incubations that contained unlabelled strychnine (10^{-4}M) were used to define non-displaceable binding to the tubes and by difference strychnine displaced [^{3}H]-strychnine binding.

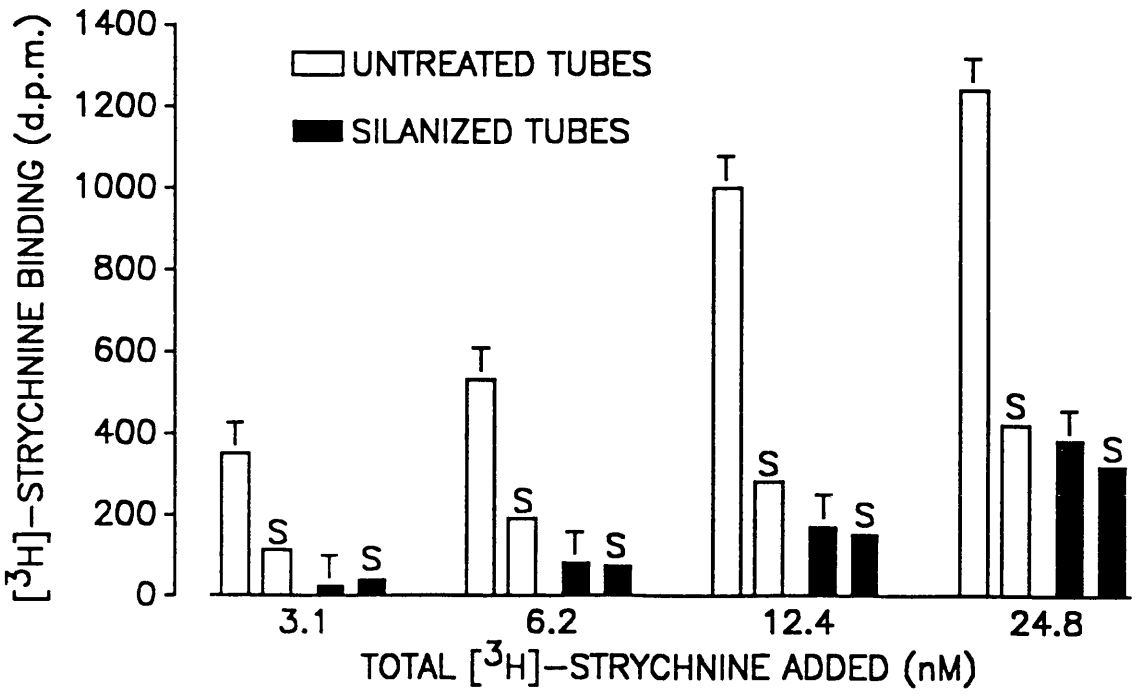


Figure 5.4: Effect of silanization on [^{3}H]-strychnine binding to assay tubes: Untreated and silanized assay tubes were incubated with [^{3}H]-strychnine at concentration shown for 2 hrs at 4°C. The assays were centrifuged, the incubation volume removed and the tubes rinsed with chilled PBS (1.2 ml). Shown are amounts of strychnine bound to tubes when incubations were performed in the absence (T) or presence of unlabelled strychnine (10^{-4}M;S).

binding to untreated tubes was displaced by unlabelled strychnine. In contrast strychnine-displaceable [^{3}H]-strychnine binding to the silanized tube was only detected when the incubations contained high ligand concentrations and at most represented approximately 10% of total bound. It was interesting to note that the dpm bound to untreated tubes in the presence of unlabelled strychnine was higher than total bound to silanized tubes. This indicated that unlabelled strychnine at a concentration of 10^{-4}M may not adequately define strychnine displaceable ligand binding to the untreated tubes. Thus preventing this phenomenon by silanization might ensure the non-specific binding is more appropriately defined.

5.2.c.2 Equilibration of strychnine binding to untreated and BD treated spinal cord membranes

An important criterion for the adequate definition of K_D and B_{max} is that ligand binding is measured at equilibrium, thus in general it is necessary to define the incubation period required for ligand binding to reach this state. In the case of BD-treated membranes, although the parameters of [^{3}H]-strychnine binding were poorly defined it is clear that BD induced binding has different characteristics to the [^{3}H]-strychnine binding to the inhibitory glycine receptor of untreated membranes. For this reason equilibration of BD induced strychnine binding was investigated.

Untreated and BD-treated membranes were incubated with [^{3}H]-strychnine for increasing periods of time at 4°C before the incubations were terminated by filtration. Non-specific binding was defined by unlabelled strychnine which displaced at least 80% of total bound to untreated and BD-treated membranes at each incubation period. There was a slight increase in the non-specific binding to BD-treated membranes for samples subjected to the longer incubation periods (data not shown) and this probably reflected the inability of 10^{-4}M unlabelled strychnine completely to displace the strychnine specifically bound.

In the untreated membrane the amount of strychnine bound remained fairly constant at each incubation period tested (fig 5.5), thus indicating the equilibration of the strychnine binding within 15 mins. In contrast specific-strychnine binding to the BD-treated membranes increased as the incubation period was extended (fig 5.5). This effect plateaued between 4 and 16 hrs, therefore strychnine binding to BD-treated membranes

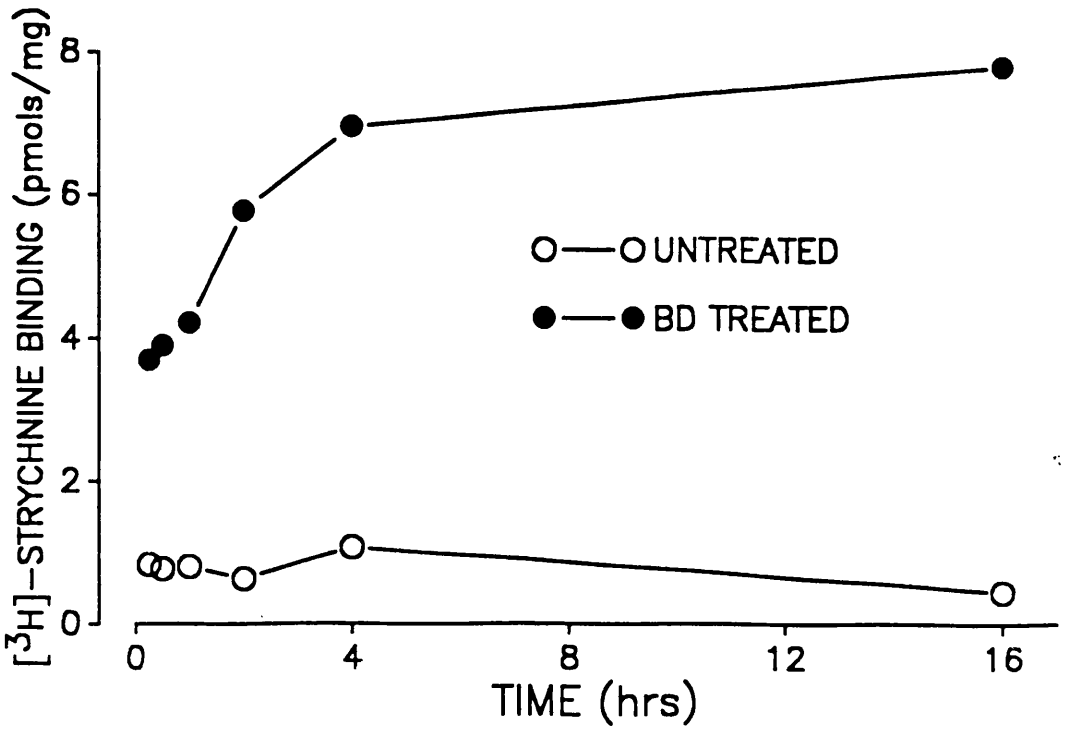


Figure 5.5: Equilibration of specific [^{3}H]-strychnine binding to untreated and BD-treated mouse spinal cord membranes: Membranes were incubated with [^{3}H]-strychnine (6 nM) for increasing periods (0.25-16 hrs) at 4°C and terminated by filtration. Incubations that contained unlabelled strychnine (10^{-4}M) defined non-specific binding. Each point reprsents the mean of triplicate estimates from a single experiment.

was taken to have equilibrated within 4 hrs at 4°C. For this reason, further studies used an incubation period of at least 4 hours when estimating strychnine binding to BD-treated membranes. Also, it is clear that previous experiments (see 5.2.a and 5.2.c) that used a 2 hr incubation period in attempting to define K_D and B_{max} were inappropriate as ligand binding would not have fully equilibrated. Therefore non-equilibration of strychnine binding in addition to previously discussed experimental limitations would have compromised the validity of the K_D and B_{max} estimated from these experiments.

5.2.c.3 Comparison of filtration and centrifugation as the methods used to separate free and bound ligand

As discussed, the fact that BD-induced strychnine binding to treated membranes exhibited a relatively low affinity for strychnine prompted experiments on the use of centrifugation as the method for termination of assays. This decision based on the generalization that ligand binding that exhibits an affinity >10 nM should be separated by centrifugation to minimize the period that bound ligand is in contact with ligand free medium in order to reduce disassociation of bound ligand. Thus the generalization is based on the assumption that low affinity binding is due to an increased rate of disassociation (Bennett and Yamamura, 1985). However, the affinity of a ligand (K_D) at equilibrium for a binding site is a consequence of a relationship between its rate of disassociation (k_{-1}) and rate of association (k_{+1}) and given by:

$$K_D = k_{-1}/k_{+1}$$

Therefore the correlation between affinity and rate of disassociation assumes a relative constant rate of association (Bennett and Yamamura, 1985). However, the equilibration of BD-induced [^{3}H]-strychnine binding suggests that the kinetics of this site could be different to those in which the rate of disassociation and affinity are directly correlated. Indeed the low affinity and slow equilibration of the BD-induced binding could be accounted for by a slow rate of association in which the rate of disassociation does not preclude the use of filtration protocols to separate free and bound ligand. This possibility was investigated by comparing the amount of [^{3}H]-strychnine bound to untreated and BD-treated membranes assayed under identical conditions but terminated by filtration or centrifugation.

The membranes were incubated with [^{3}H]-strychnine for 4 hours at 4°C before

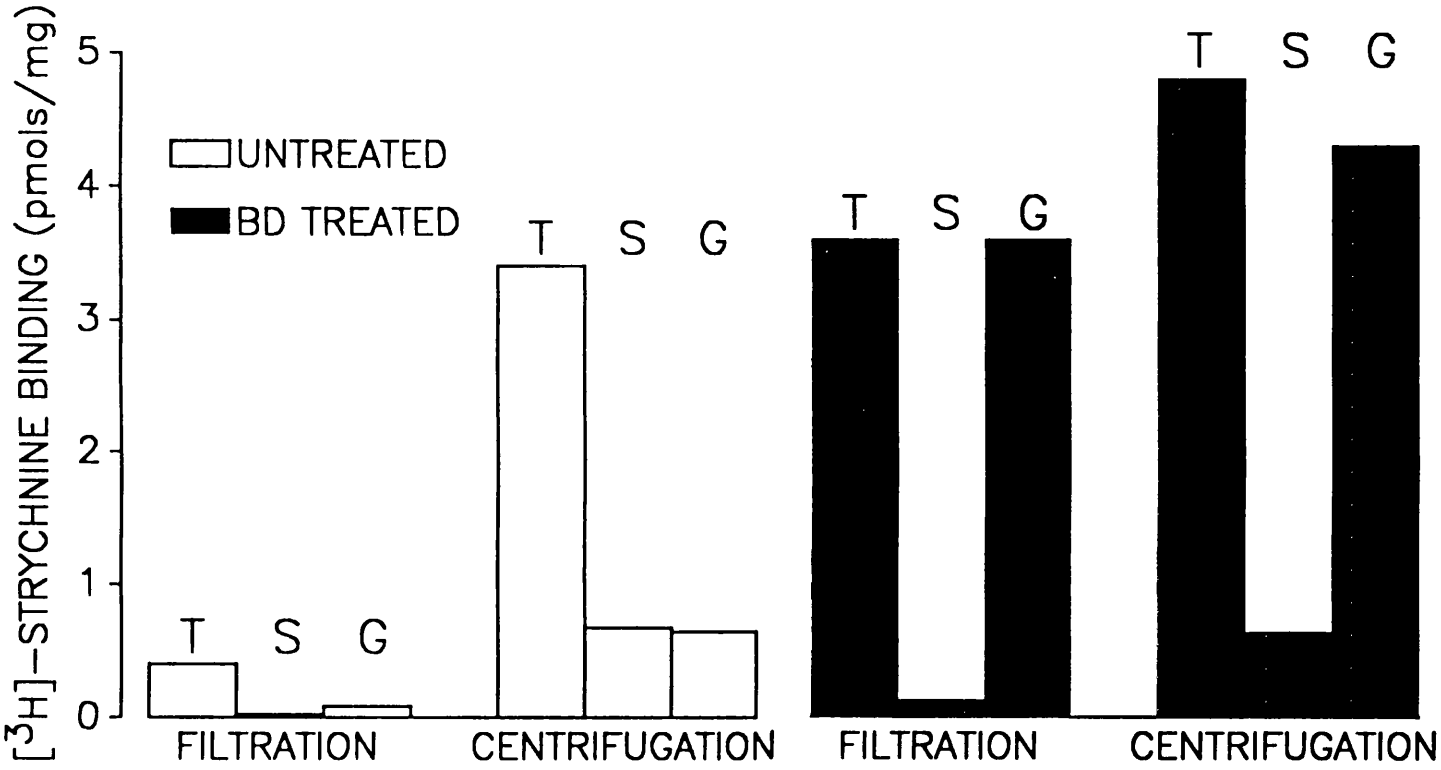


Figure 5.6: Comparison of [^{3}H]-strychnine binding to untreated and BD-treated membranes estimated by filtration or centrifugation assays: Membranes incubated with [^{3}H]-strychnine (6 nM) for 4 hours at 4°C were separated by filtration or centrifugation. Total bound (T) was compared to the amount bound in the presence of strychnine (10^{-4}M;S) or glycine (10^{-2}M;G). Silanized incubation tubes were used in the centrifugation assays. Results represent the mean of triplicate estimates from a single experiment.

being terminated (fig 5.6). Incubations that contained unlabelled strychnine (10^{-5}M) or glycine (10^{-2}M) were used to define non-specific binding and in the centrifugation assays silanized incubation tubes were used.

The results for the BD-treated membranes show that the amount of specific-strychnine bound was similar when incubations were terminated by filtration or centrifugation. This was consistent with the idea that the rate of disassociation from the BD-induced site is not as rapid as predicted from a generalization that assumes a proportional relationship between the rate of disassociation and the affinity. Further, by inference this might imply that the low affinity strychnine binding to the BD induced site has a relatively slow rate of association.

Interestingly, in the untreated membranes the amount of detectable [^{3}H]-strychnine bound was lower when incubations were terminated by filtration. The incubations terminated by centrifugation had a five fold increase in the amount of detectable specific strychnine binding. The specific binding was the same when defined by unlabelled strychnine or glycine as indicated by the proportion of total binding that was inhibited by these compounds. This suggests that the increase in binding to untreated membranes when the assays were terminated by centrifugation, was at the inhibitory glycine receptor. However, the previous estimate of the 1/2 life of high affinity strychnine binding to the rat glycine receptor (Young and Snyder, 1974a; Maksay, 1990) indicate that minimal amounts of bound [^{3}H]-strychnine should disassociate during the filtration protocol in which bound ligand was in contact with ligand free buffer for 15 secs (Bennett and Yamamura, 1985), similar to the 15-20 secs of the experiments described in this thesis. Therefore, it is possible that the increase detected by centrifugation assays relates to a population of strychnine binding sites that have different characteristics to the high affinity binding at the inhibitory glycine receptor defined by the filtration assays. This possibility prompted the use of centrifugation assays in subsequent attempts to define the parameters of equilibrium strychnine binding to untreated and BD-treated mouse spinal cord membranes.

5.2.d Inhibition of [^{3}H]-strychnine binding to untreated and BD-treated membranes by increasing concentrations of unlabelled strychnine

The previous experiments described in sections 5.2.a and 5.2.b that attempted to

define parameters of low affinity strychnine binding apparent in BD-treated membranes, were inappropriate primarily because the range of ligand concentrations were insufficient to saturate and define the BD-induced binding to spinal cord membranes. Even isotopically diluted strychnine up to 10 μM did not saturate the BD-induced binding (data not shown). Therefore, to obtain a more extensive range of ligand concentrations the inhibition of [^{3}H]-strychnine binding by increasing concentrations of unlabelled strychnine (10^{-10}M-10^{-3}M) was used to assay the saturation of ligand binding to untreated and BD - treated membranes. These experiments were subsequently analyzed as experiments in which saturation of strychnine binding sites was pursued by increasing concentrations of isotopically diluted hot ligand.

These experiments were performed under conditions optimized and described in the previous section. Consequently, centrifugation assays, silanized incubation tubes and 4 hour incubation periods were used. In addition, in view of the reservations about the definition of non-specific in the previously described procedures (see section 5.2.b) these experiments included incubations that contained 10^{-3}M unlabelled strychnine to define non-specific binding.

The membranes were incubated with [^{3}H]-strychnine (6 nM) in the presence of increasing concentrations of unlabelled strychnine and the amount specifically bound represented as percentage of specific binding to the controls membranes that contained no unlabelled strychnine (fig 5.7.a). The control values indicate that when assayed with [^{3}H]-strychnine and terminated by centrifugation the BD treatment caused an apparent two fold increase in specifically bound ligand (see fig 5.7.a). Unlabelled strychnine inhibited [^{3}H]-strychnine binding to untreated and BD-treated membranes although the right shift of the binding isotherm for the BD-treated membranes indicates the lower affinity of this binding. Indeed a comparison of the IC_{50} values, derived by EBDA for the data indicate that strychnine was approximately 300 fold less potent at inhibiting equilibrium [^{3}H]-strychnine binding to BD-treated membranes. This analysis also revealed that the Hill slopes derived from both untreated (0.87±0.06) and BD-treated (0.58±0.08) membranes were less than one which could indicate the presence of multiple strychnine binding sites in both untreated and BD treated membranes. However the Hill value of 0.87 was not considered overwhelming evidence in favour of this idea in the untreated membranes. The data was however further analyzed as saturation of binding sites by increasing concentrations of isotopically diluted ligand.

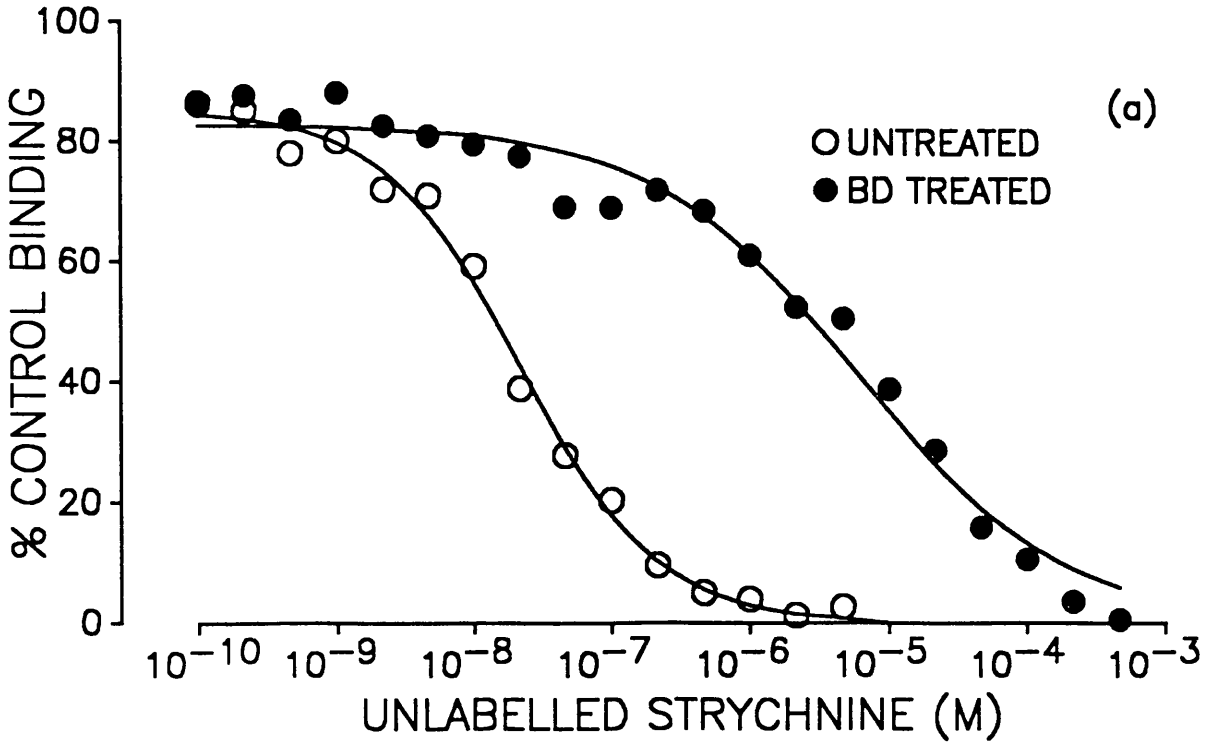
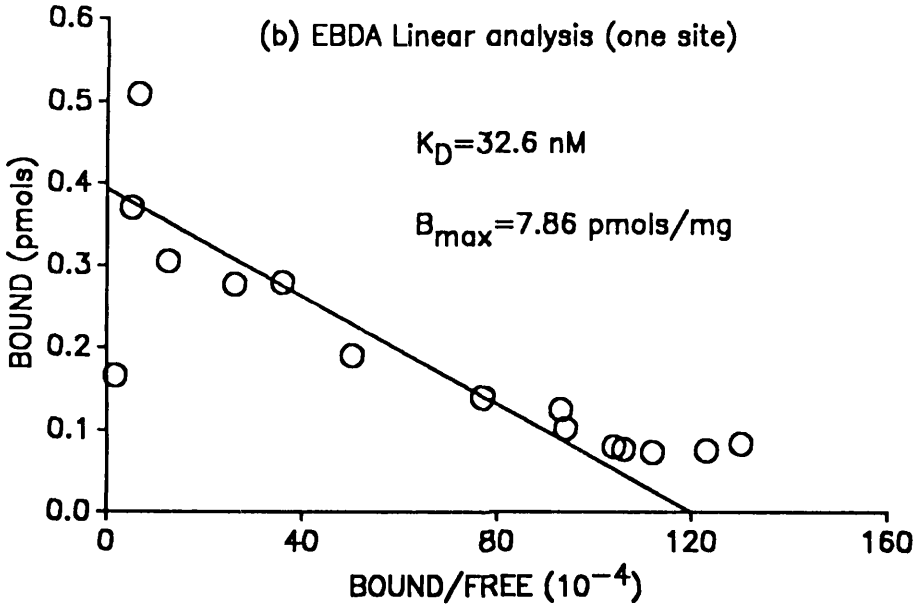
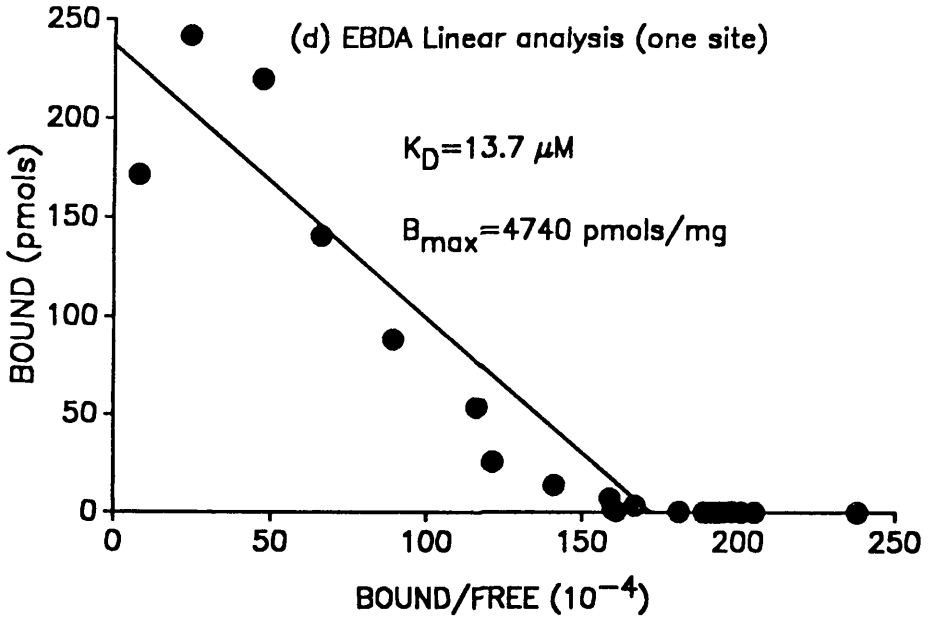


Figure 5.7: Determining equilibrium binding parameters of [^{3}H]-strychnine binding to untreated and BD-treated mouse spinal cord membranes: (a) Inhibition of [^{3}H]-strychnine binding (6 nM) to membranes (0.05 μg protein) by increasing concentrations of unlabelled strychnine. Incubations that contained no unlabelled strychnine defined control binding in untreated (4646 dpm) and BD-treated membranes (8403 dpm). Assays were incubated in silanized tubes for 4 hrs at 4°C and terminated by centrifugation. Non-specific binding was defined by incubations that contained 10^{-3}M unlabelled strychnine. Inhibition of [^{3}H]-strychnine binding was initially analyzed by EBDA and gave estimates of the IC_{50} and Hill slope. For untreated these were; 2.17(± 2.0) x 10^{-8}M and 0.87 (±0.06); treated; 5.8(± 1.0) x 10^{-6}M and 0.58 (±0.08). The results represent the mean of triplicate observations from one experiment. Data was further analyzed by considering it as saturation of binding by increasing concentrations of isotopically diluted ligand. Eadie-Hofstee plots of data for untreated (b) and BD treated (d) membranes are shown and the estimates of K_D and B_{max} derived from linear least square fits of the data by EBDA are indicated. Summary of SCAFIT analysis of data for [^{3}H]-strychnine binding to untreated (c) and BD treated (e) membranes.



MODEL	[K_A ± S.E.] (mol^{-1}) K_D^1 (nM)	B_{max}^1 ± S.E. (pmols/mg)	[K_A ± S.E.] (mol^{-1}) K_D^2 (μM)	B_{max}^2 ± S.E. (pmols/mg)	N ± S.E.	Runs Test	F value		
one site	[3.6E7 ± 5.1E6] 27.8	7.24 ± 0.60				p>0.05	3.8 (p=0.075)	6.19 (p=0.02)	6.19 (p=0.02)
one site fitted NS	[4.7E7 ± 9.0E6] 21.1	5.94 ± 0.70			0.00007 ± 0.00004	p>0.05	–	6.76 (p=0.025)	6.76 (p=0.025)
two site	[1.3E8 ± 1.3E8] 7.2	2.6 ± 1.7	[6.2E6 ± 5.7E6] 0.161	6.22 ± 1.50		p>0.05		–	–
two site fitted NS	[1.3E8 ± 1.3E8] 7.2	2.6 ± 1.7	[6.2E6 ± 5.7E6] 0.161	6.22 ± 1.50	0	p>0.05			–

(c) SCAFIT non-linear analysis



MODEL	[K_A ± S.E.] (mol^{-1}) K_D^1 (nM)	B_{max}^1 ± S.E. (pmols/mg)	[K_A ± S.E.] (mol^{-1}) K_D^2 (μM)	B_{max}^2 ± S.E. (pmols/mg)	N ± S.E.	Runs Test	F value		
one site			[9.2E4 ± 1.4E4] 10.8	3938 ± 504		p<0.01	_	0.23 (p=0.79)	0.23 (p=0.79)
one site fitted NS			[9.2E4 ± 1.4E4] 10.8	3938 ± 504	0	p<0.01	_	0.23 (p=0.79)	0.23 (p=0.79)
two site	[4.3E8 ± 5.5E9] 2.34	0.772 ± 0.270	[8.9E4 ± 1.9E4] 11.2	3700 ± 510		p>0.05		_	_
two site fitted NS	[4.3E8 ± 5.5E9] 2.34	0.772 ± 0.270	[8.9E4 ± 1.9E4] 11.2	3700 ± 510	0	p>0.05			_

(e) SCAFIT non-linear analysis

For the untreated membranes, when the data was linearized by EBDA the simple one site fit (fig 5.7.b) provided estimates of K_D (32.6nM) and B_{max} (7.86 pmol/mg) which were greater than values that have previously been estimated for [^{3}H]-strychnine binding to untreated membrane (see section 5.2.a). Indeed, visual inspection of the Eadie-Hofstee plot suggests it has a curvilinear appearance, indicating the possibility that multiple classes of strychnine binding sites exist.

The possibility of multiple classes of binding sites was given further credence when the data was analyzed by SCAFIT, because the two site model provided the best fit to the data. This was indicated by F-values when the more complex two site model was compared to the one site model. Unlike previously analyzed data (see section 5.2.b) the second site did not appear to relate to a non-specific interaction as the one site model with non-specific fitted by SCAFIT did not improve the appropriateness of the fit. Also glycine sensitivity of the increased [^{3}H]-strychnine binding detected by centrifugation assays in untreated membranes (see fig 5.6) further argues that the second site arises through a specific interaction. The estimates of the equilibrium binding parameters for the high affinity site resolved from the two site model for binding to untreated membranes corresponded well with previous estimates of strychnine binding to the inhibitory glycine receptor. The second site resolved was of lower affinity and higher capacity however the values of K_D and B_{max} do not compare well with estimates for the BD-induced binding site. This finding along with the glycine sensitivity of the increased binding detected in untreated membranes when centrifugation was used to terminate the assay suggest that this site does not underlie the BD-induced strychnine binding but is rather associated with the inhibitory glycine receptor.

The possibility that the increased specific [^{3}H]-strychnine binding in untreated membrane detected by centrifugation assays as opposed to filtration is at the inhibitory glycine receptor is compatible with the observation that saturation of glycine-specific [^{3}H]-strychnine binding to mouse spinal cord membranes is defined by a B_{max} which is 3 times greater when assays under identical conditions, were terminated by centrifugation (White, 1985) rather than filtration (Becker *et al.*,1986). However, no attempt was made to resolve the data from centrifugation assays into multiple binding sites. Indeed, this may have proved futile as the range of ligand concentrations used (0.04-35.4 nM; White, 1985) were inappropriate to saturate a low affinity site with parameters similar to those described

above. Interestingly, when a more extensive range of [^{3}H]-strychnine concentrations were used to define the equilibrium binding parameters to human spinal cord (De Montis *et al.*, 1982) detection of a low affinity strychnine binding site in addition to the high affinity binding was reported.

Two possibilities that could underlie a low affinity interaction between strychnine and the inhibitory glycine receptor, in addition to the well characterized high affinity binding are considered further. Low affinity strychnine binding could relate to an isoform of the ligand binding subunit that encodes a receptor with a reduced affinity for strychnine. Indeed, a developmentally regulated isoform of the inhibitory glycine receptor exists which has a reduced affinity for strychnine preventing its detection by filtration assays (Becker *et al.* 1988). However, when the message for the subunit that probably encodes this isoform was expressed and functionally assayed the measured affinity for strychnine (Kuhse *et al.*, 1990a) was approximately 50 fold lower than the estimated K_D of the low affinity site derived from a two site fit to the above data. In addition the developmental regulation of the low affinity site indicated that it was surpassed by an isoform with high affinity for strychnine (Becker *et al.*, 1988). The binding experiments described in the above used adult tissue where the low affinity site should be less prevalent. As the B_{max} values for the two site model indicate there is a predominance by the low affinity site, it appears unlikely that the developmentally regulated isoform of the glycine receptor subunit is responsible for the low affinity strychnine binding defined by centrifugation assays. It is possible the low affinity strychnine binding reported here is related to an undescribed isoform of the glycine receptor.

In the glycine receptor the favoured stoichiometry is $\alpha_3\beta_2$ or $\alpha_4\beta_1$ (Langosch *et al.*, 1988) and assuming the α subunit harbours the glycine and strychnine sites the glycine receptor contains at least three strychnine binding sites. Indeed this molecular structure is compatible with the idea that anion channel activation depends on 2-3 glycine molecules being bound to the receptor (Gundersen *et al.*, 1984; Schmieden *et al.*, 1989). In a pentomeric heteroligomer it would not be possible to arrange the subunits symmetrically thus a consequence of this would be non-equivalence of the nearest neighbour relation for at least one of the α subunits. This non-equivalence could manifest itself as ligand binding sites which although identical in primary sequence have different affinities for ligands. It is interesting to note that the ratio of B_{max} values for the high/low

affinity sites of approximately 1:2 and the 3 fold increase in detectable B_{max} in assays terminated by centrifugation (see previously) would be compatible with an $\alpha_3\beta_2$ arrangement in which only one of the α subunits had high affinity for strychnine.

The phenomenon of non-equivalence of agonist and competitive antagonist binding has been described at the nicotinic acetylcholine receptor where most recent evidence suggests it arises through a non-equivalence of the nearest neighbours to the proposed ligand binding subunits. In fact the neighbouring subunits may directly contribute to the ligand binding site (Blount and Merlie, 1989; Pederson and Cohen, 1990). Thus, the speculation that a similar phenomenon exists at the inhibitory glycine receptor highlights a possible functional consequence of the conserved quaternary structure of ligand gated ion channels. Such a molecular arrangement may underlie the sequential binding of glycine during receptor activation which has recently been speculated (Twyman and Macdonald, 1991).

Attempts to saturate and define the parameters of the BD-induced strychnine binding with increasing concentrations of radioligand were inadequate because of the limited range of ligand concentrations used. However, when the site was assayed by addition of increasing concentrations of unlabelled strychnine to a single concentration of [^{3}H]-strychnine, more than 90% of total binding was inhibited at the highest concentration of unlabelled strychnine which indicated that the BD-induced binding was saturating. Although the estimate from EBDA for a one site model confirmed the low affinity and high capacity of the BD-induced strychnine binding, the Eadie/Hofstee plot appeared to deviate from linearity. Inspection suggests the presence of two components, one of which would in fact define an additional high affinity strychnine binding site in BD-treated membranes. Despite the apparent multicomponent nature to the Eadie/Hofstee plot SCAFIT was not able clearly to resolve this second site in BD treated membranes. A two site model that defined a high and low affinity strychnine binding site could be fitted to the data but when this fit was compared to a one site model the F values indicated that the simple model gave the most appropriate fit. In addition to the F test, the Runs Test derived from the fits is a valuable indication of the appropriateness of a model. In BD-treated membranes, although the F-test indicate that the one site model provides the best fit, the Runs Test ($p<0.01$) indicates that the experimental points were not randomly distributed around the fitted curve suggesting an inappropriate fit. In contrast the Runs Test ($p>0.05$) for the 2 site model was compatible with an appropriate fit when this

criterion is used.

It was clear from the biochemical data presented in Chapter 4 that in addition to the induced site strychnine bound to a modified inhibitory glycine receptor in BD treated-membranes. Yet consideration of the estimated B_{max} values for the two sites indicates that the induced site was in at least 1000 fold excess over the inhibitory glycine receptor which might prevent the adequate resolution of the high affinity binding.

5.3 General discussion: speculations on the nature of the BD-induced strychnine binding based on the estimated equilibrium binding parameters for untreated and treated membrane

In addition to the points discussed, data from this chapter are considered in light of the induction of a low affinity and high capacity strychnine binding site in spinal cord membranes treated with BD. These speculations must be compatible with the data which indicate that a site with characteristics similar to the BD-induced binding could not be resolved in untreated membranes. The crudest assessment of the described phenomenon would be that BD treatment "unmasks" sites that are buried and thus undetectable in untreated membranes. Such a description has been used to explain the induction of ligand binding sites when assays were performed in high ionic strength buffer (Cuatrecasas, 1971; Young and Snyder, 1974a). However, thorough washing of membranes treated with protein modifying reagents should ensure the reagent was removed prior to assaying strychnine binding. Thus an unmasking in the manner described for high ionic strength as an explanation for the induction of strychnine binding appears unlikely.

Rather, based on the reactivity that BD and related compounds have for arginine residues in proteins, the favoured idea is that a specific protein modification underlies the induction of binding. The modification of a protein present in untreated membranes is speculated to cause a conformational change which can be assayed as an increased in strychnine binding. The increased binding has very different characteristics and predominates over strychnine binding to the inhibitory glycine receptor. The conformational dependence of the induced binding is implicated by description that the induced [^{3}H]-strychnine binding was potentiated by preincubating spinal cord membranes with unlabelled strychnine prior to and during treatment with arginine selective protein modifying reagents.

Two phenomenon that could underlie the induction of [^{3}H]-strychnine binding in BD-treated membranes are highlighted in the ensuing discussion. This serves to indicate those options which might most likely explain how protein modification could lead to an increase in ligand binding. The BD treatment could effect a change in the affinity of the putative protein for [^{3}H]-strychnine. This would equate to an induction in ligand binding if there was an increase in affinity. Conformation-dependent changes in the affinity of ligand binding proteins has been extensively documented. Important examples include the GABA induced increase in agonist benzodiazepine binding to the $GABA_A$ receptor (Martin and Candy, 1978) and increase in the affinity of desensitized ligand gated ion channels for agonist ligands (Changeaux, 1990). If a similar phenomenon underlies the effect of BD-induced strychnine binding the inability to detect a related binding site in untreated membranes could be because it has too low an affinity. A conversion of undetectable low affinity binding to one of higher affinity has been used as an explanation for the increase in [^{3}H]-GABA binding in membranes preincubated with GABA (Maksay and Ticku, 1984b).

A second option that could explain induction of binding by protein modification is the conformation dependent exposure of a strychnine binding site. In untreated membranes restricted access to the ligand binding site might explain non-detection of a site prior to BD treatment. A conformational dependent phenomenon has been shown to underlie the increase in ligand binding to the N-methyl-D-aspartate (NMDA) receptor. Thus an increase in binding of the channel blockers MK-801 and PCP to cerebral cortex membranes has been described when the incubations contained glutamate analogues or glycine, compounds that promote activation of the NMDA receptor (Kloog *et al.*, 1988a; 1988b). Thus opening the ligand gated ion channel promotes easy access of the ligands MK-801 and PCP to their binding sites which are presumed to lie within the ion channel that is buried in the membrane. Although this binding site is present in incubations in incubations that contained no added channel activators the ligands have restricted access and reach equilibration more slowly. For this reason the potentiation by channel activators is not seen if ligand binding is allowed to reach equilibrium as exposing the site simply allows a more rapid equilibration.

If a similar phenomenon underlies the BD-induced strychnine binding the inability to detect a similar site in untreated membrane could indicate that conditions used

insufficient time to allow equilibration to the unmodified protein. As shown by incubation performed overnight there was no indication that [^{3}H]-strychnine binding to untreated membranes increased thus tending to refute this suggestion. Yet, as the experiments were performed at 4°C the kinetics of binding at this temperature might not favour ligand equilibration to a site of restricted access. Another possibility is that in the unmodified state strychnine is completely prevented from gaining access to the site that is revealed by BD-treatment. Indeed a comparison of equilibrium binding to membrane bound (Kloog *et al.*, 1988a) and solubilized (Bakker *et al.*, 1991) NMDA receptors indicate, that equilibration of MK-801 binding in incubations that contained no added activators could be due to residual endogenous activators, thus in the absence of activators the ligand is unable to equilibrate with the site. The above speculations that the BD-induced binding is due to a change in affinity or access of a strychnine binding site by a conformational dependent modification should not obscure the possibility that these options may not be mutually exclusive.

A final issue that requires comment is the amount of the BD-induced site in spinal cord membranes. In the final equilibration binding experiment the estimated B_{max} in BD-treated membranes was nearly 4000 pmols/mg which represents an approximate 1000 fold higher concentration when compared to [^{3}H]-strychnine binding to the inhibitory glycine receptor in untreated membranes. The glycine receptor, assuming a B_{max} of 1 pmol/mg, a molecular weight of 310,000 and one strychnine binding site/receptor represents 0.03% of total assayed protein. Thus the BD-induced site would appear to represents a large proportion of total membrane protein. However, specifics of this cannot be quantified as the nature of the suspected protein moiety that underlies BD-induced binding can only be speculated.

Chapter Six

Pharmacological characterization of the BD-induced [^{3}H]-strychnine binding to mouse spinal cord membranes

6.1 Introduction

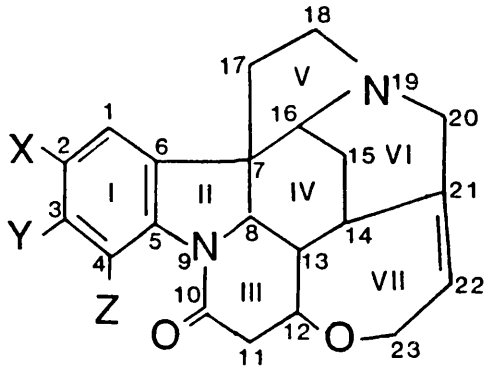
It was clear from the biochemical characterization of the [^{3}H]-strychnine binding to untreated and BD-treated spinal cord membranes that the BD-induced binding was at a site other than the inhibitory glycine receptor (see Chapter 4). This view was further vindicated by the results that show that the equilibrium binding to BD-treated membranes had different characteristics to that in untreated membranes (see Chapter 5). Therefore, to extend investigation of this apparently novel strychnine binding site the pharmacological specificity of [^{3}H]-strychnine binding to BD-treated membranes was pursued.

Two major avenues were explored in these investigations. Firstly, the structure activity relations of strychnine binding to untreated and BD-treated membranes was studied with a view to comparing ligand binding properties of the induced binding site with the inhibitory glycine receptor. The second approach concerned attempts to identify compounds that interact with the BD-induced strychnine binding site. This line of investigation was focused by reported actions of strychnine that extend beyond the well characterized antagonism of glycine at the ligand gated anion channel (see section 1.7).

6.2 Results

6.2.a Comparison of ligand recognition properties of [^{3}H]-strychnine binding to untreated and BD-treated mouse spinal cord membranes.

The ligand binding properties of the BD-induced binding were investigated by comparing the potency of strychnine and structurally related alkaloids (see Fig 6.1 and Table 6.1) to inhibit [^{3}H]-strychnine binding to treated membrane. This study also compared ligand binding properties of the BD-induced site with strychnine binding to the glycine receptor by simultaneously assaying the potency of the above compounds at inhibiting [^{3}H]-strychnine binding to untreated membrane. Additional compounds with



No.	Compound	X	Y	Z	Other modifications
1	Strychnine	H	H	H	
2	2-Aminostrychnine	NH_2	H	H	
3	2-Nitrostrychnine	NO_2	H	H	
4	Brucine	CH_3O	CH_3O	H	
5	Brucine-N-oxide	CH_3O	CH_3O	H	N → O at position 19
6	Bromodeoxyisostrychnine	H	H	H	Ring VII open C12/C23; C12-13 unsaturated; C23 becomes CH_2Br
7	Isostrychnic acid	H	H	H	Ring III open N9/C10; C10 becomes COOH, C12 and C13 inverted
8	Cacotheline	=O	=O	NO_2	Ring III open N9/C10; C10 becomes COOH
9	Diaboline	H	H	H	Ring III open C10/C12; C10 becomes $COCH_3$; OH at C12; C21-C22 saturated
10	Vomicine	H	H	OH	Ring V open C16/N19; C16 becomes C=O; CH_3 at N19
11	Weiland-Gumlich aldehyde	H	H	H	Ring III open N9/C12; OH at C12
12	N-Oxystrychninic acid	H	H	H	Ring III open N9/C10; C10 becomes COOH; N → O at position 19
13	N-Methylstrychnine	H	H	H	N-methyl at position 19

Figure 6.1 and Table 6.1: Structure of the strychnine molecule: Rings numbered and substituent positions labelled for identification of the strychnoid alkaloids listed in table 6.1.

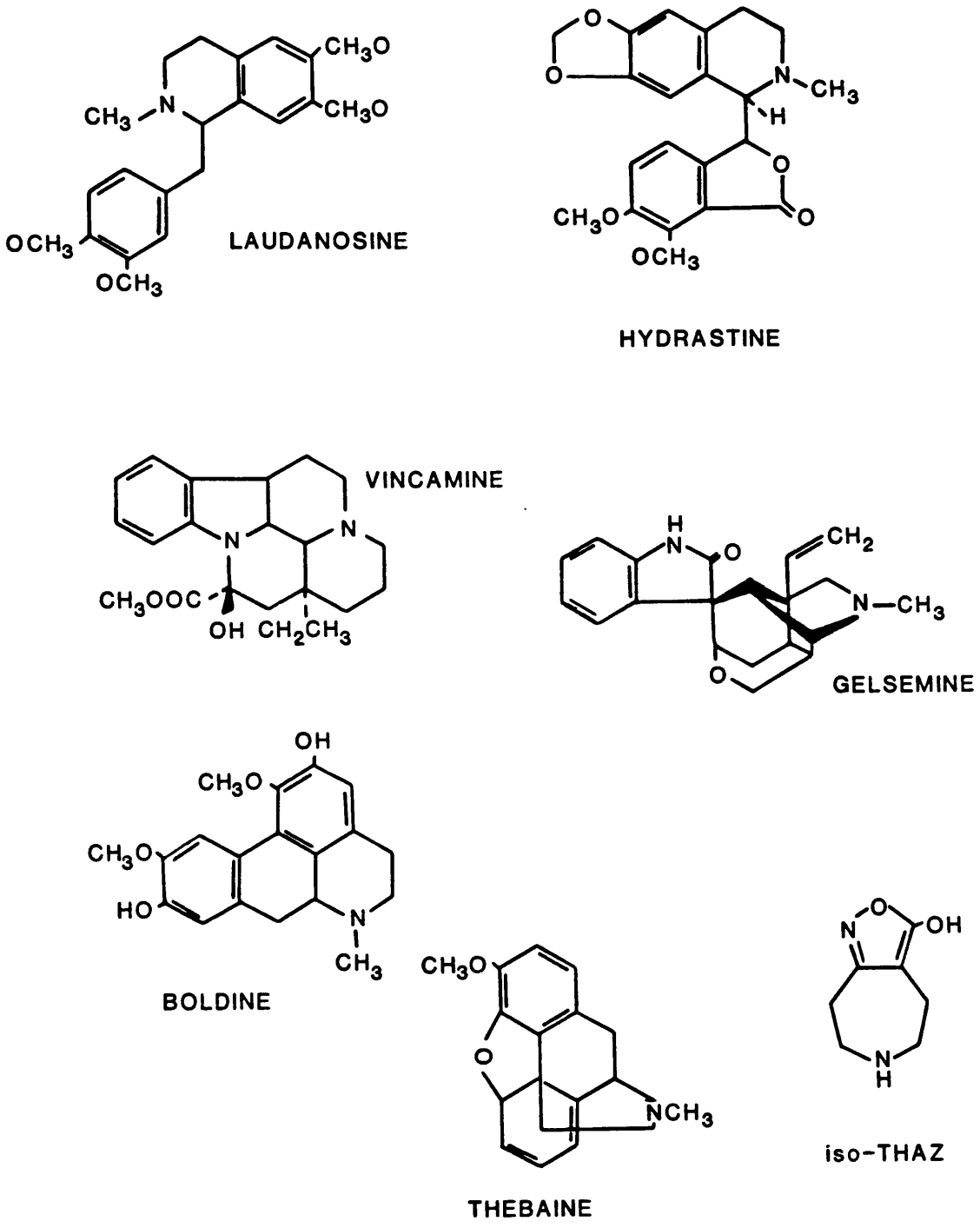


Figure 6.2: Structure of non-strychnos alkaloids and glycinergic ligands which were screened for an inhibition of [^{3}H]-strychnine binding to untreated and BD-treated membranes.

known or suspected actions at the inhibitory glycine receptor were also investigated.

Initially, compounds were screened by incubating untreated or BD-treated membranes with [^{3}H]-strychnine (6nM) in the presence of a single concentration (10^{-2} or 10^{-4}M, see table 6.2) of test compound. After 4 hours at 4°C the assays were terminated by filtration under vacuum and [^{3}H]-strychnine bound calculated as % control binding. Non-specific binding was estimated by an incubation that also contained unlabelled strychnine (10^{-4}M). For selected compounds the potency of inhibition was investigated by assaying the [^{3}H]-strychnine bound to untreated and BD-treated membranes, under conditions described above, in the presence of increasing concentrations of test compound. The IC_{50} values and the 95% confidence limits on these estimates were calculated by linear least squares fits of the data.

Of the non-alkaloid compounds screened, only the 1,2-propandiol, mephenesin was unable to inhibit [^{3}H]-strychnine binding to either untreated or BD-treated membranes (see table 6.2). In contrast the amino acids glycine, taurine, β-alanine and γ-aminobutyric acid (GABA) inhibited binding to untreated membranes but not treated membranes. This differential inhibition of [^{3}H]-strychnine binding between untreated and BD-treated was also apparent when 5,6,7,8-tetrahydro-4H-isoxazolo-[3,4]-d]-azepin-3-ol (isothaz) and 3-hydroxy-16-imino-5-17-aza-andrasto-11-one (RU-5135) were screened. The potency of the inhibition of [^{3}H]-strychnine binding to untreated membranes by these compounds is already well characterized (see for example Phelan *et al.*, 1989) and was not further investigated.

In contrast, the inhibition of [^{3}H]-strychnine binding by strychnine, related structures and other alkaloid compounds when screened at 10^{-4}M indicated a spectrum of effectiveness at untreated and BD-treated membranes. Using the percent of control binding remaining in the presence of test compound (10^{-4}M) it appeared that some compounds were more effective at inhibiting binding to untreated membranes while for others the reverse was true (see table 6.2). This was more thoroughly investigated by estimating the IC_{50} for inhibition of [^{3}H]-strychnine binding to untreated and BD-treated membranes.

Of the non-strychnos alkaloids tested (see Fig 6.2 and Table 6.2) the estimated IC_{50} values for boldine and vincamine indicated that these compounds were more potent at

Compound	% control binding in presence of drug		IC_{50} (M) [95% confidence limits]	
	Untreated	BD-treated	Untreated	BD-treated
Strychnine (1)	0	0	2.4[2.0-3.0] x 10^{-8}	1.9[0.90-4.0] x 10^{-6}
2-aminostrychnine (2)	0	37	1.4[.87-2.2] x 10^{-8}	5.5[2.5-9.5] x 10^{-6}
2-nitrostrychnine (3)	0	0	6.0[4.0-9.1] x 10^{-7}	4.2[3.5-4.9] x 10^{-6}
Brucine (4)	0	25	3.7[2.7-5.1] x 10^{-7}	6.0[3.0-9.9] x 10^{-6}
Brucine-n-oxide (5)	50	103	$>10^{-4}$	$>10^{-4}$
Br'desoxyisostrychnine (6)	0	26	7.2[5.6-9.3] x 10^{-7}	1.4[1.1-1.7] x 10^{-5}
Isostrychninic acid (7)	0	87	1.2[.91-1.7] x 10^{-7}	$>10^{-4}$
Cacotheline (8)	0	77	2.8[2.0-3.9] x 10^{-7}	$>10^{-4}$
Diaboline (9)	37	85	5.2[3.6-7.6] x 10^{-6}	$>10^{-4}$
Vomicine (10)	85	24	$>10^{-4}$	1.8[1.5-2.2] x 10^{-5}
Weiland-Gumlich ald. (11)	0	29	1.8[1.1-2.9] x 10^{-7}	$>10^{-4}$
N-oxystrychninic acid (12)	85	72	4.6[2.4-8.5] x 10^{-6}	$>10^{-4}$
N-methylstrychnine (13)	0	88	4.8[4.4-5.2] x 10^{-6}	$>10^{-4}$
Boldine	48	9	$>10^{-4}$	3.6[2.3-5.9] x 10^{-6}
Vincamine	70	14	$>10^{-4}$	9.3[6.0-10] x 10^{-6}
Gelsemine	32	25	$>10^{-4}$	$>10^{-4}$
Laudanosine	35	19	4.0[2.1-7.6] x 10^{-6}	1.4[0.97-2.0] x 10^{-6}
Hydrastine	84	75	$>10^{-4}$	$>10^{-4}$
Thebaine	25	15	6.6[5.6-7.8] x 10^{-6}	6.8[5.5-8.3] x 10^{-6}

Compounds	% control binding in presence of drugs		IC_{50} (M)	
	Untreated	BD-treated	Untreated	BD-treated
Iso-thaz	13	99	ND	ND
RU-5135	0	85	ND	ND
Glycine (10^{-2}M)	0	101	ND	ND
GABA (10^{-2}M)	23	103	ND	ND
Taurine (10^{-2}M)	6	98	ND	ND
ß-alanine (10^{-2}M)	4	103	ND	ND
Mephenesin	97	108	ND	ND

Table 6.2: Screen and relative potency of inhibition of [^{3}H]-strychnine binding to untreated and BD-treated membranes by strychnos alkaloids and other glycinergic drugs. Membranes were incubated with [^{3}H]-strychnine (6 nM, 4 hrs, 4°C) in the presence of a single concentration of drug (10^{-4}M; unless otherwise stated). Control binding was defined by an incubation that contained no unlabelled drug but an appropriate volume of drug solvent. IC_{50} values were determined by identical incubations that contained increasing concentrations of the drug. ND indicates drugs for which IC_{50} values were not estimated.

inhibiting [^{3}H]-strychnine binding to the BD-treated membrane. The potency of gelsemine and hydrastine could not be estimated from inhibition of [^{3}H]-strychnine binding from untreated and BD-treated membranes over the concentration range tested. However, using percent control binding in the presence of a single concentration of compound as the index it appeared that the potency of each compound at untreated and BD-treated membranes was similar. For laudanosine and thebaine, the final non-strychnoid alkaloids tested, only small differences in the potency of the compounds at untreated and BD-treated membranes was apparent. It is also pertinent to note the relative potency of laudanosine and thebaine at inhibiting [^{3}H]-strychnine binding to untreated membranes was at least 100 fold greater than boldine and vincamine yet in the BD-treated membranes the IC_{50} values of these compounds was very similar.

With the exception of 2-aminostrychnine at inhibiting [^{3}H]-strychnine binding to untreated membranes there was a decreased potency of the strychnine like alkaloids, when compared to strychnine, at untreated and BD-treated membranes (see table 6.2). This illustrates that structural modification introduced into the strychnine molecule (see fig 6.1 and table 6.1) effect the interaction with the binding site in both untreated and BD-treated membranes. However, the relative effects of structural alteration on the interaction at untreated and BD-treated membranes showed variation.

The IC_{50} values of strychnine and 12 related structures at inhibiting [^{3}H]-strychnine binding from untreated and BD-treated membranes indicate, in general, that these compounds are more potent at untreated membranes. As the induction of the strychnine binding BD treatment is associated with a low affinity site (see Chapter 5) the general lower potency relative to untreated membranes extends to derivatives of the strychnine molecule. The exception to this generalization was the alkaloid vomicine; this structural derivative is more potent at inhibiting [^{3}H]-strychnine binding from BD-treated membranes. The above result indicates that the structural features of strychnine that determine binding at the sites in untreated and BD-treated membranes are different.
Statistical analysis of the relationship between potency of the alkaloids at inhibiting [^{3}H]-strychnine binding to untreated and BD-treated membranes was not attempted because 6 of the compounds tested had IC_{50} values for inhibition of binding to BD-treated membranes that were > 10^{-4}M. Therefore, the potency of these compounds at BD-treated membrane could not be adequately ranked. However, a semi-quantitative description of the lack of correlation can be attempted by considering how compounds that represent

structural modifications of the most potent compound strychnine effected the relative potency for inhibiting [^{3}H]-strychnine binding to untreated or BD-treated membranes.

In this respect, although 2-aminostrychnine (compound 2) is equipotent with strychnine (compound 1) at inhibiting [^{3}H]-strychnine binding to untreated membranes it is less potent than strychnine at BD-treated membranes. In contrast when a nitro group was substituted at position 2 there was a more marked effect on the relative potency of the resulting compound 2-nitrostrychnine (compound 3) at untreated membranes. The differential effect of the group at position 2 was reflected by 2-nitrostrychnine being second in potency to strychnine at BD-treated membranes as compared to untreated membranes where it was only the seventh most potent of the compounds tested (see Table 6.2).

The principle that ligand binding at the two sites might be different is also reflected by the three compounds isostrychninic acid (compound 7), cacotheline (compound 8) and Weiland-Gumlich aldehyde (compound 11). The opening of ring III of strychnine that these compounds represents, effects the potency at both untreated and BD-treated membranes. However, the relative potency compared to strychnine was at least a 100 fold lower in BD-treated membranes but only 10 fold lower in untreated membranes. Again, this indicates dissimilarity in the ligand binding properties of the strychnine binding sites in untreated and BD-treated membranes.

In the case of brucine (compound 4) and bromodesoxyisostrychnine (compound 6) the relative change in potency when compared to strychnine, at untreated and BD-treated membranes was similar. This indicates that the differences between strychnine and these compounds were equally important in the interactions with the strychnine binding site in untreated and BD-treated membranes.

Finally, brucine-N-oxide (compound 5), diaboline (compound 9), N-oxystrychninic acid (compound 12) and N-methylstrychnine have IC_{50} values > 10^{-4}M. These compounds also have comparatively low potency at untreated membranes. Therefore, it was not possible to determine differences in their relative potency at untreated and BD-treated membranes, when compared to strychnine. For this reason it is difficult to ascertain the significance of these compounds in highlighting the comparative ligand recognition properties of the [^{3}H]-strychnine binding sites in untreated and BD-treated membranes.

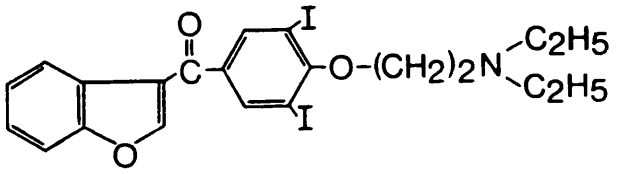
6.2.b Towards a pharmacological specificity of BD-induced [^{3}H]-strychnine binding

Strychnine has actions on nervous and other tissue, which are mediated by mechanisms other than the well characterized antagonism of glycine at its postsynaptic receptor. These include a cholinolytic action, an inhibition of GABAergic transmission, channel block at a number of cation channel types and activation of potassium conductances (see section 1.7). As the induction of [^{3}H]-strychnine binding in BD-treated membranes clearly lies at a site or sites other than the inhibitory glycine receptor, experiments focused on the non-glycinergic actions of strychnine in an attempt to characterize this induced [^{3}H]-strychnine binding. Initially this approach took the form of a screen experiment to identify compounds that interacted with the BD-induced [^{3}H]-strychnine binding site.

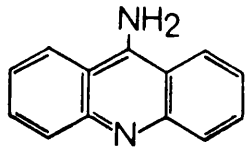
Untreated and BD-treated membranes were incubated with [^{3}H]-strychnine (6nM) in the presence or absence of a range of compounds (see Fig 6.3) with actions at sites or related sites to those that strychnine has reported actions. The assay conditions were as described previously (see section 6.2.a). The result of this screen is summarized in Table 6.3. For illustrative purposes, the compounds are classified into groups based on a site at which the action of the compound have been well characterized. Where appropriate, the clinical relevance of the drug action has been indicated. The validity of this classification is addressed in the Discussion.

In the untreated membranes, the results indicate that the drugs tested have little potency at inhibiting [^{3}H]-strychnine binding to the glycine receptor. Exceptions to this generalization might include the compounds imipramine, MK-801, 9-aminoacridine and giblencamide which inhibited at least 45% of control binding when tested at a concentration of 10^{-4}M.

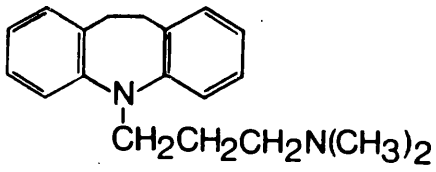
Unlike at the untreated membranes, the [^{3}H]-strychnine bound to the BD-treated membranes was sensitive to inhibition by a range of compounds tested, particularly those classified as cation channel blockers. This effect extended over several apparent subclasses to include blockers of Ca^{++}, Na^+ and K^+ channels. In addition, the BD-induced binding was inhibited by MK-801 a blocker of the cation channel associated with the N-methyl-D-aspartate (NMDA) receptor. However, a potency against the BD-induced [^{3}H]-strychnine binding was not exhibited by all channel blockers tested as indicated by the incubations



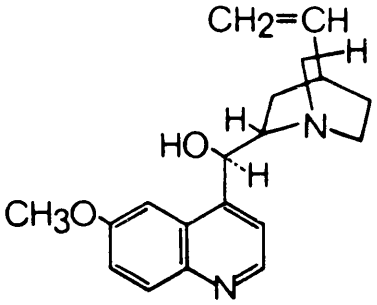
Amiodarone



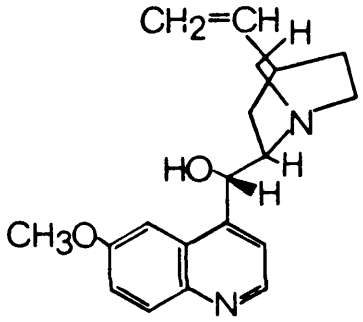
9-aminoacridine



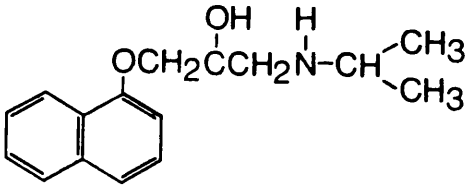
Imipramine



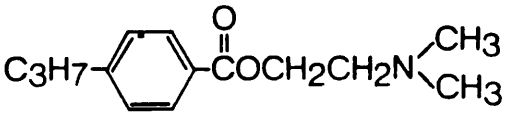
Quinine



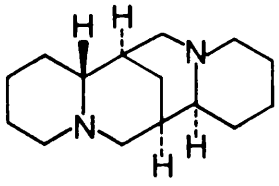
Quinidine



Propranolol



Tetracaine



Sparteine

Figure 6.3: Drug structures screened for inhibition of[^{3}H]-strychnine binding to untreated and BD-treated spinal cord membranes.

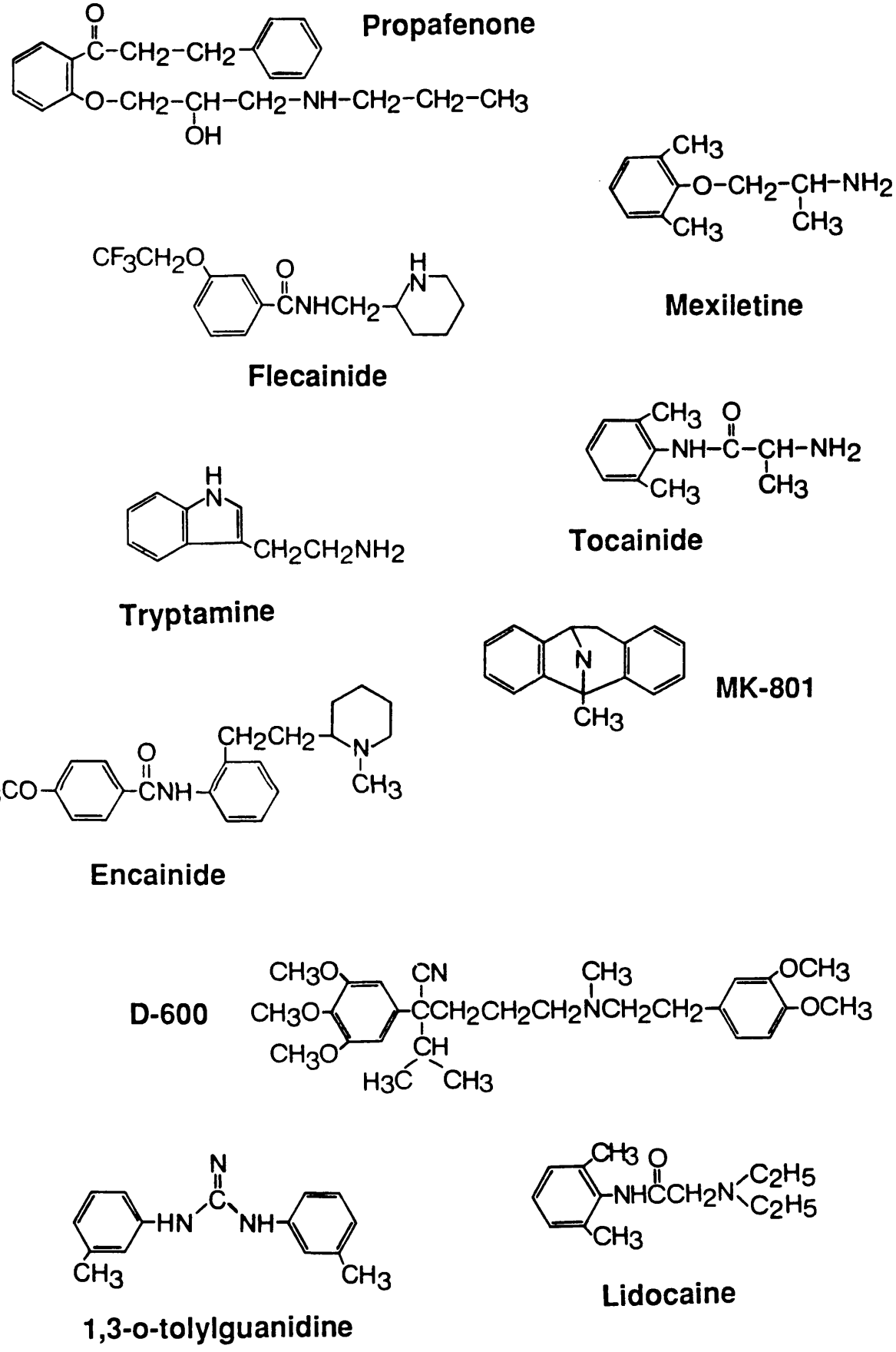


Figure 6.3 cont: Drug structures screened for inhibition of[^{3}H]-strychnine binding to untreated and BD-treated spinal cord membranes.

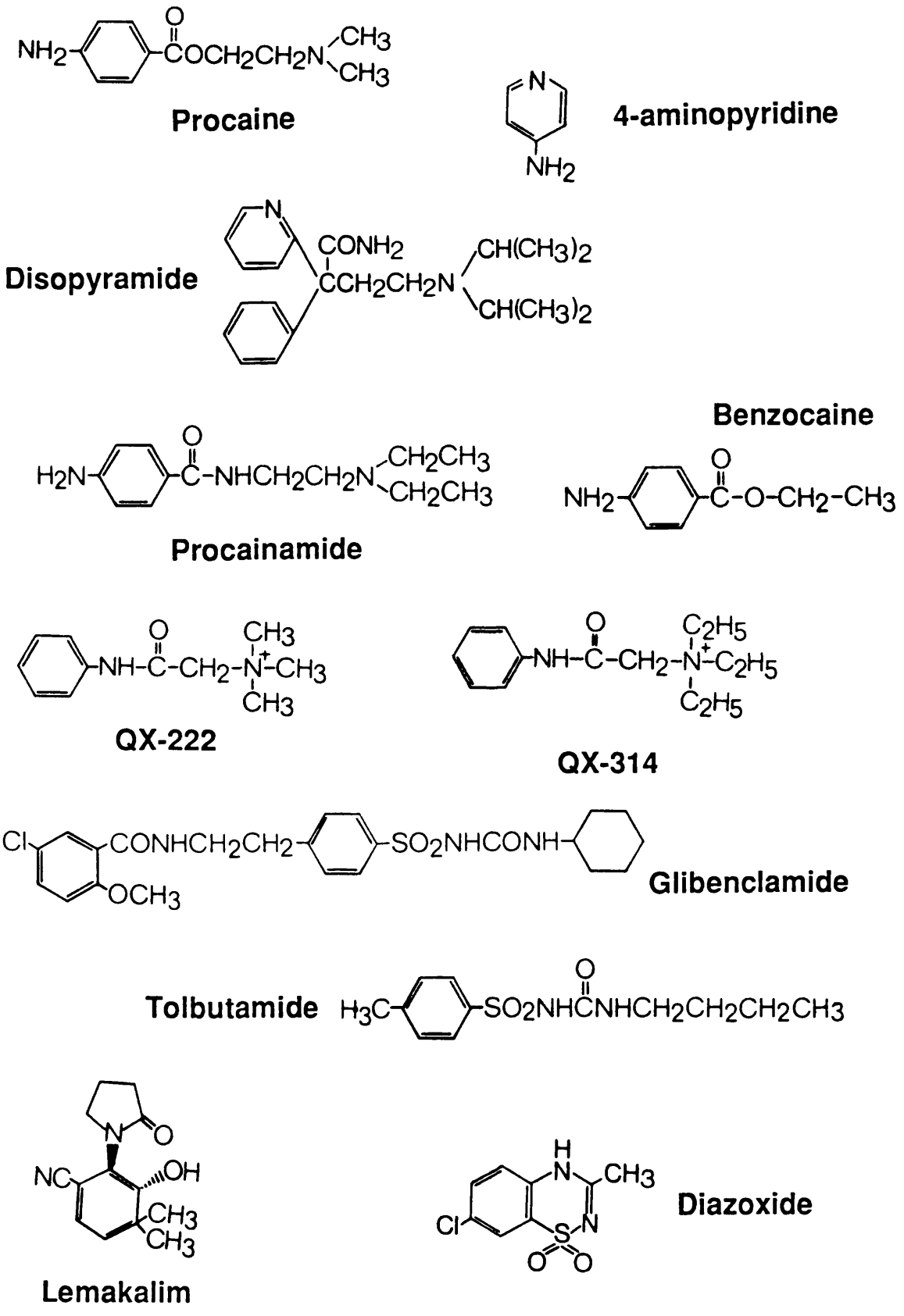
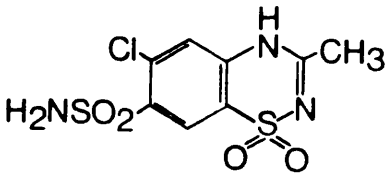
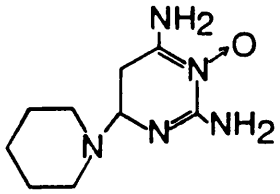


Figure 6.3 cont: Drug structures screened for inhibition of[^{3}H]-strychnine binding to untreated and BD-treated spinal cord membranes.



Chlorothiazide



Minoxidil

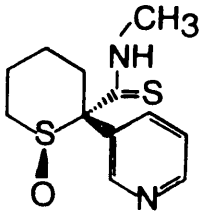


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Figure 6.3 cont: Drug structures screened for inhibition of[^{3}H]-strychnine binding to untreated and BD-treated spinal cord membranes.

that contained QX-314, QX-222, benzocaine, tetraethylammonium, giblencamide and tolbutamide. In addition, picrotoxin, a blocker of the anion channel in the $GABA_A$ receptor, did not inhibit the BD-induced [^{3}H]-strychnine binding when tested at 10^{-4}M.

Despite the inhibition of [^{3}H]-strychnine binding to BD-treated membranes by drugs which blocked Na^+ and K^+ channels toxins, with blocking actions at subclasses of the aforementioned channels did not cause an inhibition. Although at 10^{-4}M apamin caused a slight inhibition (35%) of BD-induced [^{3}H]-strychnine binding. Of the other drugs screened neither compounds which have actions as activators of cation conductances or those with cholinolytic activity inhibited the BD-induced [^{3}H]-strychnine binding. Indeed the only compounds tested other than those classified as cation channel blockers that inhibited the BD-induced [^{3}H]-strychnine binding were imipramine, propranolol, tryptamine and 1,3,o-tolyl-guanidine.

General Activity	Proposed site of action (clinical use)	Drug	% control binding	
			Untreated	BD-treated
Cholinergic antagonists	Nicotinic receptors	Tubocuraraine	142	78
		Decamethonium	74	76
		Pancuronium	140	88
		Gallamine	106	126
Channel toxins	Na^+ channels	Tetrodotoxin (10^{-6}M)	104	105
		Scorpion toxin (200 µg)	93	102
		Veratridine	93	101
	K^+ channels	Dendrotoxin (10^{-6}M)	98	112
		Apamin (10^{-6}M)	99	64
Channel blockers	Ca^{++} channels	D-600	57	0
		Nifedepine	140	35
	Na^+ channels	QX-222	111	98
		QX-314	80	104
		Benzocaine	93	104
	(Local anaesthetics and anti-arrhythmics)	Procaine	78	0
		Tetracaine	63	0
		Quinidine	109	10
		Procainamide	91	65
		Disopyramide	76	25
		Tocainide	99	0
		Mexiletine	101	14
		Lidocaine	67	0
		Flecainide	102	13
		Encainide	84	0
		Propafenone	78	70

General activity	Proposed site of action (Clinical use)	Drug	% control binding in presence of drug	
			Untreated	BD-treated
Channel blockers (cont.)	K^+ channel	9-aminoacridine	37	4
		Amiodarone	100	0
		Quinine	88	7
		4-aminopyridine	112	31
		T.E.A (10^{-3}M)	97	89
		Sparteine	94	12
		Tolbutamide	73	93
		Glibenclamide	28	90
	NMDA	MK-801	21	0
	$GABA_A$	Picrotoxin	98	87
Channel activators	K^+ channels	Lemakalim	110	113
		Minoxidil	80	84
		Diazoxide	70	93
		Chlorothiazide	74	97
		RP-52891	77	100
	Non-selective cation channel	Capsaicin	86	109
Miscellaneous	β-Adrenergic antagonist	Propranolol	83	0
	Tricyclic-antidepressant	Imipramine	62	0
	σ-Receptor ligand	1,3,-o-tolyl-guanedine	90	29
	Serotoninergic	5-HT	86	85
		Tryptamine	90	29
		Histamine	98	108

Table 6.3: Screen for inhibition of [^{3}H]-strychnine binding to untreated and BD-treated membranes by drugs with actions at sites at which strychnine has been reported to act: Membranes were incubated with [^{3}H]-strychnine (6 nM, 4 hrs, 4°C) in the presence of a single concentration of drug (10^{-4}M; unless otherwise stated). Control binding was defined by an incubation that contained no unlabelled drug but an appropriate volume of drug solvent. Assays were terminated by filtration.

6.2.c Investigation of the relative potency and molecular basis of drug interactions with the BD induced [^{3}H]-strychnine binding site

In the experiment described previously, a number of drugs exhibited an ability to inhibit the BD-induced [^{3}H]-strychnine binding to spinal cord membranes. The relative potency of these drugs was defined by comparing IC_{50} values for inhibition of [^{3}H]-strychnine binding. Further, by estimating the Hill coefficient for these competition experiments the molecular basis of the interaction with the [^{3}H]-strychnine binding to BD-treated membranes can be speculated.

The inhibition of [^{3}H]-strychnine binding by drugs which had IC_{50} values less than 10^{-4}M was investigated by adding test compounds to incubation at seven serially diluted concentrations that extend from 10^{-4}-10^{-10} or 10^{-4}M-10^{-7}M . The [^{3}H]-strychnine bound in each incubation was calculated as percent control and the data was analyzed as a Hill plot using linear least square analysis. IC_{50} values and Hill coefficients were determined along with the 95% confidence limits of these estimates.

The potencies, represented by an IC_{50} values, of the 20 compounds identified in the screen experiment (section 6.2.b) as inhibiting [^{3}H]-strychnine binding to BD-treated spinal cord membranes are shown in table 6.4. The most potent compound amiodarone was approximately 40 times more potent than procaine the least potent of the compounds tested. It also appeared that at BD-treated membranes the IC_{50} values of the eight most potent compounds were not significantly lower than strychnine although none of the compounds tested were more potent than strychnine at inhibiting [^{3}H]-strychnine binding to BD-treated membrane.

The corresponding Hill coefficients estimated from the same plots used to define IC_{50} values are also shown (see table 6.4). All compounds have Hill coefficients close to one although the absolute value for individual compounds shows a variation above and below unity. Similar Hill slopes were estimated for inhibition of [^{3}H]-strychnine binding by strychnos and other alkaloids (results not shown). Significant deviations from this general trend was shown by 9-aminoacridine which had values significantly greater than one.

Drugs	IC_{50} (M) [95% confidence limit]	Hill coefficient [± 95% confidence limit]
Amiodarone	1.10 [0.65-1.86] x 10^{-6}	0.88 [± 0.23]
9-aminoacridine	1.20 [1.07-1.35] x 10^{-6}	1.51 [± 0.14]
Imipramine	1.55 [1.26-1.91] x 10^{-6}	0.93 [± 0.10]
Quinine	1.74 [1.58-1.91] x 10^{-6}	1.01 [± 0.05]
Quinidine	2.04 [1.38-3.02] x 10^{-6}	1.08 [± 0.25]
Propafenone	2.09 [1.70-2.57] x 10^{-6}	0.96 [± 0.09]
Propranolol	2.55 [2.04-3.72] x 10^{-6}	0.85 [± 0.12]
Tetracaine	3.55 [3.09-4.07] x 10^{-6}	0.72 [± 0.04]
Sparteine	3.89 [3.24-4.68] x 10^{-6}	0.78 [± 0.07]
Mexiletine	6.03 [5.25-6.92] x 10^{-6}	0.83 [± 0.05]
Flecainide	1.07 [0.93-1.23] x 10^{-5}	1.09 [± 0.07]
MK-801	1.07 [0.91-1.26] x 10^{-5}	1.01 [± 0.07]
Tocainide	1.17 [1.00-1.38] x 10^{-5}	0.90 [± 0.08]
Encainide	1.38 [1.00-1.91] x 10^{-5}	1.12 [± 0.23]
Tryptamine	1.38 [1.02-1.80] x 10^{-5}	1.07 [± 0.17]
D-600	1.55 [1.38-1.74] x 10^{-5}	1.18 [± 0.11]
1,3-o-tolyl-guanidine	1.55 [1.31-1.86] x 10^{-5}	0.78 [± 0.09]
Lidocaine	2.09 [1.58-2.75] x 10^{-5}	1.02 [± 0.15]
4-aminopyridine	2.82 [2.29-3.36] x 10^{-5}	1.02 [± 0.15]
Procaine	4.27 [3.13-5.01] x 10^{-5}	1.04 [± 0.02]

Table 6.4: Relative potency and Hill coefficients for inhibition of [^{3}H]-strychnine binding from BD-treated membranes by drugs: BD-treated membranes were incubated in triplicate (4 hrs, 4°C) with [^{3}H]-strychnine (6 nM) in the presence of drug dilutions. Control binding was defined by incubations that contained an appropriate volume of drug solvent and non-specific binding by unlabelled strychnine (10^{-4}M). Assays were terminated by filtration.

6.2.d Drug modulation of the BD-modification that induces [^{3}H]-strychnine in treated membranes.

In Chapter 3, it was shown that the amount of [^{3}H]-strychnine binding induced in BD-treated spinal cord membranes could be potentiated if the membranes were incubated with unlabelled strychnine (10^{-4}M) prior to and during the reaction. This was taken to indicate a conformational dependence of the reaction between BD and the protein that in its modified state is responsible for the induced [^{3}H]-strychnine binding in treated membranes. The ability of BD to induce the [^{3}H]-strychnine binding site was also shown to be unaffected by the presence of glycine during the reaction (see fig 3.19). Similarly when glycine was co-incubated with strychnine during the BD reaction the presence of the amino acid had no effect on the potentiation of the BD induced [^{3}H]-strychnine effected by preincubation with unlabelled strychnine alone (results not shown). In view of the consensus reported in Chapter 4 and 5 that the BD-induced binding in spinal cord membrane occurs at sites other than the inhibitory glycine receptor, these results were not surprising. However, having identified and characterized drugs that do show a pharmacological selectivity for the BD-induced [^{3}H]-strychnine binding site it appeared appropriate to consider whether or not these compounds could modulate the reaction of BD with spinal cord membranes.

An initial experiment was performed to determine the concentration dependence for potentiation of the induced [^{3}H]-strychnine binding by the presence of unlabelled strychnine during the reaction with BD. Spinal cord membranes were preincubated with increasing concentrations of unlabelled strychnine (10^{-9}-10^{-3}M). Control membranes to which no unlabelled strychnine had been added were also studied. The preincubated membranes were divided into two equal volumes which were untreated or reacted with BD (80 mM, 20/21°C, 40 mins). The reaction was terminated, the membranes thoroughly washed and subsequently assayed for [^{3}H]-strychnine binding.

The result of this experiment is illustrated in fig 6.4. The ability of the washing procedure to remove the unlabelled strychnine added to membrane prior to assay was indicated by the constant amount of [^{3}H]-strychnine bound to untreated membranes. When spinal cord membranes were treated with BD, there was a characteristic increase in subsequent [^{3}H]-strychnine binding. In controls which were not subjected to a preincubation with unlabelled strychnine the amount bound to BD treated membranes was

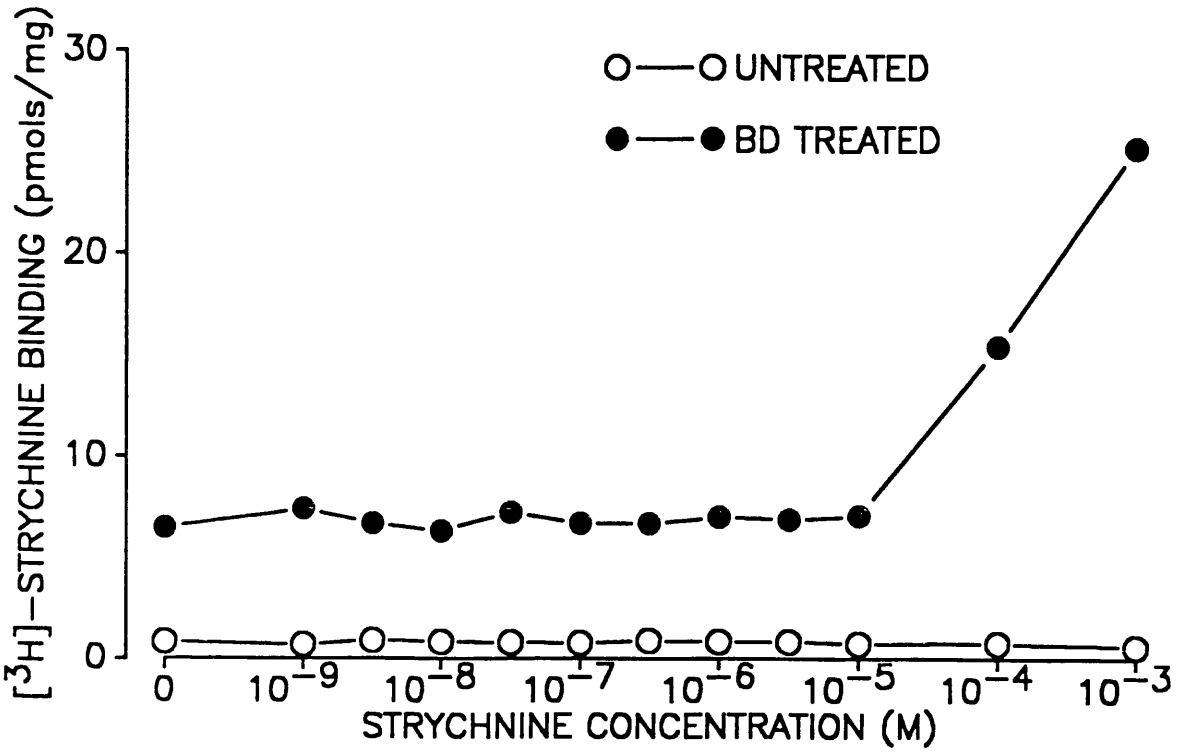


Figure 6.4: Concentration dependence of potentiation of the BD-induced [^{3}H]-strychnine binding by strychnine: Spinal cord membranes (40 vols; tissue wt./vol) incubated with increasing concentration of strychnine (20 mins, 20/21°C) were untreated or reacted with BD (80mM, 40 mins, 20/21°C). The reaction was stopped, the membranes washed and incubated in triplicate with [^{3}H]-strychnine (6nM, 2hrs, 4°C). These assays were terminated by filtration and non-specific binding defined by unlabelled strychnine (10^{-4}M).

approximately 10 fold higher than the untreated membranes. The results also show that membranes must be preincubated with strychnine concentration $>10^{-5}$M, prior to and during the BD treatment to ensure a potentiation in the BD-induced [^{3}H]-strychnine binding. Also, maximal potentiation was not reached in the membranes preincubated with 10^{-3}M strychnine, the highest concentration used in this experiment. However, strychnine at a concentration of 10^{-4}M caused a robust and reliable potentiation (>100%) of the BD-induced [^{3}H]-strychnine binding. Therefore, this was the concentration used to screen other drugs for possible modulation of the reaction of BD with spinal cord membranes.

To investigate the effect of drugs on the reaction of BD with spinal cord membranes, they were incubated prior to and during the treatment (80mM, 40 mins, 21°C) with test compound, washed and the subsequently assayed [^{3}H]-strychnine binding expressed as a percentage that measured BD-treated membranes reacted without addition of unlabelled drug. In addition, the experiment included untreated membranes and incubations that controlled for the drug solvents, distilled water, dimethylformamide or methanol. The concentration of solvent present during each treatment (1% v/v), had no effect on the amounts of [^{3}H]-strychnine binding induced by the BD-modification of spinal cord membranes.

The effects of drugs on the BD reaction have been placed into two groups (see Fig 6.5.a and 6..5.b) to coincide with the format used in the investigation described previously in this Chapter. Figure 6.5.a illustrates the effects of the strychnine-like and other alkaloids; while figure 6.5.b illustrates the effect of the drugs other than alkaloids that have been shown previously to inhibit the BD-induced [^{3}H]-strychnine binding.

As shown in fig 6.5.a most of the strychnoid and other alkaloids caused a potentiation of the BD induced [^{3}H]-strychnine binding if present during the treatment or were without effect. In addition there was a general trend which indicated that the more potent compounds at inhibiting the BD-induced [^{3}H]-strychnine binding were those that caused a potentiation (see Table 6.2). However, two compounds 2-aminostrychnine and Weiland-Gumlich aldehyde, clearly did not fit any of the generalizations documented above. These compounds rather than having no effect or potentiating, caused a decrease in the amount of [^{3}H]-strychnine binding produced by the BD treatment. This was indicated by a significant decrease in percent control binding when these drugs were present during the BD-treatment. Although this effect was most marked for 2-

Figure 6.5.a: for legend see Fig 6.5.b

aminostrychnine at the concentration tested, it was only caused a 50% decrease in the BD-induced [^{3}H]-strychnine binding.

When the drugs identified earlier as having the ability to inhibit the BD-induced [^{3}H]-strychnine binding were screened for their effects on the BD reaction a full spectrum of modulations were seen. Although propafenone, imipramine, 1,3-o-tolyl-guanidine, tryptamine and 4-aminopyridine were without effect, the remaining compounds when present during the BD reaction at a concentration of 10^{-4}M, caused either a potentiation or a inhibition of the induced [^{3}H]-strychnine binding subsequently measured in treated membranes. Of the drugs that caused a reduction, quinine, quinidine and sparteine had a similar effect to 2-aminostrychnine and Weiland-Gumlich aldehyde, in that they reduced but could not completely prevent an increased amount of [^{3}H]-strychnine when compared to untreated controls (data not shown). However, when 9-aminoacridine or amiodarone were preincubated with membranes prior to and during the BD treatment, they completely prevented the induced [^{3}H]-strychnine binding usually seen in response to BD treatment. Indeed, the amount of [^{3}H]-strychnine bound in drug incubated BD-treated membranes was not significantly ($p>0.2$) different from the corresponding untreated control membrane data not shown. Potentiation of the induced [^{3}H]-strychnine binding was indicated by drugs that caused a statistically significant increase ($p<0.05$) in percent control binding if present during the BD treatment of spinal cord membranes. These increases, ranged from the smallest caused by procaine to the largest, caused by D-600.

Crude trends in the relationship between the potency of drugs at inhibiting [^{3}H]-strychnine binding to BD-treated membranes and its ability to modulate the reaction were also apparent. In general drugs that caused a potentiation of the reaction were found to have a lower potency at inhibiting the [^{3}H]-strychnine bound to BD-treated membranes when compared to those that caused a reduction or completely prevented the BD-induced [^{3}H]-strychnine binding (see Table 6.4). This contrasted the trend shown for the alkaloid compounds.

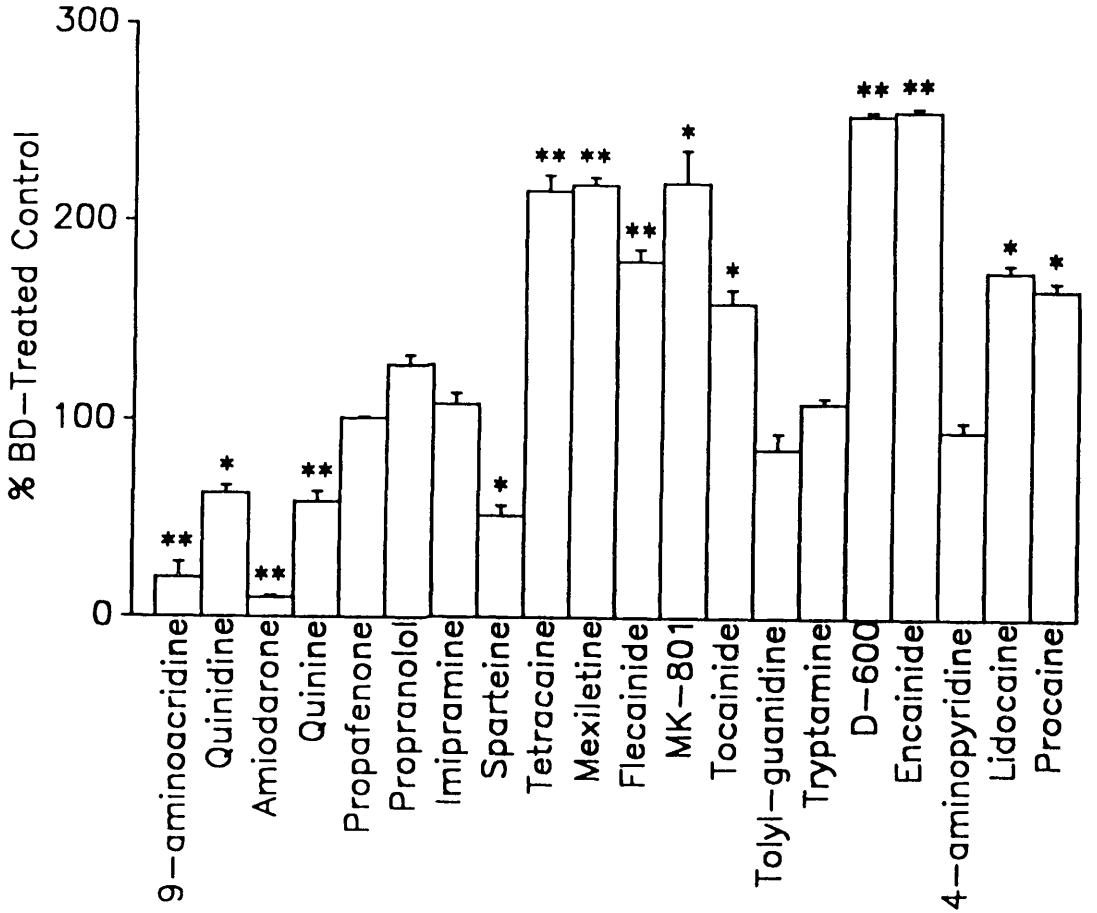


Figure 6.5.b: Effect of drug incubation prior to and during BD treatment on the amount of induced [^{3}H]-strychnine binding: Mouse spinal cord membrane were incubated (30 mins, 20/21°C) with drug (10^{-4}M) or drug solvent prior to BD treatment (80 mM, 40 mins, 20/21°C). Reaction was stopped, membranes washed and incubated in triplicate with [^{3}H]-strychnine (6nM, 4 hrs,4°C). Non-specific binding was estimated by incubations that contained strychnine (10^{-4}M). Controls defined by [^{3}H]-strychnine binding to treated membranes reacted in the absence of drugs were 9 fold higher than untreated membranes. Drug solvents present during the BD treatment had no effect on the [^{3}H]-strychnine binding to controls. Results are the mean and standard error of 3 experiments. Comparisons were made by the paired t-test, significant difference from control indicated by * $p<0.05$; ** $p<0.001$.

6.2.e Photolabelling untreated and BD-treated spinal cord membranes with [^{3}H]-strychnine to investigate the BD-induced binding site

As a further approach to characterize the nature of the BD-induced [^{3}H]-strychnine binding, the use of photoaffinity labelling was reinvestigated. In Chapter 4 this was used and experiments showed that the increased [^{3}H]-strychnine binding was not at ligand binding subunit of the inhibitory glycine receptor. Additional labelled peptides specific to BD-treated membranes were not identified. However, the photolabelling and subsequent SDS-PAGE used to analyze the labelled peptides were optimal for detecting photoincorporation at the high affinity, strychnine binding, 48 kd subunit of the inhibitory glycine receptor. Particularly, the use of 10% gels to separate components of solubilized membranes would have restricted the size range of peptides that could be resolved. Subsequent experiments described in this chapter attempted to define photolabelling under modified conditions and made use of the pharmacological specificity of the BD-induced [^{3}H]-strychnine binding that has been described earlier in this chapter.

Consequently, BD-treated and untreated membranes were incubated with 100 nM [^{3}H]-strychnine for 4 hours at 4°C before being subjected to UV-illumination (nominal peak 254 nm, 300μW/cm^2 at 15.2 cm) for 30 mins. These incubation conditions were used based on the low affinity and slow equilibration of [^{3}H]-strychnine binding to the BD-induced site (see chapter 5). The ability of strychnine, glycine and other compounds to prevent photoinduced irreversible incorporation was addressed by incubations that contained unlabelled drug in addition to [^{3}H]-strychnine.

After exposure to UV-light the membranes were chased with unlabelled strychnine (1mM, 4°C, 1 hr) and processed for gel electrophoresis or filtration. In parallel experiments, portions of the membranes used in these photolabelling procedures were assayed for reversible [^{3}H]-strychnine binding (6 nM) to confirm that they exhibited the characteristic increased binding in BD-treated membranes. The pharmacological specificity of the reversible binding was also considered with respect to the drugs used in the investigation of photoinduced irreversible [^{3}H]-strychnine binding (see fig 6.6).

6.2.e.1 Filtration of photolabelled membranes to indicate pharmacologically specific irreversible incorporation of [^{3}H]-strychnine.

Incubations subjected to photolabelling procedures described above were divided into three equal portions and subjected to filtration under vacuum to give triplicate estimates of the [^{3}H]-strychnine irreversibly incorporated into untreated and BD-treated membranes. The results for two experiments performed for this thesis are shown in Figure 6.6.

In both experiments an increased amount [^{3}H]-strychnine irreversibly bound to BD-treated membranes, compared to untreated controls, when no drugs were present during the photolabelling. However, these increases in total [^{3}H]-strychnine irreversibly incorporated, equivalent to 60% in experiment one and 11% in experiment two, were smaller than the 1200% increase in reversible [^{3}H]-strychnine binding assayed in BD-treated membranes when compared to untreated controls.

The pharmacological specificity of the reversible and photoinduced irreversible [^{3}H]-strychnine binding, indicated by comparing the amount bound in incubations that contained an excess of unlabelled drug with those that contained no drugs was considered (see figure 6.6). In both experiments strychnine at a concentration of 10^{-3}M (experiment 1) or 10^{-4}M (experiment 2) inhibited the majority of [^{3}H]-strychnine reversibly and irreversibly bound to BD-treated membranes.

When glycine (10^{-2}M) was used to investigate the pharmacological specificity of binding it was not able to inhibit [^{3}H]-strychnine bound to BD-treated membranes. This was apparent whether reversible or irreversible [^{3}H]-strychnine binding was considered. In contrast, glycine was able to inhibit the amount of [^{3}H]-strychnine reversibly or irreversibly bound to untreated membranes. The proportion of total [^{3}H]-strychnine reversibly or irreversibly bound to untreated membranes inhibited by glycine was lower than that inhibited by strychnine. However this discrepancy was most marked with respect to inhibition of irreversibly bound [^{3}H]-strychnine. Therefore, glycine appeared to be a less efficient inhibitor of the photoinduced irreversible [^{3}H]-strychnine binding to untreated membranes.

In addition, the pharmacological specificity of binding in one experiment was

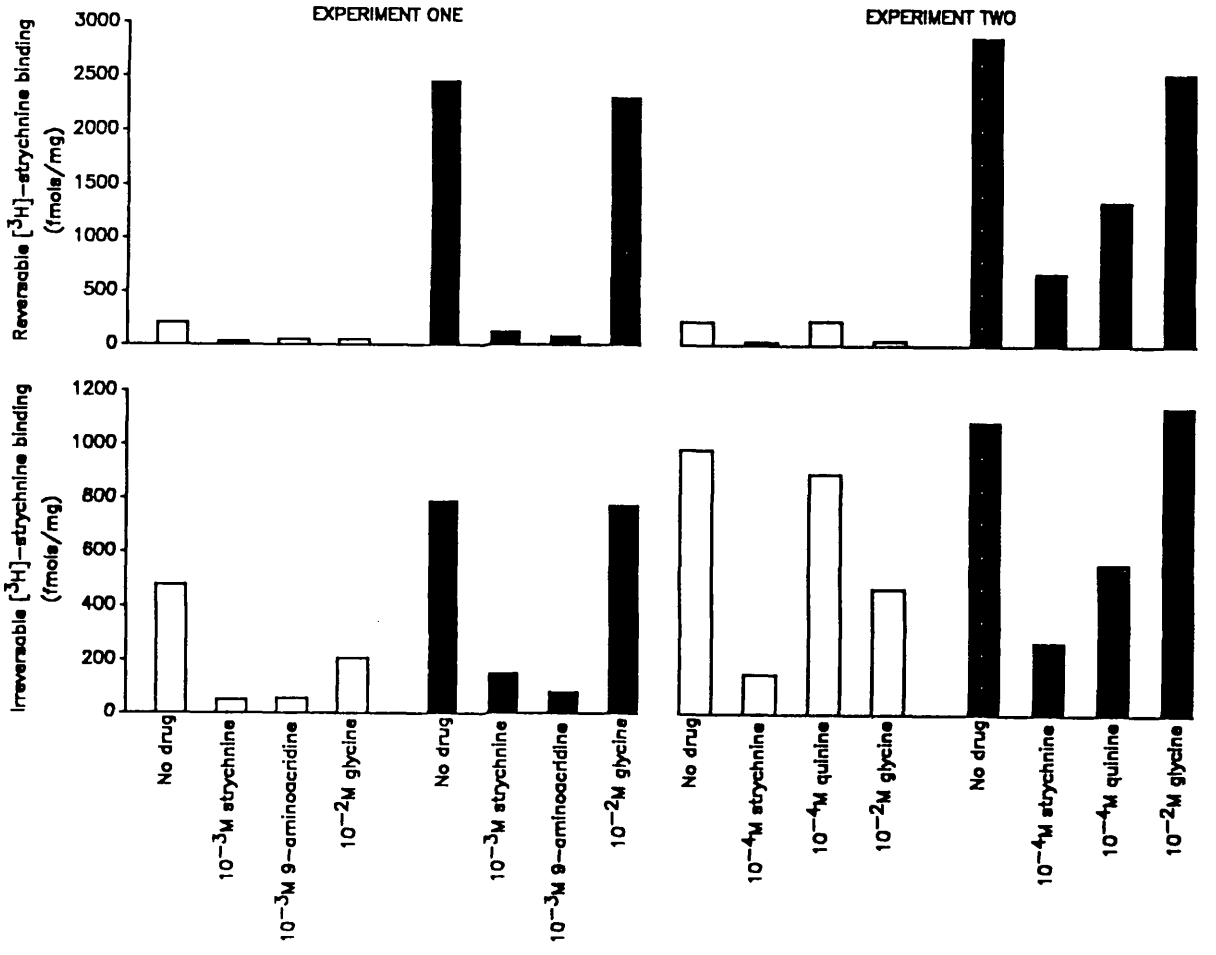


Figure 6.6: Pharmacological specificity of reversible and irreversible [^{3}H]-strychnine binding to untreated and BD-treated spinal cord membranes: Untreated (□) and BD-treated (■) membranes (400 vols, tissue wt./vol in assay) were incubated (4 hrs, 4°C) with [^{3}H]-strychnine (6nM) in the presence or absence of the unlabelled drugs indicated and then filtered to estimate reversible binding. Alternatively, membranes (20 vols; tissue wt./vol) incubated (4 hrs, 4°C) with [^{3}H]-strychnine (100nM) in presence or absence of unlabelled drugs were exposed to UV-light (nominal peak 254 nM, 30 μW/cm^2 at 15 cm). These irradiated incubations were chased with strychnine (1mM), divided into three equal volumes and washed by filtration to estimate [^{3}H]-strychnine irreversibly bound. Results from two experiments show amount bound in presence of different drugs.

investigated by 9-aminoacridine and in the other by quinine. The compound 9-aminoacridine was among the most potent of those shown to cause a selective inhibition of the [^{3}H]-strychnine bound to BD-treated rather than untreated membranes (see table 6.4). However, at a concentration of 10^{-3}M this compound inhibited a large proportion of the [^{3}H]-strychnine reversibly and irreversibly bound to both untreated and BD-treated membranes. As 9-aminoacridine did not provide sufficient specificity for the BD induced [^{3}H]-strychnine binding quinine (10^{-4}M) was considered in the second experiment. Unlike 9-aminoacridine, quinine selectively inhibited the [^{3}H]-strychnine reversibly bound to BD-treated membranes. This was confirmed by the fact that quinine caused only a minimal reduction in the [^{3}H]-strychnine irreversibly bound to untreated membranes. Despite an apparent lack of potency against the [^{3}H]-strychnine bound to the inhibitory glycine receptor in untreated membranes, quinine at a concentration of 10^{-4}M inhibited nearly 50% of the total [^{3}H]-strychnine irreversibly incorporated into BD-treated membranes.

In order to identify the peptides of spinal cord membranes that had incorporated [^{3}H]-strychnine, the photolabelled membranes were washed and solubilized overnight in 10% (v/v) SDS. These samples which included membranes photolabelled in the presence of excess unlabelled drugs, were prepared for polyacrylamide gel electrophoresis (PAGE) and analyzed by running them through a 5-20% linear gradient resolving gel. The gel was subjected to fluography to enable detection of the peptides labelled with [^{3}H]-strychnine. Samples were run in parallel with protein standards from which a calibration curve was plotted. This curve was constructed by plotting log percent gel concentration to which the standard protein had run (%T) against the log of their molecular weight. The line was fitted by linear regression. The % T of the standard protein and the peptides labelled in the samples was calculated by:

$$\% T = (R_f \times 15) + 5$$

where R_f is distance travelled by peptide relative to the dye front, which was run to the end of the gel. Using % T for samples approximate molecular weights for the labelled peptides were estimated from the calibration curve.

The pattern and pharmacological specificity of peptide labelling for photolabelled untreated and BD-treated spinal cord membranes from two individual experiments are shown (plate 6.1 and 6.2). These fluorographs represent the labelled peptides solubilized from portions of the membranes in which irreversible incorporation of [^{3}H]-strychnine upon UV-illumination was described previously (see fig 6.6).

Plate 6.1: Fluography of SDS-polyacrylamide gel electrophoresis of untreated and BD-treated membrane photolabelled with [^{3}H]-strychnine. Untreated and BD-treated membranes (20 vols; tissue wt./vol) incubated (4 hrs, 4°C) with [^{3}H]-strychnine (100nM) alone (no drug, ND) or in the presence of unlabelled strychnine (10^{-3}M, S), glycine (10^{-2}M) and or 9-aminoacridine (10^{-3}M, A) were irradiated with UV-light (nominal peak 254 nM, 30 μW/cm^2 at 15 cm) for 30 mins at 4°C. Membranes were chased with unlabelled strychnine (1mM) for 30 mins, pelleted by centrifugation and washed in Tris/HCl (pH 6.8). Washed membranes (5 μg membrane protein/μl) solubilized overnight in 10% SDS were subjected to SDS-PAGE through a 5-20% linear gradient resolving gel. Fixed gels were exposed to photographic film for 3-5 months at -70°C. Membranes used correspond to Experiment 1 in fig 6.6.

Plate 6.2: Fluography of SDS-polyacrylamide gel electrophoresis of untreated and BD-treated membrane photolabelled with [^{3}H]-strychnine. Untreated and BD-treated membranes (20 vols; tissue wt./vol) incubated (4 hrs, 4°C) with [^{3}H]-strychnine (100nM) alone (no drug, ND) or in the presence of unlabelled strychnine (10^{-4}M, S), glycine (10^{-2}M, G) and or quinine (10^{-4}M, Q) were irradiated with UV light (nominal peak 254 nM, 30 μW/cm^2 at 15 cm) for 30 mins at 4°C. Membranes were chased with unlabelled strychnine (1mM) for 30 mins, pelleted by centrifugation and washed in Tris/HCl (pH 6.8). Washed membranes (7.5 μg membrane protein/μl) solubilized overnight in 10% SDS were subjected to SDS-PAGE through a 5-20% linear gradient resolving gel. Fixed gels were exposed to photographic film for 3-5 months at -70°C. Membranes used correspond to Experiment 2 in fig 6.6.

Plate 6.1

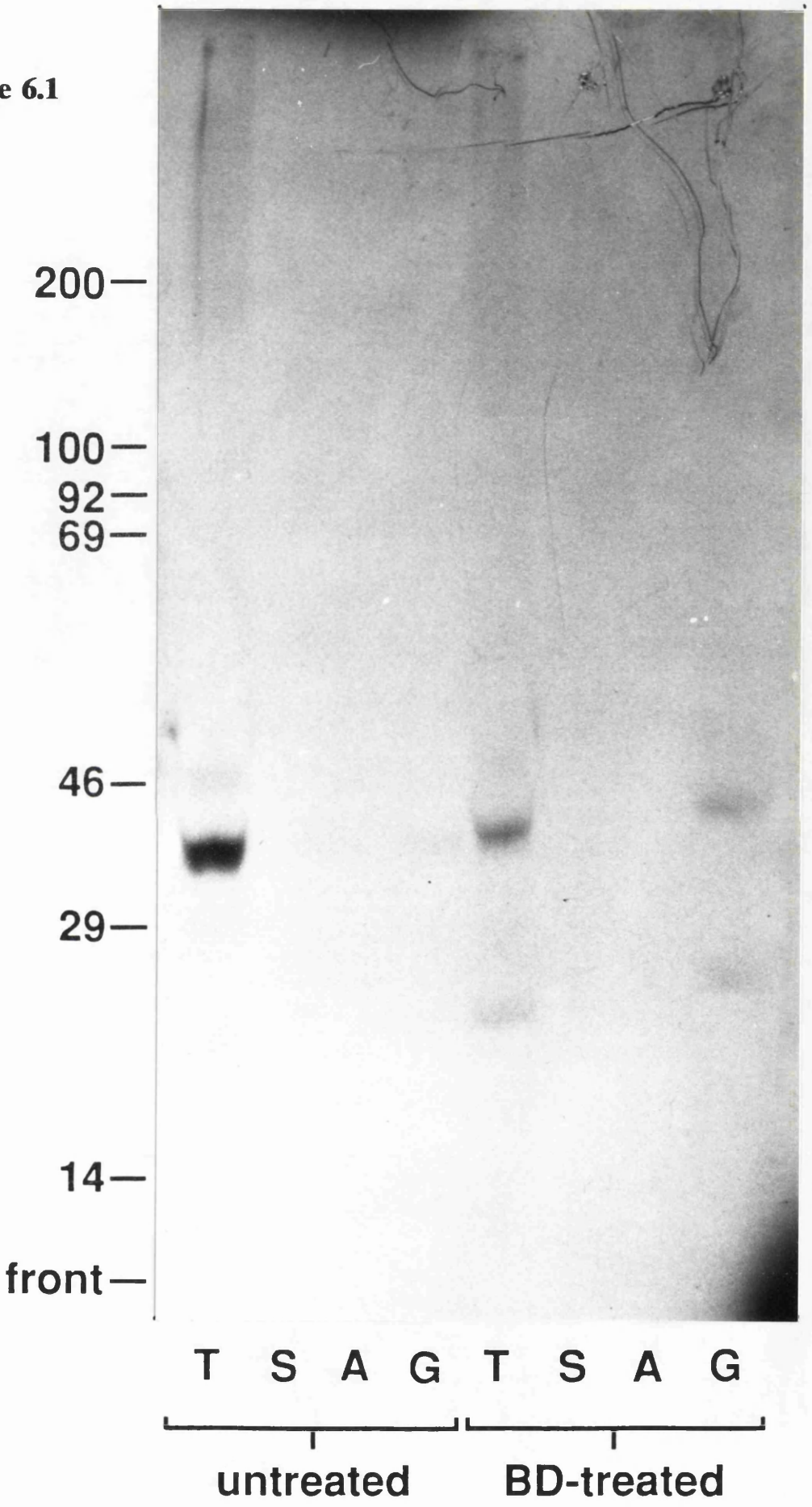
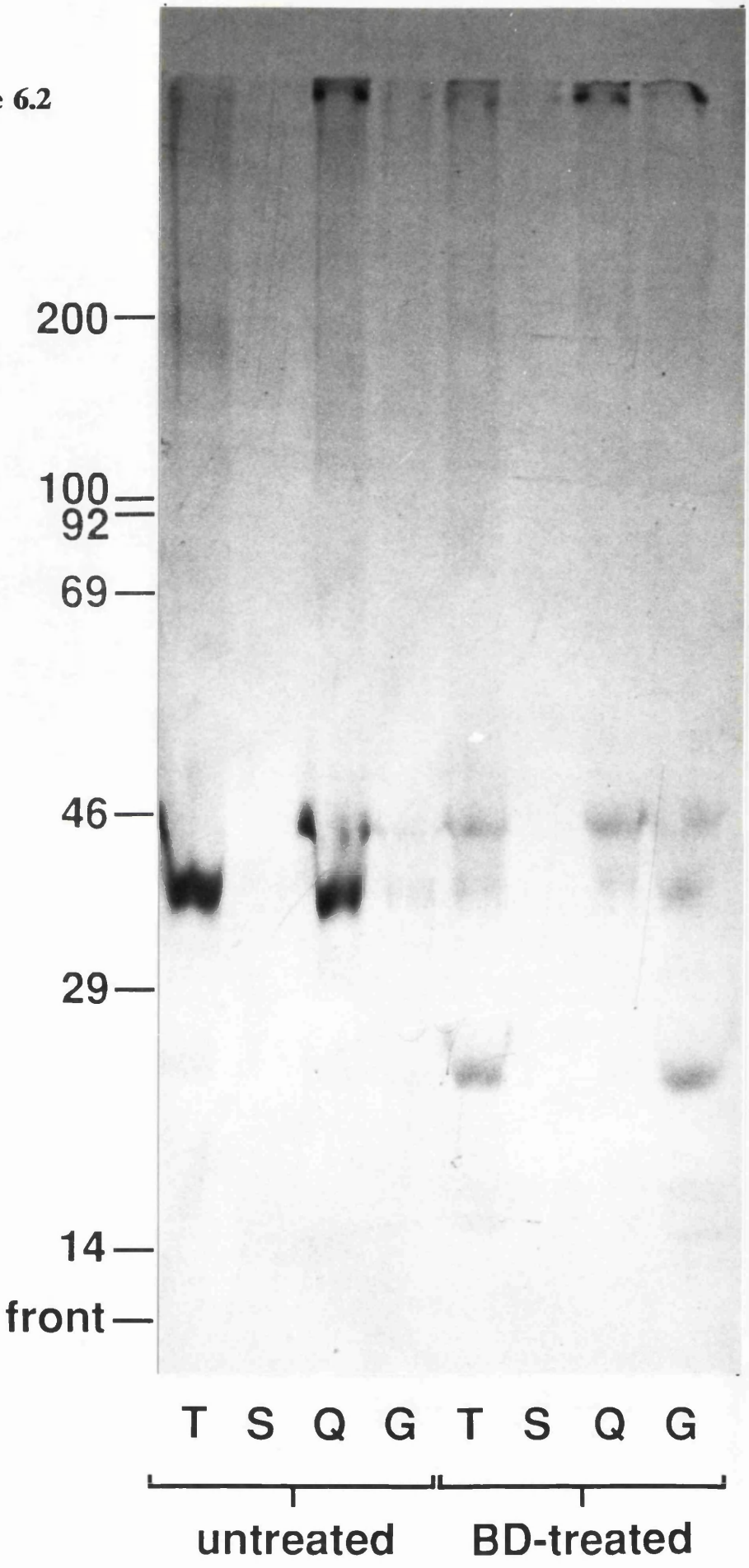


Plate 6.2



In the first experiment the fluorograph showed that in the total lane, loaded with untreated membrane photolabelled with [^{3}H]-strychnine in the absence of any drug, a single peptide was labelled. However, when the untreated membranes were photolabelled in the presence of either unlabelled strychnine (10^{-3}M), 9-aminoacridine (10^{-3}M) or glycine (10^{-2}M) incorporation of [^{3}H]-strychnine into this peptide was prevented. This result was consistent with the finding that these 3 compounds inhibited a large proportion of the [^{3}H]-strychnine irreversibly bound to the photolabelled untreated membranes when assayed by filtration. The estimated molecular weight of the labelled peptide was 35 kd.

In the BD-treated membranes photolabelled in the absence of unlabelled drugs a peptide with the same molecular weight as that detected in untreated membranes was labelled (see Plate 6.1) . However, in addition a second labelled peptide not apparent in the untreated membranes was labelled. The labelled peptide that was only detected in the BD-treated membranes had an estimated molecular weight of 23 kd. The intensity of the labelling to both peptides appeared lower when compared to the labelling of the single peptide detected in untreated membranes. When the BD-treated membranes were photolabelled in the presence of either strychnine or 9-aminoacridine labelling of both peptides detected in the total lane was prevented. In contrast, glycine did not prevent photoincorporation of [^{3}H]-strychnine into the higher molecular weight peptide whose labelling was sensitive to glycine in the untreated membranes. Also, glycine did not prevent the labelling of the low molecular weight peptide that was only detected in the BD-treated membranes. Again, this pharmacological specificity was consistent with the amounts of [^{3}H]-strychnine irreversibly bound to treated membranes when photolabelled under different drug treatments and assayed by filtration (see Fig. 6.6).

From the second gel (see Plate 6.2) in the total lane loaded with solubilized untreated membrane, labelling was in a smear which ran from an approximate molecular weight of 42 KD to 35 KD. The lowest molecular weight corresponded to the size of peptide detected in the corresponding lane of the first gel. In the lane loaded with untreated membranes that had been photolabelled in the presence of strychnine the labelling described in the total lane was no longer detectable. By way of contrast quinine did not affect this labelling. Indeed the intensity of the labelling in the lane loaded with untreated membrane labelled in the presence of quinine was very similar to the no drug lane. The inability of quinine to prevent photolabelling of the peptides detected in the

untreated, total lane, was consistent with the finding that at the same concentration it did not inhibit the reversible or irreversible [^{3}H]-strychnine bound to untreated membranes, as assayed by filtration (see fig 6.6). It also appeared from the labelled peptides in the lane loaded with membranes photolabelled in the presence of quinine, that the smear detected in the untreated total lane was due to two peptides with molecular weights of 35 kd and 42 kd respectively. When the untreated membrane photolabelled in the presence of glycine were analyzed there was a reduction in the labelling of the two peptides detected in the total untreated lane. This result was consistent with data that considered the photoinduced irreversible [^{3}H]-strychnine binding to untreated membranes assayed by filtration. In these experiments although glycine inhibited total [^{3}H]-strychnine irreversibly incorporated into untreated membranes this represented a smaller proportion than the amount of total binding inhibited by unlabelled strychnine.

As seen in the first fluorograph, in the second experiment the labelling pattern in the lane loaded with untreated membrane photolabelled in the absence of drug, was also apparent when BD-treated membranes photolabelled under identical conditions were analyzed. Although in the second fluorograph the labelling common to untreated and BD-treated membranes corresponded to a doublet. However, in addition to labelled peptides common to untreated and BD-treated membranes, a peptide with an estimated molecular weight of 23 kd was detected in the BD-treated membranes. This was similar to the molecular weight of the additional peptide labelled in BD-treated membranes from the first experiment. As in the first experiment, photolabelling in the presence of strychnine prevented incorporation of [^{3}H]-strychnine into all peptides. In contrast in the BD-treated membranes glycine failed to prevent irreversible incorporation of [^{3}H]-strychnine into the peptides that were common to untreated and BD-treated membranes despite inhibiting the labelling of these peptides in untreated membranes. Also glycine did not prevent the labelling of the peptide that could only be detected in the BD-treated membranes.

Finally, quinine which at the concentrations used in the second experiment was shown selectively to inhibit reversible [^{3}H]-strychnine binding to BD-treated membranes, specifically inhibited labelling of the 23 kd peptide. This was consistent with the description that quinine when present during the photolabelling only inhibited 50 % of the total [^{3}H]-strychnine irreversibly bound to BD-treated membranes as assayed by filtration.

6.3 Discussion

The experiments described in this chapter have used pharmacological approaches to probe and characterize the nature of the increased [^{3}H]-strychnine binding to BD-treated spinal cord membranes. Although these results have implications in their own right, they are best interpreted by also considering other aspects of the BD-induced [^{3}H]-strychnine binding that have been described in Chapters 3, 4 and 5. For this reason the foregoing discussion will address both the data described in this chapter and attempt to reconcile it with characteristics of the BD-induced [^{3}H]-strychnine binding that have been described in the preceding chapters of this thesis.

6.3.a. A comparison of the pharmacological specificity of [^{3}H]-strychnine binding to untreated and BD-treated spinal cord membranes with respect to ligands that interact at the inhibitory glycine receptor

From previous experiments (see Chapter 4) it appeared that the BD-induced [^{3}H]-strychnine binding was at a site other than the inhibitory glycine receptor. This was underlined by the pharmacological specificity exhibited by the BD-induced [^{3}H]-strychnine binding. Several compounds that have been shown to interact with the glycine receptor and in this study also inhibited the [^{3}H]-strychnine binding to untreated membranes, did not effect binding to BD-treated membranes when screened at a single high concentration. These included the amino acids ß-alanine and taurine which activate the inhibitory glycine receptor by interacting with the same (Curtis *et al.*, 1968a; Werman *et al.*, 1968; Tokutomi *et al.*, 1989) or similar recognition sites to glycine (Kuhse *et al.*, 1990a).

Similarly, RU 5135 and Iso-THAZ selectively inhibited the [^{3}H]-strychnine binding to untreated membranes. Both these compounds have been shown to antagonize the inhibitory action of glycine in spinal neurones (Krogsnaard-Larsen *et al.*, 1982; Curtis and Malik, 1985) and also inhibit [^{3}H]-strychnine binding to the inhibitory glycine receptor (Braestrup *et al.*, 1986). The site(s) on the receptor at which these compounds interact are not known but iso-THAZ inhibition of [^{3}H]-strychnine binding exhibits a differential sensitivity to protein modification similar to glycine and on this basis it has been argued that this drug interacts with the glycine recognition site (Marvizón *et al.*, 1986a). Regardless of the exact details, the inability of RU 5135 and Iso-THAZ to inhibit [^{3}H]-

strychnine binding to BD-treated membranes was in agreement with the idea that the induced site was at a site other than the inhibitory glycine receptor.

The results of one of the compounds tested, mephenesin, that did not inhibit [^{3}H]-strychnine binding to untreated or BD-treated membranes deserves further discussion. Experiments designed to investigate the mechanisms that underlie high pressure neurological syndrome (HPNS) have concluded that high pressure induced and strychnine induced convulsions are evoked through a common mechanism (Bowser-Riley *et al.*, 1988). Indeed, mephenesin is the most potent anti-HPNS drug, in addition to increasing the threshold for the production of the tremor and convulsion associated with high pressure has been shown to increase threshold for strychnine induced muscle tremor and convulsion (Bowser-Riley *et al.*, 1989). Neuroanatomical investigations (Bowser-Riley *et al.*, 1981) indicate a sub-cortical focus for the action of high pressure and is in agreement with the distribution of [^{3}H]-strychnine binding to untreated membranes. These observations have led to the suggestion that the effects of high pressure have a specific site of action at associated with the inhibitory glycine synapse. However, the inability of mephenesin to inhibit [^{3}H]-strychnine binding to the untreated membranes indicate that if this is the case, the interaction is not at the ligand recognition sites of the inhibitory glycine receptor. Also, although the BD-induced [^{3}H]-strychnine binding site has a sub-cortical distribution it is unlikely to be the site at which high pressure and strychnine have a common site of action, as mephenesin had no potency at the BD-induced site.

In contrast to the drugs discussed above, some of the ligands with actions at the glycine receptor also inhibited the [^{3}H]-strychnine binding to BD-treated membranes. The inhibition of [^{3}H]-strychnine binding to untreated membranes by thebaine (Goldinger *et al.*, 1981), laudanosine, hydrastine and vincamine has been documented previously (Phelan, 1987). In the case of the two opium derivatives thebaine and laudanosine a glycinergic site of action supports electrophysiological data (Pinto-Corrado and Longo, 1961; Curtis *et al.*, 1971b). However the ability of these four drugs to inhibit BD-induced [^{3}H]-strychnine binding was not the result of an interaction at the glycine receptor. Indeed the relative potency of these compounds at inhibiting [^{3}H]-strychnine binding to untreated and BD-treated membranes when compared to each other and to unlabelled strychnine suggested that the site in each case had different ligand binding properties.

The suggestion that the ligand binding properties of the [^{3}H]-strychnine binding sites in untreated and BD-treated membranes are different is better illustrated by comparing the potency of strychnine and related alkaloids at the two sites. In the case of the inhibition of [^{3}H]-strychnine binding to untreated membranes by strychnos alkaloids the relative potencies is in good agreement with a previous study (Phelan *et al.*, 1989). The additional strychnos alkaloid tested here was the quarternerized form of strychnine, N-methylstrychnine. The potency that N-methylstrychnine exhibited for inhibition of [^{3}H]-strychnine binding to untreated membranes was in agreement with one previous publication (Márvizón *et al.*, 1986a) that used rat membranes but was of lower affinity than another study that used pigeon membranes (Le Fort *et al.*, 1978). The structure activity relations for inhibition from untreated membranes agree with previous conclusions that substitutions in the aromatic ring or modification of ring VII are the least disruptive changes in the structure of strychnine. The configuration of ring III and the nitrogen at position 19 are more important determinants of ligand recognition at the inhibitory glycine receptor, as indicated by the more marked effect on the relative potency at inhibiting [^{3}H]-strychnine binding when compounds that have structural modifications in these regions of the strychnine molecule are tested (Phelan *et al.*, 1989).

N-methylstrychnine, a compound in which the nitrogen corresponding to position 19 of strychnine is methylated and thus carries a permanent positive charge maintains a potency at untreated membranes. This would agree with previous speculations that if structures with disruption in this region of the strychnine molecule maintain a positive charge they can still interact with the inhibitory glycine receptor (Phelan *et al.*, 1988). Careful evaluation of the potency of this drug with estimates that vary from μM (this chapter) to nM (Le Fort *et al.*, 1978) may have important repercussions for the proposed idea that aromatic residues working through a cationic-π interaction might be important in recognition of strychnine's positive charge (see discussion Chapter 3). This is based on the theoretical ideas concerning the recognition properties of such a site. It might be predicted that quarternerized structures might easily be tolerated and the potency of the resultant compound in relation to the tertiary compound only slightly effected (Dougherty and Stauffer *et al.*, 1990).

In general terms the structure activity relations at BD-treated membranes was similar to those described in untreated membranes. Thus, substitutions of the aromatic ring

(2-aminostrychnine, 2-nitrostrychnine, and brucine) or modification of ring VII (bromodeoxyisostrychnine) caused the least deleterious effects on potency. However, as for the untreated membranes if the configuration of ring III is changed (isostrychninic acid, cacotheline, diaboline, Weiland-Gumlich aldehyde) or the nitrogen at position 19 modified (Brucine-n-oxide, n-oxystrychninic acid) the potency of the resultant compounds was more markedly effected. In the case of BD-treated membranes this was reflected by these compounds having IC_{50} values $>10^{-4}$M.

Despite the crude correlation in determinants of the structure of strychnine important in ligand binding to the sites in untreated and BD-treated membranes there were variations which clearly indicated that the ligand binding properties of the two sites are different. This is exemplified by specific examples from the generalized structural modification that was the least disruptive for the potency of compounds, namely modifications that result in substitutions of the aromatic ring of strychnine. Thus, substitution of an amino group at position 2 of the aromatic ring had no effect on the relative potency at untreated membranes of 2-aminostrychnine when compared to strychnine. Non-disruptive accommodation of an amino substitution to the structure has facilitated the usefulness of strychnine as a molecular probe for the inhibitory glycine receptor (see Chapter 1). In contrast the relative potency of 2-aminostrychnine at BD-treated membranes was more markedly effected so that it was only the fourth most potent strychnos alkaloid tested. This pattern was reversed when the compound tested had position 2 of strychnine molecule substituted with a nitro rather than an amino group. The relative potency of 2-nitrostrychnine being most effected at untreated membranes. Such discrepancies in the relative potency of modified structures of strychnine at inhibiting [^{3}H]-strychnine binding to untreated and BD-treated membranes was apparent when comparing the relative potencies of compounds in which ring III (isostrychnic acid, cacotheline) or position 19 (vomicine) were modified.

The pharmacology of the BD-induced site displays two sets of characteristics that distinguish it from the strychnine binding site at the inhibitory glycine receptor. Firstly, [^{3}H]-strychnine binding to BD-treated membranes was insensitive to several glycinergic ligands. Secondly the relative potency of those compounds that effected a binding to both treated and untreated membranes indicate distinct ligand binding properties of the two sites. Thus the results agree with previous experiments that concluded that the BD induced binding is at sites other than the inhibitory glycine receptor.

The distinct pharmacology displayed by the BD-induced [^{3}H]-strychnine binding, although to a chemically modified site, would also tend to rule out the possibility that this binding is a result of an isoform of the glycine receptor whose affinity for strychnine is undetectable in the unmodified state. Such a possibility deserves discussion in view of the description of the pharmacology of a developmentally regulated isoform of the glycine receptor (Becker *et al.*, 1988). This isoform has an apparent affinity for [^{3}H]-strychnine which is undetectable in ligand binding studies. In addition it has recently been shown that a single amino acid exchange in the message that probably codes for this isoform, represented by a glutamate to a glycine at position 205 (see Fig 1.3) shifts the apparent affinity for strychnine which would make it high enough to be detected by ligand binding assays (Kuhse *et al.*, 1990a). However, the mutation does not coincide with selectivity of BD for arginine residues. In addition the possibility that an undescribed isoform of the glycine receptor underlies the BD-induced binding is unlikely in view of its abundance and the pharmacological specificity described and discussed in the proceeding sections.

6.3.b A pharmacological specificity for the [^{3}H]-strychnine binding site in BD-treated membranes

A plethora of actions of strychnine not associated with the inhibitory glycine receptor (see section 1.7) highlight that this alkaloid molecule contains structural determinants that allow it to interact with a wide variety of protein domains. The possibility that BD-induced binding might involve strychnine interacting with such a domains investigated. Thus, an inhibition of [^{3}H]-strychnine binding was screened for by compounds which have actions at sites that underlie some of the non-glycinergic actions of strychnine. For most of such compounds tested, if they had an effect it was specific to BD-treated membranes. However, five compounds Imipramine, 9-aminoacridine, MK-801, D-600 and giblencamide caused an approximate 50% inhibition of [^{3}H]-strychnine binding to control untreated membranes. Only in the case of imipramine has an action at the inhibitory glycine receptor been reported (Le Fort *et al.*, 1978). Although these interactions were of low affinity ($IC_{50} > 10^{-4}M$) it is interesting to speculate that polycyclic nature of these compounds, a structural feature they share with strychnine, is of importance. However, the significance of this effect was not further investigated and the remaining discussion is concerned with the pharmacological specificity exhibited by the BD-induced [^{3}H]-strychnine binding.

The BD-induced [^{3}H]-strychnine binding was not inhibited by any nicotinic antagonist tested, 5-HT, GABA or picrotoxin. Although, any speculation regarding this site must be tempered by the knowledge that it represents a modified state, these results indicate that the BD-induced [^{3}H]-strychnine binding is not at sites with which the above nicotinic, gabaergic and serotoninergic ligands interact. However, this cannot rule out the possibility that other sites on the nicotinic receptor, $GABA_A$ or subclasses of 5-HT receptors might underlie the BD-induced [^{3}H]-strychnine binding. As these receptors have been implicated in the actions of strychnine (Faber and Klee, 1974; 1977; Davidoff *et al.*, 1969). However, the distribution of these receptors argues against them underlying the BD induced [^{3}H]-strychnine binding. Thus, autoradiographs that illustrated the distribution of binding sites associated with the $GABA_A$ (Palacios *et al.*, 1981; Bowery *et al.*, 1987) and the nicotinic receptor (Clarke *et al.*, 1985) do not parallel the distribution of binding sites associated with the BD-induced [^{3}H]-strychnine binding in the central nervous system (see Chapter 4). In the case of 5-HT receptors they have been shown to exist in several non-neural tissue preparations (Bradley *et al.*, 1986) whereas an induced [^{3}H]-strychnine binding was not detected in membranes from striated muscle, lung, heart, spleen, kidney or ileum when treated with BD (see section 4.2.a). The above studies do not highlight more subtle changes that can exist in the distribution of isoforms of the receptors. However, in situ hybridization with message that codes for isoforms of the nicotinic (Deneris *et al.*, 1991) or $GABA_A$ (Lüddens and Wiseden, 1991) receptor subunits do not reveal the pattern of distribution exhibited by the BD-induced binding sites. In any case, the possibility that modification of these receptors underlies the BD-induced binding is also unlikely as this binding is at least 1000 fold more abundant than sites associated with the nicotinic (Yoshida *et al.*, 1979), $GABA_A$ (Biscoe and Fry, 1984) or 5-HT receptors (Bradley *et al.*, 1986; Kilpatrick *et al.*, 1987).

Several cation channel openers were screened on the basis of reports that strychnine at concentrations supermaximal to those required for an antagonism at the glycine receptor caused an activation of potassium conductances dorsal root ganglion cells (Aibra *et al.*, 1991) or an associated hyperpolarization of rabbit pulmonary artery (Cook and Quast, 1990). However, of the K^+ channel activators tested (for review see Edwards and Weston, 1990) none were able to inhibit [^{3}H]-strychnine binding to BD-treated membranes. In addition tolbutamide and giblencamide specific blockers of the ATP sensitive potassium channel (Cook and Quast, 1990), which have been shown to interact

with a lemakalim binding site (Quast and Webster, 1989), did not inhibit the BD-induced [^{3}H]-strychnine binding. These results suggest that sites at which potassium channel activators have been reported to act do not underlie the BD-induced [^{3}H]-strychnine binding. However, this does not completely discount that the BD-induced [^{3}H]-strychnine binding is associated with the site that underlies potassium channel activation, as although the membrane hyperpolarising properties of strychnine have been likened to that of other K^+ openers (Cook and Quast, 1990), an interaction between these compounds and strychnine has not been reported. Therefore, the actions of strychnine could represent a novel potassium channel opening activity.

The strychnine activation of K^+ channels was represented in freshly disassociated dorsal root ganglion (DRG) cells and preliminary data using cultured DRG and neuronal cell line indicated the response was specific to freshly dissociated cells (M. Duchen personal communication). If this was extended to represent a selective distribution to DRG cells it could provide explanation for the caudal rostral distribution of the BD-induced [^{3}H]-strychnine binding. This is based on the premise that the membrane properties expressed by these terminals in the brainstem and spinal cord are the same as those at the cell bodies. Thus it can be envisaged that BD modification of the strychnine channel opening activity in DRG could underlie the distribution of the BD-induced [^{3}H]-strychnine binding to membranes prepared from different regions of the CNS.

It is more difficult to reconcile the association of BD-induced [^{3}H]-strychnine binding with the potassium channel opening activity on the basis of the abundance of the BD-induced site. Although it should be noted that the low noise (personal communication M. Duchen) of the large strychnine-induced K^+ channel opening (Aibra *et al.*, 1991) is consistent with ideas that a relatively abundant low conductance channel being involved, it is questionable as to whether such activity could explain a binding site that appears to represent such a large percent of total membrane protein.

As strychnine activated a cation current in DRG cells which serve a sensory function (Shepherd, 1974), inhibition of [^{3}H]-strychnine binding to treated membranes was screened for by capsaicin. This drug selectively depolarizes sensory neurons by activating a non-selective cation conductance (Marsh *et al.*, 1987) and at higher concentrations blocks K^+ conductances in the DRG neurones (Peterson *et al.*, 1987). However, when

screened at 10^{-4}M, it did not inhibit [^{3}H]-strychnine binding to BD-treated membranes which indicates that this binding was not associated with capsaicin recognition sites.

In contrast to the compounds discussed above, a range of drugs with reported cation channel blocking activities inhibited the BD-induced [^{3}H]-strychnine binding when screened at 10^{-4}M. According to the classification, these included channel blockers of voltage-gated Na^+, K^+ and Ca^{++} channels. In addition, MK-801 which blocks the NMDA subtype of ligand gated glutamate channels (Foster and Wong, 1987), inhibited the increased [^{3}H]-strychnine binding to BD-treated membranes. These results are consistent with reports that strychnine can cause channel block of voltage gated sodium (Shapiro, 1977b, Cahalan and Almers, 1979) and potassium (Shapiro, 1977a; Cook,1988) conductances and NMDA channels (Bertolino and Vincini, 1988); although there are no direct reports of strychnine blocking voltage gated calcium channel this action has been implied from investigation of the excitability strychnine treated ganglia of *Aplysia* (Klee *et al.*, 1973). However, rather than indicating that modification of several different major subclasses of cation channels underlies the BD-induced [^{3}H]-strychnine binding, these results likely reflect common structural determinants of the strychnine and other drug molecules, which allow them to interact across major subclasses of cation channels, also being important in the interaction with the BD-induced binding site.

In this respect, although the site of action of local anaesthetics and class 1 antiarrhythmic for impulse conduction block is discussed in terms of their ability to interact with voltage gated sodium channels (Strichartz and Ritchie, 1987; Vaughan-Williams, 1989) several of these drugs have well documented blocking actions at subclasses of potassium channels (Cook and Quast, 1990; Strichartz and Ritchie, 1987). In addition some of the drugs classified as local anaesthetics and antiarrhythmic have actions at voltage gated calcium channels (Herman and Gorman, 1984) and the cation channel of the nicotinic acetylcholine receptor (Adams, 1977; Koblin and Lester, 1979). Indeed, an overlap in activity of major subclasses of cation channels is also apparent when the other groups of drugs that inhibited the increased [^{3}H]-strychnine binding to BD-treated membranes were considered. Thus, the class III antiarrhythmic, amiodarone, which prolongs repolarization by blocking K^+ conductances, has a class 1 type action on cardiac Na^+ channels (Honjovetal *et al.*, 1991). Such cross-reactivity has been reported for other compounds tested and listed as K^+ channel blockers (Yeh, 1979). This also extends to

drugs classified as Ca^{++} blockers, as D-600, at concentrations slightly higher than required to produce Ca^{++} channel block (Gola and Ducreux, 1985) inhibited K^+ conductances.

It is also important in relation to defining a possible site at which the BD-induced binding is occurring, to stress that the blocking effects of many of the drugs that inhibited BD-induced [^{3}H]-strychnine binding extends to subclasses of K^+ channels (Cook, 1988). At some subclasses this coincides with a reported blocking action of strychnine (Cook and Haylett, 1985).

A specificity of the inhibition of [^{3}H]-strychnine binding by cation channel blocker encompasses the NMDA receptor antagonist MK-801. The uncompetitive action of this drug relates to an interaction with the cationic channel of the NMDA receptor (Macdonald and Nowak, 1990). This probably relates to structural determinants of these drugs that are common to cationic channel blockers rather than indicating that the induced binding occurs at the NMDA receptor. As the distribution of the BD-induced [^{3}H]-strychnine binding does not coincide with that of the NMDA receptor (Bowery, 1987).

In a similar way, the inhibition of [^{3}H]-strychnine binding to BD-treated membranes by drugs classified as miscellaneous probably relates to structural determinants that are common to cationic channel blockers. This conclusion is justified by reported actions of these drugs at cation channels or conductances. Thus, the best characterized action of the tricyclic antidepressant, Imipramine is to prevent uptake of catecholamines (Baldessarini, 1990). However this drug has reported class 1 antiarrhythmic actions and can interact with the cation channel of the NMDA receptor (Bakker *et al.*, 1991). Similarly, propranolol a high affinity ß-adrenergic antagonist, in common with related compounds, can at higher concentrations exsert class I and class III antiarrythmic actions by interacting with Na^+ and K^+ channels (Ijzerman and Soudijn, 1989). Finally 1,3,-o-tolyl-guanidine a drug initially developed as a specific high affinity sigma receptor ligand (Weber *et al.*, 1986), can at higher concentrations than required for this action, block ligand-activated K^+ conductances (Bobker *et al.*, 1989) and the cation channel of the nicotinic acetylcholine receptor (Galligan *et al.*, 1989).

It is important to note that the potency of the drugs that inhibited the BD-induced [^{3}H]-strychnine binding as defined by IC_{50} is consistent with reports that cation channel

blocking activity occur in the μM range (see references listed above).

In contrast to the inhibition of the BD-induced [^{3}H]-strychnine binding by cation channel blocking drugs, when some toxins with greater channel subclass specificity were screened they did not inhibit binding. Thus dendrotoxin, which is relatively specific for a subclass of slowly inactivating voltage gated potassium channels (Dolly, 1988) did not inhibit binding. Apamin, a toxin specific for a subclass of Ca^{++} activated K^+ conductances inhibited a small proportion of the BD-induced [^{3}H]-strychnine binding. It is interesting to note that previously strychnine was shown to inhibit iodinated apamin binding to hepatocytes where it was speculated that the cationic charge of strychnine was the basis of this interaction (Cook and Haylett, 1985). However, the nature of the interaction was not further investigated and it is unlikely that the inhibition of [^{3}H]-strychnine binding is related to this interaction. Firstly, the concentration of Apamin required to produce the small inhibition at the BD-induced site suggests a non-specific interaction. Secondly the apamin binding site exists at a 1,000,000 fold lower concentration than the BD-induced [^{3}H]-strychnine binding (Strong, 1990). Also the BD-induced [^{3}H]-strychnine binding could not be identified in liver (see section 4.2.a).

In a similar way, the BD-induced [^{3}H]-strychnine binding was screened for inhibition by toxins with specific actions at voltage gated sodium channels (Catterall, 1980). However, tetrodotoxin, water extracted scorpion toxin (*Leurius quinquestriatus herbreus*) at a concentration previously shown to modulate sodium channel function (Catterall, 1975), or veratridine, failed to inhibit the BD-induced [^{3}H]-strychnine binding. As the recognition site on the channel protein for these toxins are distinct from each other and from a putatative local anaesthetic site this does not totally exclude the possibility that BD-induced binding is on this protein. However, two independent pieces of evidence suggest this possibility is unlikely. Firstly, the site to which persistent activators of Na^+ channel function bind is coupled allosterically to the putative cation channel blocking site (Postma and Catteral, 1984) and unless the allosteric modulation is modified by BD-treatment an inhibition of [^{3}H]-strychnine binding by veratridine might be expected. Secondly the abundance of the BD-induced [^{3}H]-strychnine binding is too high to be accounted for by an interaction with voltage gated sodium channel (Catterall *et al.*, 1979). In addition, as crude scorpion toxin was used in the above experiments it should be noted that two other K^+ channel toxins, charybdotoxin and leiurotoxin were inadvertently

screened because they are minor components of this scorpion toxin (Strong, 1990). Therefore, the BD-induced [^{3}H]-strychnine binding has a specificity for drugs which like strychnine have been shown to act as cation channel blockers.

Indeed a consideration of structure activity relations indicate that features of drug structure that underlie cation channel blocking activity may also be important in the drug interactions with BD-induced [^{3}H]-strychnine binding site. The compounds that inhibited the BD-induced binding can all be classified as cationic amphipathic drugs (CADS). This is the general structure that is the basis of local anaesthetic drug action (Courtney and Strichatrz, 1987). The local anaesthetics feature an aromatic ring joined to an amine group by a linking arm. The link ensures that there is some separation between the hydrophobic moiety associated with the aromatic group and the hydrophillic moiety associated with the amine group. Also the cationic charge associated with the tertiary amine group has a pK value that ensures that the drug molecule will exist in a equilibrium between uncharged and cationic drug moieties.

These structural determinants have been highlighted in the strychnine molecule (Shapiro, 1977a; 1977b) and exist in drugs which have been shown to inhibit [^{3}H]-strychnine binding to BD-treated membranes. Even in the case of sparteine where there is no aromatic moiety the polycyclic ring structure should provide hydrophobicity to ensure an ampipathic nature.

The potential ability to undergo proton exchange appears an important determinant of the interaction at the BD-induced [^{3}H]-strychnine binding site. This is indicated by the loss of the inhibition of [^{3}H]-strychnine binding by lidocaine and strychnine when their structures are modified so that the tertiary amine group is quarternerized by methylation. The resulting structures QX-314 and N-methylstrychnine carry a permanent positive charge. This may be more than a simple reduction in hydrophobicity as the hydrophobic local anaesthetic benzocaine that lacks a protonatable amine does not inhibit the BD-induced [^{3}H]-strychnine binding. This points to both uncharged and cationic forms and an ability to interconvert as important determinants of the interaction at the induced binding site. Similar features have been postulated to be an important for some mechanisms of cation channel block (Schwarz *et al.*, 1977).

If the interaction of CADS at the BD-induced [^{3}H]-strychnine binding site and cation channel are controlled by similar structural determinants, the significance of changes in drug structure to relative potency is likely to be complex. Although various structures might be expected to impart different recognition properties on a drug, structural modification also effects physicochemical properties that might be important for drug interaction. Molecular size, hydrophobicity and pKa of amine functions show marked variation among structurally related CADS and these properties in turn can underlie differences in relative anaesthetic potency (Courtney and Strichartz, 1987). The BD-induced binding site has been postulated to exist in a hydrophobic environment and the ability of drugs to undergo proton exchange have been implicated in CADS interactions at this site. Therefore, changes in hydrophobicity and pKa of CADS structures may also be important in determining the relative potencies at the BD-induced [^{3}H]-strychnine binding.

The relative hydrophobicity has been used to explain the increased anaesthetic potency of tetracaine which exceeds that of the structurally related drug procaine by a factor of 50 (Courtney and Strichartz; 1987). In this respect it is relevant to note that tetracaine is 10 fold more potent at inhibiting [^{3}H]-strychnine binding to the BD-induced binding site. The possibility that changes in the hydrophobic nature of CAD serves as a general determinant of structure-activity relations at BD-induced site is indicated by comparing quinidine and procainamide. Quinidine a relatively potent inhibitor of the BD-induced [^{3}H]-strychnine binding. In contrast the lowered hydrophobicity of procainamide is reflected by its low potency at inhibiting the BD-induced [^{3}H]-strychnine binding (Vaughan-Williams, 1989).

In contrast to the above, the CADS, tocainide and encainide which have similar hydrophobicities to procainamide (Vaughan-Williams, 1989) but were more potent at inhibiting [^{3}H]-strychnine binding. This indicates that other criteria in addition to hydrophobicity were important in determining drug potency at the BD-induced site. This possibility is further exemplified by lidocaine which despite a relative hydrophobic nature (Vaughan-Williams, 1989) is among the least potent drugs at inhibiting BD-induced [^{3}H]-strychnine binding that had a definable IC_{50}. One factor that may cause the relative low potency is the pKa value of this drug (Vaughan-Williams, 1989). The electron withdrawing effect of the linking amide group causes a relatively electropositive terminal amine in lidocaine which does not readily undergo protonation (Courtney and Strichartz,

1987). As discussed this aspect of CADS structure may have an important role in their interaction with the BD-induced [^{3}H]-strychnine binding. Alternatively, as has been discussed for cation channel block, the carbonyl group of the amide link in lidocaine protrudes away from the aromatic ring. This has been speculated to effect close apposition of the aromatic function with a hydrophobic region at the channel binding site. This speculation may be relevant to BD-induced [^{3}H]-strychnine binding as this has been postulated to exist in a hydrophobic environment.

The discussion of structure activity-relations at the BD induced [^{3}H]-strychnine binding site although limited to some specific examples highlights possible similarities with the interaction of CADS in cation channel block. Therefore structural determinants and variations in physicochemical properties of CADS can conspire to determine the relative potency of drug inhibition of [^{3}H]-strychnine binding to BD-treated membranes.

6.3.c Significance of the pharmacological specificity exhibited by the BD induced [^{3}H]-strychnine binding site

Present experimental evidence is inadequate to allow the assumption that modification of a cation channel protein underlies the increased [^{3}H]-strychnine binding to BD-treated membranes, let alone define if a previously characterized channel is involved. A major problem is that if strychnine is interacting through determinants that impart local anaesthetic activity, a number of possible sites might underlie the induced binding. For the purpose of discussion, these are considered as more general drug interactions with membrane phase or membrane proteins and a more specific interaction with drug recognition sites other than cation channels. It should be noted that the principles that underlie these interactions may not be mutually exclusive from each other or an interaction with cation channel proteins.

The structural determinants that are associated with CADS also allow them to act as membrane perturbants via direct interaction with the lipid phase. Indeed, experiments with liposomes indicate that two distinct interactions might underlie the association with the lipid phase (Wang *et al.*, 1983). Drug molecule in its uncharged form can thus partition into the lipid or in its cationic form orientate in the membrane with its positive charge interacting with the ionic charge of certain phospholipid head groups. With respect

to BD- induced [^{3}H]-strychnine binding to crude spinal cord membranes it is possible to envisage that treatment with membrane permeable reagent could fluidize the membrane component and thus facilitate a previously undetected interaction with CADS as assayed by an increase in [^{3}H]-strychnine binding. Such membrane perturbant actions have been proposed to underlie the mechanism of general anaesthesia (Courtney and Strichartz, 1987). Interestingly, BD has features in common with other general anaesthetics (Franks and Lieb, 1984). This explanation might explain the relative abundance of the BD-induced [^{3}H]-strychnine binding. However, several lines of evidence argue against this possibility. First, washing procedures used after BD treatment should ensure complete removal of the unreacted reagent prior to [^{3}H]-strychnine binding assay. Secondly, if either cationic alignment or hydrophobic partitioning underlie the BD-induced binding then quarternerized drugs (QX-222, QX-314 and N-methylstrychnine) or hydrophobic anaesthetics (benzocaine) would have been expected to exert a subsequent inhibition of [^{3}H]-strychnine binding to BD-treated membranes. Yet, neither class were effective when tested up to a concentration of 10^{-4}M. Thirdly the phenomenon would be expected to generalize to membrane preparations from different tissue sources. However, BD-induced [^{3}H]-strychnine was not seen in any of the tissues tested other than CNS. Finally, when spinal cord membranes were incubated with increasing concentrations of BD or the membrane perturbant benzyl alcohol an increased [^{3}H]-strychnine binding over untreated controls was only seen in BD-treated membranes (results not shown). Thus membrane perturbation by the protein modifying reagent do not seem to explain the increased [^{3}H]-strychnine binding.

In addition to the interaction between CADS and ion channel in what is envisaged to be a fairly well defined manner at the cation pore (Miller, 1989) it has been intimated that these drugs interact with a more diverse range of membrane bound proteins by an interaction at the boundary between lipid and protein (Casanovas *et al.*, 1985). It should be noted that a similar site may exist on cation channel proteins in addition to the better defined interaction with ion pore (Heidman *et al.*, 1983; Strichartz and Ritchie, 1987). Although in this respect the archetypal hydrophobic local anaesthetic (benzocaine) did not inhibit the BD-induced [^{3}H]-strychnine binding. However, a related site at a protein lipid interphase could underlie the induced binding if treatment with BD modified the environment in such a way as to favour local anaesthetic binding. This might be envisaged to involve a change in protein conformation that effected access to the site or affinity of

the interaction at the site (see Discussion chapter 5). This scenario might provide an attractive explanation because by its general nature could involve several classes of membrane proteins and thus explain the abundance of the BD-induced [^{3}H]-strychnine binding. However, it might also be expected to involve membrane proteins located in several tissue types and this was not reported. In addition, the idea is incompatible with the experimental observation that indicate a specific and single class of protein is involved in BD-induced [^{3}H]-strychnine binding (see later). Yet these discrepancies could be accommodated in this explanation if BD was specifically modifying the lipid protein interphase of a membrane protein that was particular to membranes of central tissue.

A further manner in which BD treatment of neuronal membrane could increase subsequent [^{3}H]-strychnine binding envisages a protein recognition site for CADS on a membrane protein which when modified is unmasked. This envisages a specific protein rather than a point of interaction between the lipid and protein. This idea has been extensively addressed in the previous discussion with receptor sites at which strychnine has reported actions. However, there are possible interactions with sites at which an interaction with strychnine has not been investigated but through its CAD determinants may be able to interact. For example, sub-classes of P450 found to have an unevenly distributed activity in the CNS have been shown to have a specificity for CADS (Tyndale *et al.*, 1991). Although the ligand recognition properties of this enzyme do not exactly match that of the BD-induced [^{3}H]-strychnine binding, this example illustrates that other CAD recognition sites exist that could feasibly underlie the phenomenon.

6.3.d Experimental observations that indicate a specific site underlies the BD-induced [^{3}H]-strychnine binding

It is clear that definition of the BD-induced [^{3}H]-strychnine binding site as an acceptor site for CADS is of limited value in directly defining the nature of the site. However, two experimental observations indicate that a specific protein site underlies the phenomenon. These observations are important because they are incompatible with some of the ideas that were outlined in the above discussion.

Firstly, the inhibition of [^{3}H]-strychnine binding to BD-treated membranes by increasing concentration of CADS was described by curves that in general had Hill slopes

close to one. This generalization also holds for the strychnine related alkaloids (data not shown). The exception to this was 9-aminoacridine and will be addressed later. This result indicates that the interaction between [^{3}H]-strychnine and CADS is a simple competition from the same site. If a broad spectrum of sites were responsible for the induced binding the Hill slopes detected would have been significantly less than one. A remaining possibility is that several physically distinct sites are induced by the BD treatment but they have very similar affinities for CADS. However biochemical evidence suggests that this is not the case.

Experiments that further investigated the photoinduced irreversible incorporation of [^{3}H]-strychnine into untreated and BD-treated membranes suggest a specific protein site underlies the induced binding. In the case of untreated membranes the pharmacological specificity of irreversible [^{3}H]-strychnine binding and peptide labelling was consistent with incorporation of ligand at the inhibitory glycine receptor. However the size of the photolabelled peptide resolved by 5-20% gradient gels in untreated membranes from two experiments was consistently smaller than when photolabelled membranes were resolved through a 10 % gel (see Chapter 4). Discrepancies in the estimated peptide size can occur when comparing protein resolved by a single concentration and a linear gradient gel (Hames, 1983). In this case, it cannot explain the discrepancy, as the amino acid sequence of the strychnine binding subunit predicts a size of at least 48 kd (Grenningloh *et al.*, 1987) which is significantly larger than the peptides labelled in the untreated membrane in either gel one or two. Therefore, it is likely that some degradation has occurred as a result of the procedures used to photolabell the membranes. Similarly this degradation could explain the doublet labelling of the inhibitory glycine receptor in gel two which had a smeared appearance.

When membranes were incubated with [^{3}H]-strychnine and exposed to UV-light the amount irreversibly incorporated into BD-treated membranes was higher than untreated membranes. However, the increase was not as great as the amount of induced [^{3}H]-strychnine binding assayed in BD-treated membranes. This is consistent with experiments in Chapter 4 which indicated that the efficiency of photolabelling at BD-treated membranes is considerably lower than at untreated membranes.

The pharmacology of the irreversibly bound [^{3}H]-strychnine binding to BD-treated

membranes indicate that it was inhibited by strychnine but not glycine. Where as incubation with quinine during the photolabelling reduced by about 50% the amount of [^{3}H]-strychnine irreversibly incorporated into BD-treated membranes. When the photolabelled BD-treated membranes were solubilized and resolved through a 5-20% linear gradient gel it was clear that [^{3}H]-strychnine could still irreversibly incorporate into the inhibitory glycine receptor. However, as indicated in previous experiments (see Chapter 4) the receptor in BD-treated membranes had modified characteristics. However, in addition to peptides associated with the glycine receptor, low molecular weight peptide was shown irreversibly to incorporate [^{3}H]-strychnine in the BD-treated membranes. In both gels the apparent molecular peptide was 23 kd. Importantly, the pharmacology of this labelling was consistent with it having recognition properties similar to the reversible [^{3}H]-strychnine binding in BD-treated membranes. Thus, glycine did not inhibit labelling of this peptide. In contrast quinine inhibited labelling of the peptide specific to BD-treated membranes without effecting the labelling of the peptides associated with the glycine receptor. This result explains how quinine inhibited only 50% of the [^{3}H]-strychnine that was photoincorporated onto BD-treated membranes as the total irreversibly bound is made up of labelling at the glycine receptor and a second site which can only be detected in treated membranes.

The relative intensity of labelled peptides suggests that irreversible binding to the glycine receptor made up 50% of the total [^{3}H]-strychnine irreversibly incorporated into BD-treated membranes when photolabelled in the absence of drug. This is in contrast to the amount of reversible [^{3}H]-strychnine binding to the glycine receptor in BD-treated which accounts for less than 0.1% of total binding. Photolabelling of a site in BD-treated membranes may also account for the observation made in previous photolabelling experiments in which labelled peptides were subsequently resolved through 10% mini-gels. The untreated and BD-treated membranes labelled in the absence of drugs appeared irreversibly to incorporate similar amounts of [^{3}H]-strychnine. Yet, in lanes loaded with BD-treated membranes the intensity of labelling at sites associated with the inhibitory glycine receptor was consistently lower than in untreated membranes. This indicated that activity associated with other components of the BD-treated membranes may have existed. This can be explained by the peptide resolved by the 5-20% gradient gels that was shown to be specific to BD-treated membranes. The labelling of this peptide in the experiments described in this chapter would have been favoured by the modified incubation conditions used prior to exposure with UV-light. However, failure to detect this component when

10% gels were used more likely relates to the peptides of an approximate weight of 23 kd running at the dye front, as indicated by molecular weight markers.

Thus the present experiments have identified a low molecular weight peptide which binds [^{3}H]-strychnine with a specificity compatible to the reversible binding in BD-treated membranes. This and previously described observations form the focus for further discussion. This concerns experiments that have defined a protein binding site at axonal membranes that like the BD-induced [^{3}H]-strychnine binding has an apparent specificity for CADS (Greenberg and Tsong, 1982). The characteristics of this site are of particular interest as they have features that can reconcile aspects of the BD-induced [^{3}H]-strychnine binding that cannot be explained by evoking an interaction at other sites which strychnine has been reported to act (see section 1.7). The site described by Greenberg and Tsong (1982) was detected by an assay system that utilized changes in fluorescence of quinacrine that occur when the free drug becomes bound. In this way an interaction between quinacrine and bovine axonal membrane protein was reported by several local anaesthetic drugs. The interaction with quinacrine is specific to axonal membranes and therefore particularly enriched in brain regions that contain the highest levels of white matter. If the same site underlies the BD-induced [^{3}H]-strychnine binding it explains the regional distribution that is prevalent in spinal cord and brainstem (see Chapter 4). These regions are rich in nerve tracts and, by extension white matter. In addition, as for BD-induced [^{3}H]-strychnine no interaction with quinacrine was detected with membranes prepared from several different non-neuronal tissues (Greenberg and Tsong, 1982). By quantitating the change in fluorescence that occurs on quinacrine binding, the site was shown to have a B_{max} between 5-10 nmol/mg membrane protein and a K_D of approximately 1 μM. The B_{max} is in excellent agreement with that estimated for [^{3}H]-strychnine binding to BD-treated membranes (see Chapter 5). This confirms the existence of local anaesthetic binding site in untreated membrane that are at least 1000 fold more abundant than voltage or ligand gated channels previously described (see earlier discussion). Thus, the quinacrine site offers an attractive explanation for apparent abundance of the BD-induced [^{3}H]-strychnine binding. However, the K_D for the quinacrine site indicates a higher affinity than strychnine binding to BD-treated membranes. This could be a true reflection of the affinity of quinacrine or relate to differences in the assay systems used to define quinacrine binding to axonal membranes and [^{3}H]-strychnine binding to BD-treated membranes. It should also be noted that the concentrations of quinacrine used to define the binding isotherms were insufficient to saturate the site which could lead to poor estimates of K_D

(Greenberg and Tsong, 1982).

At present, the pharmacological specificities of the BD-induced [^{3}H]-strychnine binding and quinacrine binding are difficult to directly compare as there is limited cross-over in the drugs tested in both assay systems. However, where cross-over exists there is a good agreement in potency of drugs that inhibit. In both assays, tetracaine is a more potent inhibitor than lidocaine and procaine which in turn are almost equipotent (Greenberg and Tsong, 1982; *cf* table 6.4). Similarly, ligand binding in both assay systems was insensitive to TEA but inhibited by aminopyridines. Despite these correlative trends discrepancies exist in the potency of the drugs determined by IC_{50} in the two assay systems. However, this could be either a function of the fundamental differences in the assay systems (see later discussion) or the binding site having modified characteristics when treated with BD. Indeed, the latter is predicted as the induced binding site described in this thesis can only be detected subsequent to BD treatment of the membrane preparation.

Further, compatibility between the CAD binding site, assayed by quinacrine fluorescence or [^{3}H]-strychnine binding to BD-treated membranes is indicated by observations that suggests in each instance the binding site lies in a hydrophobic environment. In the case of quinacrine this is indicated by a blue shift in the fluorescence spectrum on drug binding (Greenberg and Tsong, 1982). In BD-treated membranes the evidence is not as direct but taken to be the case based on the requirement for membrane permeable arginine-specific reagents to induce the [^{3}H]-strychnine binding (see Chapter 3) and the slow equilibration of the induced binding (see Chapter 5). The hydrophobic environment of the binding site may also underlie the apparent sensitivity of quinacrine binding (Greenberg and Tsong, 1984) and [^{3}H]-strychnine binding in BD-treated membranes (Chapter 4) to the presence of detergents.

However, there are discrepancies between characteristics of quinacrine binding and the BD-induced [^{3}H]-strychnine binding that might indicate that the two phenomenon do not have a common origin. Firstly, the question as to why an interaction with [^{3}H]-strychnine is only apparent after BD-treatment needs to be addressed. It could relate to the differences in the assay systems used. The quinacrine fluorescence assay does not suffer from the need to incorporate a separation step which can preclude measurement of

low affinity binding (see Chapter 5). Equally quinacrine may exhibit high enough affinity for the site to allow its use as a ligand to detect an interaction with the CAD site in an unmodified state. In contrast, [^{3}H]-strychnine binding assays may only be used when the CAD site has been modified to allow its detection by radioligand binding assays. In this respect the potency of those drugs that have been examined in both assays is higher for inhibition of BD-induced [^{3}H]-strychnine binding. This might relate to modified characteristics of the CAD site after BD treatment.

The nature of the interaction of CADS defined in the two assay systems deserves discussion. Quinacrine binding to axonal membranes was shown to be co-operative as indicated by the Hill coefficient of 2 derived from a saturation binding isotherm. In contrast, the Hill slopes derived from the inhibition of [^{3}H]-strychnine binding to BD-treated membranes by increasing concentration of strychnine, related alkaloids and CAD gave coefficients close to one. Variation in the experimental protocol used to define the Hill slopes might underlie this difference. However, it is interesting to note that 9-aminoacridine had Hill coefficients significantly greater than one, suggesting some co-operativity in its interaction with the [^{3}H]-strychnine binding site. 9-aminoacridine is structurally similar to quinacrine, perhaps indicating the nature of the interaction is different for sub-classes of CADS. In this respect it would be interesting to compare Hill slopes for drugs that have had their potency defined by inhibition of quinacrine fluorescence and [^{3}H]-strychnine binding to BD-treated membranes. Unfortunately, the Hill coefficients with regard to CAD inhibition of quinacrine fluorescence were not indicated (Greenberg and Tsong, 1982). Alternatively, it could be that co-operativity is modified by the BD-treatment. With respect to quinacrine binding the co-operativity was lost when the site was detergent solubilized (Greenberg and Tsong, 1984).

Finally biochemical characteristics of the quinacrine binding and the BD-induced [^{3}H]-strychnine binding are considered. A protein component with an apparent molecular weight of 16 kd has been identified by detergent solubilization and affinity chromatography, as the quinacrine binding site (Greenberg and Tsong, 1984). The protocol depended on the identification of sodium cholate (1% w/v) as a detergent that could solubilize the quinacrine binding site in an active form. In contrast, experiments described earlier illustrated that the BD-induced [^{3}H]-strychnine binding was sensitive to inhibition by detergents which included sodium cholate (1% w/v). An apparent discrepancy appears to exist in sensitivity of the quinacrine and BD-induced [^{3}H]-strychnine binding. It is

possible that phosphatidylcholine present during attempts to solubilize the BD-induced [^{3}H]-strychnine binding effected the nature of the sodium cholate micelles which consequently led to an inactivation of the BD-induced [^{3}H]-strychnine binding. Alternatively, the sensitivity of CAD site to detergent might be altered when modified with BD such that the CAD binding activity as detected by [^{3}H]-strychnine binding is sensitive to inhibition by sodium cholate.

Using photolabelling procedures, [^{3}H]-strychnine was shown specifically to incorporate into a 23 kd peptide in addition to the inhibitory glycine receptor in BD-treated membranes. Thus, similarly to quinacrine binding the BD-induced [^{3}H]-strychnine binding was associated with a low molecular protein component of the membrane preparation. However, the apparent weights of these peptides identified by different procedures appear not to be identical. Differences in the gel system used to resolve peptides might explain this discrepancy although it is difficult to discuss this issue as it is not clear under what conditions the quinacrine binding activity was resolved (Greenberg and Tsong, 1984). It is also possible that the peptide identified by solubilization and affinity purification of quinacrine binding activity has undergone a degree of degradation and the true weight is underestimated. In this respect it is interesting to note a band with a molecular of 23 KD is among the major peptide components of axonal membrane preparations (Greenberg and Tsong, 1984). Equally the discrepancy could relate to a species difference. Bovine axonal membranes were used to investigate the quinacrine fluorescence and crude murine spinal cord membranes in the characterization of BD induced [^{3}H]-strychnine binding.

If the CAD binding site assayed by quinacrine fluorescence and [^{3}H]-strychnine binding are a related phenomena the low efficiency of photoincorporation into BD-treated membranes must be reconciled. The low efficiency is indicated by the low proportion of reversible [^{3}H]-strychnine binding that can be irreversibly incorporated into BD-treated membranes when incubations are exposed to UV-light and contrasts a relatively efficient process at the inhibitory glycine receptor (see section 4.2.c Graham *et al.*, 1983). In earlier discussion it was argued that the lack of energy transfer from the induced site might underlie the lower efficiency at BD-treated membranes. However, if the two CAD sites are related phenomena, this is incompatible with evidence that indicates that such a process occurs between protein and quinacrine in axonal membrane (Greenberg and

Tsong, 1982). Possible explanation of this apparent discrepancy could be that BD-modification of CAD site effects the energy transfer process. Alternatively the concentration of [^{3}H]-strychnine used when the BD-treated membranes were photolabelled was only 100 nM and this concentration is still 100 fold lower than the estimated K_D for the induced site and would preclude an efficient photolabelling process.

6.3.e Conformational dependence of the reaction of BD with spinal cord membranes.

Modulation of the amount of BD-induced binding by the presence of strychnine during the reaction was taken to indicate conformational dependence of the reaction (see Chapter 3). Interestingly, this has been extended to include several other strychnos alkaloids and CADS that were shown to have a pharmacological specificity for the BD-induced [^{3}H]-strychnine binding site.

A group of these drugs were like strychnine in that they significantly increased the induced [^{3}H]-strychnine binding if present during the BD treatment. In contrast, another group of drugs significantly decreased or as in the case of 9-aminoacridine or amiodarone completely abolished the induced [^{3}H]-strychnine binding when present during the BD-treatment. It is unlikely that this effect is caused by an inactivation of BD by direct reaction with the drug molecule as the protein modifying reagent is in 800 fold molar excess. A further group of the drugs tested for modulation of the reaction had no significant effect when present at 10^{-4}M during the BD treatment. In the case of the strychnos alkaloids, those drugs that were without effect were also among the least potent at inhibiting BD-induced [^{3}H]-strychnine binding and it might be speculated that they would effect the reaction if present at a higher concentration. In this respect it is interesting to speculate that equilibration of drugs prior to BD treatment is to an unmodified form of the site. This idea is consistent with the threshold concentration of strychnine (10^{-5}M) that will potentiate the BD-reaction being at least 20 fold higher than the estimated IC_{50} for inhibition of BD-induced [^{3}H]-strychnine binding by strychnine.

Structural features of the drugs that might determine the modulation they exhibit are not obvious. For example, although the CADS that decrease the amounts of induced [^{3}H]-strychnine binding if present during the BD treatment are polycyclic there are also other polycyclic compounds that potentiate or have no effect on the reaction. However, as for the potency of inhibition of [^{3}H]-strychnine binding from the induced site the

strychnos alkaloids offer the most controlled way of considering the effects of drug structure on modulation of the reaction. Thus, 2-nitrostrychnine was like strychnine and potentiated the amount of increased [^{3}H]-strychnine binding if present during the reaction. In contrast the structurally related drug 2-aminostrychnine, which is more hydrophillic in nature (Mackerer *et al.*, 1977), decreased the amount of BD-induced [^{3}H]-strychnine binding if present during the reaction. Therefore, as discussed with respect to potency of compounds at inhibiting BD-induced binding, physicochemical as well as structural properties may give rise to the spectrum of modulation when different drugs are present during the BD treatment.

6.3.f Devising models to explain observed aspects of the BD-induced [^{3}H]-strychnine binding: parallels in the actions of CADS

In addition to indicating how the BD modification could induce a previously undetectable [^{3}H]-strychnine binding site, a scheme has to include explanation as to how drugs that exhibit a pharmacological specificity for the induced binding site, can also modulate the reaction of BD with membranes. Two possible schemes are proposed and where appropriate their relevance to the experimental observations that have been made is indicated. In each case a covalent modification of a putative protein is speculated to modify the [^{3}H]-strychnine binding properties of this moiety to reveal a previously undetected binding site, as has been discussed previously (see Chapter 5).

In the first scheme, all drugs that inhibit the BD-induced binding are envisaged to bind to the same site. This is consistent with most of the drugs inhibiting the BD-induced binding with a Hill slope close to one. Also, the arginine residue that is modified is not found at the ligand binding site, consistent with the idea that in general drugs that inhibit the induced binding do not protect against the effects of BD treatment when present during the reaction. Although all the CADS bind to the same site, in doing so they promote distinct protein conformations. The CADS can bind and promote conformational changes in the unmodified protein, although as indicated by assays in untreated membrane, [^{3}H]-strychnine binding to the unmodified site cannot be detected. However, the distinct conformations that different CADS promote cause the arginine residue, that is modified to reveal the induced [^{3}H]-strychnine binding, to become more or less reactive. This would explain the positive and negative modulation of the amount of BD-induced

[^{3}H]-strychnine binding. As the modifiable residue has been speculated to exist in a hydrophobic environment it is speculated that conformational changes in protein could effect the reactivity of the residue by making it more or less exposed.

The second scheme envisages two CAD recognition sites associated with the protein molecule. The first site is the one revealed by the BD modification and binds all classes of drugs that inhibit the BD-induced [^{3}H]-strychnine binding. In addition a second site, of extremely low affinity binds CAD to promote conformational changes in the protein. Again these either increase or decrease reactivity of the residues whose modification underlies the induced binding. Unlike the first site, the binding characteristics are not modified in a way that enables its detection by ligand binding assays. This is required as it appears that [^{3}H]-strychnine binding in BD-treated membranes is represented by a single site. The different possibilities do not exist in isolation from each other or other possible schemes that might explain the characteristics of the BD-induced [^{3}H]-strychnine binding.

The general concept that conformational transitions are important in ligand protein interactions, as outlined in the above three schemes, is consistent with the ideas concerning the functioning of regulatory enzymes and haemoglobin (Monod *et al.*, 1965). These ideas have been extended to include regulation of ion channel function (Hille, 1984a; Changeaux, 1990). With respect to BD-induced [^{3}H]-strychnine binding it is interesting that local anaesthetics or CAD interactions with ion channels are controlled by conformational transitions in these proteins (Hille, 1977; Changeaux, 1990). Present experimental evidence precludes positive identification of a cation channel as the site of BD-induced binding. However, it is pertinent to highlight the similarities between the schemes above and the proteins at which CAD actions have been best defined namely cation channels. This discussion has been generalized to voltage-gated and ligand-gated cation channels because there appear to be striking similarities in the way CADS interact with sodium channels or nicotinic acetylcholine receptors (Adams, 1981; Strichartz and Courtney, 1987).

Whether the channel is gated by voltage or agonist the respective stimulations lead to a change in protein conformation from a resting state to an open sate in which the conducting pathway, a water filled pore, that transverses the membranes becomes

accessible to ions (Unwin, 1990). Indeed, the open channel conformation has been proposed to interact with CADS. These drugs enter the channel pore and are thought sterically to plug the conducting pathway (Adams, 1981; Strichartz and Ritchie, 1987). This mechanism has intriguing parallels with the BD-induced binding as it indicates there is a conformational dependent interaction governed by access of the drug to a previously inaccessible binding site. This mechanism is of particular relevance to quarternerized permanently charged CADS as they require a hydrophillic route to gain access to a membrane buried binding site within the channel pore (Hille, 1977). In contrast, it has been proposed that tertiary amine CADS may gain access to the binding site by partitioning into the membrane phase in a uncharged form before interacting at the channel pore in their cationic form (Schwarz *et al.*, 1977). This has obvious similarities with the proposed idea that both charged and uncharged forms of CADS were required for inhibition of the BD-induced [^{3}H]-strychnine binding. In this respect it is pertinent to highlight that quarternerized CADS QX-222, QX-314 and N-methylstrychnine did not inhibit the BD- induced [^{3}H]-strychnine binding therefore the modification of the putative protein may involve more than a conformation that simply provides the ligand access to the binding site.

Subsequent to the open or active conformation, voltage-gated sodium channels become inactivated. This is a conformation in which the ion channel is in a closed state distinct from the resting conformation (Hille, 1970). Although it is a closed state, experimental evidence suggests that this conformation exhibits a higher affinity for CADS (Hille, 1977). This has parallels to the proposal that BD treatment might induce [^{3}H]-strychnine binding by covalently stabilizing a conformation with a high enough affinity to be detected by conventional binding assays. The role of conformational transitions in the interaction between CAD and cation channels is described by the modulated receptor hypothesis (see Strichartz and Ritchie, 1987).

An analogous conformation to the inactivated state is seen in ligand gated ion channels, as exemplified by the nicotinic acetylcholine receptor. This desensitized conformation (Katz and Thesleff, 1957) in which the conducting pathway is closed exhibits a higher affinity for agonist ligands than at rest (Weiland *et al.*, 1977). CADS have been shown not only to speed up desensitization in the presence of agonist but also directly to modulate receptor conformation (Heidmann *et al.*, 1983). Indeed such

stabilization of a non-conducting conformation has been speculated to provide an allosteric rather than a steric mechanism of cation channel block (Neher and Steinbach, 1978).

In addition to inhibition of the BD-induced [^{3}H]-strychnine binding, the experimental observations presented in this chapter indicate that CAD can modulate the amount of induced binding if present during the treatment. These drug modulations of the modification reaction which indicate conformational dependence may be analogous to phenomenon that have been described for the interaction with CADS with cation channels.

As pointed out above, there are at least 3 conformational states associated with the cation channels. Although interconversion of these states depends on activity, even at rest, an equilibrium will exist between the different states (Heidmann and Changeux, 1979; Courtney and Strichartz, 1987). The CAD stabilize a non-resting conformation thus when applied at rest they effect the equilibrium so that the proportion of channels in the desensitized conformation (Heidmann *et al.*, 1983) or the inactivated conformation (Courtney, 1975) is increased. The proportions seem to vary with different CADS (Heidmann *et al.*, 1983). However it has also been shown that when some CADS (usually polycyclic in nature) interact with the sodium channel they retard transition to the inactivated state (Stricartz and Ritchie, 1987; Hille, 1984a). In addition, the majority of CADS vary proportions of resting nicotinic receptors to shift to a desensitized state there are some CADS (procaine and tetracaine) that appear to have no effect on this transition (Blanchard *et al.*, 1979; Boyd and Cohen, 1984). It is possible to envisage how different CAD structures could favour distinct conformational states.

In the schemes devised to explain aspects of the experimental observations associated with the BD-induced [^{3}H]-strychnine binding it is speculated that distinct sites for CADS might exist. At sodium channels, similar multiple site models have been devised in an attempt to reconcile differences in the actions of quaternerized, tertiary and permanently uncharged local anaesthetics (Strichartz and Ritchie, 1987). A site in addition to that in the channel pore which binds hydrophobic drugs has been proposed and an interaction here stabilizes the inactivated conformation (Strichartz and Ritchie, 1987). At the nicotinic acetylcholine receptor located in the *torpedo* electroplax, a CAD site additional to that associated with the channel pore has been defined by ligand binding (Heidmann *et al.*, 1983). This site is of low affinity and high capacity possibly associated

with the lipid protein interphase. Indeed an interaction at this site has been shown to modulate protein conformation by stabilizing the desensitized states (Heidmann *et al.*, 1983). Thus a scheme which evokes multiple binding sites at a protein that interacts with CADS has some precedent at cation channels.

Finally, it is interesting to note that chemical modification of nerve preparation have been shown to effect conformational transitions associated with cation channel function (Hille 1984b) which in turn could have consequences for the interaction with CADS. Of particular interest is the observation that an irreversible modification by arginine selective reagents removed the inactivation process associated with the sodium channel (Eaton *et al.*, 1978). This was consistent with the proposal that pronase treatment, which also removed inactivation, was digesting at an arginine residue associated with the cytoplasmic domain of the channel protein (Armstrong *et al.*, 1973). This conclusion has been supported by experiments which have modified, by selective mutations, the characteristics of a wild type sodium channel expressed in *Xenopus* oocytes. The mutations involved cleavage of the proposed cytoplasmic loop that linked the last two of four homologous domains that make up a functional sodium channel (Stühmer *et al.*, 1989). The mutants express sodium channels which when compared to wildtype or native sodium channels have much slower inactivation kinetics. The region deleted by the mutation contains several lysine and arginine residues. More recent experiments using mutants of subunits of A type potassium channels indicate that similarly positively charged with proposed cytoplasmic locations may be important in inactivation (Hosli *et al.*, 1990; Baldwin *et al.*, 1991).

Molecular cloning has identified other arginine residues that have significance for conformational transitions underlying function of voltage-gated cation channels. This relates to a conserved sequence in homologous domains of sodium (Noda *et al.*, 1984) and calcium channels (Tanabe *et al.*, 1987) that have also been identified in subunits that make up the family of A-type potassium channels (Tempel *et al.*, 1987). This S4 sequence is a putative transmembrane α-helix structure that has arginine or lysine residues at every third amino acid position. The unprecedented sequence is speculated to form part of the voltage sensor of these channels (see Guy and Conti, 1990). This interpretation is supported by mutation and expression experiments with regard to sodium (Stühmer *et al.*, 1989) and potassium channels (Papazian *et al.*, 1991). Although such experiments also

highlight that the sequence has a role in maintaining ion channel structure (Liman *et al.*, 1991).

A direct significance of these examples given above with respect to the BD-induced [^{3}H]-strychnine binding is not clear. However, these examples serve to illustrate that the residue with which BD has a selective reactivity, would appear to play a pivotal role in functionally important conformational transitions of ion channels. In turn these conformational transitions are central to the modulation of CADS interaction with cation channels, the site at which their actions are best defined.

Present experimental data is phenomological and centres on a binding site that is induced by treating a crude neuronal membrane preparation with an arginine selective protein modifying reagent. The induced site appears specific to neuronal tissue and interacts with drugs that have important clinical use. However, to understand if this has any biological significance requires the identification of the structure that underlies this induced binding and assaying the functional significance of this structure.

Preliminary biochemical investigations indicate a protein with a subunit of 23 kd might underlie the induced binding although the sensitivity of the induced [^{3}H]-strychnine binding to detergent has precluded initial attempts to purify this apparently abundant protein. The molecular cloning of different classes of ion channels suggests these are made up of subunits or homologous domains that are between 25-60 kd (Miller, 1989). This, in addition to previously discussed evidence argues against BD-induced binding being associated with cation channels that have to date been characterized at the molecular level.

It could be that the BD-induced binding represents a novel cation channel activity something that has also been discussed for the previously described quinacrine binding activity (Greenberg and Tsong, 1984). In this respect it is interesting to note the recent identification of a potassium conductance activity associated with an mRNA that codes for a relatively small peptide. An expression cloning strategy was used and the message predicts a protein sequence of 15 kd associated with a potassium channel activity in kidney (Tokumi *et al.*, 1988). More recent mutation experiments support the idea that this represents a novel channel forming subunit rather than an ancillary protein that controls

activity of an associated channel (Goldstein and Miller, 1991). Sensitivity towards some potassium channel blockers was investigated but this was not extended to the study of CADS (Goldstein and Miller, 1991). The novel channel activity does not seem to be represented in nervous tissue (Takumi *et al.*, 1988) therefore its tissue distribution argues against it underlying the BD-induced binding or quinacrine binding activity. However, it is possible that a related protein might exist that awaits identification.

As speculated in discussions of the significance of the quinacrine binding activity (Greenberg and Tsong, 1984), the BD-induced binding could be a regulatory protein that associates with cation channels to modulate their function. However, the abundance of the induced binding argues against it being analogous to the small subunits that have been found associated with the sodium and calcium channels (Agnew *et al.*, 1984; Catterall 1988) which may serve to modulate their function (Auld *et al.*, 1988).

Finally, the nicotinic acetylcholine receptor constitutes the cation channel whose structural features are best defined (Changeux, 1990). This has enabled relatively defined mutations at points in the subunits that are modelled to constitute the pore-lining, the site at which CADS probably interact (Leonard *et al.*, 1988; Charnet *et al.*, 1990; Revah *et al.*, 1991). The consequence of these mutations have been taken in support of the idea that there is a relatively loose association between α-helical arrangements of the protein and the hydrophobic and cationic determinants of CAD structures. This arrangement could generalize to other cation channels with which CADS interact (Charnet *et al.*, 1990). In view of the potential importance of α-helical arrangements in membrane protein structures (Albers, 1989), other sites with which CADS might interact might be reasonably expected to exist.

Therefore, to identify if the BD modification represents the stabilization of a functionally relevant conformation of a protein requires further experimentation designed to purify and functionally assay the protein concerned.

Summary

The convulsant alkaloid strychnine is a potent antagonist of the action of glycine at the inhibitory postsynaptic receptor. Although the alkaloid interacts at other sites that mediate neuronal excitability the selective potency for the inhibitory glycine receptor ensures this is the primary site of action. This has led to extensive and successful use of the alkaloid in the characterization of the glycinergic transmitter system particularly the postsynaptic receptor. Strychnine has been central to procedures that led to the purification of the inhibitory glycine receptor which in turn led to the cloning and sequencing of receptor subunits that make up a functional receptor. More recently use of these sequences have established existence of isoforms of receptor subunits which have indicated a more extensive role for glycine in the nervous system than previously indicated by studies that focused on the use of strychnine as a label for the inhibitory glycine receptor.

The primary sequence of the proposed ligand binding subunit of the glycine receptor has provided a framework on which to postulate protein domains associated with function. Despite this, there remains much dispute concerning the nature of the interaction between strychnine and glycine at receptor recognition sites. Experimental investigations of the inhibition of [^{3}H]-strychnine binding by increasing concentrations of glycine have led to three distinct models to describe this interaction. In chapter 3 it was shown that failure to control for the [^{3}H]-strychnine binding to glass fibre used to terminate binding assays could account for one discrepancy that exists between different models. The possible reasons for other deviations from a favoured model in which glycine acts as a fully competitive inhibitor of [^{3}H]-strychnine binding described by Hill coefficients equal to one, are also discussed.

The relationship between strychnine and glycine recognition sites at the inhibitory glycine receptor was further probed by considering the effects of residue selective protein modifying reagents on parameters of [^{3}H]-strychnine binding. The specificity of these effects on the strychnine and glycine recognition sites was considered by combining ligand protection with these studies. The results suggest that both glycine and strychnine protect against the decreased [^{3}H]-strychnine binding produced by 3 protein modifying reagents with distinct residue selectivities. This argues that strychnine and glycine share

common recognition sites. However, the ability to protect against the disruption of glycine's ability to inhibit [^{3}H]-strychnine binding, subsequent to treatment, was not always common to glycine and strychnine. This is taken to indicate that residues outside a common recognition site are important to integrity of the glycine inhibition of [^{3}H]-strychnine binding. These results are discussed in terms of strychnine and glycine interacting through a common but conformationally distinct recognition sites, · model that is contrary to those previously proposed, but compatible with data on which these earlier models were based.

The protein modifying reagents, tetranitromethane, diethylpyrocarbonate and N-bromosuccinimide, that decreased [^{3}H]-strychnine binding have a selective reactivity for tyrosine, histidine and tryptophan under the conditions used. This indicates a possible role for aromatic residues in ligand recognition at a common agonist/antagonist site. A possible significance of such residues for recognition of the cationic charge associated with strychnine and glycine is highlighted in view of recent studies at the nicotinic acetylcholine receptor.

Amino acid selective protein modifying reagents were used to investigate ligand recognition properties of a strychnine binding polyclonal antisera that has previously been shown to have pharmacological specificity similar to the strychnine binding conformation of the glycine receptor. [^{3}H]-strychnine binding to the antisera was reduced by reagents that also decreased binding to the glycine receptor. Further, protection studies revealed that some of the detected inhibition of antisera binding was mediated by modifications at its strychnine recognition sites. The significance of this result is speculated upon in terms of common determinants at the recognition site of antisera and the postsynaptic glycine receptor.

This chapter also characterizes the reactivity of the arginine selective reagent 2,3-butanedione (BD). This membrane permeable protein modifying reagent when used to treat mouse spinal membranes caused a concentration dependent increase in subsequent [^{3}H]-strychnine binding. The increased binding was inhibited by unlabelled strychnine (10^{-4}M) but unlike untreated membranes was not inhibited by glycine (10^{-2}M). The ability to induce such binding is shared by a sub-class of arginine selective reagents which are membrane permeable and planar in structure. The result is discussed in terms of modification of membrane buried arginine residue(s) to induce a [^{3}H]-strychnine binding

not apparent in untreated membranes. Chapters Four, Five and Six describe and discuss approaches designed to characterize the nature of the BD-induced [^{3}H]-strychnine binding.

In Chapter 4 a number of approaches were used to investigate a possible connection between the BD-induced [^{3}H]-strychnine binding and the inhibitory glycine receptor. The distribution of the strychnine binding sites revealed by BD treatment appears to parallel that of high affinity sites for this ligand in untreated membranes: both parameters being highest in brain stem and spinal cord and lower in more rostral regions. However, comparison of this relationship within a region of the CNS indicate the correlation does not arise because the induced binding occurs at structures associated with the inhibitory glycine receptor. This was also indicated by studies that compared amounts of [^{3}H]-strychnine binding to untreated and BD-treated spinal cord membranes from the *spastic* mouse. This mutant has a 80% decrease in high affinity [^{3}H]-strychnine binding sites yet the amount of binding to BD-treated membranes from *spastic* and unaffected littermates was the same.

The distinct nature of the BD-induced site was further indicated by studies that used reversible [^{3}H]-strychnine binding to affinity purified glycine receptors or photoinduced irreversible incorporation into the ligand binding subunit to assay an interaction at the receptor. In either case BD treatment did not cause an increase in [^{3}H]-strychnine binding. Rather treatment resulted in a reduction in total binding and the ability of glycine to inhibit total [^{3}H]-strychnine binding. These results are discussed in terms of arginine residues that have a role in ligand interactions at the glycine receptor.

The distinct nature of the BD-induced binding was also indicated by its relative sensitivity to detergents which were used in preliminary attempts to solubilize this binding. This is compatible with the idea that the induced [^{3}H]-strychnine binding site resides in a hydrophobic environment.

In chapter 5 experiments designed to optimize the measurement of the equilibrium binding parameters of [^{3}H]-strychnine binding to BD-treated membranes indicated the induced binding equilibrated relatively slowly at 4°C. The amount of [^{3}H]-strychnine bound to BD-treated membranes was similar in assays terminated by filtration or centrifugation. These results are probably due to a slow on and off rate. However, no

attempt was made to directly measure the disassociation or association rate constants.

Saturation isotherms for [^{3}H]-strychnine binding to untreated and BD-treated membranes were analyzed by a non-linear least square fit program in an attempt to resolve a site that underlies the BD-induced binding in untreated membrane. This proved unsuccessful. Similarly, despite evidence from biochemical experiments that [^{3}H]-strychnine binding to a modified form of the glycine receptor existed in BD-treated membranes, this site could not be resolved from the parameters that defined binding to the induced site. A predominance of the BD-induced [^{3}H]-strychnine binding over that to the inhibitory glycine receptor probably explain this apparent discrepancy. The BD-induced binding was defined by a K_D of approximately 10 μM and a B_{max} of approximately 4 nmols/mg protein.

Experiments that considered saturation of [^{3}H]-strychnine binding to untreated membranes under conditions that favoured detection of low affinity binding was resolved into two sites by non-linear least square fit analysis. However, the binding capacity of the low affinity site and an apparent sensitivity to inhibition by glycine indicated this was not the phenomenon underlying the BD-induced [^{3}H]-strychnine binding. Rather, this observation is discussed in terms of a low affinity site associated with the inhibitory glycine receptor.

The induction of a low affinity and high capacity [^{3}H]-strychnine binding site in BD-treated membranes is rationalized by evoking a conformational change in a putative binding protein. This in turn causes a change in affinity or access to a binding site for [^{3}H]-strychnine binding that exists but is not detected in untreated membranes.

In chapter 6 experiments define the distinct pharmacological specificities of the [^{3}H]-strychnine binding to untreated and BD-treated membrane. Thus, several compounds that inhibit [^{3}H]-strychnine binding to untreated membranes have no effect at the BD-induced site. Moreover, those compounds that do inhibit the binding at both sites confirm the distinct nature of ligand binding as there is no apparent correlation between their potencies relative to strychnine at inhibiting [^{3}H]-strychnine binding to untreated and BD-treated membranes.

In chapter 1, non-glycinergic actions of strychnine were discussed and these were

used to focus investigation that attempted to define the pharmacological specificity of the BD-induced site. The BD-induced [^{3}H]-strychnine binding was inhibited by a class of compounds with a general cationic amphipathic drug structure (CADS). The IC_{50} values for this inhibition ranged from 1-100 μM for 20 different drugs tested. In contrast these compounds did not inhibit [^{3}H]-strychnine binding to untreated membranes with sufficient potency to enable detection of IC_{50} values. Related structures, that were quarternerized and carried a permanent positive charge or those that were uncharged, failed to inhibit BD-induced [^{3}H]-strychnine binding. This is discussed in terms of the interconversion between uncharged and charged forms of the drugs being an important feature of the interaction at the BD-induced site. Further discussion of the structure activity relations considered both structural and physico-chemical characteristics that might be important in the interaction at the BD-induced site.

The CADS that inhibit BD-induced [^{3}H]-strychnine binding represent a group of drugs that have found extensive use as local anaesthetics and antiarrhythmics. A use-dependent block of cation and particularly sodium channels is proposed to underlie their clinical usefulness. A similar activity has previously been described for the alkaloid strychnine which also has a CAD structure. The significance of an interaction at such a site as possibly underlying the BD-induced [^{3}H]-strychnine binding are discussed. CADS have a spectrum of activities at subtypes of ion channels, other membrane proteins and membrane sites. Therefore a plethora of CNS sites could feasibly underlie the BD-induced binding. However, the inhibition of [^{3}H]-strychnine binding to BD-treated membranes by the majority of CADS is described by Hill slopes close to one. This was taken to indicate that a single class of sites underlies this phenomenon. This result is consistent with experiments that indicate that in BD-treated membranes [^{3}H]-strychnine binding was irreversibly incorporated upon exposure to UV light into the inhibitory glycine receptor and an additional peptide. The additional labelling in BD-treated membranes was sensitive to inhibition by the CAD, quinine and has the same pharmacological specificity as the BD-induced [^{3}H]-strychnine binding.

The labelled peptide had an apparent molecular weight of 23 kd. This is inconsistent with an interaction with any previously characterized ion channel. However a previously described low affinity and high capacity CAD binding site of unknown function has several characteristics that are compatible with the BD-induced [^{3}H]-strychnine binding site. The possibility that these are related phenomenon is discussed.

In chapter 3 it was shown that when membranes were incubated with strychnine during the BD treatment a potentiation of the induced [^{3}H]-strychnine binding was observed. This is discussed in terms of a conformational dependent modification. In Chapter Six this was further investigated by studying modulation of the BD-reaction with strychnos alkaloids, other alkaloids and CADS that inhibited the BD-induced binding. The results indicate that the induced binding was potentiated, unaffected, reduced or completely prevented depending on the drug present during the BD treatment. It appeared that the more potent CADS at inhibiting [^{3}H]-strychnine binding to BD-treated membranes reduced or prevented the induced binding if present during the treatment. The result is discussed in terms of drugs interacting and causing distinct protein conformations which lead to an increase or decrease in the reactivity of the residue that is modified to induce [^{3}H]-strychnine binding. The ability of CADS to induce functionally distinct conformations when they interact with voltage gated sodium channels and the nicotinic acetylcholine receptor is highlighted.

An attempt to rationalize the different aspects of the BD-induced [^{3}H]-strychnine binding is attempted by postulating 2 models that might be compatible with present data. However, it is clear that unravelling a possible biological significance of this CNS specific binding site, that is sensitive to clinically important drugs requires further experimentation. The most direct approach would be attempts to identify the structure whose modification by BD leads to the induced [^{3}H]-strychnine binding. Therefore at a time when the importance of strychnine as a molecular probe for the inhibitory glycine receptor seems to be diminished the alkaloid may provide the impetus that leads to the identification of another CNS structure. At present this is defined by some interesting characteristics whose functional significance remains unclear.

References

Adams, P.R. (1977). Voltage jump analysis of procaine action at frog end plate. J. Physiol. **268:** 291-318.

Adams, P.R. (1981). Acetylcholine receptor kinetics. J. Memb. Biol. **58:** 161-174.

Agnew, W.S. (1984). Voltage regulated Na^+ channel molecules. Annu. Rev. Physiol. **46:** 517-530.

Aibara, K., Oonuma, M. and Akaike, J. (1991). Strychnine-induced potassium current in isolated dorsal root ganglion cells of the rat. Br. J. Pharmacol. **102:** 492-496.

Akagi, H. and Miledi, R. (1988). Heterogeneity of glycine receptors and their messenger RNAs in rat brain and spinal cord. Science **242:** 270-273.

Akagi, H., Patton, D.E. and Miledi, R. (1989). Discrimination of heterogenous mRNAs encoding strychnine sensitive glycine receptors in *Xenopus* oocytes by antisense oligonucleotides. Proc. Nat. Acad. Sci. **86:** 8103-8107.

Akagi, H., Hirai, K. and Hisinum, F. (1991). Functional properties of strychnine-sensitive glycine receptors expressed in *Xenopus* oocytes injected with a single mRNA. Neuroscience Res. **11:** 28-40.

Albers, R.W. (1989). Cell membrane structure and functions. In Basic Neurochemsitry. Eds. G. Siegel, B. Agranoff, R.W. Albers and P. Molinoff. Raven Press.

Altschuler, R.A., Betz, H., Parakkal, Reeks, K. and Wenthold, R.J. (1986). Identification of glycinergic synapses in cochlear nucleus through immunocytochemical localization of the postsynaptic receptor. Brain Res. **369:** 316-320.

Alzari, P.M., Lascombe, M-B. and Poljak, R.J. (1988). Three-dimensional structure of antibodies. Ann. Rev. Immunol. **6:** 555-580.

Andres, S.F. and Atassi, M.Z. (1973). Immunochemistry of sperm-whale myoglobin. Conformation and Immunochemistry of derivatives prepared by reaction with Diazonium-1H-tetratzole. Evaluation of the specificity of the reagent. Biochemistry **12:** 942-948.

Aprison, M.H. and Werman, R. (1965). The distribution of glycine in cat spinal cord and roots. Life Sci. **4:** 2075-2083.

Aprison, M.H., Shank, R.P. and Davidoff, R.A. (1969). A comparison of the concentration of glycine, a transmitter suspect, in different areas of the brain and spinal cord in seven different vertebrates. Comp. Biochem. Physiol. **28:** 1345-1355.

Aprison, M.H. and Daly, E.C. (1978). Biochemical aspects of transmission at inhibitory synapses: The role of glycine. In: Advances in Neurochemistry. Eds. B.W. Agranoff and M.H. Aprison. Plenum Press, New York.

Aprison, M.H., Lipkowitz, K.B. and Simon, J.R. (1987) Identification of a glycine like fragment on the strychnine molecule. J. Neurosci. Res. **17:** 209-213.

Aprison, M.H. (1990). The discovery of the neurotransmitter role of glycine. In: Glycine Neurotransmission. Eds. O.P. Ottersen and J. Storm-Mathisen. John Wiley and Sons.

Araki, T., Yamano, M., Murakami, T., Wanaka, A., Betz, H. and Tohyama, M. (1988) Localization of glycine receptors in the rat central nervous system: An immunocytochemical analysis using monoclonal antibody. Neuroscience **25:** 613-624.

Armstrong, C.M., Benzanilla, F. and Rojas, E. (1973). Destruction of sodium conductance inactivation in squid axons perfused with pronase. J. Gen. Physiol. **62:** 375-391.

Ascher, P. and Gachelin, G. (1967). Role du colliculus superieur dans L'elaboration de response matrices a des stimulations visuelles. Brain Res. **3:** 327-342.

Auld, V.J., Goldin, A.L., Krafte, D.S., Marshall, J., Dunn, J.M., Catterall, W.A., Lester, H.A., Davidson, N. and Dunn, R.J. (1988). A rat brain Na^+ channel α subunit with novel gating properties. Neuron **1:** 449-461.

Bakker, M.H.M., McKernan, R.M., Wong, E.H.F. and Foster, A.C. (1991). [^{3}H]-MK-801 binding to N-methyl-D-aspartate receptors solubilized from rat brain: effects of glycine site ligands, polyamines, Ifenprodil and Desipramine. J. Neurochem. **57:** 39-45.

Baldessarini, B. (1990). Drugs and the treatment of psychiatric disorders. In: Goodman and Gilman. The Pharmacological Basis of Therapeutics 8th Edition. Eds A.G. Gilman, T.W. Rall, A.S. Nies and P. Taylor. Pergamon Press.

Baldwin, T.J., Tsaur, M-L., Lopez, G.A., Jan, Y.N. and Jan, L.Y. (1991). Characterization of a mammalian cDNA for an inactivating voltage sensitive K^+ channel. Neuron. **7:** 471-483.

Barkas, T., Mauron, A., Roth, B., Alliod, C., Tzartos, S.J. and Ballivet, M. (1987). Mapping the main immunogenic region and toxin-binding site of the nicotinic acetylcholine receptor. Science, **235:** 77-80.

Barnard, E.A., Darlison, M.G. and Seeburg, P. (1987). Molecular biology of the $GABA_A$ receptor: the receptor/channel superfamily. TINS **10:** 502-509.

Barron, S.E. and Guth, P.S. (1987). Use and limitations of strychnine as a probe in neurotransmission. TIPS **8:** 204-206.

Barton, G.J. and Sternberg, M.J.E. (1987). A strategy for the rapid and multiple alignment of protein sequences. Confidence levels from tertiary structure comparison. J. Mol. Biol. **198:** 327-337.

Barton, G.J. and Sternberg, M.J.E. (1987). Evalulation and improvements in the automatic alignment of protein sequences. Protein Engineering **1:** 89-94.

Basbaum, A.I. (1988). Distribution of glycine receptor immunoreactivity in spinal cord of the rat: Cytochemical evidence for a differential glycinergic control of lamina I and V nociceptive neurons. J. Comp. Neurol. **278:** 330-336.

Becker, C-M., Hermans-Borgmeyer, I., Schmitt, B. and Betz, H. (1986). The glycine receptor deficiency of the mutant mouse spastic: evidence for normal glycine receptor structure and localization. J. Neurosci. **6:** 1358-1364.

Becker, C-M. and Betz, H. (1987). Strychnine: useful probe or not. TIPS **8:** 379-380.

Becker, C-M, Hoch, W. and Betz, H. (1988) Glycine receptor heterogeneity in rat spinal cord during postnatal development. EMBO J. **7:** 3717-3726.

Becker, C-M., Hoch, W. and Betz, H. (1989). Sensitive immunoassay shows selective association of peripheral and integral membrane proteins of the inhibitory glycine receptor complex. J. Neurochem. **53:** 124-131.

Becker, C-M. (1990). Disorders of the inhibitory glycine receptor: the spastic mouse. FASEB. J. **4:** 2767-2774.

Benavides, J., López-Lahoya, J., Valdivieso, F. and Ugarte, M. (1981). Postnatal development of synaptic glycine receptors in normal and hyperglycinemic rats. J. Neurochem. **37:** 315-320.

Bennett, Jr., J.P. and Yamamura, H.I. (1985) Neurotransmitter hormone or drug receptor binding methods. In Neurotransmitter receptor binding. Eds. H.I. Yamamura, S.J. Enna, and M. Kuhar. N.Y. Raven Press.

Berek, C. and Milstein, C. (1987) Mutation drift and repertoire shift in the maturation of the immune response. Immunol. Rev. **96:** 23-39.

Bertolino, M. and Vicini, S. (1988). Voltage-dependent block by strychnine of N-methyl-D-aspartic-acid-activated cationic channels in rat cortical neurones in culture. Mol. Pharmacol. **34:** 98-103.

Betz, H. (1985). The glycine receptor of rat spinal cord. Exploring the site of action of the plant alkaloid strychnine. Angew. Chem. Int. Ed. Engl. **24:** 365-370.

Betz, H. (1987). Biology and structure of the mammalian glycine receptor. TINS **10:** 113-117.

Betz, H. and Becker, C-M. (1988). The mamalian glycine receptor: biology and structure of a neuronal chloride channel protein. Neurochem. Int. **13:** 137-146.

Betz, H. (1990). Homology and analogy in transmembrane channel design: lessons from synaptic membrane proteins. Biochemistry **29:** 3591-3599.

Betz, H. (1991). Glycine receptors: heterogeneous and widespread in the mammalian brain. TINS **14:** 458-461.

Beyer, C., Banas, C. Gomara, P. and Komisharuk, B.R. (1988). Prevention of convulsant and hyperalgesic action of strychnine by intrathecal glycine and related amino acids. Pharmacol. Biochem and Beh. **29:** 73-78.

Biscoe, T.J. and Curtis, D.R. (1966). Noradrenaline and inhibition of Renshaw cells. Science **151:** 1230-1231.

Biscoe, T.J. and Fry, J.P. (1984). GABA and Benzodiazepine receptor in neurologically mutant mice. In: Actions and interaction of GABA and Benzodiazepines. Ed. N.G. Bowery. Raven Press, New York.

Biscoe, T.J. and Duchen, M.R. (1986). Synaptic physiology of spinal motorneurones of normal and *spastic* mice: an in vitro study. J. Physiol. **379:** 275-292.

Blanchard, S.G., Elliot, J. and Raftery, M.A. (1979). Interaction of local anaesthetics with *Torpedo california* membrane bound acetylcholine receptor. Biochemistry **18:** 1875-1883.

Blount, P. and Merlie, J.P. (1989). Molecular basis of the two nonequivalent ligand binding sites of the muscle acetylcholine receptor. Neuron **3:** 349-357.

Bobker, D.H., Shen. K-Z, Surprenant, A. and Williams, J. T. (1989). DTG and (+)-3-PPP inhibit a ligand activated hyperpolarization in mammalian neurons. J. Pharmacol. Exp. Ther. **251:** 840-845.

Bormann, J. (1990). Patch clamp studies of glycine-gated chloride channels. In: Glycine Neurotransmission. Eds. O.P. Ottersen and J. Storm-Mathisen. John Wiley and Sons.

Bowery, N., Hill, D.R. and Hudson, A.L. (1983). Characteristics of $GABA_B$ receptor binding sites on rat whole brain synaptic membranes. Br. J. Pharmacol. **78:** 191-206.

Bowery, N.G., Hudson, A.L. and Price, G.W. (1987). $GABA_A$ and $GABA_B$ receptor sites distribution in the rat central nervous system. Neuroscience **20:** 365-383.

Bowery, N.G. (1987). Glycine binding sites and NMDA receptors in brain. Nature, **326:** 338.

Bowman, W.C. and Rand, W.C. (1980) Principles of drug action. 39.1-39.69. In: Textbook of pharmacology. Eds. W.C. Bowman and W.C. Rand.

Bowser-Riley, F., Paton, W.D.M. and Smith, E.B. (1981). Experiments to investigate the neuronal origin of the high pressure neurological syndrome. Br. J. Pharmacol. **74:** 820.

Bowser-Riley, F., Daniels, S. and Smith, E.B. (1988). Investigations into the origin of the high pressure neurological syndrome: the interaction between pressure strychnine and 1,2-propandiols in the mouse. Br. J. Pharmacol. **94:** 1069-1076.

Bowser-Riley, F., Daniels, S., Hill, W.A.G. and Smith, E.B. (1989). An evaluation of the structure-activity relationship of a series of analogoues of mephhenesin and strychnine on the response to pressure in mice. Br. J. Pharmacol. **96:** 789-794.

Boyd, N.D. and Cohen, J.B. (1984). Desensitization of membrane bound *Torpedo* acetylcholine receptor by amine non-competitive antagonists and aliphatic alcohols studies of [^{3}H]-acetylcholine binding and $^{22}Na^+$ fluxes. Biochemistry **23:** 4023-4033.

Bradford, M.M. (1976) A rapid and sensitive method for the quantification of microgram quantities of protein using the principle of protein dye binding. Anal. Biochem. **72:** 248-254.

Bradley, K., Easton, D.M. and Eccles, J.C. (1953). An investigation of primary or direct inhibition. J. Physiol. **122:** 474-488.

Bradley, P.B., Engel, G., Fenuik, W., Fozard, J.R., Humphrey, P.P.A., Middlemiss, D.N., Mylecharane, E.J., Richardson, B.P. and Saxena, P.R. (1986). Proposals for the classification and nomenclature of functional receptors for 5-hydroxtryptamine.Neuropharmacol. **25:** 563-576.

Braestrup, C., Nielsen, M. and Krogsgaard-Larsen, P. (1986). Glycine antagonists structurally related to the 4,5,6,7-Tetrahydroisoxazolo [5,4-c] pyridin-3-ol inhibit binding of [^{3}H]-strychnine to rat brain membranes. J. Neurochem. **47:** 691-696.

Bristow, D.R., Bowery, N.G. and Woodruff, G.N. (1986). Light microscopic autoradiographic localization of [^{3}H]-glycine and [^{3}H]-strychnine binding sites in rat brain. Eur. J. Pharmacol. **126:** 303-308.

Bürgisser, E. (1984). Radioligand-receptor binding studies: what's wrong with Scatchard analysis? TIPS **5:** 142-144.

Cahalan, M.D. (1978). Local anaesthetic block of sodium channels in normal and pronase treated squid giant axons. Biophys. J. **23:** 285-311.

Cahalan, M.D. and Almers, W. (1979). Block of sodium conductance and gating current in squid giant axons poisoned by quarternary strychnine. Biophys. J. **27:** 57-74.

Casanovas, A.M, Labat, C., Courriere, P. and Oustine, J. (1985). Interaction of local anaesthetics with cytochrome oxidase studied with fluorescence quenching. Biochem. Pharmacol. **34:** 663-8.

Catterall, W.A. (1975). Co-operative activation of action potential Na^+ ionophore by neurotoxins. Proc. Natl. Acad. Sci. **72:** 1782-86.

Catterall, W.A., Morrow, C.S. and Harthshone, R.P. (1979). Neurotoxin binding to receptor sites associated with voltage sensitive sodium channels in intact lysed and detergent solubilized brain membrane. J. Biol. Chem. **25:** 11379-11387.

Catterall, W.A. (1980). Neurotoxins that act on voltage sensitive sodium channels in excitable membranes. Ann. Rev. Pharmacol. Toxicol. **20:** 15-43.

Catteral, W.A. (1988). Structure and function of voltage sensitive ion channels. Science **242:** 50-61.

Changeux, J-P. (1990). The nicotinic acetylcholine receptor: an allosteric protein prototype of ligand gated ion channels. TIPS **11:** 485-492.

Charnet, P., Labarca, C., Leonard, R.J., Vogelaar, N.J., Czyzylk, L., Gouin, A., Davidson, N. and Lester, H.A. (1990). An open-channel blocker interacts with adjacent turns of α-helices in the nicotinic acetylcholine receptor. Neuron. **2:** 87-95.

Clarke, P.B.S., Schwartz, R.D., Paul, S.M., Pert, C.B. and Pert, A. (1985). Nicotinic binding in rat brain: Autoradiographic comparison of [^{3}H]-acetylcholine, [^{3}H]-nicotine and [^{125}I]-α-ßungarotoxin. J. Neurosci. **5:** 1307-1315.

Cohen, J.B. and Changeaux, J-P. (1973). Interaction of a fluorescent ligand with membrane-bound cholinergic receptor from *Torpedo marmorata*. Biochemisty **12:** 4855-4864.

Cohen, L.A. (1970) Chemical modification as a probe of structure and function. In: The enzymes (Vol. 1; 3rd edition). Ed. P.O. Boyer. Academic Press.

Conti-Tronconi, B.M., Tang, F., Diethelm, B.M., Spencer, S.R., Reinhardt-Maelicke, S. and Maelicke, A. (1990). Mapping of a cholinergic binding site by means of synthetic peptides, monoclonal antibodies and α-Bungarotoxin. Biochemistry **29:** 6221-6230.

Cook, N.J. and Haylett, D. (1985). Effects of apamin, quinine and neuromuscular blockers on calcium activated potassium channels in guinea pig hepatocytes. J. Physiol. **358:** 373-394.

Cook, N.J. (1988). The pharmacology of potassium channels and their therapeutic potential. TIPS **9:** 21-28.

Cook, N.S. and Quast, U. (1990). Potassium channel pharmacology. In: Potassium channels, structure, classification, function and therapeutic potential. Ed. N.S. Cook, Ellis Horwood Ltd.

Coombes, J.S., Curtis, D.R. and Eccles, J.C. (1955). The specific ionic conductances and the ionic movements across motor neuronal membranes that produce the inhibitory post-synaptic potential. J. Physiol. **130:** 326-373.

Courtney, K.R. (1975). Mechanisms of frequency dependent inhibition of sodium currents in frog myelinated nerve by GEA-968, a new lidocaine derivative. J. Pharmacol. Exp. Ther. **195:** 225-236.

Courtney, K.R. and Strichartz, G.R. (1987). Structural elements which determine local anaesthetic activity. In: Local anaesthetics. Ed. G.R. Strichartz. Springer-Verlag.

Cuatrecasas, P. (1971). Properties of the insulin receptor of isolated fat cell membrane. J. Biol. Chem. **246:** 7265-7274.

Curtis, D.R. and Watkins J.C. (1960). The excitation and depression of spinal neurones by structurally related amino acids. J. Neurochem. **6:** 117-141.

Curtis, D.R., Hosli, L. and Johnston, G.A.R. (1967a). Inhibition of spinal neurones by glycine. Nature. **215:** 1502-1503.

Curtis, D.R., Hosli, L., Johnston, G.A.R. and Johnston, I.H. (1967b). Glycine and spinal inhibition. Brain Res. **5:** 112-114.

Curtis, D.R. Hosli, L. and Johnston, G.A.R. (1968a). A pharmacological study of the depression of spinal neurones by glycine and related amino acids. Exp. Brain Res. **6:** 1-18.

Curtis, D.R., Hosli, L., Johnston, G.A.R. and Johnston, I.H. (1968b). The hyperpolarization of spinal motoneurones by glycine and related amino acids. Exp. Br. Res. **5:** 235-258.

Curtis, D.R., Duggan, A.W. and Johnston, G.A.R. (1969). Glycine, strychnine, pictrotoxin and spinal inhibition. Brain Res. **14:** 759-762.

Curtis, D.R., Duggan, A.W. and Johnston, G.A.R. (1971a). The specificity of strychnine as a glycine antagonist in the mammalian spinal cord. Exp. Brain Res. **12:** 547-565.

Curtis, D.R., Duggan, A.W. Felix, D. and Johnston, G.A.R. (1971b). Bicuculline, an antagonist of GABA and synaptic inhibition in spinal cord of the cat. Brain Res. **32:** 69-96.

Curtis, D.R. and Johnston, G.A.R. (1974). Amino acid transmitters in the mammalian central nervous system. Ergebn. Physiol. **69:** 98-188.

Curtis, D.R. and Malik, R. (1985). Glycine antagonism by RU 5135. Eur. J. Pharmacol. **110:** 383-384.

Damle, V.N. and Karlin, A. (1980). Effects of agonists and antagonist on the reactivity of the binding site disulphide in acetylcholine receptor from *Torpedo california.* Biochemistry **19:** 3924-3932.

Davidoff, R.A., Graham, L.T. Jr., Shank, R.P., Werman, R. and Aprison, M.H. (1967). Changes in amino acid concentration associated with loss of spinal interneurons. J. Neurochem. **14:** 1025-1031.

Davidoff, R.A., Aprison, M.H. and Werman, R. (1969). The effects of strychnine on the inhibition of interneurons by glycine and γ-aminobutyric acid. Int. J. Neuropharmacol. **8:** 191-194.

Davies, D.R., Sheriff, S. and Padlan, E.A. (1988) Antibody-Antigen Complexes. J. Biol. Chem. **263:** 10541-10544.

De Blasi, A., O'Reilly, K. and Motulsky, H.J. (1989). Calculating receptor number from binding experiments using same compound as radioligand and competitor. TIPS **10:** 227-229.

DeFeudis, F.V. and Schiff, N. (1975). Comparison of binding affinities and capacities of gamma-aminobutyric acid and glycine in synaptosome-enriched fractions of rat cerebral cortex and spinal cord. Exp. Neurol. **48:** 325-335.

de Montis G., Beaumont, K., Javoy-Agid F., Agid, Y., Constandinis, J., Lowenthal, A. and Lloyd, K.G. (1982). Glycine receptors in the human substantia nigra as defined by [^{3}H]-strychnine binding. J. Neurochem. **38:** 718-724.

Deneris, E.S., Connolly, J., Rogers, S.W. and Duvoisin, R. (1991). Pharmacological and functional diversity of neuronal nicotinic acetylcholine receptors. TIPS **12:** 34-40.

Dennis, M., Giraudat, J., Kotzyba-Hibert, F., Goeldner, M., Hirth, C., Chang, J-Y., Lazure, C., Chretien, M. and Changeaux, J-P. (1988). Amino acids of the *Torpedo marmorata* acetylcholine α-subunit labelled by a photoaffinity ligand for the acetylcholine binding site. Biochemistry. **27:** 2346-2357.

Dolly, J.O. (1988). Potassium channels-what can protein chemistry contribute? TINS **11:**186-188.

Dougherty, D.A. and Stauffer, D.A. (1990) Acetylcholine binding by a synthetic receptor: Implications for biological recognition. Science **250:** 1558-1560.

Duggan, M.J. and Stephenson, F.A. (1987) Diethylpyrocarbonate modification of the central benzodiazepine binding site of bovine brain. Biochem. Soc. Trans. **15:** 1151.

Dusser de Barenne, J.G. (1933). The mode and site of action of strychnine on the nervous system. Physiol. Rev. **13:** 325-335.

Eadie, G.S. (1952). The inhibition of cholinesterase by physostigmine and prostigmine. J. Biol. Chem. **146:** 85-93.

Eaton, D.C., Brodwick, M.S., Oxford, G.S. and Rudy, B. (1978). Arginine-specific reagents remove sodium channel inactivation. Nature **271:** 473-476.

Eccles, J.C., Fatt, P. and Koketsu, K. (1954). Cholinergic and inhibitory synapses in a pathway from motor-axon collaterals to motoneurones. J. Physiol. **216**: 524-562.

Edwards, F.A., Konnerth, A., Sakmann, B. and Takahashi, T. (1989). A thin slice preparation for patch clamp recordings from neurones of mammalian central nervous system. Pflügers Arch. **414:** 600-612.

Edwards, G. and Weston, A.H. (1990). Structure-activity relationships of K^+ channel openers. TIPS. **11:** 417-422.

Ehrlich, R.S. and Colman, R.F. (1977). The role of arginine in the triphosphopyridine nucleotide dependent Isocitrate Dehydrogenase of pig heart. Biochemistry **16:** 3378-3383.

Eyzaguirre, J. (1987) Chemical modification of enzymes, an overview. The use of group specific reagents. In: chemical modification of enzymes. Ed Eyzaguirre, J. Ellis Horwood Ltd.

Faber, D.S. and Klee, M.R. (1974). Strychnine interactions with acetylcholine, dopamine and serotonin receptors in *Aplysia* neurons. Brain Res. **65:** 109-126.

Falke, J.J. and Chan, S.I. (1986). Molecular mechanisms of Band 3 inhibitors. 2. Channel blockers. Biochemistry **25:** 7895-7898.

Fields, R. (1972) The rapid determination of amino groups with TNBS 464-468. In: Meths. Enzymol. vol. 25. Eds. C.H.W. Hirs and S.N. Timasheff. Acad. Press.

Findlay, J.B.C. and Evans, W.H. (1987). Protein modifying reagents (Appendix IV). In: Biological membranes: A practical approach. Eds J.B.C. Findlay and W.H. Evans. IRL Press.

Fodor, G. and Kovács, O. (1949). A synthesis of Adrenalin-like compounds. J. Am. Chem. Soc. **71:** 1045-1048.

Foster, A.C. and Wong, E.H.F. (1987). The novel anticonvulsant MK-801 binds to the activated state of N-methyl-D-Aspartate receptor in rat brain. Br. J. Pharmacol. **91:** 304-309.

Franks, N.P. and Lieb, W.R. (1984). Do general anaesthetics act by competitive binding to specific receptors. Nature **310:** 599-601.

Frostholm, A. and Rotter, A. (1985). Glycine receptor distribution in mouse CNS. Autoradiographic localization of [^{3}H]-strychnine binding sites. Brain Res. Bull. **15:** 473-486.

Frumkes, T.E., Miller, R.F., Slaughter, M. and Dacheux, R.F. (1981). Physiological and pharmacological basis of GABA and glycine action on neurons of mud puppy retina. Amacrine mediated inhibitory influences on ganglion cell receptive field organization: A model. J. Neurophysiol. **45:** 783-804.

Fry, J.P. and Phelan, P.P. (1986). Interactions of glycine receptor ligands in the normal mouse spinal cord. J. Physiol. **373:** 21P.

Galligan, J., Campbell, B.G., Kavanaugh, P. and North, R.A. (1989). 1,3-Di(2-tolyl-guanidine blocks nicotinic responses in guinea pig myenteric neurons. **251:** 169-174.

Galzi, J-L., Revah, F., Black, D., Goeldner, M., Hirth, C. and Changeaux, J-P. (1990). Identification of a novel amino acid tyrosine 93 within the cholinergic ligands binding site of the acetylcholine receptor by photoaffinity labeling. J. Biol. Chem. **265:** 10430-10437.

García-Calvo, M., Ruíz-Gómez, A., Vásquez, J., Morato, E., Valdivieso, F. and Mayor Jr., F. (1989). Functional reconstitution of the glycine receptor. Biochemistry **28:** 6405-6409.

Giraudat, J., Dennis, M., Heidmann, T., Chang, J.V. and Changeux, J.P. (1986). The structure of the high affinity site for noncompetitive blockers of the acetycholine receptor: serine-262 of the δ subunit is labelled by [^{3}H]-chlorpromazine. Proc. Natl. Acad. Sci. USA **83:** 2719-2723.

Glazer, A.N. (1976). The chemical modification of proteins by group specific and site specific reagents. In: The proteins (3rd edition). Eds. H.,Neurath, R.L. Hill and C.L. Boeder.

Goeldner, M.P. and Hirth, C.G. (1983). Specific photoaffinity labelling induced by energy transfer: Application to irreversible inhibition of acetylcholinesterase. Proc. Nat. Acad. Sci. **77:** 6439-6442.

Gola, M. and Ducreux, C. (1985). D-600 a direct blocker of Ca-dependent K^+ currents in Helix neurones. Eur. J. Pharmacol. **117:** 311-322.

Goldinger, A., Müller, W.E. and Wollert, U. (1981). Inhibition of glycine and GABA receptor binding by several opiate agonists antagonists. Gen. Pharmacol. **12:** 477-479.

Goldstein, A., Lewis, A. and Kalman, S.M. (1974). Molecular mechanisms of drug action. In: Principles of Drug Action. The basis of Pharmacology, 2nd Edition. John Wiley and Sons.

Goldstein, S.A.N. and Miller, C. (1991). Site specific mutation in a nominal voltage dependent K^+ channel alter ion selectivity and open channel block. Neuron **7:** 403-408.

Graham, D., Pfeiffer, F. and Betz, H. (1981). UV light induced crosslinking of strychnine to the glycine receptor of rat spinal cord membranes. Biochem. Biophys. Res. Comm. **102:** 1330-1335.

Graham, D., Pfeiffer, F. and Betz, H. (1983). Photoaffinity-labelling of the glycine receptor of rat spinal cord. Eur. J. Biochem. **131:** 519-525.

Graham, D., Pfeiffer, F., Simler, R. and Betz, H. (1985). Purification and characterization of the glycine receptor of pig spinal cord. Biochemistry **24:** 990-994.

Graham, L.T., Shank, R.P., Werman, R. and Aprison, M.H. (1967). Distribution of some synaptic transmitter suspects in cat spinal cord. J. Neurochem. **14:** 465-472.

Greenberg, M. and Tsong, T.Y. (1982). Binding of quinacrine, a fluorescent local anaesthetic probe to mammalian axonal membranes. J. Biol. Chem. **257:** 8964-8971.

Greenberg, M. and Tsong, T.Y. (1984). Detergent solubilization and affinity purification of a local anaesthetic binding protein from mammalian axonal membranes. J. Biol. Chem. **259:** 13241-13245.

Grenningloh, G., Rienitz, A., Schmitt, B., Methfessel, C., Zensen, M., Beyreuther, K., Gundelfinger, E.D. and Betz, H. (1987). The strychnine binding subunit of the glycine receptor shows homology with nicotinic acetylcholine receptors. Nature **328:** 215-220.

Grenningloh, G., Pribilla, I., Prior, P., Multhaup, G., Beyreuther, K., Taleb, O. and Betz, H. (1990a). Cloning and expression of the 58 kd ß subunit of the inhibitory glycine receptor. Neuron **4:** 963-970.

Grenningloh, G., Schmieden, V., Schofield, P.R., Seeburg, P.H., Siddique, T., Mohandas, T.K., Becker C-M. and Betz, H. (1990b). Alpha subunit variants of human glycine receptor: primary structures, functional expression and chromozomal localization of the corresponding genes. EMBO J. **9:** 771-776.

Grippon, J-C. and Hofmann, T. (1981). Inactivation of aspartyl proteinases by butane-2,3-dione. Modification of tryptophan and tyrosine residues and evidence against reaction of arginine residues. Biochem. J. **193:** 55-65.

Grossberg, A.L. and Pressman, D. (1968). Modification of arginine in active sites of antibodies. Biochemistry **7:** 272-279.

Gundersen, C.B., Miledi, R. and Parker, I. (1984) Properties of human brain glycine receptors expressed in *Xenopus* oocytes. Proc. R. Soc. Lond. **221:** 235-244.

Guy, H.R. and Conti, F. (1990). Pursuing the structure and function of voltage gated channels. TINS **13:** 201-206.

Haga, T., Haga, K. and Hume, E.C. (1990). Solubilization, purification and molecular characterization of receptors: principles and strategy. In: Receptor biochemistry: A practical approach. Ed. E.C. Hume. IRL Press.

Halasz, N., Ljungdahl, A. and Hökfelt, T. (1979). Transmitter histochemistry of the rat olfactory bulb. III. Autoradiographic localization of [^{3}H]-GABA. Brain Res. **167:** 221-240.

Hames, B.D. (1983). An introduction to polyacrylamide gel electrophoresis. In: Gel electrophoresis of proteins. Eds. BD Hames and D. Rickwood. IRL Press.

Hamill, O.P., Bormann, J. and Sakmann, B. (1983) Activation of multiple-conductance state chloride channels in spinal neurones by glycine and GABA. Nature **305:** 805-808.

Heidmann, T. and Changeaux, J-P. (1979). Fast kinetic studies on the allosteric interactions between acetylcholine receptor and local anaesthetic binding sites. Eur. J. Biochem. **94:** 281-296.

Heidmann, T., Oswald, R.E. and Changeux, J-P. (1983). Multiple sites of action for non-competitive blockers on acetylcholine rich membrane fragments from *Torpedo marmorata*. Biochemistry **22:** 3112-3127.

Herman, A. and Gorman, A.L.F. (1984). Action of quinidine on ionic currents of molluscan pacemaker neurons. J. Gen. Physiol. **83:** 919-940.

Hill, A.V. (1910). The possible effects of the aggregation of the molecules of haemoglobin on its dissociation curves. J. Physiol. **XL:** iv-vii.

Hille, B. (1970). Ionic channels in nerve membranes. Prog. Biophys. Mol. Biol. **21:** 1-32.

Hille, B. (1977). Local anaesthetics: Hydrophilic and hydrophobic pathways for the drug receptor reaction. J. Gen. Physiol. **69:** 497-515.

Hille, B. (1984a). Mechanisms of block. In: Ionic channels of excitable membranes. Sinauer Associate.

Hille, B. (1984b). Modifiers of gating. In: Ionic channels of excitable membranes. Sinauer Associates.

Hjemland, L.M. (1986). The design and synthesis of detrgents for membrane biochemistry. In: Meths. Enzymol. vol. 124. Ed. P.M. Conn. Academic Press.

Hoch, W., Betz, H. and Becker, C-M. (1989). Primary cultures of mouse spinal cord express the neonatal isoform of the inhibitory glycine receptor. Neuron **3:** 339-348.

Hofstee, B.H.J. (1952). On the evaluation of the constants V_m and K_m in enzyme reactions. Science **116:** 329-331.

Hollmann, M., O'Shea-Greenfield, A., Rogers, S.W. and Heinemann, S. (1989). Cloning by functional expression of a member of the glutamate receptor family. Nature **342:** 643-648.

Honjo, H., Kodama, I., Kamiya, K. and Toyama, J. (1991). Blocking cardiac sodium channels by amiodarone studied by using Vmax of action potential in single ventricular myocytes. Br. J. Pharmacol. **102:** 651-656.

Hoshi, T., Zagotta, W.N. and Aldrich, R.W. (1990). Biophysical and molecular mechanisms of shaker potassium channel inactivation. Science **250:** 533-538.

Ijzerman, A.P. and Soudijn, W. (1989). The antiarrhythmic properties of ß adrenoreceptor antagonists. TIPS **10:** 31-37.

Imoto, T. and Yamada, H. (1989) Chemical modification. In: Protein function a practical approach. Ed T Creighton. IRL Press.

Ishida, Y. (1977). Intralaminar thalamic responses to somatic and fastigial stimulation: cortical inhibition and its blockade by strychnine in cats. Neurpharmacol. **16:** 163-170.

Johnson, J.W. and Ascher, P. (1987). Glycine potentiates the NMDA response in cultured mouse brain neurons. Nature **325:** 529-531.

Johnston, G.A.R. (1968). The intraspinal distribution of some depressant amino acids. J. Neurochem. **15:** 1013-1017.

Johnston, G.A.R. and Iverson, L.L. (1971). Glycine uptake in the central nervous system slices and homogenates: Evidence for different uptake mechanisms in spinal cord and cerebral cortex. J. Neurochem. **18:** 1951-1961.

Jordon, C.C. and Webster, R.A. (1971). Release of acetylcholine and [^{14}C]-glycine from the cat spinal cord *in vivo*. Br. J. Pharmacol. **43:** 441P.

Jordan, L.M. and Phillis, J.W. (1972). Acetylcholine inhibition in the intact and chronically isolated cerebral cortex. Br. J. Pharmacol. **45:** 584-595.

Kaneda, M., Wakamori, M. and Akaike, N. (1989). GABA-induced chloride current in rat isolated Purkinje cells. Am. J. Physiol. **256:** C1153-1159.

Kao, P.N. and Karlin, A. (1986) Acetylcholine receptor binding site contain a disulphide cross link between adjacent half cystinyl residues. J. Biol. Chem. **261:** 8085-8088.

Karlin, A. (1969) Chemical modification of the active site of the acetylcholine receptor. J. Gen. Physiol. **54:** 254s-264s.

Katz, B. and Thesleff, S. (1957). A study of 'desensitization' produced by acetylcholine at the motor end-plate. J. Physiol. **138:** 63-80.

Keinanen, K., Wisden, W., Sommer, B., Werner, P., Herb, A., Vedoom, T.A., Sakmann, B. and Seeburg, P.H. (1990). A family of AMPA-selective glutamate receptors. Science **249:** 556-560.

Kellaris, K.V. and Ware, P.K. (1989). Assessment of the number of free cysteines and the isolation and identification of cystine containing peptides from acetylcholine receptor. Biochemistry **28:** 3469-3482.

Kelly, J.S. and Krnjèvic, K. (1969). The action of glycine on cortical neurones. Exp. Brain Res. **9:** 155-163.

Kilpatrick, G.J., Jones, B.J. and Tyers, M.B. (1987). Identification and distribution of $5HT_3$ receptor in rat brain using radioligand binding. Nature **330:** 746-748.

Klee, M.R. and Faber, D.S. (1971). The effect of strychnine on acetylcholine and dopamine receptors in *Aplysia* neurons. Experientia **27:** 8-9.

Klee, M.R., Faber, D.S. and Heiss, W.D. (1973). Strychnine and pentylenetctrazol-induced changes of excitability in *Aplysia* neurons. Science **179:** 1133-1136.

Kloog, Y., Nadler V. and Sokolovsky M. (1988a) Mode of binding of [^{3}H]-dibenzocycloalkenimine (MK-801) to the N-methyl-D-aspartate (NMDA) receptor and its therepeutic implications. FEBS Lett. **230:** 167-170.

Kloog, Y., Haring, R. and Sokolovsky, M. (1988b). Kinetic characterization of the phencyclidine-N-methyl-D-aspartate receptor interaction: evidence for a steric blockade of the channel. Biochemisty **27:** 843-848.

Koblin, D.D. and Lester, H.A. (1979). Voltage-dependent and voltage-independent blockage of acetylcholine receptors by local anaesthetics in *Electrophorus* Electroplaques. Mol. Pharmacol. **15:** 559-580.

Konigsberg, W. (1972). Reduction of disulphide bonds in proteins with dithiothreitol. In: Methods in Enzymology vol. 25. Eds. C.H.W. Hirs and S.N. Timasheff. Acad. Press.

Krnjèvic, K., Randic, M. and Straughan, D.W. (1966). Pharmacology of cortical inhibition. J. Physiol. **184:** 78-105.

Krogsgaard-Larsen, P., Hjeds, H., Curtis, D.R., Lean, J.D. and Peet, M.J. (1982). Glycine antagonists structurally related to muscimol, THIP, or isoguvacine. J. Neurochem. **39:** 1319-1324.

Kuhse, J., Schmieden, V. and Betz, H. (1990a). A single amino acid exchange alters the pharmacology of the neonatal rat glycine receptor subunit. Neuron **5:** 867-873.

Kuhse, J., Schmieden, V. and Betz, H. (1990b). Identification and functional expression of a novel ligand binding subunit of the inhibitory glycine receptor. J. Biol. Chem. **265:** 22317-22320.

Kuhse, J., Kuryatov, A., Maulet, Y., Malosio, M.L., Schmieden, V. and Betz, H. (1991). Alternative splicing generates two isoforms of the α2 subunit of the inhibitory glycine receptor. FEBS letters **283:** 73-77.

Kumar, K.N., Tilakaratne, N., Johnson, P.S., Allen, A.E. and Michaelis, E.K. (1991). Cloning of cDNA for the glutamate binding subunit of an NMDA receptor complex. Nature **354:** 70-73.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature **227:** 680-6

Langosch, D., Thomas, L. and Betz, H. (1988) Conserved quaternary structure of ligand gated ion channels: The postsynaptic glycine receptor is a pentamer. Proc. Nat. Acad. Sci. **85:** 7394-7398.

Larson, M.D. (1969). An analysis of the action of strychnine on the recurrent IPSP and amino acid induced inhibitions in cat spinal cord. Brain Res. **15:** 185-200.

Le Fort, H., Henke, H. and Cuénod, M. (1978) Glycine specific [^{3}H]-strychnine binding in the pigeon CNS. J. Neurochem. **30:** 1287-1291.

Leonard, R.J., Labarca, C.G., Charnet, R., Davidson, N. and Lester, H.A. (1988). Evidence that M2 membrane spanning region lines the ion channel pore of the nicotinic receptor. Science **240:** 1177-1181.

Light, A., Hardwick, B.C., Hatfield, L.M. and Sondack, D.L. (1969) Modification of a single disulphide bond in trypsinogen and the activation of carboxy methyl derivative. J. Biol. Chem. **244:** 6289-6296.

Liman, E.R., Hess, P., Weaver, F. and Koren, G. (1991). Voltage sensing residues in the S4 region of a mammalian K^+ channel. Nature **353:** 752-755.

Logan, W.J. and Snyder, S.H. (1972). High affinity uptake systems for glycine glutamic and aspartic acids in synaptosomes of rat central nervous tissues. Brain Res. **42:** 413-431.

Lüddens, H. and Wisden, W. (1991). Function and pharmacology of multiple $GABA_A$ receptor subunits. TIPS. **12:** 49-51.

Macadam, S. (1856). Lecture on strychnine. Pharmaceutical Journal. **August-September:** 1-12.

MacDonald, J.F. and Nowak, L. (1990). Mechanisms of blockade of excitatory amino acid receptor channels TIPS. **11:** 1167-1172.

Mackerer, C.R., Kochman, R.L., Shen, T.F. and Hershenson, F.M. (1977). The binding of strychnine and analogs to synaptic membranes of rat brainstem and spinal cord. J. Pharmacol. Exp. Ther. **201:** 326-331.

Maelike, A. (1989). Common structural principles of ion channel proteins. In: molecular biology of neurotransmitters and ion channels. Ed A. Maelike. Springer-Verlag.

Maksay, G. and Ticku, M.K. (1984a) Characterization of γ-aminobutyric acid-benzodiazepine receptor complex by protection against inactivation by group specific reagents. J. Neurochem. **42:** 1715-1727.

Maksay, G. and Ticku, M.K. (1984b). Pretreatment with GABA and modulatory ligands enhances GABA receptor binding. Eur. J. Pharmacol. **104:** 185-188.

Maksay, G. (1990) Dissociation of Muscimol, SR 95531 and strychnine from $GABA_A$ and glycine, receptors respectively suggests similar cooperative interactions. J. Neurochem. **54:** 1961-1966.

Malosio, M.L., Grenningloh, G., Kuhse, J., Schmieden, V., Schmitt, B., Prior, P. and Betz, H. (1991a). Alternative splicing generates two variants of the α1, subunit of the inhibitory glycine receptor. J. Biol. Chem. **266:** 2048-2053.

Malosio, M-L., Marquèze-Pouey, B., Kuhse, J. and Betz, H. (1991b). Widespread expression of glycine receptor subunit mRNAs in adult and developing rat brain. EMBO. J. **10:** 2401-2409.

Marsh, S.J., Stansfield, C.E., Brown, D.A., Davey, R. and McCarthy, D. (1987). The mechanism of action of capsaicin on sensory c-type neurons and their axons *in vitro*. Neuroscience **23:** 275-289.

Martin, I.L. and Candy, J.M. (1978). Facilitation of benzodiazepine binding by sodium chloride and GABA. Neuropharmacol. **17:** 993-998.

Maruhashi, J., Otani, T., Takahashi, H. and Yamada, M. (1956). On the effect of strychnine upon the myelinated nerve fibres of toads. Jap. J. Physiol. **6:** 175-189.

Marvizón J.C.G., Vázquez J., García-Calvo M., Mayor F., Jr., Ruíz-Gómez A., Valdivieso F. and Benavides J. (1986a). The glycine receptor: pharmacological studies and mathematical modelling of the allosteric interaction between the glycine and strychnine binding sites. Mol. Pharmacol. **30:** 590-597.

Marvizón, J.C.G., García-Calvo, M., Vásquez, J., Mayor, Jr., F. Ruíz-Gómez, A., Valdiviesco, F. and Benavides, J. (1986b). Activation and inhibition of [^{3}H]-strychnine binding to the glycine receptor by Eccles' anions: Modulatory effects of cations. Mol. Pharmacol. **30:** 598-602.

Marvizón, J.C.G. and Skolnick, P. (1988). Enhancement of t-[^{35}S]-butylbicyclophosphorothionate and [^{3}H]-strychnine binding by monovalent anions reveals similarities between γ-aminobutyric acid and glycine-gated chloride channels. J. Neurochem. **50:** 1632-1639.

McCarthy, M.P. and Stroud, R.M. (1989). Conformational states of the nicotinic acetylcholine receptor from *Torpedo california* induced by binding agonist, antagonist and local anaesthetics. Equilibrium measurements using tritium hydrogen exchange. Biochemistry **28:** 40-48.

McCormick, D.J. and Atassi, M.Z. (1984). Localization and synthesis of acetylcholine binding site in the α chain of *Torpedo california* acetylcholine receptor. Biochem. J. **224:** 995-1000.

McPherson, G.A. (1983) Analysis of radioligand binding experiments. A collection of computer programs for the IBM PC. J. Pharmacol. Meths. **14:** 213-228.

Melchoir, W.B. and Fahrney, D. (1970). Ethoxyformylation of proteins; reaction of ethoxyformic anhydride with α chymotrypsin, pepsin and pancreatic nuclease at pH 4. Biochemistry **9:** 251-258.

Miles, E.W. (1977) Modification of histidyl residues in proteins by diethylpyrocarbonate. In: Meths. Enzymol. Eds. C.H.W. Hirs and S.N. Timasheff. Acad. Press.

Miller, C. (1989). Genetic manipulation of ion channels: A new approach to structure and mechanism. Neuron **2:** 1195-1205.

Monod, J., Wyman, J. and Changeux, J-P. (1965). On the nature of allosteric transitions: A plausible model. J. Mol. Biol. **12:** 88-118.

Moriyoshi, K., Masayuki, M., Ishii, T., Shigemoto, R., Mizuno, N. and Nakanishi, S. (1991). Molecular cloning and characterization of the rat NMDA receptor. Nature **354:** 31-37.

Mulder, A.H. and Snyder, S.H. (1974). Potassium induced release of amino acids from cerebral cortex and spinal cord slices of the rat. Brain Res. **76:** 297-308.

Muller, W.E. and Snyder, S.H. (1978). Strychnine binding associated with synaptic glycine receptors in rat spinal cord membranes: Ionic influences. Brain Res. **147:** 107-116.

Munson, P.J. and Rodbard, D. (1980). LIGAND: A versitile computerized approach for the characterization of ligand binding systems. Anal. Biochem. **100:** 201-220.

Murakoshi, T. and Otsuka, M. (1985). Respiratory reflexes in an isolated brainstem-lung preparation of the newborn rat: possible involvement of γ-aminobutyric acid and glycine. Neurosci. Letts. **62:** 63-68.

Nahmias, C., Strosberg, D. and Emorine, L.J. (1988). The immune response toward ß-adrenergic ligands and their receptors. VIII Extensive dversity of V_H and V_L genes encoding anti-alprenolol antibodies. J. Immunol. **140:** 1304-1311.

Neher, E. and Sakmann, B. (1976). Single-channel currents recorded from membrane of denervated frog muscle fibres. Nature **260:** 779-802.

Neher, E. and Steinbach, J.H. (1978). Local anaesthetics transiently block currents through single acetylcholine receptor channels. J. Physiol **227:** 153-176.

Neubig, R.R. and Cohen, J.B. (1979). Equilibrium binding of [^{3}H]-tubocurarine and [^{3}H]-acetylcholine by *Torpedo* postsynaptic membranes: Stoichiometry and ligand interactions. Biochemistry **18:** 5464-5475.

Noda, M., Shimizu, S., Tanabe, T., Takai, T., Kayano, T., Ikeda, T., Takahasi, H., Nakayama, H., Kanaoka, Y., Minamino, N., Kangawa, K., Masuo, H., Raftery, M.A., Hirose, T., Inayama, S., Hayashida, H., Migata, T. and Numa, S. (1984). Primary structure of *Electrophorus electricus* sodium channel deduced from cDNA sequence. Nature **312:** 121-127.

Ohnishi, M., Kawagishi, T., Abe, T. and Hiromi, K. (1980). Stopped-flow studies on the chemical modification with N-bromosuccinimide of model compounds of trptophan residues. J. Biochem. **87:** 273-279.

Okada, Y., Onoue, K., Narashima, S. and Yamamura, Y. (1963) Studies on enzymes-antienzymes system II N-bromosuccinimide modified bacterial α-amylase. J. Biochem. **54:** 477-483.

Owen, A.G.W. and Sherrington, C.S. (1911). Observation of strychnine reversal. J. Physiol. **43:** 232-241.

Padlan, E.A. (1977) Structural basis for the specificity of antibody-antigen reactions and structural mechanisms for the diversification of antigen-binding specificities. Quarterly Reviews of Biophysic. **10:** 35-65.

Palacios, J.M., Wamsley, J.K. and Kuhar, M.J. (1981). High affinity $GABA_A$ receptors autoradiographic localization. Brain Res. **222:** 285-307.

Papazian, D., Timple, L., Jan, Y.N. and Jan, L.Y. (1991). Alterations of voltage dependence of a shaker potassium channels by mutation in the S4 sequence. Nature **349:** 305-310.

Pedersen, S.E. and Cohen, J.B. (1990) d-Tubocurarine binding sites are located at α-γ and α-δ subunit interfaces of the nicotinic acetylcholine receptor. Proc. Natl. Acad. Sci. USA. **87:** 2785-2789.

Petersen, M., Pierau, Fr-K. and Weyrich, M. (1987). The influence of capsaicin on membrane currents in dorsal root ganglion neurones of guinea pig and chicken. Pflüggers Arch. **409:** 403-410.

Pfeiffer, F. and Betz, H. (1981). Solubilization of the glycine receptor from rat spinal cord. Brain Res. **226:** 273-279.

Pfeiffer, F., Graham, D. and Betz, H. (1982). Purification by affinity chromatography of the glycine receptor of rat spinal cord. J. Biol. Chem. **257:** 9389-9393.

Pfeiffer, F. (1983). Ph.D thesis, Universitat München, München FRG.

Pfeiffer, F., Simler, R., Grenningloh, G. and Betz, H. (1984). Monoclonal antibodies and peptide mapping reveal structural similarities between subunits of the glycine receptor of rat spinal cord. Proc. Natl. Acad. Sci. **81:** 7224-7227.

Phelan, P.P. (1987). Interactions of strychnine with glycine receptors in mouse spinal cord. Ph.D Thesis. University of London.

Phelan, P.P., Fry, J.P., Martin, I.L. and Johnston, G.A.R. (1989) Polyclonal antibodies to the glycine receptor antagonist strychnine. J. Neurochem. **52.** 1481-1486.

Phillis, J.W. and York, D.H. (1967). Strychnine block of neural and drug induced inhibition in cerebral cortex. Nature **216:** 922-923.

Pinto-Corrado, A. and Longo, V.G. (1961). An electrophysiological analysis of the convulsant action of morphine, codeine and thebaine. Arch. Int. Pharmacodyn. **132:** 255-269.

Postma, S.W. and Catterall, W.A. (1984). Inhibition of binding of [^{3}H]-Batrachotoxinin-A-20-α-Benzoate to sodium channels by local anaesthetics. Mol. Pharmacol. **25:** 219-227.

Probst, R., Corlés, R. and Palacios, J.M. (1986). The distribution of glycine receptors in the human brain. A light microscopic autoradiographic study using [^{3}H]-strychnine. Neurosci. **17:** 11-35.

Quast, U. and Webster, C. (1989). Sulphonylureas and tetralyammonium ions as inhibitors of cromakalim stimulated $^{56}Rb^+$ efflux. Naunyn-Schmiedelberg's Arch. Pharmacol. **339:** (suppl. R64).

Ratnam, M., Le Nguyen, D.L., Sargent, P.B. and Lindstrom, J. (1986). Transmembrane topology of nicotinic acetylcholine receptor: Immunochemical tests contradict theoretical predictions based on hydrophobicity profiles. Biochemistry **25:** 2633-2643.

Rees, A.R. (1987). The antibody combining site: retrospect and prospect. Immunol. Today **8:** 44-45.

Revah, F., Bertrand, D., Galzi, J-L, Dévillers-Thiéry, A., Mulle, C., Hussy, N., Bertrand, S., Ballivet, M. and Changeux, J-P. (1991). Mutations in the channel domain alter densensization of a neuronal nicotinic receptor. Nature **353:** 846-848.

Riordan, J.F. and Valee, B.L. (1972a). Diazonium salts as specific reagents and probes of protein conformation. In: Meths. Enzymol. vol. 25. Eds: C.H.W. Hirs and S.N. Timasheff. Acad. Press.

Riordan, J.F. and Vallee, B.L. (1972b). Nitration with tetrenitromethane. In: Meths. Enzymol. vol. 25. Eds. C.H.W. Hirs and S.N Timasheff. Acad. Press.

Riordan, J.F. and Valee, B.L. (1972c) O-Acetyltyrosine. In: Meths. Enzymol. vol. 25. Eds. C.H.W. Hirs and S.N. Timasheff. Acad. Press.

Riordan, J.F. (1979). Arginyl residues and anion binding sites in proteins. Mol. and Cell. Biochem. **26:** 71-91.

Roberts, P.J. and Mitchell, J.F. (1972). The release of amino acids from the hemisected spinal cord during stimulation. J. Neurochem. **19:** 2473-2481.

Ruíz-Gómez, A., Fernandez-Shaw, C., Valdivieso, F. and Mayor Jr., F. (1989) Chemical modification of the glycine receptor with fluoresein isothiocyanate specifically affects the interaction of glycine with its binding site. Biochem. Biophys. Res. Comm. **160:** 374-381.

Ruíz-Gómez, A., Morato, E., García-Calvo, M., Valdivieso, F. and Mayor Jr., F. (1990). Localization of the strychnine binding site on the 48 kd subunit of the glycine receptor. Biochemistry **29:** 7033-7040.

Schaeffer, J.M. and Anderson, S.M. (1981). Identification of strychnine binding sites in the rat retina. J. Neurochem. **36:** 1597-1600.

Schmieden, V., Grenningloh, G., Schofield, P.R. and Betz, H. (1989). Functional expression in *Xenopus* oocytes of the strychnine binding 48 kd subunit of the glycine receptor. EMBO. J. **8:** 695-700.

Schmitt, B., Knaus, P., Becker, C-M. and Betz, H. (1987). The Mr 93,000 polypeptide of the postsynaptic glycine receptor complex is a peripheral membrane protein. Biochemistry **26:** 805-811.

Schröder, S., Hoch, W., Becker, C-M., Grenningloh, G, and Betz, H. (1991). Mapping of antigenic epitopes on the α1 subunit of the inhibitory glycine receptor. **30:** 42-47.

Schulz, G.E. and Schirmer, R.H. (1979). Amino Acids. In: Principles of protein structure. Eds G.E. Schulz and RH. Schirmer. Springer-Verlag.

Schwarz, W., Palade, P.T. and Hille, B. (1977). Local anaesthetics: Effect of pH on use-dependent block of sodium channels in frog muscle. Biophys. J. **20:** 343-368.

Seitanidou, T., Triller, A. and Korn, H. (1988). Distribution of glycine receptors on the membrane of a central neuron: An immunoelectron microscopic study. J. Neurosci. **8:** 4319-4333.

Shank, R.P. and Aprison, M.H. (1970). Method for multiple analyses of concentration and specific radioactivity of individual amino acids in nervous tissue extracts. Anal. Biochem. **35:** 136-145.

Shapiro, B.I., Wang, C.M. and Narahashi, T. (1974). Effects of strychnine on ionic conductances of squid axon membrane. J. Pharmacol. Exp. Ther. **188:** 66-76.

Shapiro, B.I. (1977a). Effect of strychnine on the potassium conductance of the frog Node of Ranvier. J. Gen. Physiol. **69:** 897-914.

Shapiro, B.I. (1977b). Effect of strychnine on the sodium conductance of the frog Node of Ranvier. J. Gen. Physiol. **69:** 915-926.

Shepherd, G.M. (1974). The synaptic organization of the brain, an introduction. Oxford University Press.

Sherrington, C.S. (1905). On reciprocal innervation of antagonistic muscles. Eighth note. Proc. Roy. Soc. B. **76:** 269-297.

Sigel, E. and Barnard, E.A. (1984). A γ-aminobutyric acid/benzodiazepine receptor complex from bovine cerebral cortex. Improved purification with preservation of regulatory sites and their interactions. J. Biol. Chem. **259:** 7219-7223.

Sokolovsky, M., Fuchs, M. and Riordan, J.F. (1970). Reaction of teranitromethane with tryptophan and related compounds. FEBS letter **7:** 167-170.

Sontheimer, H., Becker, C-M., Pritchett, D.B., Schofield, P.R., Grenningloh, G., Kettenmann, H., Betz, H. and Seeburg, P.H. (1989). Functional chloride channels by mammalian cell expression of rat glycine receptor subunit. Neuron. **2:** 1491-1497.

Spande, T.F., Witkop, B., Degani, Y. and Patchornik, A. (1970). Seletive cleavage and modification of peptides and proteins. In: Advances in protein chemistry. Eds. G.B. Anfunsen, J.T. Edsall and F. Richards. Acad. Press.

Srinivasan, Y., Guzikowski, A.P., Haugland, R.P. and Angelides, K.S. (1990). Distribution and lateral mobility of glycine receptor on cultured spinal cord neurones. Neuroscience **10:** 985-995.

Strader, C.D., Sigal, I.S. and Dixon, R.A.F. (1989). Structural basis of ß-adrenergic receptor function. FASEB J. **3:** 1825-1832.

Strichartz, G.R. and Ritchie, J.M. (1987). The actions of local anaesthetics on ion channels of excitable tissues. In: Local Anaesthetics Handbook of Experimental Pharmacology. 8th Ed. G.R. Strichartz, Springer-Verlag.

Strong, P.N. (1990). Potassium Channel Toxins. Pharmac. Ther. **46:** 137-162.

Stühmer, W., Conti, F., Suzuki, H., Wang, X., Nodar, U., Yahagi, N., Kubo, M. and Numa, S. (1989). Structural parts involved in activation and inactivation of the sodium channel. Nature **339:** 597-603.

Takumi, T., Ohkubo, H., and Nakanishi, S. (1988). Cloning of a membrane protein that induces a slow voltage gated potassium current. Science **242:** 1042-1045.

Tanabe, T., Takeshima, H., Hikami, A., Flockerzi, V., Takahasi, H., Kanagawa, K., Kojima, M., Matusuo, H., Hirose, T. and Numa, S. (1987). Primary structure of the receptor for calcium channel blockers from skeletal muscle. Nature **328:** 313-318.

Tempel, B.L., Papazian, D.M., Schwarz, T.L., Jan, Y.N. and Jan, L.Y. (1987). Sequence of probable potassium channel component encoded at shaker locus of *Drosophila*. Science **237:** 770-774.

Ten Bruggencate, G. and Engberg, I. (1968). Analysis of glycine actions on spinal interneurons by intracellular recording. Brain Res. **11:** 446-450.

Thornton, J.M. (1981) Disulphide bridges in globular proteins. J. Mol. Biol. **151:** 261-287.

Tokutomi, N., Kaneda, M. and Akaike, N. (1989). What confers specificity on glycine for its receptor site. Br. J. Pharmacol. **97:** 353-60.

Triller, A., Clauzeaud, F., Pfeiffer, F., Betz, H. and Korn, H. (1985). Distribution of glycine receptors at central synapses: An immunoelectron microscopy study. J. Cell. Biol. **101:** 683-688.

Triller, A., Clauzeaud, F. and Korn, H. (1987). Gamma-aminobutyric acid containing terminals can be opposed to glycine receptors at central synapses. J. Cell. Biol. **104:** 947-956.

Tunnicliff, G. (1979). Inhibition by pyridoxal-5'-phosphate of γ-aminobutyric acid receptor binding to synaptic membranes of cat cerebellum. Biochem. Biophys. Res. Comm. **87:** 712-718.

Twyman, R.E. and Macdonald, R.L. (1991). Kinetic proprties of the glycine receptor main and subconductance states of the mouse spinal cord neurones in culture. J. Physiol. **435:** 303-331.

Tyndale, R.F., Sunahara, R., Inaba, T., Kalow, W., Gonzalez, F.J. and Niznik, H.B. (1991). Neuronal cytochrome P45011D1 (Debrisoquine/sparteine-Type): Potent inhibition of activity by (-)-Cocaine and nucleotide sequence identity to human hepatic P450 gene CYP2D6. Mol. Pharmacol. **40:** 63-68.

Unnerstall, J.R. (1990) Computer-assisted analysis of binding data. In: Methods in neurotransmitter receptor analysis. Eds. H.I Yamamura, S.J. Enna, and M.J. Kuhar. Raven Press Ltd.

Unwin, N. (1989). The structure of ion channels in membranes of excitable cells. Neuron **3:** 665-676.

Uyeda, K. (1969) Reaction of Phosphofructokinase with maleic anhydride, succinic anhydrude and pyridoxal-5'-phosphate. Biochemistry **8:** 2366-2373.

Valdes, F. and Orrego, F. (1975). Interactions of glycine with brain-cortex membrane fragments: kinetics, ionic requirements and amino acid specificity. Brain Res. **97:** 277-290.

van den Pol, A.N. and Gorc, T. (1988). Glycine and glycine receptor immunoreactivity in brain and spinal cord. J. Neurosci. **8:** 472-492.

Vaughan-Williams, E.M. (1989). Classification of antiarrhythmic actions. In: Antiarrhythmic drugs. Ed. E.M. Vaughn Williams. Springer-Verlag.

Vincent, J.P., Lazdunski, M. and Delage, M. (1970). On the use of tetranitromethane as nitration reagent. The reaction of phenol side chain in bovine and porcine trypsinogens and trypsins. Eur. J. Biochem. **12:** 250-257.

Wang, H.K., Earnest, J. and Limbacher, H.P. (1983). Local anaesthetic membrane interaction: A multiequilibrium model. Proc. Nat. Acad. Sci. **80:** 5297-5301.

Washizu, Y., Bonewell, G.W. and Terzulo, C.A. (1961). Effect of strychnine upon the electrical activity of an isolated nerve cell. Science **133:** 333-334.

Weber, E., Sonders, M., Quarum, M., McLean, S., Pou, S. and Keana, J.F.W. (1986). 1,3-Di (2-[5-^{3}H]-toly) guanidine: A selective ligand that labels σ-type receptors for psychotomimetic opiates and antipsychotic drugs. Proc. Nat. Acad. Sci. **83:** 8784-8788.

Weiland, G., Georgia, B., Lappi, S., Chignell, C.F. and Taylor, P. (1977). Kinetics of agonist mediated transitions in state of the cholinergic receptor. J. Biol. Chem. **252:** 7648-7656.

Werman, R., Davidoff, R.A. and Aprison, M.H. (1966a). Glycine and postsynaptic inhibition in cat spinal cord. The Physiologist, **9:** 318.

Werman, R., Davidoff, R.A. and Aprison, M.H. (1966b). The inhibitory action of cystathione. Life Sci. **5:** 1431-1440.

Werman, R., Davidoff, R.A. and Aprison, M.H. (1967a).Inhibition of motoneurones by ionophoresis of glycine. Nature **214:** 681-683.

Werman, R., Davidoff, R.A. and Aprison, M.H. (1967b). Evidence for glycine as the principal transmitter mediating postsynaptic inhibition in the spinal cord of the cat. J. Gen. Physiol. **50:** 1093-1094.

Werman, R., Davidoff, R.A. and Aprison, M.H. (1968). Inhibitory action of glycine on spinal neurons in cat. J. Neurophysiol. **31:** 81-95.

White, W.F. and Heller, A.H. (1982). Glycine receptor alteration in the mutant mouse *spastic*. Nature **298:** 655-657.

White, W.F. (1985). The glycine receptor in the mutant mouse *spastic (spa)*. Strychnine binding charecteristics and pharmacology. Brain Res. **329:** 1-6.

White, W.F., Brown, K.L. and Frank, D.M. (1989). Glycine binding to rat cortex and spinal cord: binding characteristics and pharmacology reveal distinct populations of sites. J. Neurochem. **53:** 503-512.

White, W.F., O'Gorman, S. and Roe, A.W. (1990). Three dimensional autoradiographic localization of quench-corrected glycine receptor specific activity in mouse brain using [^{3}H]-strychnine as the ligand. J. Neurosci. **10:** 795-813.

Wilkin, G.P., Csillag, A., Balazs, R., Kingsbury, A.E., Wilson, J.E. and Johnson, A.L. (1981). Localization of high affinity [^{3}H]-glycine transport sites in the cerebellar cortex. Brain Res. **216:** 11-33.

Williams, B.L. and Wilson, K. (1981). Chromatographic techniques. In: A biologist's guide to principles and techniques of practical biochemistry. Eds. B.L. Williams and K. Wilson. Arnold.

Woodward, R.B., Cava, M.P., Ollis, W.D., Hunger, A., Daeniker, H.U. and Schenker, K. (1954). The total synthesis of strychnine. J. Am. Chem. Soc. **76:** 4749-4753.

Wu, S.H. and Oertel, D. (1986). Inhibitory circuitory in the ventral cochlear nucleus is probably mediated by glycine. J. Neurosci. **6:** 2691-2706.

Yakushiji, T., Tokutomi, N., Akaike, N. and Carpenter, D.O. (1987). Antagonists of GABA responses studied using internally perfused frog dorsal root ganglion neurones. Neuroscience **22:** 1123-1133.

Yeh, J.Z. (1979). Dynamics of 9-aminoacridine block of sodium channels in squid axons. J. Gen. Physiol. **73:** 1-21.

Yokoto, T., Nishikawa, N. and Nishikawa, Y. (1979). Effects of strychnine of different classes of trigeminal subnucleus caudalis neurons. Brain Res. **168:** 430-434.

Yoshida, K. and Imura, H. (1979). Nicotinic cholinergic receptors in brain synaptosomes. Brain Res. **172:** 453-459.

Young, A.B. and Snyder, S.H. (1973). Strychnine binding associated with glycine receptors of the central nervous system. Proc. Nat. Acad. Sci. **70:** 2832-2836.

Young A.B. and Snyder S.H. (1974a). Strychnine binding in rat spinal cord membranes associated with the synaptic glycine receptor: cooperativity of glycine interactions. Mol. Pharmacol. **10:** 790-809.

Young, A. B. and Snyder, S.H. (1974b). The glycine synaptic receptor: evidence that strychnine binding is associated with the ionic conductance mechanism. Proc. Nat. Acad. Sci. USA. **71:** 4002-4005.

Zarbin, M.A., Wamsley, J.K. and Kuhar, M.J. (1981). Glycine receptor light microscopic autoradiographic localization with [^{3}H]-strychnine. J. Neurosci. **1:** 532-547.

Zukin, S.R., Young, A.B. and Snyder, S.H. (1975). Development of the synaptic glycine receptor in chick embryo spinal cord. Brain Res. **83:** 525-530.