STUDIES ON THE MODULATORY ROLE OF GLYCINE AT THE NMDA RECEPTOR COMPLEX IN SUPRASPINAL REGIONS OF RODENT BRAIN

ALAN EDWARD FLETCHER

THE WORK PRESENTED IN THIS THESIS WAS COMPLETED AT DEPARTMENT OF PHARMACOLOGY, SCHOOL OF PHARMACY, BRUNSWICK SQUARE, LONDON AND AT THE BIOCHEMISTRY DEPARTMENT, IMPERIAL COLLEGE OF SCIENCE, TECHNOLOGY AND MEDICINE, SOUTH KENSINGTON, LONDON.

PRESENT ADDRESS:

MERCK SHARP AND DOHME,
NEUROSCIENCE RESEARCH CENTRE,
TERLINGS PARK,
HARLOW.
ESSEX.

THESIS SUBMITTED IN PART FULFILMENT FOR THE DEGREE OF DOCTOR OF PHILOSOPHY FROM THE UNIVERSITY OF LONDON, APRIL 1991.

Abstract.

A quantitative autoradiographical technique has been employed to investigate the regional distribution of the strychnine-insensitive glycine receptor in rat brain. A heterogenous pattern of binding was observed throughout the regions studied, the highest binding being localised in the CA1 stratum radiatum and the dentate gyrus molecular layer. An evaluation of the pharmacological specificity of the receptor revealed a single population of sites, which displayed a complex agonist/antagonist structure activity relationship.

Quantitative autoradiography has been used to evaluate the enhancement of ^{3}H-MK-801 binding produced by glutamate and glycine in a number of discrete brain regions. The study was extended to include the effect of extended preincubation times on the regional enhancement. In addition, the effects of the NMDA antagonists, 3-[(±)-2-carboxypiperazin-4-yl]-1-phosphonic acid (CPP) and 7 chloro kynurenic acid (7 ClKYN) on the regional binding of ^{3}H-MK-801 has been evaluated, together with an autoradiographical demonstration of the competitive nature of these antagonists. The lack of regional variation observed throughout the studies suggest that, at least in the regions studied, the relationship between the sites on the NMDA receptor complex is uniform.

An *in vitro* slice preparation has been used to evaluate the nature of glycine release in rat cerebellum and hippocampus. High K^{+} (30-55mM) stimulated release of endogenous and tritiated glycine from cerebellar slices was calcium

dependent. In contrast ^{3}H-glycine release from hippocampal slices was calcium independent. Excitatory amino acid stimulated release of endogenous and tritiated glycine release from cerebellar slices in a dose dependent manner. In addition a pharmacological and autoradiographical determination of the strychnine-insensitive glycine receptor in the cerebellum was localised (> 90%) in the granule cell layer.

A preliminary investigation of NMDA receptor mediated enhancement of cGMP levels in slices (chopped and handcut) of rat (8-day and adult) and gerbil is described, together with an evaluation of NMDA-mediated enhancement in cortical cell cultures.

Dedicated to the memory of

Peter and Betty Morgan.

Acknowledgements.

I would firstly like to thank my supervisors Prof Norman Bowery and Dr Graham Wilkin for their help, guidance and encouragement throughout the past three years. Special thanks go to Richard Davey, Christine Knott, Janet Maguire, Dave MaCarthey and Gerard Pratt for keeping me on the straight and narrow and ensuring that I made it to the end of my Ph.D.

I would also like to express my grateful thanks to Derek King for educating me in the traditions of the School, and for providing the excellent art work presented in this thesis. Where would we be without you ?????

In addition my thanks go to Michelle Qume for her patience and assistance during the compilation of the manuscript, and to Mark, Sarah and Paul for keeping me sane, fed and watered during the months of writing.

Thanks also are due to all members of Imperial college and SOP for making my stay an enjoyable experience. In addition I would like to thank Dr John Garthwaite, University of Liverpool for the generous gift of antisera used during this study.

Finally I would like to thank me parents for their unlimited support and encouragement and without whom I would not have been able to complete this thesis.

CONTENTS

Experimental.

2: Characterisation of the Strychnine-insensitive glycine receptor.

3: Agonist and Antagonist modulation of ^{3}H-MK-801 binding.

List of Figures

List of Tables

Abbreviations.

ACBC	aminocyclobutane carboxylic acid
ACCP	1-Amino,1,3,dicarboxycyclopentane
Ach	acetyl choline
ACPC	aminocyclopropane carboxylate
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propanoic acid
ANOVA	analysis of variants
AP4	2-amino-4-phosphonobutanoate
AP5	2-amino-5-phophonopentanoate
ASP-AMP	β-D-aspartylamino methyl phosphonate
Ba	barium
BMAA	β-N-methyl-amino-L-alanine
B_{MAX}	receptor number
BOAA	β-N-Oxalylamino-L-alanine
CCPG	cis-D,3,4-cyclopropylglutamate
Cd	cadmium
cGMP	cyclic-3-5-guanosine monophosphate
CGP 37849	DL-(α)-2-amino-4-methyl-5-phosphono-3-pentanoic acid
CGP 39551	Ethyl DL-(α)-2-amino-4-methyl-5-phosphono-3-pentanoate
CGS 19755	1-cis-2-carboxypiperdine-4-yl methyl-1-phosphonate
7-Cl KYN	7-chloro kynurenic acid
CMO	cis,2,4,methanoglutamate
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CNS	central nervous system
Co	cobalt
CPP	3-((-)-2-carboxypiperazine-4-yl)propyl-1-phosphonate
CPP-ene	3-((-)-2-carboxypiperazine-4-yl) propenyl-1-phosphonate
CSF	cerebral spinal fluid
D-CA	D-cysteic acid
DA	dissociative anaesthetic
DAA	α-aminoadipate

DAP	D-aminopimelic acid
DAS	D-aminosuberate
DG	1,2 diacylglycerol
DGG	γ-D-glutamylglycine
DQCA	6.7 Dichloro-3-hydroxy-1-quinoxalate carboxylic acid
DNA	deoxyribonucleic acid
DNQX	6-7-dinitroquinoxaline-2-3-dione
DTG	Di-O-tolyl-guanidine
EAA	excitatory amino acid
EDRF	endothelium derived relaxing factor
EGTA	ethyleneglycol-bis-(β-amino ethyl ether) N,N'-tetracetic acid
EPSC	excitatory postsynaptic current
EPSP	excitatory postsynaptic potential
GABA	γ-aminobutyric acid
GDEE	glutamate diethyl ester
GLU-AMP	γ-D-glutamyl amino methyl phosphonate
GTP	guanosine 3-5-triphosphate
^{3}H-	tritium
HA-966	3-amino-1-hydroxy-2-pyrrolidone
HCSA	Homocyteine sulfinic acid
5-HT	5 Hydroxy tryptamine
IP_3	inositol-1,4,5,triphosphate
IPSP	inhibitory postsynaptic potential
JSTX	Joro spider toxin
Kd	dissociation constant
KH	Krebs Henseleit
L-BMAA	β-N-methylamino-L-alanine
LI	immunoreactivity
LTP	long term potentiation
Mg^{2+}	magnesium
mins	minutes
MK-801	(+)-5-methyl-10,11-dihydro-5H-dibenzo-[a,d]cyclohepten-5,10-imine

	maleate
MNQX	6,8,dinitroquinoxaline
mRNA	messenger ribonucleic acid
mV	milli volts
NAAG	N-acetyl aspartyl glutamate
Ni	nickel
NMA	N-methyl aspartate
NMDA	N-methyl D-aspartate
NMLA	N-methyl-L-aspartate
NMMA	N-mono-methyl-L-arginine
NO	nitric oxide
NO_2	nitrogen dioxide
NPC 12626	2-amino-4,5,(1,2-cyclohexyl)-7-phosponoheptanoic acid
PCP	phencyclidine
PI	phosphoinositol
PKC	protein kinase C
pS	pico seemen
PTX	Pertussis toxin
S.A.	specific activity
S-(L)CA	S-(L) cysteic acid
SCN^-	thiocyanate
sec	second
SEM	standard error of the mean
SKF 10047	N-allylnormetazocine
TCAD	tricyclic antidepressant
TCP	N-(1-thienyl)-cyclohexyl-3,4,piperidine
TRANS-ACPD	trans-1-amino-cyclopentyl-1,3-dicarboxylate
Tris	2-amino-2-hydroxymethyl propane-1,3,diol.
Zn^{2+}	zinc

CHAPTER 1 - GENERAL INTRODUCTION.

Chapter 1.

1.1: Historical developments.

Today the acidic amino acid L-glutamate is widely accepted as the major excitatory amino acid mediating fast synaptic transmission in the mammalian central nervous system. The origins of this statement stem from early scientific discoveries of Hayashi (1954), that the L-isomeric forms of the amino acids glutamate and aspartate exhibited a degree of convulsant activity, and the subsequent observations that both these acids displayed excitatory and depolarising properties on single cat spinal cord neurones (Curtis *et al.*,1960). A lack of stereoselectivity however indicated that these effects were not receptor mediated. The synthesis of a number of analogues of glutamate and aspartate, including a range of N-alkyl enantiomers (Watkins,1962) and the structure activity reports of Curtis and Watkins (1963) enabled elucidation of the requirements for excitation in neurones, based on a so-called "3-point attachment" receptor model. Compounds which exhibited excitatory properties possessed a number of common features; an acidic group in an α position to an amino group (preferably primary) and 2 or 3 carbon atoms distinct to another acidic group, subsequent side chain attachment decreased the excitatory properties. Of the analogues tested, the most potent and stereo selective was N-methyl D-aspartate (NMDA).

The initial suggestion of multiple receptor existence originated with observed differential potencies of L-glutamate in different thalamic regions (McLennan *et al.*,1968). Similarly L-glutamate and L-aspartate were reported to display

small, yet significant differences in excitatory potentials on spinal cord neurones (Duggan,1974). NMDA and kainic acid (a conformationally restricted form of glutamate) displayed greater "disparity" than glutamate and aspartate on dorsal horn interneurones and ventral horn Renshaw calls (McCulloch *et al.*,1974), confirming the earlier suggestions of Johnston *et al.*,(1974) that conformationally restricted agonists may lead to differential receptor interaction, with the suggestion of "glutamate" and "aspartate" preferring sites, currently recognised as quisqualate and NMDA receptors. Major advances in the identification of excitatory amino acid (EAA) receptor classes came with the introduction of selective antagonists, glutamate diethyl ester (GDEE) (Haldeman and McLennan,1972) and 3-amino-1-hydroxy-2-pyrrolidone (HA-966) (Curtis *et al.*,1973). The former depressed L-glutamate responses in rat central neurones, whilst the latter inhibited homocysteate responses to a greater extent than those of glutamate in spinal cord. In addition pentobarbitone, chlorpromazine and the tricyclic antidepressant (TCAD) amitryptyline were all reported to possess preferential antagonist properties for EAA receptors (Evans *et al.*,1977). Definitive receptor separation was defined later in the same decade using the selective antagonists Mg^{2+} (Evans *et al.*,1978), HA-966 (Evans *et al.*,1978) and D-α aminoadipate (DAA) a selective NMDA blocker (Biscoe *et al.*,1977). All displayed a similar pattern of antagonism to NMDA-induced depolarisations, whilst having little or no effect on responses to quisqualate and kainate, findings confirmed in subsequent electrophysiological (Davies *et al.*,1979; McLennan and Lodge,1979) and radioligand binding studies (Michaelis *et al.*,1974; Foster and Roberts,1978;

London and Coyle,1979). Early binding studies were prone to inconsistencies attributed to the presence of Cl^- ions in the assay buffers. These ions were found to increase glutamate binding by means of promoting interaction with a specific uptake system in preference to a receptor (Mena *et al.*,1982), resulting in a pharmacologically distinct profile from that ascribed to EAA receptors (Fagg *et al.*,1982).

Receptor autoradiographical studies (Monaghan *et al.*,1983) provided differential separation of ^{3}H-glutamate binding to kainate, NMDA and quisqualate receptors. The distributions and pharmacological profiles of each EAA receptor coincided with the radioligand binding of ^{3}H-AMPA for quisqualate (Honore *et al.*,1982; Monaghan *et al.*,1984), ^{3}H-kainate for kainate (London and Coyle,1979; Monaghan and Cotman,1982) and ^{3}H-AP5 (2-amino-5-phosphonopentanoate),a potent and selective NMDA antagonist previously described by Davies *et al.*,(1981), for NMDA (Monaghan *et al.*,1984; Olverman *et al.*,1984). Over the past decade many new and selective agents have been developed, these will be discussed later.

1.2: Current receptor classification.

Current understanding of the synaptic responses elicited by EAAs, suggests the existence of at least 5 different receptor subtypes. As previously described three of these receptors, NMDA, kainate and quisqualate were classified during the 1970's and early 80's and are defined after their respective selective agonists. The classical quisqualate receptor has recently been renamed the AMPA

receptor after the more selective ligand α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (Krogsgaard-Larsen *et al.*,1980). The existence of a fourth subtype, the L-AP4 (2-amino-4-phosphonobutyrate) receptor, was suggested following the antagonistic profile of AP4 at excitatory synapses in brain and spinal cord (Foster and Fagg,1984). Recently links between a fifth receptor, the metabotropic receptor, and phosphoinositol (PI) metabolism have been suggested (Sladeczek *et al.*,1985; Schoepp and Johnson,1988), however research into this particular subtype remains in its infancy.

1.3: N-methyl D-aspartate receptor complex.

The N-methyl D-aspartate subtype of glutamate receptor has been the focus of research into the involvement of EAAs in neurological function and synaptic transmission due to the availability of a wide range of selective agonists and antagonists and the advancements in electrophysiological and neuroanatomical techniques. The use of these selective agents and high affinity radioligands, has enabled researchers to elucidate the detailed structure of this complex ionotropic receptor which, in addition to a transmitter recognition site, possesses a number of functional domains, each with discrete ligand binding sites. To date this complex is thought to contain 5 distinct sites which include;

i) Transmitter recognition or binding site for L-glutamate.

ii) Regulatory or coactivator site for glycine.

iii) Channel located dissociative anaesthetic site for phencyclidine and related compounds.

iv) Voltage dependent Mg^{2+} binding site.

v) Inhibitory divalent cation site for Zn^{2+} and TCAD.

An additional site for the polyamines has also been postulated, however the location and whether this site is a distinct entity remains unclear. A current visualisation of the NMDA receptor complex is shown in Figure 1.1.

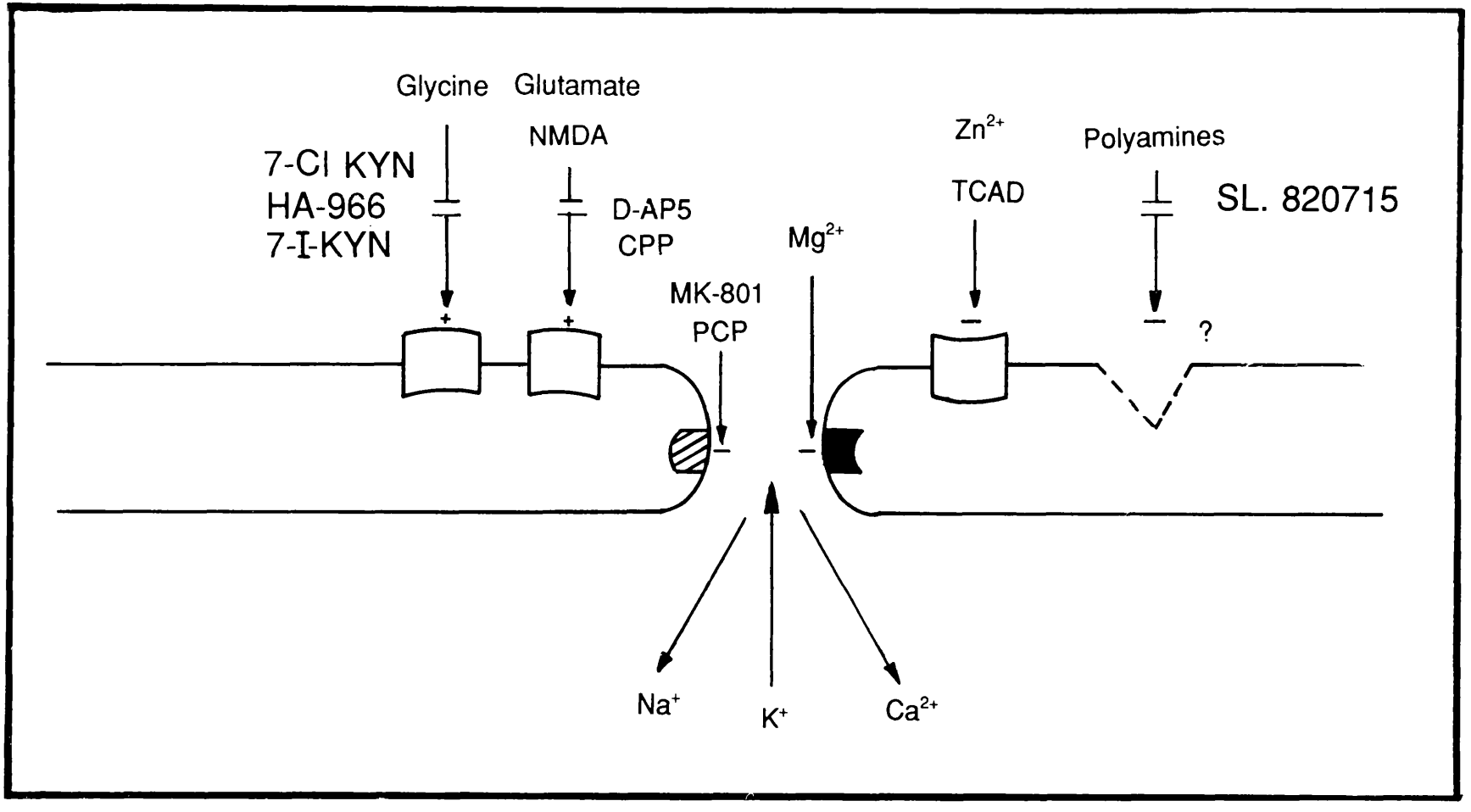
1.3.1: Selective NMDA agonists and antagonists.

1.3.1.1: Competitive glutamate agonists.

Unlike antagonists, the structure activity relationships of agonists cannot be readily deduced from electrophysiological studies. Thus acquisition of knowledge requires a comparison of the effects of small structural differences on activity in a number of preparations (Watkins,1989). A number of selective NMDA agonists have been described, however L-glutamate remains the ligand of highest affinity. In addition to NMDA, two synthetic analogues of aspartate, namely NMLA and the racemic mixture NMA, have been shown to possess agonistic activity (Curtis *et al.*,1961). A number of sulphur-containing analogues of L-glutamate also act at NMDA receptors. Homocysteine sulfinic acid (HCSA) (Curtis *et al.*,1961) displayed actions which were inhibited by AP5, whilst L-HSCA, S-(L) cysteic acid (S-(L)CA) and D-cysteic acid (D-CA) can be considered as mixed agonists whose actions are predominantly mediated via NMDA receptors (Do *et al.*,1986; McLennan and Lodge,1979; Turski *et al.*,1987). Ibotenate, quinolinate and N-acetyl aspartyl glutamate (NAAG) are all weak agonists (Johnston *et al.*,1968; Martin and Lodge,1987; Joels *et al.*,1987) although some doubt exists as to whether NAAG elicits its actions via NMDA or L-AP4 receptors (Westbrook *et al.*, 1986). Recently, a number of new

Fig 1.1: A schematic illustration showing the various sites of interaction at the NMDA receptor-ion channel complex.

The effect on channel conductance of binding at each site is denoted by (+) representing an increase and (-) a decrease in conductance. Antagonists are indicated by two bars crossing arrow. Abbreviations as listed in text.


Glycine
Glutamate
NMDA
7-Cl KYN
HA-966
7-I-KYN
D-AP5
CPP
MK-801
PCP
Mg2+
Zn2+
TCAD
Polyamines
SL. 820715
?
Na+
K+
Ca2+

agonists have been reported, these include: 1-amino,1,3,dicarboxycyclopentane (ACCP) (Monahan and Michel,1987) and Cis-D,3,4-cyclopropylglutamate (CCPG) (Monahan *et al.*,1990). Both compounds have been reported to possess similar potencies to glutamate whilst exhibiting a greater degree of selectivity for NMDA receptors.

1.3.1.2: Competitive glutamate antagonists.

Much of our current understanding of the role and structure of NMDA receptors arises as a direct result of the development of selective antagonists. Since the introduction of the early antagonists eg: D-aminopimelic acid (DAP) and D-α-aminoadipate (DAA) (Evans *et al.*,1978; Collingridge and Davies,1979) a number of more potent compounds have been developed. The phosphonic carboxylic acid derivative D-2-amino-5-phosphonovalerate (AP5) (Evans *et al.*, 1982) formed by the replacement of the α-carboxy terminal of D-L-α-aminoadipate (DLAA), and D-2-amino-7-phosphonovalerate (AP7) (synthesised by corresponding substitution of D-aminosuberate (DAS)) are but two of a number of potent selective agents which include the peptides α-D-aspartylaminomethyl phosphonate (ASP-AMP) and α-D-glutamylamino-methyl phosphonate (GLU-AMP) (Jones *et al.*,1984), guanosine 3-5-triphosphate (GTP) (Monahan *et al.*,1988), and the structurally more rigid analogue of AP7, 3-((-)-2-carboxypiperazin-4-yl)propyl-1-phosphonate (CPP) (Lehmann *et al.*,1987). More recently Lehmann *et al.*,(1988) have described the antagonist activity of 1-cis-2-carboxypiperdine-4-yl methyl-1-phosphonate (CGS 19755), the CPP equivalent of AP5, whilst 2-amino-4,5,(1,2-cyclohexyl)-7-

phosphonoheptanoic acid (NPC 12626) antagonises glutamate responses in rat and gerbil (Ferkany *et al.*,1989). In addition a number of orally active antagonists CGP 37849 (DL-(α)-2-amino-4-methyl-5-phosphono-3-pentanoic acid), CGP 39551 and CPP-ene (3-((-)-2-carboxypiperazine-4-yl) propenyl-1-phosphonate) have been reported (Herrling *et al.*,1989; Fagg *et al.*,1990; Lowe *et al.*,1990).

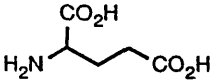
1.3.1.3: Non-competitive antagonists.

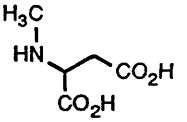
Non-competitive NMDA receptor antagonist is a term which can be applied to a series of compounds exerting their actions at sites other than the transmitter recognition site. As previously mentioned the receptor complex is thought to be comprised of 4 additional sites at which antagonists may exert their effects. These include the glycine site for 7-chlorokynurenic acid (7ClKYN) and HA-966 (Kemp *et al.*,1988), the dissociative anaesthetic site for phencyclidine (PCP), TCP and MK-801 (Loo *et al.*,1986; Wong *et al.*,1986) and the divalent cation sites for Mg^{2+} and Zn^{2+}, which appear to act at distinct sites (Ault *et al.*,1980; Mayer and Westbrook,1987). In addition antagonists which interact with the polyamine site have also been reported as non competitive NMDA antagonists, these include SL-820715 (Roa *et al.*,1989) and ifenprodil (Schoemaker *et al.*,1990).

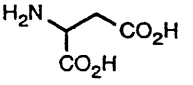
Figure 1.2 illustrates the structures of a number of the current agonists and antagonists of the NMDA receptor.

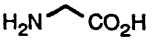
Fig 1.2: Structures of a number of selective agonist and antagonists which interact with the various sites associated with the NMDA receptor-ion channel complex.

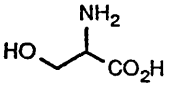
NMDA Agonists / Antagonists

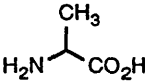

Glutamate

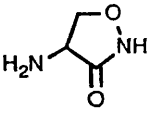

NMDA

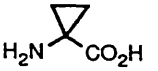

Aspartate

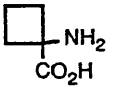

Glycine

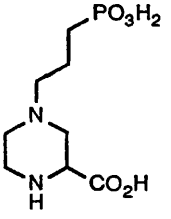

D-Serine

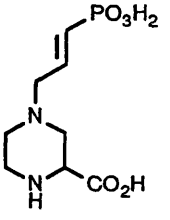

D-Alanine

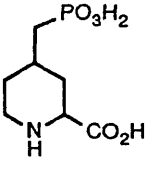

D-Cycloserine

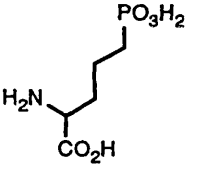

ACPC

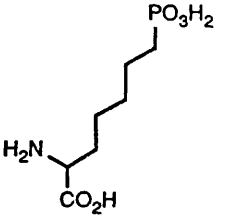

ACBC

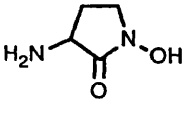

CPP

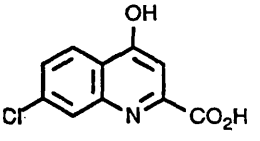

CPP-ene

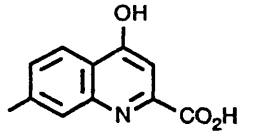

CGS 19755

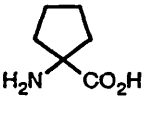

D-AP5

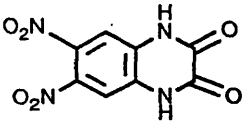

D-AP7

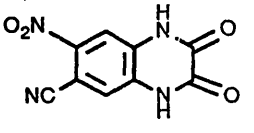

(R) - HA966

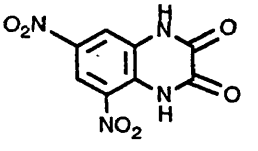

7-ClKyn

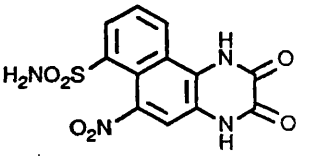

7-Iodo-Kyn

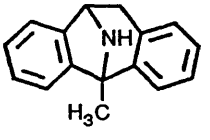

Cycloleucine

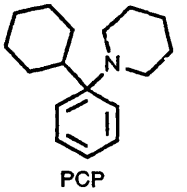

DNQX

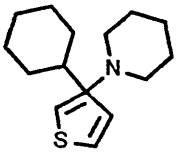

CNQX

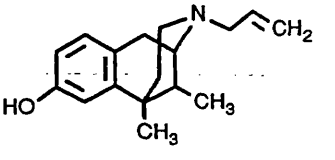

MNQX


NBQX


MK - 801


PCP


TCP

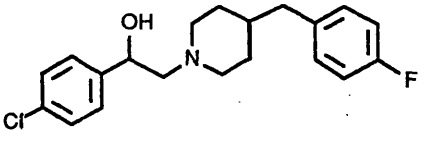

SKF 10,047

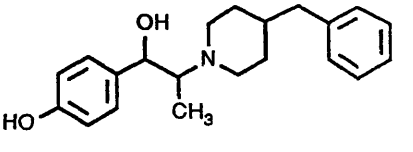
$H_2N(CH_2)_4NH(CH_2)_3NH_2$

Spermidine

$H_2N(CH_2)_4NH_2$

Putrescine


SL 820715


Ifenprodil

1.3.2: Receptor binding sites on the NMDA complex.

1.3.2.1: Glutamate recognition site.

The biochemical characterization of this site has been aided through the availability of a number of selective radiolabelled agonists eg: ^{3}H-glutamate and antagonists eg: CPP, CGS 19755 . The ability of ^{3}H-glutamate to bind to a site associated with the NMDA receptor in a Cl^-/Ca^{2+} independent manner was first reported by Fagg *et al.,(*1982), however the specificity of ^{3}H-glutamate as a selective ligand has been questioned following reports of interactions with other types of EAA receptors namely AMPA and kainate. Foster and Fagg (1984) demonstrated that glutamate displayed the greatest degree of specific binding and the highest affinity within a number of ^{3}H-ligands (L-aspartate, L-AP5 and NMDA) confirming earlier reports by Olverman *et al.,(1984)*. Furthermore the extent of the non-NMDA interaction has been attributed to as low as 10% of the total binding in both postsynaptic density and synaptic membrane preparations (Monahan and Michel,1987; Foster and Fagg,1987). ^{3}H-NMDA has been reported to display a high degree of non-specific binding together with a low affinity for the receptor (Snodgrass,1979) and has therefore been of little use as an NMDA receptor ligand .

Radiolabelled antagonists have proved to be useful ligands of the NMDA site. The original antagonist of choice ^{3}H-APV, has been superseded by ^{3}H-CPP and CGS 19755 which exhibit higher affinity for the receptor (Lehmann *et al.,*1987; 1988). ^{3}H-APV was used in a number of early experiments but it had a limited use due to rapid dissociation and a high degree of non-specific binding.

1.3.2.2: Glycine binding site.

The presence of a distinct glycine binding site in supraspinal regions of mammalian CNS (central nervous system) was first reported by Kishimoto *et al.*,(1981). This receptor appeared to differ from the classical inhibitory receptor associated with the spinal cord (Young and Snyder,*1973*) since it was insensitive to strychnine. The physiological significance of this receptor was elucidated following the discovery that glycine could modulate NMDA induced conductance in cultured mouse neurones (Johnson and Ascher,1987). Moreover autoradiographical evidence (Bristow *et al.*,1986; Bowery,1987) demonstrated a direct correlation between the anatomical localisation of this receptor with that previously reported for ^{3}H-glutamate (Monaghan and Cotman,1985). These observations favoured a close association between NMDA and glycine. A more detailed appraisal of the strychnine-insensitive binding site can be found later.

1.3.2.3: Dissociative anaesthetic binding site.

Biochemical and physiological evidence have demonstrated the interaction of phencyclidine with two distinct sites in the CNS (Largent *et al.*,1986). The first σ site can be labelled with selective ligand ^{3}H-SKF 10047 (N-allylnormetazocine) (Tam and Cook,1984) and the more potent di-O-tolyl-guanidine (DTG) (Weber *et al.*,1986). A second high affinity and stereoselective phencyclidine preferring site was demonstrated by Vigon *et al.*,(1983). Evidence suggests that this latter site is associated with the NMDA receptor (Coan and Collingridge,1987a; Kemp *et al.*,1987). Selective ligands include ^{3}H-PCP, the thienyl derivative ^{3}H-TCP (N-(1-thienyl)-cyclohexyl-3,4,piperidine) (Loo *et*

al.,1986) and the most potent, ^{3}H-MK-801 (Wong *et al.*,1986). Recent observations (Loo *et al.*,1986) of interactions between ^{3}H-TCP binding and selective NMDA ligands have suggested a channel localisation for this receptor site and the evidence for this concept will be presented later.

1.3.3: NMDA receptor ion channel.

The individual properties of the NMDA receptor complex are usually attributed to the associated ion channel through which selective agents gate their responses. The NMDA ion channel is a 50pS channel which displays a non-linear conductance between -80 and +60 mV (Ascher *et al.*,1988). Other conductance states have been demonstrated however the transition state data analysis favours a single channel population (Cull-Candy and Usowicz,1987; Jahr and Stevens,1987). Reports of negative slope conductance for NMDA and L-aspartate induced currents, consistent with a region of voltage dependence (MacDonald *et al.*,1982; Mayer and Westbrook,1985), provided a major breakthrough in our understanding of NMDA receptor properties. Major agonist currents were greatest at moderate depolarising potentials (-30 to -20 mV) and reversed at about 0 mV (Ascher *et al.*,1988). The voltage dependence was attributed to a blockade of the ion channel by Mg^{2+} (Ascher *et al.*,1988; Nowak *et al.*,1984), at potentials more negative than -30 mV. Ion permeability studies revealed the channel to be equipermeable to Na^{+}, K^{+} and Cs^{+} (Ascher *et al.*,1988), but in addition to other EAA receptor channels, the NMDA channel displayed a permeability to Ca^{2+}. Voltage clamp studies with Ca^{2+} sensitive dyes demonstrated that NMDA induced channel opening allowed the

influx of Ca^{2+} in a manner independent of the voltage dependent Ca^{2+} channel entry (Mayer *et al.*,1987). Furthermore the reversal-potential of NMDA induced currents was altered by fluctuations in extracellular Ca^{2+} levels (Ascher *et al.*,1988; Jahr and Stevens,1987). This influx of calcium is associated with the physiological effects of NMDA eg: excitotoxicity and long term potentiation (LTP). In addition the probability of channel opening has been demonstrated to be modified by glycine (Johnson and Ascher,1987).

1.3.3.1: Mechanisms of channel blockade.

1.3.3.1.1: Mg^{2+} and divalent cations.

The ability of Mg^{2+} ions to inhibit NMDA agonist evoked depolarisations was first demonstrated in spinal cord preparations (Ault *et al.*,1980). Subsequent voltage clamp studies on cultured neurones revealed the inhibition to be non-competitive and voltage dependent (MacDonald *et al.*,1982). Blockade by Mg^{2+} is characterised by a "fast open channel" blockade, present at negative membrane potentials. Single channel currents are interrupted by transient blockade, such that the currents appear as bursts of brief openings, separated by brief closures (Ascher *et al.*,1988). The open time was demonstrated to be dependent upon the Mg^{2+} concentration, whilst the closure time remained independent (Ascher *et al.*,1988). Mg^{2+} blockade fails to fulfil earlier criteria for fast channel blockade (Neher and Steinbach,1978) since the burst duration, in the presence of elevated Mg^{2+}, is either unaltered or decreased (Ascher *et al.*,1988) in contrast to the characteristic increase associated with a fast channel blockade. Explanations of this phenomenon are wide ranging ; Mg^{2+}

blockade has been likened to the closure of the channel prior to removal of the ion, the onset of an additional slow channel blockade (Ascher *et al.*,1988) or as most recently suggested the excitation of closed channels (MacDonald and Nowak,1990).

One additional aspect of the Mg^{2+} blockade that remains unresolved is the localisation of the site of interaction within the channel. Explanation of the voltage dependence of open channel blockade was initially proposed by Woodhull (1973), with the strength of the dependence related to the distance travelled by the blocker molecule across a linear, and assumed constant, transmembrane electric field. On this basis the Mg^{2+} binding site is hypothesised to be situated toward the cytoplasmic side of the cell deep in the channel (Ascher and Nowak,1988a). This original observation is incompatible with the report of Ascher *et al.*,(1988) who demonstrated that NMDA blockade by intracellular Mg^{2+} showed an inverse voltage dependence compared to that for extracellular Mg^{2+}. These data point to the possible existence of two binding sites for Mg^{2+} or a single site mid way up the channel (Johnson and Ascher,1990).

In addition to the electrophysiological data, biochemical evidence exists which demonstrates the Mg^{2+} blockade of the action of NMDA. In cultured mouse neurones Mg^{2+} inhibits NMDA receptor induced Ca^{2+} influx (Murphy *et al.*,1987a) cyclic-3-5-guanosine monophosphate (cGMP) formation (Novelli *et al.*,*1987*) and arachidonic acid release (Lazarewicz *et al.*,1988).

Cation permeation through NMDA channels displays a high correlation with the ease of hydration of the individual cation (Coan and Collingridge,1987) Antagonistic cations (Ni^{2+},Co^{2+},Mg^{2+}) were found to dehydrate slowly, in contrast to ions which possessed a fast rate of dehydration and displayed greater permeability (Ca^{2+},Ba^{2+},Cd^{2+}).

Zn^{2+} attenuation of NMDA-agonist induced depolarisations in cortical neurones has been reported (Peters *et al.*,1987). In contrast to Mg^{2+}, the non-competitive inhibition by Zn^{2+} of NMDA induced responses in hippocampal neurones displayed voltage dependency (Westbrook and Mayer,1987). This would suggest a distinct site of action for Zn^{2+}, probably located outside the channel. Recently, Zn^{2+} has been reported to inhibit NMDA receptors via an non-competitive interaction with glycine binding (Yeh *et al.*,1990), whilst having opposing effects on NMDA and non-NMDA receptors expressed in Xenopus oocytes (Rassendren *et al.*,1990). These findings provide further evidence to support the concept of a Zn^{2+} binding site associated with the NMDA receptor complex.

1.3.3.1.2: Blockade by Phencyclidine.

Early reports of a non-competitive antagonism of NMDA receptor function by the dissociative anaesthetics ketamine and phencyclidine (Lodge *et al.*,1982) have been substantiated in a number of preparations (Duchen *et al.*,1985; Harrison and Simmonds,1985; Thompson *et al.*,1985). Furthermore TCP and MK-801 display greater selectivity and potency than PCP (Wong *et al.*,1986). The actions of these compounds have been shown to be use and voltage

dependent (Mayer *et al.*,1988; Honey *et al.*,1985; Woodruff *et al.*,1987), consistent with a blockade of the cation channel associated with the NMDA receptor, at a site situated inside the channel. Support for this concept arises from electrophysiological studies on single channels. PCP and MK-801 reduce channel opening frequency and mean opening time without affecting conductance (Bertolino *et al.*,1988; Huettner and Bean,1988). Similarly Mg^{2+} inhibits MK-801 blockade in a voltage dependent manner, suggesting a close anatomical association of the respective sites within the channel (Huettner and Bean,1988). Radioligand binding data acts to confirm the association of the PCP binding site with the NMDA receptor, and demonstrates the use dependent activation of this site (Loo *et al.,1986*). A number of complex site interactions have been described involving PCP, and these will be discussed at length in subsequent chapters.

Anatomical localisation of the phencyclidine binding site remains a subject of speculation (as is the case of Mg^{2+}). Original suggestions (Mayer *et al.,* 1988), based on the Woodhull model of electric field, placed the site deep in the channel. By contrast the reports of Sernagor *et al.,(*1989) of a voltage dependent blockade by neutral tricyclic antidepressants, suggests that the voltage dependency arises from a source other than the influence of the electric field on the molecule. Furthermore Huettner and Bean (1988) have demonstrated a voltage independent component to the association rate of MK-801, a factor inconsistent with a site located deep within a channel. The most recent hypothesis places the site near the mouth of the channel (MacDonald

and Nowak,1990).

1.3.4: Colocalisation of NMDA, PCP and Glycine binding sites.

Evidence indicates that the allosteric modulation of the NMDA receptor is due to a colocalistion of NMDA, PCP and glycine binding sites on the receptor complex. Functional NMDA receptors expressed in Xenopus oocytes displayed inhibition by PCP and potentiation by glycine (Kushner *et al.*,1988; Kleckner and Dingledine,1988). In addition the autoradiographical distribution reported for L-glutamate (Monaghan and Cotman,1985), AP5 (Monaghan *et al.*,1984) and CPP (Jarvis *et al.*,1987; Olverman *et al.*,1986) binding to NMDA, the binding of glycine to strychnine-insensitive receptors (Bristow *et al.*,1986; MacDonald *et al.*,1990; Chapter 2) and the distribution of TCP (Sircar and Zukin,1985; Contreara *et al.*,1986; Maragos *et al.*,1988) and MK-801 (Bowery *et al.*,1988) binding sites display a high degree of correlation (Bowery,1987). Receptor solubilisation studies (Ambar *et al.*,1988; McKernan *et al.*,1989) also provide direct support for colocalisation within the complex.

A more detailed discussion of this concept is presented in chapters 2 and 3.

1.3.5: NMDA receptor heterogeneity

Discrepancies in the regional distributions of ^{3}H-glutamate and ^{3}H-CPP binding in brain have led Monaghan and colleagues (1988) to propose the concept of agonist or antagonist preferring subtypes of NMDA receptors. The highest density of agonist preferring sites, as labelled by ^{3}H-glutamate, were located in the CA1 of the hippocampal formation, anterior cingulate and perihinal

cortex, striatum and cerebellum. In contrast ^{3}H-CPP binding revealed a higher degree of antagonist sites in the I-V laminae of the cerebral cortex, the parietal / temporal cortices and in the CA3 of the hippocampus. NMDA agonists and antagonists displayed complimentary regional variation in their ability to displace ^{3}H-glutamate binding (Monaghan *et al.*,1988).

Glycine has also been demonstrated to alter the affinity of agonists and antagonists at the glutamate recognition site (Monaghan *et al.*,1988) and is proposed to mediate the shift from antagonist to agonist preferring forms of the receptor. However interconversion fail to explain the regional discrepancies since the two NMDA binding sites retain their differing distributions independent of the presence of glycine (Monaghan *et al.*,1988). These latter observations promote the idea of two anatomically distinct sites, each displaying separate states depending upon the interaction with the glycine site. It has been proposed that the apparent receptor heterogeneity results from genetically distinct isoforms, as suggested for the GABA-A complex (Olsen and Snowhill,1983) or alternatively a variety of post-translational factors are involved (Monaghan *et al.*,1989).

1.3.6: Polyamine site.

The polyamines, spermine and spermidine, have been reported to increase the binding of ^{3}H-MK801 by a mechanism enhanced by glutamate and glycine and blocked by NMDA antagonists (Ransom and Stec,1988). The co-operative nature of this modulation suggests that the polyamines act at a distinct site

and may modulate NMDA receptor function by a means of an intracellular mechanism. Subsequent structure activity studies have confirmed these initial observations and provided additional information about this modulatory site (Sacaan and Johnson,1989). The efficacy of receptor activation has been linked to the number of nitrogen atoms in the polyamine backbone. Whilst the number of methylene groups separating the nitrogen atoms is the major determinant of affinity (Sacaan and Johnson,1989). Recent studies have characterised a binding site for ^{3}H-spermidine in rat brain membranes (Mantione *et al.*,1990) and synaptosomal membrane preparations (Mantione *et al.*,1990).

The ability of spermine to enhance ^{3}H-glycine binding to strychnine-insensitive receptors (Sacaan and Johnson,1989), in a non-competitive manner, provides further evidence to support the presence of a distinct site for the polyamines. This enhancement is manifested as an increase in the affinity of ligand binding and not inhibited by either NMDA or glycine antagonists .

It has also been reported that SL 820715 and ifenprodil may function as antagonists at the polyamine site (Roa *et al.*,1989: Schoemaker *et al.*,1990), although evidence to demonstrate the non competitive action of ifenprodil has been reported (Reynolds and Miller,1989). Moreover the latter antagonist displays an affinity for the σ binding site (Karbon *et al.*,1990).

At present, whilst a distinct site of the action for the polyamines is suspected,

the presence of such a site on the NMDA receptor complex remains unsubstantiated.

1.4: Non-NMDA receptors.

Detailed investigation of kainate and AMPA receptors has been hindered by the lack of selective agonists and antagonists.

1.4.1: Quisqualate/AMPA receptors.

The excitant properties of quisqualate were initially described in crayfish, frog, rat and cat (Johnston *et al.*,1974; Biscoe *et al.*,1976; Shinozaki and Konishi,1970; Shinozaki and Shibuya,1974). The first differential evidence of a separate receptor specific for quisqualate, arose from antagonistic studies. Glutamate diethyl ester (GDEE) was reported to inhibit response of quisqualate in preference to kainate in central neurones (Haldeman and McLennan,1972; McLennan and Liu,1982), whilst the receptor displayed a distinctive agonist profile; quisqualate > glutamate > kainate (Honore *et al.*,1982). Development of a more selective agonist AMPA, which displayed a greater affinity for quisqualate receptors than quisqualate itself (Krogsgaard-Larsen *et al.*,1980) has led to the renaming of the receptor. The most potent antagonists of this receptor centre around the quinoxaline family, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and 6-7-dinitroquinoxaline-2-3-dione (DNQX) have both been reported to antagonise responses at AMPA receptors (Drejer and Honore,1988) however these compounds also interact competitively with the strychnine-insensitive glycine receptor (Birch *et al.*,1988a). Other

compounds which have been reported to act on AMPA receptors include the toxins Joro spider toxin (JSTX) (Usherwood and Duce,1985) and philanthotoxin 435 (Jones *et al.*,1989).

Receptor binding studies have revealed a probable postsynaptic location for the AMPA site (Fagg and Matus,1984; Foster and Fagg,1987) and an identical agonist profile to that of quisqualate. Furthermore, unlike quisqualate, AMPA has no effect on kainate binding (Honore *et al.*,1982; Krogsgaard-Larsen *et al.*,1980). The possible existence of subtypes of the AMPA receptor have been proposed following the discovery that in the presence of thiocyanate ions (SCN^-) (Honore and Neilsen,1985) ^{3}H-AMPA affinity is increased, prompting Honore and colleagues (1982) to suggest the presence of high and low affinity interconverting receptor forms. Moreover the observed increase in AMPA affinity following high energy radiation suggests an alternative explanation may lie in the presence of a high molecular weight subunit responsible for down regulation of the receptor (Murphy *et al.*,1987b).

Electrophysiological investigation of the ion channel associated with the receptor have also provided conflicting results. In general AMPA receptors activate low conductance channels (<20pS) (Ascher and Nowak,1988b) in hippocampus and cerebellum. This channel is equipermeable to Na^+ and K^+ (Ascher and Nowak,1988b; Vyklicky *et al.*,1988) and impermeable to Ca^{2+} (Mayer and Westbrook,1987). AMPA has additionally been reported to operate a fast kinetic, medium conductance channel (15 to 30 pS) (Ascher and

Nowak,1988b). Furthermore Tang *et al.*,(1989) described the presence of a high conductance (35pS) channel in hippocampus, which displayed fast kinetics, reversed at 0 mV, with little voltage dependence. This latter channel was suggested by the authors to mediate the fast quisqualate receptor excitatory postsynaptic current (EPSC), but remains to be proven.

1.4.2: Kainate receptors.

As outlined earlier, investigation of kainate receptors has been hampered by the lack of selective agents. A number of agonists have been described and these include kainate (Shinozaki and Konishi,1970), domoate (Davies *et al.*,1979; Evans *et al.*,1987) and bromowillardine (Agrawal and Evans,1986). Antagonists include γ-D-glutamylglycine (DGG) (Davies and Watkins,1981), kynurenate (Ganong *et al.*,1983) and the quinoxaline derivatives, CNQX and DNQX (Honore *et al.*,1988), in addition to the spider toxin JSTX (Akaike *et al.*,1987). Quisqualate has also been reported to activate kainate receptors furthering the belief that it is a partial agonist for the kainate site (London and Coyle,1979; Slevin *et al.*,1983).

Receptor binding data for ^{3}H-kainate have revealed a two population site displaying high and low affinities for kainate (Foster and Fagg,1984). These sites are thought to occupy a postsynaptic location and display similar pharmacological profiles (Foster *et al.*,1981). Furthermore Monaghan *et al.*,(1985) suggested that a small population of ^{3}H-glutamate sites possessed kainate ligand sensitivity. Additionally Greenamyre *et al.*,(1985a) proposed that

the low affinity kainate binding site may be equivalent to the high affinity AMPA binding site.

Kainate channels display identical permeability properties to those previously described for AMPA, and have a low conductance (4pS) with an open time of between 0.5 and 3 ms (Ascher and Nowak,1988b). However it has been suggested that the observed channel properties are the net result of the activation of two separate channels (Ascher and Nowak,1988b). Kainate has also been reported to activate a small population of higher conductance channels (Cull-Candy *et al.*,1988) attributed to a non-specific action at NMDA receptors.

1.4.2.1: Are Kainate and AMPA receptors distinct ?

The complex interactions reported between the kainate and AMPA receptors has led to the suggestion that these two receptors are in fact one entity. Quisqualate decreases kainate currents (Ishida and Neyton,1985; Mayer and Vyklicky,1989) and influences kainate binding (Slevin *et al.*,1983), however the actual nature of their individual responses, observed electrophysiologically, is quite distinct. Quisqualate displays a rapid, fast acting response (Perequansky and Grantyn,1989) whilst kainate produces a slow, more sustained response (Trussell *et al.*,1988). The concept that both may act at the same receptor has led to a number of hypotheses. The variation in response may be due to initial quisqualate activation causing receptor desensitisation and that quisqualate blockade of kainate responses relates directly to this

desensitisation (Collingridge and Lester,1989). Alternatively both agents compete directly for the same site, with quisqualate displaying less efficacious tendencies when bound (Collingridge and Lester,1989). Despite these ideas, evidence for an independent mechanism of activation for both agonists does exist (Perequansky and Grantyn,1989).

1.4.3: AP4 receptor.

This fourth type of EAA receptor was identified through the antagonist actions of L-AP4 at synapses assumed to use glutamate as a neurotransmitter. AP4, an analogue of glutamate in which the α-carboxy moiety has been replaced by a phosphonic acid group, potently and stereoselectively blocked lateral perforant path-evoked excitations of dentate gyrus granule cells in a superfused hippocampal slice preparation (Koerner and Cotman,1981). In addition AP4 was found to suppress synaptic activity in a number of other pathways including the lateral-olfactory tract evoked potentials in olfactory cortical slices (Collins,1982; Hearns *et al.*,1986), the mossy fiber-CA3 synapse in guinea pig hippocampal slices (Yamamoto *et al.*,1983; Lanthorn *et al.*,1984) and the monosynaptic dorsal root evoked ventral root potentials of the spinal cord of the cat (Davies and Watkins,1982) and rat (Evans *et al.*,1982). These electrophysiological studies demonstrated that AP4 inhibited excitatory responses evoked by kainate, quisqualate and NMDA. Studies on retinal transmission (Neal *et al.*,1981; Slaughter and Miller,1981) revealed that the AP4 actions were consistent with those of the endogenous transmitter at ON-bipolar cells.

Further investigations of the receptor are hampered by the lack of selective agents. To date relatively few compounds have displayed any activity at this receptor, L-serine-O-phosphate (Ganong and Cotman,1982) and cyclic analogues of 2-amino-4-phosphonobutanoic acid (Crooks *et al.*,1986) have been reported to exhibit moderate agonist properties. No selective antagonists have been reported. Current evidence as to the anatomical localisation of the receptor point toward a presynaptic location, indeed it is believed that this receptor may function as an autoreceptor controlling the release of glutamate from nerve terminals (Cotman *et al.*,1986; Anson *et al.*,1987; Forsythe and Clements,1988).

1.4.4: Metabotropic/ACPD receptor.

Accumulating evidence points to the existence of a novel class of EAA receptor that is coupled to the hydrolysis of membrane phosphoinositides (PI) (Sladeczek *et al.*,1985; Nicoletti *et al.*,1986a; Sugiyama *et al.*,1987). Enhanced hydrolysis of PI leads to the formation of two distinct second messengers, inositol-1,4,5,triphosphate (IP_3) and 1,2 diacylglycerol (DG) (Michell,1975) which act, in the case of IP_3, at intracellular receptors located on the endoplasmic reticulum to increase the intracellular concentration of Ca^{2+} (Berridge,1984; Nishizuka,1984). DG has been demonstrated to activate protein kinase C in the presence of Ca^{2+} and phosphatidylserine (Putney,1987; Nishizuka,1986).

Glutamate induced oscillating chloride currents were first demonstrated (Gundersen *et al.*,1984) in oocytes injected with rat brain mRNA, these

currents were mimicked by IP_3 injection (Parker and Miledi ,1987) and blocked by ethyleneglycol-bis-(β-amino ethyl ether) N,N-tetraacetic acid (EGTA) and pertussis toxin (PTX) (Sugiyama *et al.*,1987). Furthermore cross-desensitisation studies using IP_3 and glutamate (Sugiyama *et al.*,1987) indicated that IP_3 was indeed a mediator of these responses. Pharmacological analysis revealed that glutamate, ibotenate and quisqualate were able to induce IP_3 synthesis in brain slices (Baudry *et al.*,1986) and injected oocytes (Sugiyama *et al.*,1987). Subsequent studies in cultured striatal neurones (Sladeczek *et al.*,1985), cerebellar granule cells (Nicoletti *et al.*,1986b), hippocampal brain slices (Nicoletti *et al.*,1988a) and neocortex (Godfrey *et al.*,1988) confirmed the quisqualate enhancement of IP_3 production. Recently a new selective agonist TRANS-ACPD (trans-1-amino-cyclopentyl-1,3-dicarboxylate) has been described to be 100-fold more potent than glutamate (Palmer *et al.*,1989), whilst carboxycyclopropyl glycine (Nakagawa *et al.*,1990) and β-N-methylamino-L-alanine (L-BMAA) (Copani *et al.*,1990) have high affinity for this site. Cha *et al.*,(1990) have demonstrated ^{3}H-glutamate binding to this receptor but other EAA agonists NMDA, kainate and AMPA, and the selective antagonists CNQX and JSTX were without effect on brain slice preparations (Nicoletti *et al.*,1986a) and on oocytes (Verdoon and Dingledine,1988; Palmer *et al.*,1988). Additionally Murphy and Miller (1988) demonstrated that the elevation of intracellular calcium concentrations by quisqualate and glutamate in single neurones was independent of the extracellular calcium concentration. These observations are consistent with a receptor mediated release of calcium from endoplasmic reticulum. Recent electrophysiological evidence suggests that the

metabotropic receptor may gate a K^+ channel (Charpak *et al.*,1990). The slow excitation observed in the hippocampus following the receptor activation was demonstrated to be as a result of the inhibition of a Ca^{2+} and voltage-gated K^+ current. The physiological significance of such a receptor remains to be classified, however, reports that NMDA receptor activation in neonatal rat hippocampal slices inhibited quisqualate-induced IP_3 formation in a Ca^{2+} dependent manner (Palmer *et al.*,1988) has invited speculation that this mechanism represents a negative regulation of synaptic plasticity in the visual cortex (Bear *et al.*,1987) proposed to occur via an excitotoxic process (Schmidt *et al.*,1987).

1.5: Physiological relevance of EAA receptor activation.

Excitatory amino acids have been linked with a number of physiological states including long term potentiation, status epilepticus, excitotoxicity and neurodegeneration.

1.5.1: Long term potentiation (LTP).

LTP is a phenomenon, first described by Bliss and colleagues, in which periods of high frequency stimulation (tetanus) induce a sustained enhancement in the synaptic response. This apparent increase in synaptic efficacy together with the demonstration of afferent fibre heterosynaptic co-operativity (Bliss and Gardner-Medwin,1973) have implied an associative nature of LTP. A concept supported by the subsequent demonstration of tetanised path specificity (Andersen *et al.*,1980). Moreover these associative properties of LTP have been

suggested to represent an underlying mechanism for learning and memory (Collingridge and Bliss,1987).

LTP has been observed in a number of pathways including all the major excitatory pathways in the hippocampus eg: Schaffer-collateral-commissural pathway, mossy fibre projection (Alger and Teyler,1976; Schwartzkroin and Wesler,1975). NMDA receptor activation has been described as an absolute requirement for LTP induction (Collingridge and Lester,1989). Evidence to support this statement is substantial. The selective NMDA antagonist AP5 reversibly blocks LTP induction in the association-commissural pathway of CA1/3 (Collingridge,1985; Errington *et al.*,1987), a feature also displayed by the non-competitive NMDA inhibitors PCP, ketamine, MK-801 and 7-chloro kynurenic acid (Stringer *et al.*,1983; Coan and Collingridge,1987b). In addition AP5 has been demonstrated to inhibit LTP induced by pairing low frequency shocks with depolarisation of the post synaptic cell (Gustafsson *et al.*,1987) or via brief tetani delivered at the theta frequency (Diamond *et al.*,1988). Glycine has also been reported to synergistically potentiate LTP in the hippocampus (Trauck and Ashbeck,1990). The late phase of LTP has also been shown to require NMDA receptor activation during tetanic stimulation (Reyman *et al.*,1989). Two further aspects of LTP that can be attributed to the involvement of NMDA receptors are the high voltage dependence (MacDonald *et al.*,1982) and enhanced extracellular Ca^{2+} entry (Mayer *et al.*,1987).

1.5.1.1: Current concept of LTP.

During normal low frequency transmission the evoked excitatory postsynaptic potential (EPSP) is mediated by non-NMDA receptors, probably AMPA (Andreasen *et al.*,1989), whilst NMDA receptor activation provides little or no contribution due to the high voltage blockade (MacDonald *et al.*,1982).

The release of neurotransmitter (probably glutamate) from the presynaptic afferent fibres activates postsynaptic AMPA and NMDA receptors (Collingridge and Davies,1989). Under normal low frequency synaptic transmission cell depolarisations and concurrent IPSP (inhibitory post synaptic potentials) activation (mediated through GABA A and B receptors) prevent NMDA receptor activation since rapid hyperpolarisation moves the membrane to a state of maximal Mg^{2+} blockade (Collingridge and Davies,1989). During high frequency stimulation membrane depolarisation is sufficient to enable removal of the Mg^{2+} blockade and thus allow NMDA receptor activation. This in turn induces an increase in synaptic efficacy and the induction of LTP. Reasons for the extended membrane depolarisation are unclear, however a frequency dependent fatigue of IPSP, involving an inhibitory GABA feedback mechanism mediated through GABA B autoreceptors has been proposed (Davies *et al.*,1990). This current hypothesis fulfils the criterion proposed by Hebb (1949) that synaptic strengthening requires simultaneous pre and post synaptic activity. In the case of NMDA neurotransmitter release (pre) and decreased Mg^{2+} blockade (post synaptic) make up the individual components.

Although NMDA receptor activation is generally thought to be essential for the induction of LTP (Collingridge and Lester,1989), much disagreement exists about the post induction maintenance phase. NMDA induced LTP does not possess an indefinite lifespan (Collingridge *et al.*,1983), furthermore potentiated and nonpotentiated EPSPs are inhibited by CNQX (nonNMDA antagonist) and not AP5 (Davies *et al.*,1989; Collingridge *et al.*,1983) suggesting a nonNMDA receptor mediated maintenance. Neurochemical evidence (Collingridge *et al.*,1989; Errington *et al.*,1987) indicates that an increase in neurotransmitter release and the resultant increase in receptor activation is sufficient to maintain LTP. In contrast electrophysiological studies (Muller *et al.*,1988; Kauer *et al.*,1988) favour a postsynaptic mechanism involving AMPA receptors alone. More recently Collingridge and colleagues, following the demonstration that LTP in CA1 neurones displayed a delayed increase in sensitivity to AMPA agonists, proposed the idea that LTP may indeed be initially maintained by increased transmitter release with the subsequent postsynaptic contribution (Davies *et al.*,1989).

Another area of speculation surrounds the mechanism through which NMDA activation brings about changes in synaptic efficiency. The debate centres around the possible activation of a number of second messengers which appear to be involved in the induction and maintenance of LTP. LTP has been associated with enhanced Ca^{2+} entry (Wroblewski and Danysz, 1989), a factor consistent with NMDA receptor cation permeability. Secondary biochemical changes resulting from increased intracellular Ca^{2+} (phosphorylation of GAP-43

protein,a substrate for PKC (Linden *et al.*,1988)), and the activation of Ca^{2+} sensitive kinases (Smith,1987) and proteases (Lynch and Baudry,1984) may prove sufficient to produce persistent changes. Endothelium derived relaxing factor (EDRF) (Garthwaite *et al.,1988*) and arachidonic acid metabolites (Dragunow *et al.*,1988) have been associated with LTP, and have been proposed to act as intercellular messengers communicating pre and postsynaptic changes in membrane status.

1.6: EAA in Epilepsy.

Epilepsy is a chronic neurological disorder characterised by periodic and unpredictable occurring seizures. The latter being defined as transient alterations in behaviour due to disordered, synchronous, rhythmic firing of a CNS neuronal population (Traynelis and Dingledine,1988). Seizures can be divided into either partial or generalized, the former have a local onset and can be further subdivided into simple (conscious) or complex (unconscious) whilst the latter have no evidence of local onset and subdivide into tonic-clonic (grand mal) or absence (petit mal) (Traynelis and Dingledine,1988).

A number of *in vivo* and *in vitro* animal models have been developed to further the insights into this disease. The majority are based around two susceptible areas, the hippocampus and the neocortex. These include models of burst-firing (Chamberlin *et al.*,1990), electrographic seizures (Kawasaki *et al.*,1990) and finally *in vivo* seizure models such as kindling (McNamara *et al.*,1988; Sato *et al.*,1988) and audiogenic seizures (Croucher *et al.*,1982).

EAA appear to be implicated in all models, however the specific receptor population involved is dependent on the surrounding neuronal circuitry. This is reflected in the ability of AP5 (Mody *et al.*,1987), 7-chloro-kynurenic acid (Singh *et al.*,1990a) and MK-801 (Slater *et al.*,1985) to inhibit spontaneous hippocampal CA1 seizure formation observed in an Mg^{2+} containing environment (Mody *et al.*,1987; Coan and Collingridge,1985). The AMPA antagonist CNQX being without effect. In contrast, CA3 seizures, caused by increasing the K^+ concentration, are sensitive to CNQX and not AP5 (Aram *et al.*,1989).

It has been suggested that the released neurotransmitter acts at both NMDA and AMPA receptors, and the relative contribution of each is dependent upon the degree of voltage-dependent block and the circuitry activated (Aram *et al.*, 1989). Little evidence exists for AMPA receptor involvement in any of the electrographic seizure models so far reported, whilst evidence points to a role for NMDA receptors in the induction or establishment of the epileptic state rather than increased expression of previously established seizure (Dingledine *et al.*, 1990). This concept is further evident from *in vivo* studies where the anticonvulsant activities of AP5 and AP7 (Croucher *et al.*,1982) and other competitive and non-competitive antagonists have been demonstrated to vary considerably in a number of models (Lehmann *et al.*,1988; Meldrum *et al.*,1982), reflecting the receptor population involved.

NMDA antagonists can prevent induction of kindling (McNamara *et al.*,1988; Sato *et al.*, 1988), a state linked to LTP, whilst displaying reduced anti-convulsant properties (McNamara *et al.*,1988).

An increased participation of NMDA receptors in synaptic transmission (Mody *et al.*,1987) has been proposed to explain the apparent reduction in anti-convulsant activity. Alternatively it has been suggested that the reduction in anticonvulsant activity may be explained through an increased sensitivity of NMDA neurones to glutamate (Morrisett *et al.*,1989).

The reported observations of increased receptor (NMDA and associated glycine binding site) numbers in some studies (Yeh *et al.*,1989; Johnston *et al.*,1989) remains controversial following reports (Vezzani *et al.*,1990) that receptor numbers remain unaltered for NMDA, glycine and indeed AMPA.

There is no doubt, however that EAA antagonists have therapeutic potential as either anti-convulsant or anti-epileptogenesis agents.

1.7: EAA in neurotoxicity.

EAA have for some time been believed to be involved in excitotoxicity within a cell. The potent neurotoxic properties of NMDLA were first reported by Olney,(1978). Conformation of these early findings have paralleled the advances of the NMDA receptor complex. Today it is widely accepted that excitatory amino acid transmitters, released at a synapse, possessed the ability

to destroy receptive postsynaptic neurones. The main transmitter is thought to be glutamate, however other candidates include aspartate, quinolinate and homocysteate (Mewett *et al.*,1983; Recaseris *et al.*,1982).

A noticeable aspect of toxicity associated with EAA is the wide degree of variation in vulnerability between brain regions (Garthwaite,1989). Of the five main EAA receptors, NMDA, kainate and AMPA can be directly associated with toxicity whilst the fourth, the AP4 receptor, may be involved through interactions with calcium although this remains to be seen. NMDA is particularly toxic to differentiating neurones of the hippocampus, whilst quisqualate (or AMPA) shows selectivity for the CA3 pyramidal cells (Garthwaite and Garthwaite,1989a). Similarly in the developing cerebellum, differentiating granule cells are susceptible to NMDA but not kainate, a trend reversed in golgi cells (Garthwaite and Garthwaite,1986; Hajos *et al.*,1986). These regional variations are not confined to different areas, since similar differentials have been associated with particular neuronal populations (Garthwaite and Garthwaite,1989a). In general undifferentiated neurones show a resistance to NMDA toxicity eg: dentate gyrus (Garthwaite and Garthwaite,1989b), cerebral cortical neurones (Choi *et al.*,1987) and cerebellar golgi cells (Garthwaite and Garthwaite,1984:1986), which is depreciated in the adult state. Some regions however remain resistant throughout (Garthwaite and Garthwaite,1984). Non-NMDA toxicity requires excitatory afferent innervation to induce any effect (Rakio,1971), which explains the increase in particular neuronal vulnerability with age. Morphological investigations have

revealed distinct differences in the characteristics associated with NMDA and nonNMDA toxicity (Meldrum and Garthwaite,1990). The former is characterized by a rapid necrotic profile typified by mass swelling and vacuolation of the cytoplasm, rapid mitochondrial expression and focal clumping of nuclear chromatin. In contrast AMPA/quisqualate toxicity is accompanied by dark staining of the soma and nuclei, and microvacuolation of the cytoplasm leading to a "studded" effect (Meldrum and Garthwaite,1990).

Currently two main factors have been proposed to explain the differential characteristics and regional vulnerability of EAA toxicity. The first relates to the receptor population present in the individual regions and the second to the possible involvement of Ca^{2+}. Current ideas point to the existence of two types of NMDA toxicity. Acute toxicity was originally related to a Cl^- dependent mechanism, in which Cl^- ions passively diffuse into the cell accompanied by the passage of H_2O and cations and this results in osmotic lysis and cell death (Olney,1976). This mechanism was particularly apparent in culture systems and is widely accepted as a phenomenon peculiar to cultured cells (Garthwaite and Garthwaite,1989b). The demonstration of Ca^{2+} dependent Cl^- independent NMDA toxicity in pyramidal neurones of CA1,CA3 and granule cells of the dentate gyrus (Garthwaite and Garthwaite,1989b) has promoted the idea that a Ca^{2+} overload of the cell buffering systems may ultimately lead to cell death. The Ca^{2+} permeability of the NMDA receptor channel is well established (Mayer *et al.*,1987), thus activation may lead to an excessive influx of Ca^{2+} and a resultant overloading of the buffering systems (Griffiths *et al.*,1984), a factor

potentiated by the presence of glycine (Patel *et al.*,1990). Furthermore a good correlation exists between the sites of irreversible neuronal damage and calcium accumulation (Meldrum and Garthwaite,1990). The effects of calcium influx are also well documented and these include pathological changes in organelles, activation of a number of destructive enzymes, production of free radicals and corresponding alterations in protein, membrane and DNA ultrastructure (Wolff *et al.*,1986; Imaly and Linn,1988).

1.7.1: Delayed NMDA toxicity.

Delayed NMDA toxicity is characterised by a progressive slow degeneration subsequent to sublethal administration of a toxic agent. Several mutually cooperative factors are thought to be involved. It has been suggested that neurones undergo a period of enhanced vulnerability following non-lethal exposure to a toxic agent, and its subsequent addition results in an enhanced effect (Foster *et al.*,1988; Garthwaite,1989). The extended vulnerability has been hypothesised to coincide with the delay in reinstatement of the calcium buffering systems. Alternatively Ca^{2+} influx promotes the activation of an enzymic cascade, similar to that described for acute toxicity (Meldrum and Garthwaite,1990), or the toxicity may result from a preferential loss of inhibitory neurones, removing the inhibitory feedback mechanism, with a subsequent amplification of the NMDA response (Meldrum and Garthwaite, 1990).

1.7.2:Kainate toxicity.

As already mentioned, the increased vulnerability of neuronal populations to non-NMDA toxicity is age dependent, leading to the conclusion that an important, if not essential, aspect of kainate toxicity is initial excitatory afferent stimulation (Campochiaro and Coyle,1978). A Ca^{2+} dependent Cl^- independent mechanism has also been demonstrated for kainate (Koh *et al.*,1990),and the recent reports of sub types of kainate activated channels with varying Ca^{2+} permeability (Iino *et al.*,1990) may provide an insight into the importance of Ca^{2+} influx via voltage dependent channels.

1.7.3: AMPA toxicity.

AMPA toxicity can also be divided into two particular types. Acute AMPA toxicity has been suggested to develop subsequent to a long exposure to a low concentration of agonist (Meldrum and Garthwaite,1990). However, in general, very little is known about this particular type of toxicity. Delayed AMPA toxicity has been linked to an initial period of receptor desensitisation following the initial insult (Garthwaite and Garthwaite,1989b). These observations suggest that the secondary degeneration may occur via the presence of endogenous, rather than exogenous agents. In addition. a possible role for voltage dependent Ca^{2+} influx has also been proposed (Weiss *et al.*,1990).

1.7.4: EAA-induced toxicity.

EAA-induced toxicity has been linked to a number of acute and chronic neurodegenerative disorders. The role of EAA in the acute disorder status epilepticus was discussed earlier. Glutamate excitotoxicity has been implicated in hypoglycaemic neurodegeneration, an impaired glutamate uptake system (Rothman and Olney,1987) and selective striatal neuronal protection by AP7 (Wieloch,1985) implicates the possible involvement of excessive glutamate accumulation and NMDA receptors. Similarly excessive glutamate accumulation is suspected to be responsible for the neuronal degeneration associated with cerebral ischaemia, a disorder which can include transient global ischaemia, irreversible focal ischaemia and hypoxia (Meldrum,1985; Benveniste *et al.*,1985; Rothman and Olney,1986). Various lesion studies have demonstrated a reduction in glutamate (Crepal *et al.*,1988), phencyclidine (Leach *et al.*,1988) and glycine (unpublished data) receptors following ischaemic damage, a phenomenon readily prevented by selective NMDA antagonists including CPP, CGS-19755 and MK-801 (Boast *et al.*,1988; Gill *et al.*,1987; Simon *et al.*,1984). An involvement of AMPA receptors (Westerberg *et al.*,1987; Sheardown *et al.*,1990) cannot be discounted. Acute disorder excitotoxicity is not confined to NMDA and AMPA receptors since kainate receptors are the suspected site of action for the human disorder domoic acid poisoning. Domoic acid, a potent analogue of kainic acid, possesses excitotoxic actions at the kainate receptors (Davies *et al.*,1979;), and the areas affected correspond to areas of high kainate receptor density, eg: cortical laminae *V* and *VI*, hippocampal regions CA3 and amygdala (Carpenter *et al.*,1990).

The distribution pattern of neuronal loss induced by injection of quinolinic acid (Beal *et al.*,1986) displays marked similarities to those observed in the chronic disorder Huntingtons Chorea. Preferential degeneration of specific glutamatergic pathways in the striatum (Olney,1979) has been observed and those neurones are suspected to be of the NMDA type (Young *et al.*,1988). Furthermore the actions of quinolinic acid can be inhibited by selective NMDA antagonists (Stone *et al.*,1981; Foster *et al.*,1985). However elevated levels of quinolinic acid were absent from patients showing this disorder (Reynolds *et al.*,1987) and it remains to be established if the above observations substantiate a role for EAA-induced excitotoxic mechanisms. A similar unsubstantiated role for the involvement of EAA excitotoxicity in Alzheimers disease has been proposed. Reports of decreased glutamate, quisqualate and TCP binding sites (Greenamyre *et al.*,1985:1987a) and decreased glutamate and aspartate uptake sites (Cross *et al.*,1987; Procter *et al.*,1988) have been disputed (Cowburn *et al.*,1988) and it would appear that receptor density decreases occur late in the disease state and are accompanied by massive neuronal degeneration (Hardy and Cowburn,1987).

Additionally, tentative links between EAA toxins and chronic neurological disorders have been proposed for β-N-oxalyamino-L-alanine (BOAA), a plant toxin (Rao *et al.*,1964) acting via AMPA receptors (Bridges *et al.*,1989). Furthermore non-NMDA receptors have been linked as possible sites of action of α-methyl carbonate, the functional excitotoxic derivative of β-N-methyl-amino-L-alanine (BMAA) in amyotrophic lateral sclerosis or Guan disease

(Weiss *et al.*,1989).

1.8: Inhibitory glycine receptors.

The neutral amino acid glycine was first proposed to be an inhibitory neurotransmitter in 1965 (Aprison and Werman). Neurochemical and pharmacological evidence, including the release of this amino acid (Hopkin and Neal,1971) and its inhibitory actions on motoneurones (Werman *et al.*,1968; Curtis,1963), have confirmed a role for glycine as the major inhibitory neurotransmitter in the spinal cord (Aprison and Daly,1978: Aprison,1990). Glycine, and the other major inhibitory amino acid γ-aminobutyric acid (GABA) mediate their inhibitory actions through a mechanism of postsynaptic membrane hyperpolarisation involving increased chloride and decreased potassium conductance (Curtis,1963; Aprison and Werman, 1965; Davidoff *et al.*,1967). The resultant hyperpolarization serves to antagonise the excitatory potential, whilst the released glycine is removed by diffusion or interaction with a high affinity uptake system (Johnston and Iversen,1971; Neal,1971). This feedback mechanism is employed in Renshaw cells and many other inhibitory interneurones in the spinal cord (Curtis,1963) and functions to regulate tonic inhibitory control of spinal action. The alkaloid, strychnine, was found to be a selective and potent antagonist of the inhibitory actions of glycine (Tebecis and DiMaria,1972) and as a consequence has been employed as a preferential ligand in receptor localisation studies (Young and Snyder,1973). High densities of strychnine-sensitive glycine receptors were reported in the spinal cord, in rostral portions of the brain stem and in the retina (Zarbin *et*

al., 1981; Frostholm and Rotter, 1985; Probst *et al.*, 1986) whilst GABA receptors were localised in the forebrain and cortex (Bowery *et al.*, 1987).

The solubilisation (Pfeiffer and Betz, 1981) and subsequent purification (Pfeiffer *et al.*, 1982) of the glycine receptor, paralleled with the development of monoclonal antibodies (Pfeiffer *et al.*, 1984) and immunocytochemical techniques (Triller *et al.*, 1985; Altshuler *et al.*, 1986; Ottersen *et al.*, 1988) have confirmed earlier pharmacological and anatomical findings. Furthermore cDNA cloning techniques have enabled elucidation of the primary structure of the receptor (Greeningloch *et al.*, 1987; Betz, 1987; Betz and Becker, 1988).

Although glycine is well established as an inhibitory neurotransmitter in the spinal cord, little evidence exists for a similar role within supraspinal regions. Glycine receptors are known to exist in higher centres (Bristow *et al.*, 1986) and the amino acid has been reported to potentiate responses mediated through the excitatory NMDA receptor (Johnson and Ascher, 1987), an observation contradictory to our understanding of the distinct roles of excitatory and inhibitory neurotransmitters. These initial findings have served as a springboard for research into the possible excitatory role for the classically regarded, inhibitory amino acid, glycine.

1.8.1: Excitatory actions of glycine.

Autoradiographical and receptor binding evidence points to the presence of a distinct glycine receptor in supraspinal regions, which displays a marked

insensitivity to strychnine (Bristow *et al.*,1986; DeFaudis *et al.*,1978; Kishimoto *et al.*,1981). deFaudis and colleagues (1978) proposed that sodium dependent glycine binding reflected binding to both populations of glycine receptors, whilst independent binding was a measure of the strychnine-insensitive population rather than an interaction with sodium dependent uptake sites, as was originally thought (Johnson and Iversen,1971; Kishimoto *et al.*,1981). These sites appeared to lack any physiological role, however in parallel with electrophysiological studies, Bowery (1987) described a close correlation between strychnine-insensitive sites and those of NMDA (Monaghan and Cotman,1985) implying an association with the excitatory complex, a suggestion that was to prove correct.

As previously mentioned, studies on patch-clamp cultured cells revealed the presence of an endogenous compound of low molecular weight, capable of potentiating NMDA responses (Johnson and Ascher,1987). Detailed investigation identified this endogenous mediator as glycine, acting on strychnine-insensitive receptors. Single channel analysis revealed that glycine potentiation could be attributed to an increased frequency of channel opening with no effect on conductance or duration of open time (Johnson and Ascher, 1987). Attempts to reproduce the potentiation in other "intact" systems were initially unsuccessful, a factor attributed to the presence of high endogenous levels of glycine causing glycine site saturation (Ascher and Johnson,1989).

The demonstration of glycine receptor antagonism by kynurenate (Kessler *et al.*,1988:1989a; Watson *et al.*,1988) has been reported in a variety of

preparations, these include cortical wedges (Kemp *et al.*,1988; Fletcher and Lodge,1990), hippocampal slices (Watson *et al.*,1988), spinal cord (Birch *et al.*,1988) and cultured neurones (Ascher *et al.*,1989; Kemp *et al.*,1988). Moreover, the antagonism can be reversed by glycine. This strychnine-insensitive reversal was mimicked by D-serine and alanine but not GABA (Fletcher *et al.*,*1989*). In addition kynurenic acid possesses low affinity for the glutamate site on the NMDA receptor complex. (Fletcher *et al.*,1989; Kemp *et al.*,1987, Burton *et al.*,1988).

Despite the relatively recent discovery of the strychnine-insensitive glycine receptor a number of antagonists have already been described. The GABA analogue, HA-966, originally described by Bonta *et al.*,(1971), inhibited NMDA evoked responses in a glycine sensitive manner (Fletcher and Lodge,1988; Foster and Kemp,1989). Recent reports (Singh *et al.*,1990b) indicate that the R(+)-enantiomer of HA-966 is responsible for the antagonism, whilst the S(-)-enantiomer displays marked sedative and muscle relaxant properties in vivo. It has been suggested that HA-966 is best regarded as a low efficacy partial agonist at the glycine site (Foster and Kemp,1989; Singh *et al.*,1990b; Kloog *et al.*,1990). Another partial agonist at the glycine receptor is aminocyclopropane carboxylate (ACPC) (Marvizon *et al.*,1989), in contrast aminocyclobutane carboxylate (ACBC) is described as a weak antagonist (Hood *et al.*,1989a). The most potent and selective full glycine antagonists described to date are 7-chlorokynurenic acid (7-ClKYN) (Kemp *et al.*,1987) and its 5 iodo analogue (Foster *et al.*,1990). The inhibitory effects are completely glycine sensitive and no residual NMDA response was observed (Foster and Kemp,1989). The

quinoxaline derivatives CNQX and DNQX , originally described as non-NMDA antagonists (Fletcher and Lodge,1988), have been reported as potent glycine antagonists (Birch *et al.*,1988). Similarly 6,7,dichloro-3-hydroxy-1-quinoxalate carboxylic acid has been described as a weak, yet selective, glycine antagonist (Snell *et al.*,1988), whilst 6,8,dinitroquinoxaline (MNQX), a structurally similar derivative of DNQX, displays a greater selectivity for the glycine receptor than its counterpart (Sheardown *et al.*,1990).

Antagonism studies have implied an absolute requirement for glycine prior to NMDA activation, a problem addressed and confirmed by Kleckner and Dingledine (1988), through studies on mRNA injected oocytes. In an apparent glycine free environment NMDA evoked channel opening was abolished, an observation recently confirmed by D'Angelo *et al.,(*1990) in cerebellar granule cells. The concept of absolute requirement is disputed by Mayer *et al.,*(1989), who proposed enhancement of receptor activation via a reduction in NMDA-induced desensitisation. Glycine did not affect the onset of desensitisation but enhanced the recovery, in a manner inversely proportional to its concentration (Mayer *et al.*,1988). The concept of NMDA receptor desensitisation was first described (Mayer and Westbrook,1985; Vyklicky *et al.*,1986) prior to the elucidation of glycine as a mediator of NMDA receptor function, and was related, at least in part, to the elevation of intracellular calcium levels. Furthermore glycine has recently been reported to modulate NMDA receptor desensitisation in cultured mouse neurones (Vyklicky *et al.*,1990). However, the findings of Ascher *et al.,(*1990) contradict this theory since their results

indicate a glycine independent component to NMDA desensitisation.

The ability of glycine to potentiate NMDA responses has been further demonstrated in a number of pharmacological studies in vivo, including potentiation of NMDA evoked increases in cerebellar cGMP levels (Dansyz *et al.*,1989), PI hydrolysis (Nicoletti and Canonico,1989), release of noradrenaline from hippocampal slices (Ransom and Deschenes,1988) and acetylcholine and dopamine from striatal slices (Ranson and Deschenes,1989; Crawford and Roberts,1989).

Electrophysiological advances have been paralleled by biochemical demonstration of glycine potentiation. Following the study by Johnson and Ascher (1987), Reynolds *et al.*,(1987) reported glycine enhancement of NMDA induced calcium permeability in mouse striatal neurones, which is consistent with glycine affecting NMDA channel opening. This has recently been confirmed by Oliver *et al.*,(1990a). In addition Foster and Wong (1987) reported links between glycine and sodium influx.

Confirmatory evidence of glycine interaction with the NMDA receptor complex arose from reports of glycine enhancement of ^{3}H-TCP and ^{3}H-MK-801 binding (Snell *et al.*,1987; Reynolds *et al.*,1987; Wong *et al.*,1987; Bonhaus *et al.*,1987). The latter effects were described as strychnine insensitive and agonist dependent (a concept discussed earlier) and the effects were inhibited by the NMDA antagonists CPP and AP5. In addition the action of glycine could be

mimicked by D-serine and alanine (Bonhaus *et al.*,1987; Snell *et al.*,1988; Ransom and Stec,1988), cycloserine, glycine methyl ester, O-phospho-D-serine (Snell *et al.*,1988; Johnson *et al.*,1988) and aminocyclopropane-1-carboxylic acid (Hodd *et al.*,1989a; Nadler *et al.*,1988). Kinetic evaluation attributed the action of glycine to an increase in the rate of association and dissociation of ^{3}H-TCP and ^{3}H-MK-801, in line with the idea of increased channel opening (Kloog *et al.*,1988a; Reynolds and Millar,1988; Ransom and Stec,1988). Whilst glycine potentiation of ^{3}H-MK-801 binding in solubilised receptor preparations confirmed the presence of a glycine domain as an integral part of the NMDA receptor complex (McKernan *et al.*,1989).

Glycine receptor regulation reflects the integrated nature of the NMDA receptor complex. Magnesium has been demonstrated to increase glycine binding (Marvison and Skolnick,1988) whilst NMDA, glutamate AP5 and CPP also interact with glycine binding (Johnson *et al.*,1988) reflecting the reciprocal interaction between recognition sites (Monaghan *et al.*,1988; Kaplita and Ferkany,1990; Hood *et al.*,1990). A similar reciprocal interaction appears to exist between glutamate, glycine and the polyamine, spermidine (Ransom and Stec,1988) whilst another polyamine ,spermine, has recently been observed to enhance strychnine-insensitive glycine binding in rat membranes (Sacaan and Johnson,1990). At present the significance of these observations remains unclear, since the site of action of the polyamines is, as yet, unresolved, although a recent report of ^{3}H-ifenprodil binding to a polyamine sensitive site in the rat cerebral cortex (Schoemaker *et al.*,1990) may provide new evidence.

The novel non competitive NMDA antagonists ifenprodil and SL 820715 were initially regarded as glycine antagonists (Rao *et al.*,1989), however their lack of selectivity has since been elucidated (Karbon *et al.*,1990; Schoemaker *et al.*,1990).

1.9: Project overview.

A brief outline of the objectives of this project is given below :-

The discovery of the ability of glycine to modulate NMDA receptor activation (Johnson and Ascher,1987), through a distinct strychnine-insensitive glycine binding site (Kishimoto *et al.*,1981; Bristow *et al.*,1986) has generated an interest into the possible role of glycine in the CNS. The primary aim of this project was to investigate the interaction between glycine and NMDA receptors in the supraspinal regions of the rat.

A quantitative autoradiographical technique has been employed to investigate the regional distribution of the strychnine-insensitive glycine receptor in rat brain. Moreover this technique was modified to permit a pharmacological characterisation of the receptor. These data provide the basis for the study presented in Chapter 2.

The close association of glycine and NMDA receptors (Chapter 2) was extended to provide the basis for the study described in chapter 3. The activation of the NMDA receptor is accompanied by an opening of the associated ion channel. The degree of channel opening is reflected in the binding profile of specific

ligands thought to associate with the activated form of the receptor eg: MK-801 (Wong *et al.*, 1986). Moreover receptor activation has been suggested to display an absolute requirement for glycine (Kleckner and Dingledine, 1988). The main aim of this study was to investigate whether the apparent interdependence between glutamate, glycine and receptor activation displayed any regional variation. To this end a quantitative autoradiographical procedure has been used to evaluate the enhancement of ^{3}H-MK-801 binding produced by glutamate and glycine in a number of discrete brain regions. The study was extended to encompass the effects of a number of NMDA receptor antagonists on the regional binding of ^{3}H-MK-801.

The possible role for glycine in the cerebellum forms the basis of the study presented in chapter 4. Immunocytochemical studies have revealed a co-localisation of glycine and GABA within the same neuronal population (Golgi neurones) (Ottersen *et al.*, 1988). The ability of these neurones to release glycine (endogenous and exogenous) in response to K^{+} induced depolarisation was evaluated. For means of comparison the exogenous release of glycine in the hippocampus was also investigated. Preliminary data of the ability of selective EAA agonists to stimulate glycine release from cerebellar sections is also described. In addition to the release studies, an evaluation of the possible sites of action for the released glycine, the strychnine-insensitive glycine receptor is presented. On the basis of these data obtained in this study, together with that described in the literature, a hypothetical model for the role of glycine in the cerebellum is presented.

The final chapter outlines the possible areas for future research resulting from this present study, together with the results of a preliminary investigation into the interaction of NMDA receptors and the cGMP second messenger system.

CHAPTER 2 - CHARACTERIZATION OF THE STRYCHNINE-INSENSITIVE GLYCINE RECEPTOR

Chapter 2.

2.1: Introduction.

Many of the major advances in EAA research over the past decade have centred around the study of receptors and their function. In the 3 years since the discovery of the modulatory role of glycine by Johnson and Ascher (1987), our understanding of this concept has been furthered by detailed electrophysiological and biochemical studies. The latter investigations have involved predominantly the use of radioligand binding to membrane preparations (either whole brain or specific regions). While providing a useful and accurate means of elucidating the pharmacological properties of a particular receptor, the membrane binding assay provides little information as to the anatomical localisation. The need for such information has warranted the development of a second technique, receptor autoradiography, which, when used in association with other studies can be a powerful pharmacological tool.

2.1.1: Receptor autoradiography.

Autoradiography is an old technique preceding and contributing to the discovery of radioactivity. In general it may be regarded as an elaboration or refinement of the receptor binding assay. The technique as we know it dates from Labold's demonstration of the distribution of radioactive iodine in sections of thyroid in the 1940's. Whilst the first use of the technique to study receptors involved the use of ^{14}C-curare to study cholinergic nicotinic receptors (Waser and Luthi,1962). The introduction of ^{3}H-ligands led to a number of *in vivo* investigations into the distribution of dopamine, opiate and cholinergic

muscarinic receptors (Kuhar and Yamamura, 1975; Pert *et al.*,1975; Kuhar *et al.*,1978), although access and metabolism problems hampered these early studies. The development of an alternative approach to receptor autoradiography (Young and Kuhar,1979) based on the combination of two previously described techniques, that of "slide mounted sections" (Polz-Tejera *et al.*,1975) and "emulsion apposition" (Roth *et al.*,1974) formed the foundations of what is today regarded as coverslip autoradiography. The use of this method to distinguish subtypes of α adrenoceptor proved more successful than previous attempts. It removed the need to immerse the radioactive section in photographic emulsion, thus reducing ligand diffusion into tissue with a resultant increase in resolution.

A further alternative approach emerged with the development of ^{3}H-sensitive film (LKB Ultrofilm), first used by Palacios *et al.*,(1981). The ease of handling of the emulsion coated film at all times during the autoradiographic process proved a distinct advantage over the more cumbersome and unreliable coverslip technique. Similarly so-called "bulk exposures" of sections proved more efficient and economical. The loss of resolution, compared with coverslips, is counteracted by the increased ease of densitometric analysis, since the underlying problem of interference from a permanently attached section is removed.

Over the past decade receptor autoradiography has been employed to evaluate the existence of a number of receptor subtypes including GABA-A and-B

(Bowery *et al.*,1987: Wilkin *et al.*,1981a), β-adrenoceptors (Palacios *et al.*,1982),excitatory amino acid receptors (Monaghan and Cotman,1985; Bowery *et al.*,1988),and the receptors for 5-hydroxytryptamine (5-HT) (Pratt and Bowery,1989; Kilpatrick *et al.*,1988). The latter serves to illustrate a major advantage of receptor autoradiography over other receptor binding techniques. One of the receptor subtypes for 5-HT is the 5-HT_3 receptor which appear to be located in high density in the nucleus tractus solitarius, a discrete brain region adjacent to the area postrema. Localisation of the receptor subtype relied almost exclusively on the use of receptor autoradiography (Pratt and Bowery 1989), since receptor binding studies failed to distinguish specific binding in such a discrete brain region (Kilpatrick *et al.*,1988).

Like many other scientific techniques, receptor autoradiography is not without its limitations. The requirement of selective high affinity ligands is uppermost, furthermore accurate correlation between the methodology employed (particularly washing) and the ligand affinity is essential to prevent the total elimination of the bound ligand, along with the unbound ligand, prior to processing. In addition the aqueous and lipid solubility of the ligands must be considered particularly in the case of low affinity ligands. Moreover any process that requires a purification step prior to the production of optimal binding renders receptor autoradiography redundant. By far the most limiting drawback is the time scale involved in using this technique. Exposure times of several weeks, even months, are often required to obtain a suitable autoradiogram.

Despite these limitations receptor autoradiography provides a powerful alternative to homogenate binding, particularly with regard to the localisation of binding sites, a fact that will be demonstrated during this, and subsequent chapters.

The visualisation of receptor distribution, through receptor autoradiography, has proved invaluable in elucidating the complex nature of the NMDA receptor ionophore. A comparison of the anatomical distribution, based on previous data for NMDA (Monaghan and Cotman,1985) and glycine (Bristow *et al.*,1986) revealed a close correlation of these receptors (Bowery,1987). Moreover this correlation was extended to include the dissociative anaesthetic site following the reported distribution of another ligand, ^{3}H-MK-801 (Bowery *et al.*,1988).

Today it is widely accepted that the NMDA complex possesses a variety of binding sites which include a strychnine insensitive glycine site (see earlier). However despite its functional significance, information as to the distribution of the site remains superficial. In contrast to the strychnine-sensitive glycine site (described in depth by Zarbin *et al.*,1981), little is known about the regional densities of the strychnine-insensitive glycine receptor throughout the CNS. To this end, the data presented in this chapter represent an in depth investigation of glycine binding to strychnine insensitive receptors in rat brain.

A light microscopic autoradiographic technique has been used to investigate the anatomical distribution of these receptors in the CNS. In addition the

pharmacological specificity of the receptor has been studied by means of displacement curves using a number of known agonists and antagonists.

2.2: Methods.

2.2.1: Light microscopic ^{3}H-glycine autoradiography.

Male rats (Sprague-Dawley, 200-250g) were killed by decapitation, the brains rapidly removed and frozen in isopentane (cooled to just above its freezing point in liquid nitrogen). Ten micron cryostat sections of each brain were mounted on glass slides and stored at -20°C for up to 3 weeks. Slide mounted sections were brought to room temperature and incubated for 60 minutes in 50mM Tris-Citrate buffer (pH 7.4), at 20°C, to allow for the dissociation of possible endogenous inhibitors, and allowed to air dry. After drying 100µl of Tris-citrate buffer (pH 7.4) containing 100nM ^{3}H-glycine (43.5 Ci/mmol) was applied over each section at 4°C with or without unlabelled drug. This was left in contact for 20 minutes unless otherwise stated. After incubation the radiolabel solution was rapidly aspirated off and the section rapidly "dip" washed in ice cold Tris citrate buffer, followed by a 10 second wash in fresh Tris-citrate buffer. The sections were finally "dip" washed in deionised water (4°C) and dried in a stream of cool air.

To determine the optimal conditions for binding, and for the assessment of displacing compounds, sections were immersed in "Optiphase Safe" solution and the bound radioactivity measured by scintillation spectrometry. Non-specific binding was determined by addition of 100µM glycine to the incubation solution in the optimisation and autoradiographical studies or the addition of varying concentrations of displacers where appropriate.

2.2.2: Autoradiogram generation.

Autoradiograms were generated by placing the dried section in contact with tritium sensitive film (LKB Utrofilm) for 4-6 weeks at 20°C. The film was developed with D19 (Kodak Ltd.) for 2-3 minutes at 20 °C, fixed in Unifix for 5-6 mins and washed for 15 mins in water (23°C).

2.2.3: Densitometric analysis of autoradiograms.

Photographic images obtained were analyzed by means of a computerized routine on a Quantimet 970 image analyser (Cambridge Instruments). Regional densities were converted to the corresponding ligand concentration by reference to tritium standards prepared by Amersham International.

2.3: Results.

Optimisation experiments.

2.3.1: Effect of pH, incubation buffer and temperature.

^{3}H-Glycine binding to strychnine-insensitive glycine binding sites exhibited a marked variation corresponding to alterations in pH (Fig 2.1). Optimal pH levels (7-7.4) produced specific binding of 82%, which decreased rapidly to 15% at acidic values (pH 4). In contrast a more alkaline pH had less influence with specific binding levels of 65% still being observed at pH 10.

The effect of 3 incubation buffers, Tris-acetate, Tris-citrate and Tris-HCl were investigated. Highest specific binding (85%) was observed with Tris-citrate, with binding reduced to 73% and 61% of total by Tris-acetate and Tris-HCl respectively. All buffers contained 50mM Tris-base, lowering the concentration to 5mM failed to provide any beneficial alteration in the total:background ratio.

Raising the incubation temperature from 4°C to 24°C produced a 43% reduction in specific binding relative to that obtained at 4°C.

2.3.2: Effect of incubation and wash time.

The influence of varying the incubation times between 5 and 120 min in Tris-Citrate buffer (pH7.4, 4°C) was evaluated (Fig 2.2). Total binding increased over the initial 10 minute period, and then remained unaltered for at least 120 minutes. Optimal specific binding, corresponding to 75% of total activity associated with each section, was obtained after 20 mins. Background levels

Fig 2.1: The effect of pH on strychnine-insensitive ^{3}H-glycine binding to rat brain sections (parasagittal).

Slices were incubated with 100 nM ^{3}H-glycine at pH values from 4 to 10. Non-specific binding was determined by the additional presence of 100µM glycine. Data represents the mean specific binding as a percentage of the total binding from 3 determinations.

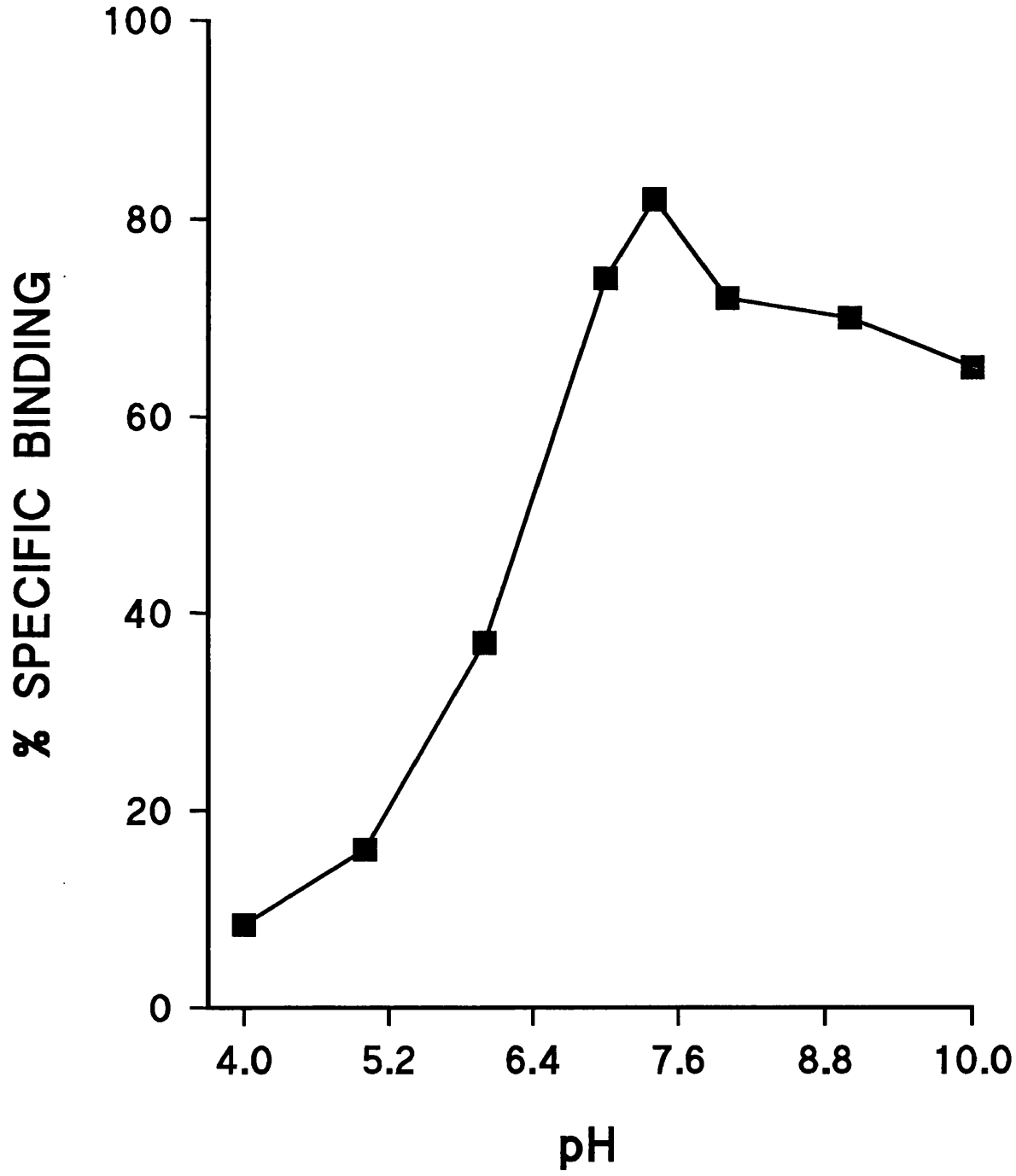
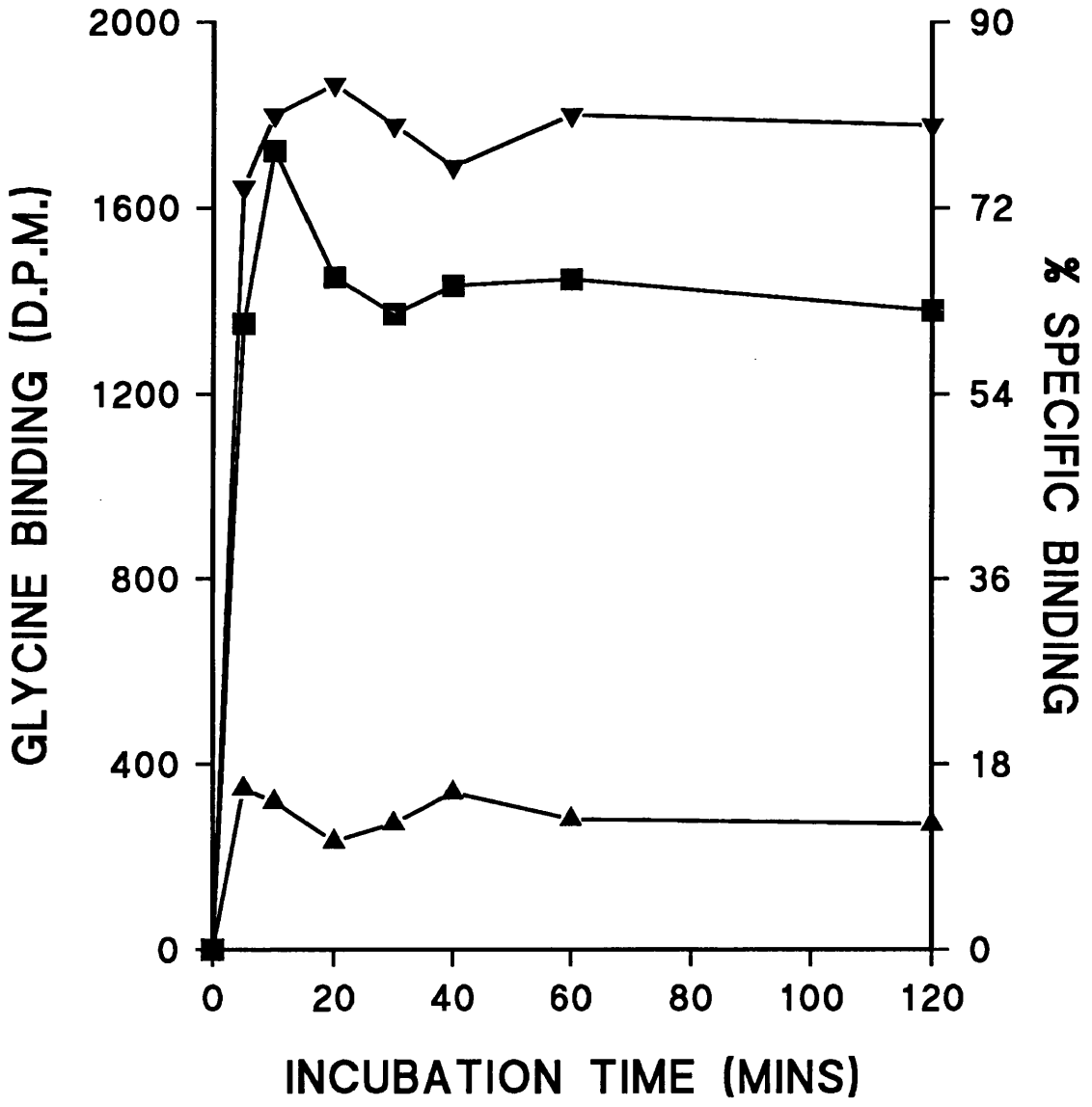

100
80
60
40
20
0
% SPECIFIC BINDING
4.0
5.2
6.4
7.6
8.8
10.0
pH

Fig 2.2: Time course of the binding of ^{3}H-glycine to parasagittal sections of rat brain.

Sections were incubated with 100 nM ^{3}H-glycine for the time periods shown on the abscissa scale. Each point represents the mean of 3 experiments. The SEM was < 10% of the mean in all cases. Total binding (■) was determined in ^{3}H-glycine alone, whereas background (▲) was determined in the additional presence of unlabelled glycine (100μM). The values representing specific binding (▼) have been calculated as a percentage of the total binding. Binding values on the left ordinate represent d.p.m values associated with a single section.


2000
1600
1200
800
400
0
GLYCINE BINDING (D.P.M.)
90
72
54
36
18
0
% SPECIFIC BINDING
0
20
40
60
80
100
120
INCUBATION TIME (MINS)

exhibited little variation, with values remaining between 10-15% of total binding throughout.

Wash times ranging from a rapid "dip" wash (<1 sec) to 120 secs were investigated (Fig 2.3). The optimal rinse time was 10 sec in Tris-citrate buffer at 4°C. After this time the specifically bound ligand represented 77% of total radioactivity associated with each section. A degree of specific binding could be detected after washing for 120 secs but this amounted to only 20% of total radioactivity, a decrease of approx 75% of that observed at 10 sec. Periods greater or less than 10 secs failed to improve the specific : background ratio. Rinsing sections at 24°C failed to provide any improvement in the binding ratio.

2.3.3: Saturation of ^{3}H-glycine binding.

Saturation experiments for ^{3}H-glycine binding were performed over the range 10 - 1000nM glycine. Scatchard analysis of saturation data (Fig 2.4) illustrated, under the experimental conditions employed, that glycine binds to a single, saturable site with a K_D of 196 ± 47 nM, maximal binding (B_{MAX}) being 17.6 ± 2.5 pmol/mg tissue. The Hill coefficient from these data was .93 ± .08 indicating that no cooperativity occurs between sites.

2.3.4: Inhibitors of ^{3}H-glycine binding.

The ability of a number of compounds to displace ^{3}H-glycine binding to strychnine-sensitive glycine receptors was investigated (Table 2.1).

Fig 2.3: Wash curves of ^{3}H-glycine binding to parasagittal sections of rat brain.

Slices were incubated with 100nM ^{3}H-glycine for 20 min (4°C) and subsequently washed for the time period shown on the abscissa scale. Each point is the mean of 3 separate determinations. The SEM in all cases was <10 % of the mean value. The total binding (■) was obtained in the presence of 100nM ^{3}H-glycine alone, whilst background (▲) binding was determined in the additional presence of 100 µM glycine. The specific binding (▼) has been calculated as a % of the total binding. Binding values on the left ordinate represent d.p.m associated with a single section.

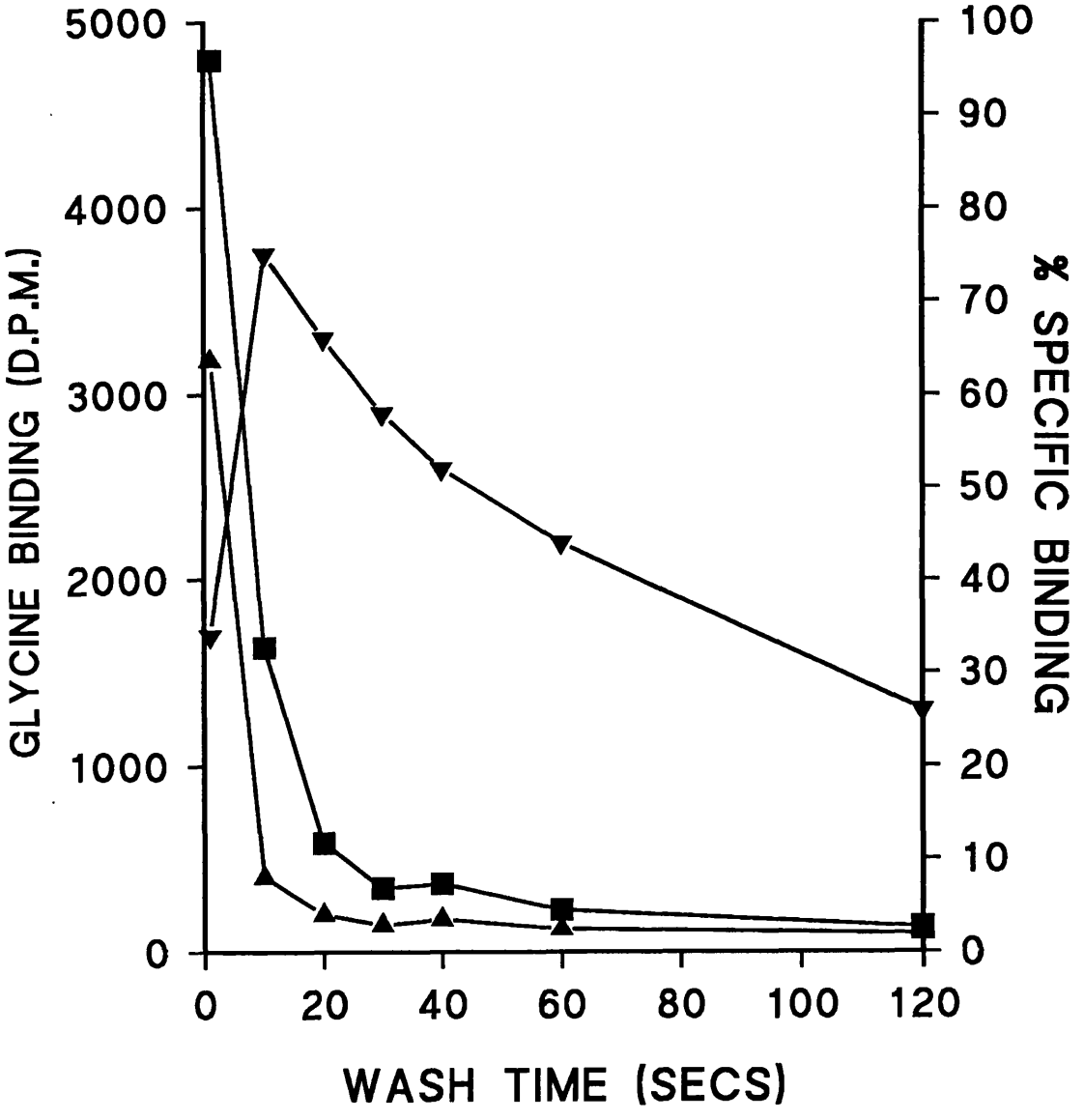

5000
4000
3000
2000
1000
0
GLYCINE BINDING (D.P.M.)
100
90
80
70
60
50
40
30
20
10
0
% SPECIFIC BINDING
0
20
40
60
80
100
120
WASH TIME (SECS)

Fig 2.4: Scatchard analysis of ^{3}H-glycine binding to parasagittal sections of rat brain.

Sections were incubated with 100nM ^{3}H-glycine and from 5nM to 10 µM glycine ; non-specific binding was determined in the presence of 100µM unlabelled glycine. The graph represents the mean data obtained from 5 separate experiments. Data were analysed using a non-linear least squares curve fitting model. These data from rat brain were best fitted by a single site model.

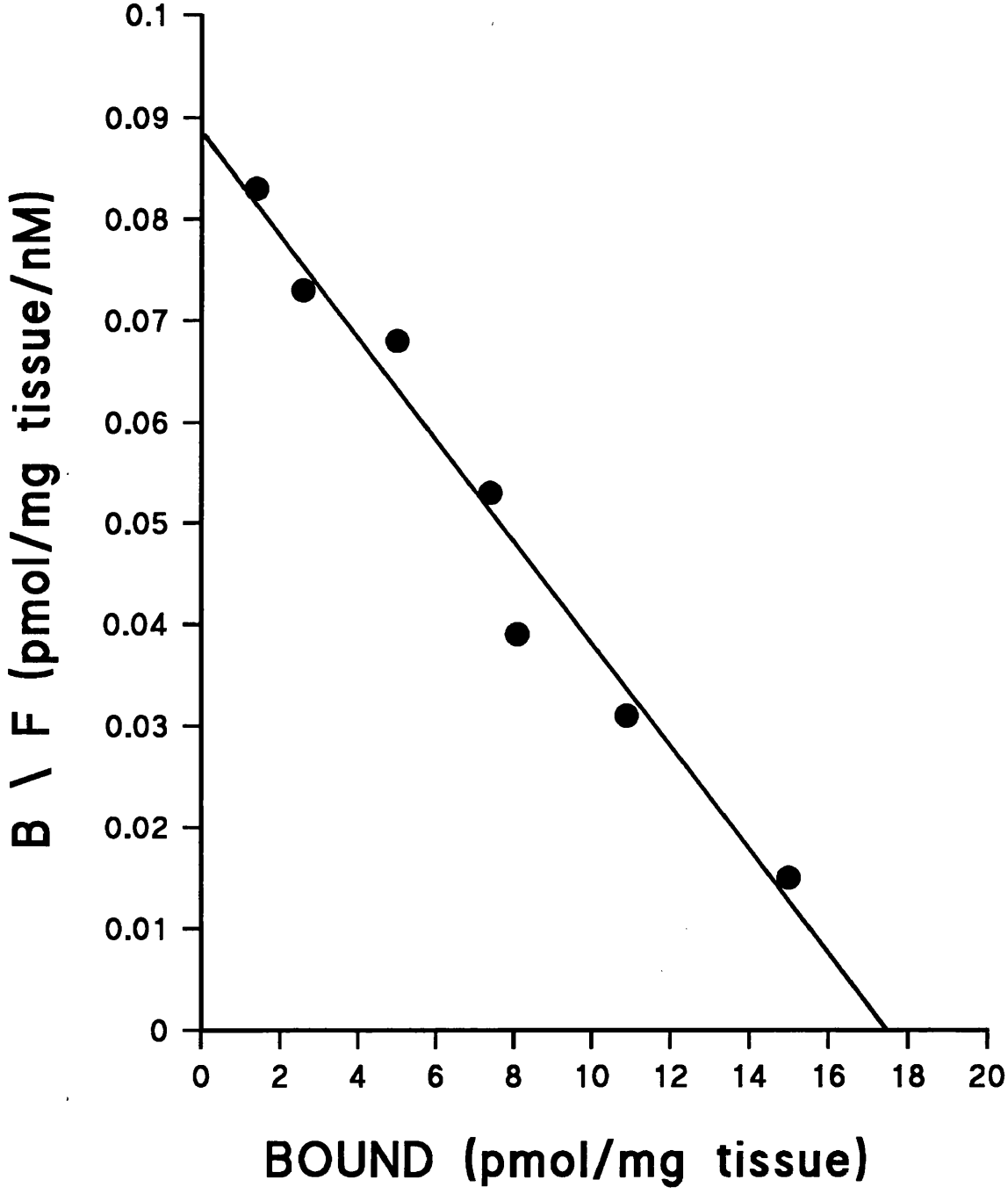

0.1
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
B \ F (pmol/mg tissue/nM)
0
2
4
6
8
10
12
14
16
18
20
BOUND (pmol/mg tissue)

Table 2.1: The influence of glycine analogues and other compounds on ^{3}H-glycine binding to rat parasagittal sections.

Data represent mean IC_{50} ± SEM (μM) from 3 - 5 experiments. Slices were incubated with 100nM ^{3}H-glycine in the presence or absence of varying concentrations of displacer. Non-specific binding was determined in the presence of 100 μM unlabelled glycine.

COMPOUND	IC_{50} ± SEM (μM)
GLYCINE	.28 ± .17
TAURINE	> 1000
D-SERINE	.33 ± .21
L-SERINE	56.20 ± 11.16
D-CYCLOSERINE	2.45 ±.48
L-CYCLOSERINE	479.10 ± 9.61
L-GLUTAMIC ACID	> 1000
NMDA	> 1000
DNQX	. 26 ± .11
CNQX	2.38 ± .71
7 Cl KYN	.78 ± .16
HA-966	21.20 ± 4.47
AP5	> 1000
AP7	> 1000
STRYCHNINE	> 1000
CPP	> 1000
MK-801	> 1000
GABA	> 1000
2-PYRROLIDINONE	> 1000
IFENPRODIL	> 1000

Among the simple amino acids glycine (IC_{50} =.23 ± .17 µM) and D-serine (.33 ± .21 µM) were found to be the most potent displacers of glycine binding. Whereas the L isomer of serine was 173 fold less potent than the D-form (56.2 ± 11.16 µM) (Fig 2.5). In general the D-amino acids were more potent than the corresponding L forms, also shown with D-cycloserine (2.45 ± .48 µM) and L-cycloserine (479.10 ± 9.61 µM). The majority of the amino acids, with the exception of GABA, glutamate and NMDA, produced a complete inhibition at concentrations up to 3mM. The rank order for displacement was :-

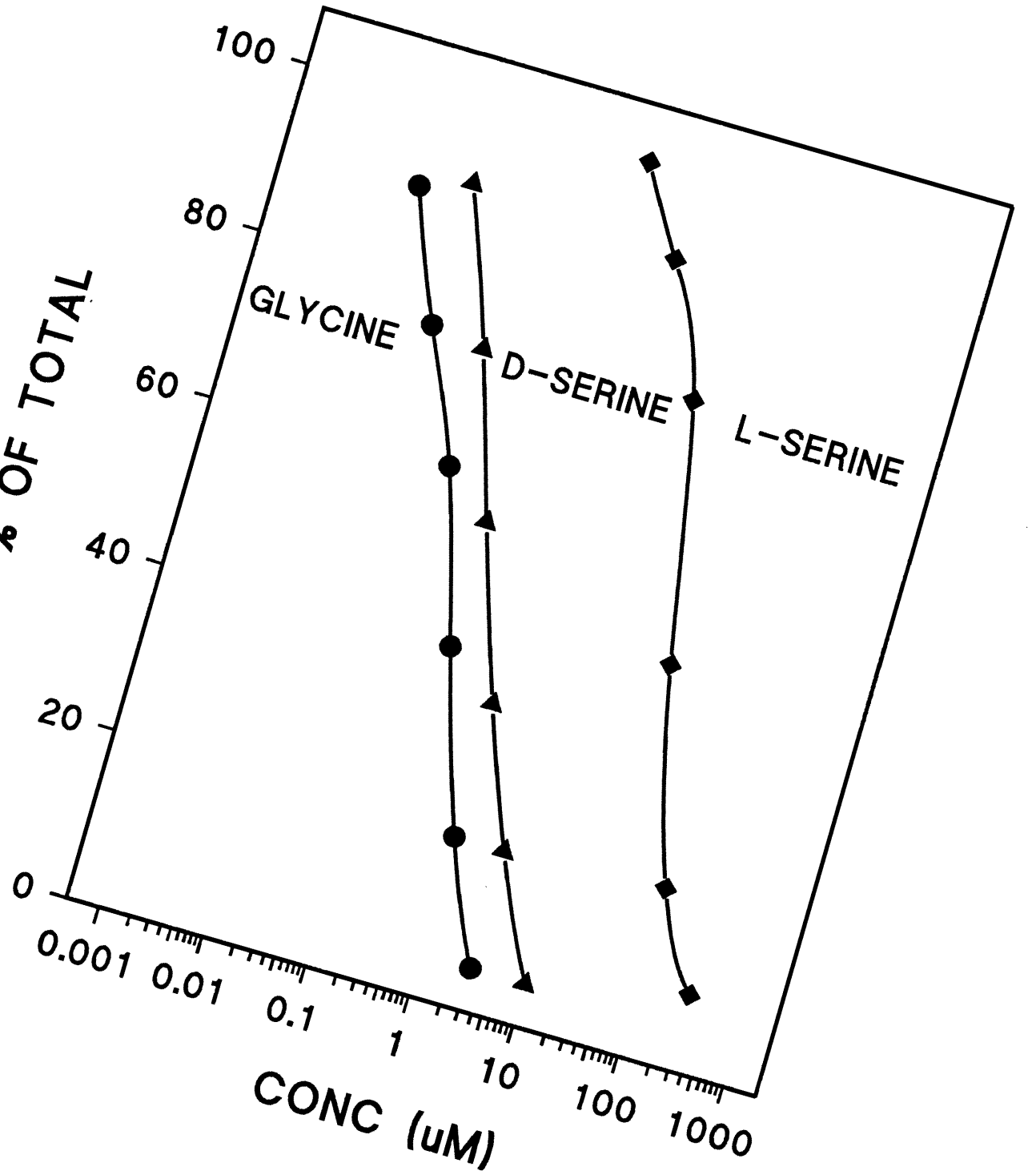
Glycine > D-serine > D-cycloserine > L-serine > L-cycloserine.

Two competitive glycine antagonists HA-966 and 7- Chlorokynureninc acid and two AMPA antagonists CNQX and DNQX were found to inhibit binding in a dose dependent manner. Of these compounds DNQX and 7-ClKYN were the most effective IC_{50} values of .26 ± .11 and .78 ± .16 µM respectively. CNQX (2.38 ± .71 µM) was 10 fold weaker and HA966 (21.20 ± 4.47 µM) some 80 fold less potent than CNQX.

None of the NMDA receptor agonists (L-glutamate and NMDA) or antagonists (CPP and MK-801) significantly reduced binding, with the exception of DL AP5 (APV) which produced a 43% displacement at 3mM. Strychnine failed to significantly alter binding and similarly taurine produced no inhibition at concentrations up to 3mM. Tetramethylpyrazine, GABA and 2-pyrrolidinone failed to alter glycine binding over the concentration range of 100nM to 10mM.

Fig 2.5: Displacement of strychnine-sensitive ^{3}H-glycine binding to parasagittal sections of rat brain by D (▲) and L (♦) serine.

Sections were incubated with 100 nM ^{3}H-glycine in the presence or absence of varying concentrations of displacer. Each point represents the mean of 3 sections obtained from a single experiment. Similar results were obtained in 4 separate determinations. Specific binding is defined as a percentage of the total binding.


100
80
60
40
20
0
% OF TOTAL
GLYCINE
D-SERINE
L-SERINE
0.001
0.01
0.1
1
10
100
1000
CONC (uM)

2.3.5: Distribution of strychnine-insensitive glycine binding sites.

The autoradiographical distribution of strychnine-insensitive ^{3}H-glycine binding in rat brain indicate a distinct regional heterogeneity. A degree of specific binding was detected in all brain regions, with levels increasing corresponding to a rostral- caudal progression. The results of quantitative analysis are shown in Tables 2.2 and Figs 2.6, 2.7 and 2.8. The areas exhibiting the highest density of binding were those of the stratum molecular of the dentate gyrus (539 ± 34 fmol / mg tissue) and the CA1 stratum radiatum of the hippocampal formation (473 ± 44: 100%) with the lowest being localised in the habenula nucleus (26 ± 3).

A distinct laminal distribution of glycine binding was present within the olfactory bulb; the external plexiform layer showing the highest binding (39% of that observed in CA1) for the region, with decreased densities in the deeper glomerular layer (21%) and the more superficial, internal granule layer (19%) regions. Higher levels of binding were located in the primary olfactory cortex (74%) and the anterior olfactory nuclei exhibited a homogeneous distribution of sites between the medial (79%) and lateral (77%) nuclei.

The cerebral cortical regions displayed intermediate and high levels of glycine binding throughout the structure, with a high degree of variation existing between the cortices. The highest binding of all cortical regions was detected in the cingulate nuclei, the outer layers of which possessed 79% of the total binding density, with a reduction to 60% in the inner layers . The frontal

Table 2.2: Regional distribution of strychnine-insensitive ^{3}H-glycine binding sites in sections of rat brain.

Values are presented as the mean ± SEM (n = 5) and as a percentage relative to stratum radiatum of the CA1 hippocampal formation. The values were measured by densitometric analysis with reference to tritium standards apposed to the same films. Background binding was subtracted in all cases.

Table 2.2

		^{3}H-glycine bound fmol\mg tissue	
	Abreviations	Mean ± SEM	% CA1
Olfactory region			
Glomerular layer	GL	99 ± 7	21
External plexiform layer	EPL	185 ± 28	39
Anterior olfactory nucleus - medial	Med AON	373 ± 31	79
Anterior olfactory nucleus - lateral	Lat AON	364 ± 28	77
Internal granule layer	IGL	89 ± 6	19
Primary olfactory cortex	POC	350 ± 27	74
Cortex			
Entorhinal layer - outer	O ENT	161 ± 10	34
Entorhinal layer - middle	M ENT	179 ± 13	38
Entorhinal layer - inner	I MET	175 ± 9	37
Frontoparietal layer - outer	O FrPa	303 ± 32	64
Frontoparietal layer - middle	M FrPa	274 ± 29	58
Frontoparietal layer - inner	I FrPa	232 ± 26	49
Frontal layer - outer	O FRCX	283 ± 27	60
Frontal layer - middle	M FRCX	265 ± 24	56
Frontal layer - inner	I FRCX	170 ± 14	36
Cingulate layer - outer	O CICX	374 ± 33	79
Cingulate layer - inner	I CICX	283 ± 25	60
Basal ganglia			
Caudate putamen - medial	Med-CPu	194 ± 13	41
Caudate putamen - lateral	Lat-CPu	179 ± 16	38
Caudate putamen - anterior	Ant-CPu	274 ± 24	58
Caudate putamen - posterior	Post-CPu	302 ± 27	64
Globus pallidus - anterior	Ant-GP	47 ± 5	10
Globus pallidus - postMwior	Post-GP	66 ± 8	14
Accumbens nucleus	Acb	193 ± 36	41

Table 2.2 continued

	Specific ^{3}H-glycine bound fmol/mg tissue		
	Abreviations	Mean ± SEM	% CA1
Biencephalic nuclei.			
Medial dorsal	MD-THAL	242 ± 21	51
Anterior ventral	AV-THAL	227 ± 18	48
Ventral lateral	VL-THAL	199 ± 20	42
Ventral posterior medial	VPM-THAL	208 ± 21	44
Habenula	HB	26 ± 3	5
Lateral geniculate	LG	180 ± 19	38
Medial geniculate	MG	231 ± 21	49
Amygdala.- overall	Amg	114 ± 22	24
Hippocampus.			
CA1. lacunosum moleculare	SLM-CA1	345 ± 39	73
CA1. stratum radiatum	SR-CA1	473 ± 44	100
CA1. stratum pyramidale	SP-CA1	322 ± 28	68
CA1. stratum oriens	SO-CA1	373 ± 27	79
CA2. stratum radiatum	SR-CA2	270 ± 35	57
CA2. stratum pyramidale	SP-CA2	99 ± 21	21
CA2. stratum oriens	SO-CA2	204 ± 17	43
CA3. stratum radiatum	SR-CA3	350 ± 49	74
CA3. stratum pyramidale	SP-CA3	180 ± 15	38
CA3. stratum oriens	SO-CA3	312 ± 21	66
Dentate gyrus - molecular	MDG	539 ± 34	122
Dentate gyrus - granular	GDG	203 ± 14	43
Brain stem.			
Central gray	CG	34 ± 3	7
Inferior colliculus	IC	47 ± 5	10
Substantia nigra	SNR	61 ± 7	13
Cerebellum.			
Molecular layer	Mol-Cb	43 ± 6	9
Granular layer	Gr-Cb	185 ± 11	39

Fig 2.6 : Autoradiograms of strychnine-insensitive ^{3}H-glycine binding to rat brain sections.

Sections were incubated with 100 nM ^{3}H-glycine and autoradiograms generated as described in Methods. Abbreviations as listed in Table 2.2.

(A = Anterior: B = Medial: C = Posterior)

Figures correspond to plates 13, 30 and 38 of The Rat Brain by Paxinos and Watson (1982).

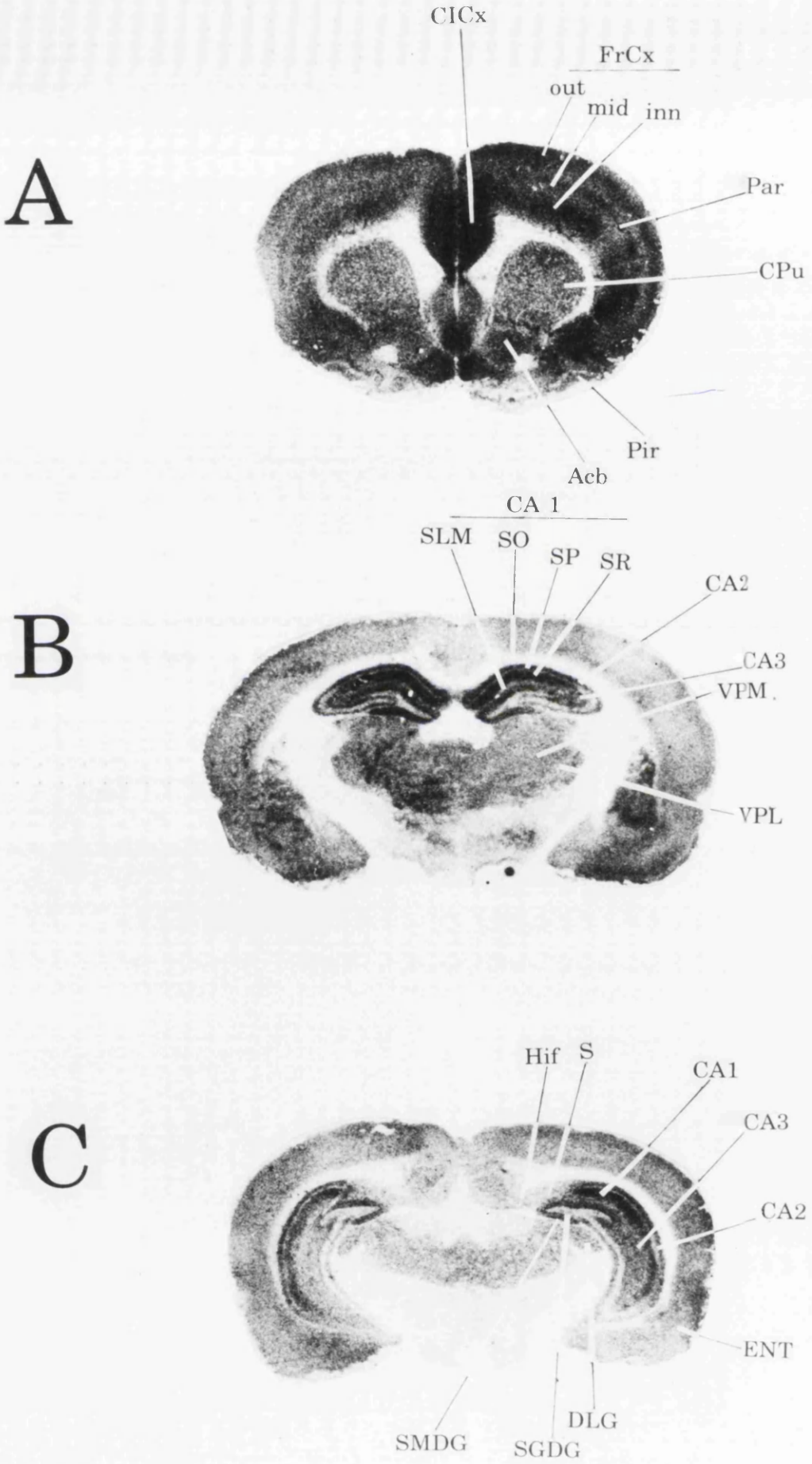

A
CICx
FrCx
out
mid
inn
Par
CPu
Pir
Acb
B
CA 1
SLM
SO
SP
SR
CA2
CA3
VPM
VPL
C
Hif
S
CA1
CA3
CA2
ENT
DLG
SMDG
SGDG

Fig 2.7: Autoradiograms of strychnine-insensitive ^{3}H-glycine binding to rat brain sections.

Sections were incubated with 100 nM ^{3}H-glycine and autoradiograms generated as described in Methods. Abbreviations as listed in Table 2.2.

(A = Parasagittal: B = Horizontal)

Figures correspond to plates 84 and 101 of The Rat Brain by Paxinos and Watson (1982).

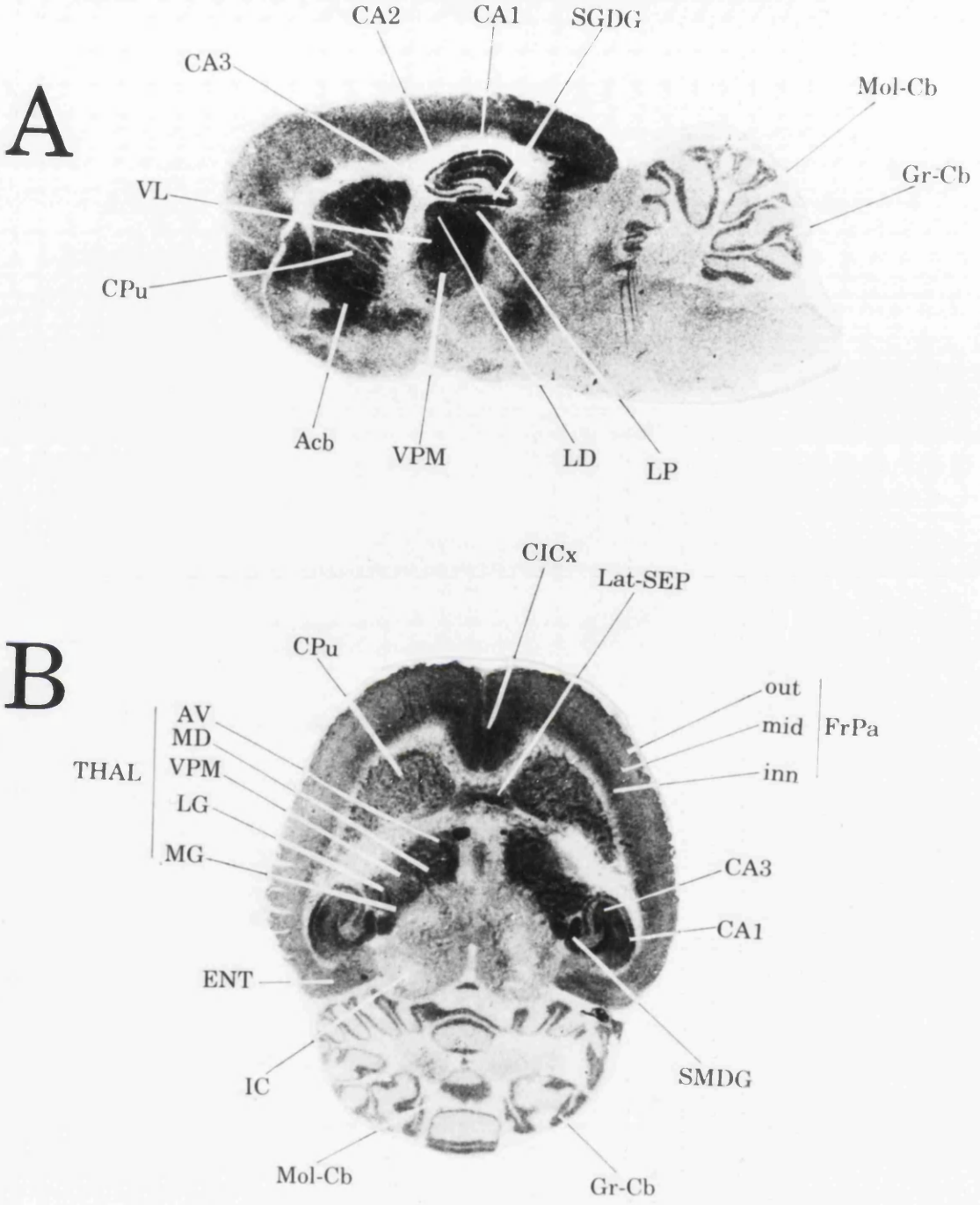
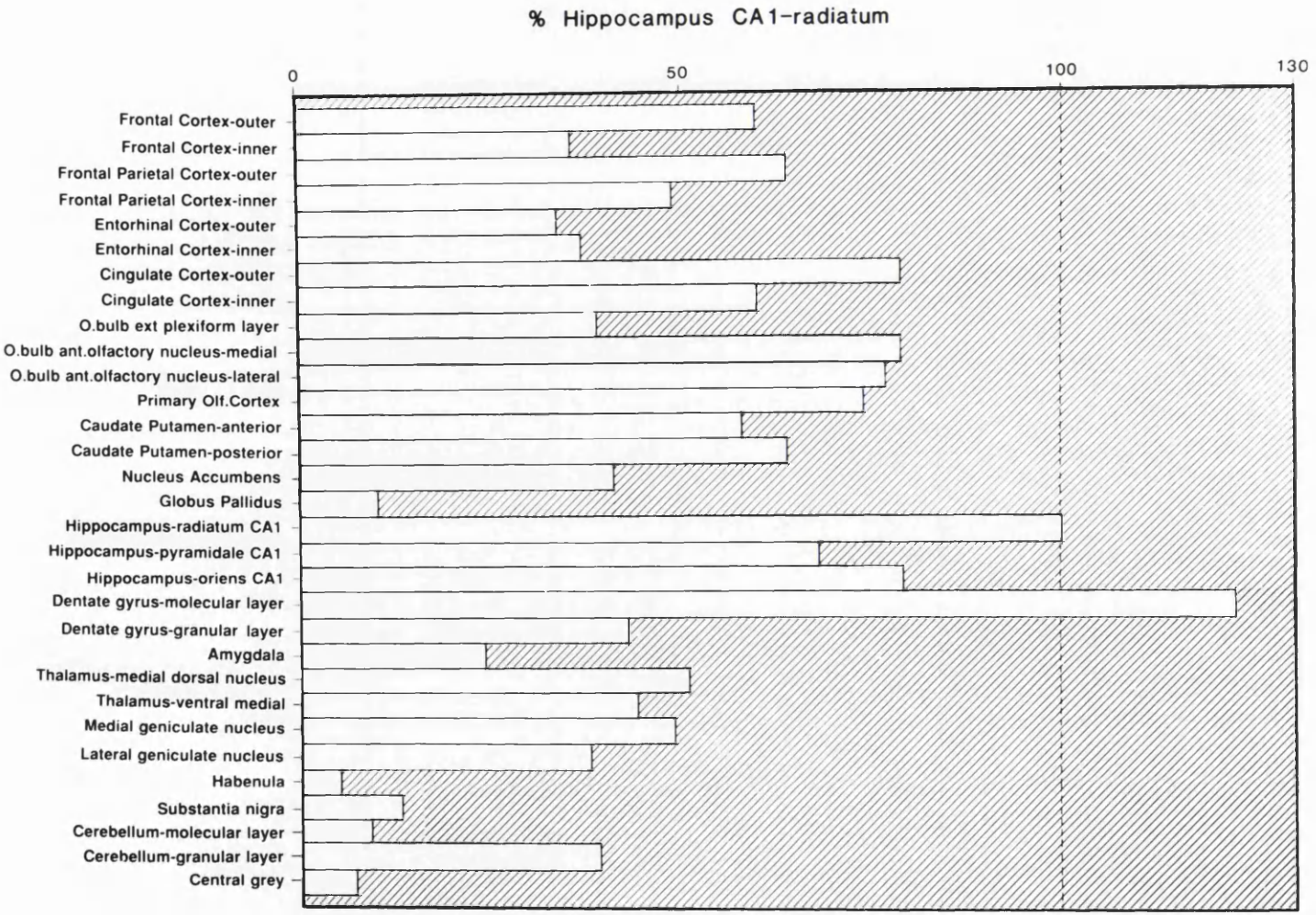

A
CA2
CA1
SGDG
CA3
Mol-Cb
Gr-Cb
VL
CPu
Acb
VPM
LD
LP
B
CICx
Lat-SEP
CPu
out
mid
inn
FrPa
AV
MD
THAL
VPM
LG
MG
CA3
CA1
ENT
IC
SMDG
Mol-Cb
Gr-Cb

Fig 2.8: Comparative regional densities of ^{3}H-glycine binding in rat brain sections.

Density values for each brain region from at least 3 sections each from 4 rats. Values were determined by densitometric analysis and calibrated by reference to tritium standards (Amersham plc) apposed to the same films.

REGIONAL DISTRIBUTION OF ^{3}H GLYCINE BINDING SITES IN RAT BRAIN



cortex displayed a progressive decrease in binding from outer to inner (outer layer 60% through to inner layer 36%). This pattern was repeated, if somewhat less marked in the frontoparietal cortices (outer layer 64%, inner layer 49%). The layers of the entorhinal cortex exhibited a lower density of binding with respect to the other regions, with intermediate levels being observed in a homologous pattern throughout the layers (outer 34%, inner 37%).

The basal ganglia exhibits a marked heterogeneity, the highest levels being located in the caudate putamen, which itself displayed an increase in density in more posterior areas with intermediate levels in the nucleus accumbens (41%) and the lowest density in the globus pallidus (12%).

In contrast, a more universal distribution was present in all areas of the biencephalic nuclei, which range from 42% of total CA1 in the ventral lateral regions through 48% in anterior ventral thalamic regions to a maximum of 51% in the medial dorsal nuclei. A similar pattern was observed in the geniculate nuclei, which exhibited densities of 38% in lateral and 49% in medial zones. The habenula nuclei, as previously indicated, contained the lowest binding of any region, with only 5% of that found in the CA1 of the hippocampus. The amygdala formation displayed slightly higher levels of binding, but still only 24% of CA1.

The region of highest density present in the brain was found within the hippocampal formation. The stratum radiatum of the CA1 was the most intensely labelled (473 ± 44 fmol / mg tissue) with the stratum oriens (79%) and the pyramidal cell layer (68%) showing lower binding intensities. This cellular pattern was repeated in other hippocampal regions, with the CA3 being generally more densely populated than CA2. In all cases the pyramidal cell layer exhibited a lower degree of binding compared with the other areas. The granular cell layer of the dentate gyrus was relatively low in binding sites (43%) compared with the molecular layer (122%) of the same region which exhibited the highest density in the brain.

The distribution observed in the dentate gyrus was reversed in the cerebellum, with the granule cell layer showing intermediate levels of binding (39%) whilst the molecular layer exhibited much lower binding (9%). Very low levels of strychnine insensitive glycine binding sites were located in all regions of the brain stem (central grey (7%), inferior colliculus (10%) and substantia nigra (13%).

2.4: Discussion.

2.4.1: Pharmacological analysis.

A direct comparison between the widespread literature and the data presented in this chapter reveals a close correlation to previous studies. Furthermore the pharmacological selectivity, saturation data, scatchard analysis and regional distribution fulfils the criteria for the presence of the physiologically defined strychnine-insensitive glycine receptor in rat brain sections.

The binding of ^{3}H-glycine to strychnine-insensitive glycine receptors in rat brain sections exhibited an equilibrium dissociation constant (K_D) of 196 ± 47nM and a B_{MAX} of 17663 ± 2581 fmol/mg tissue (Fig 2.4). These values, and the subsequent IC_{50} values (Table 2.1), compare favourably to those previously reported by Kishimoto *et al.*,(1981) and Bristow *et al.*,(1986) for binding to brain homogenates and to the EC_{50} values for glycine enhancement of NMDA stimulation of ^{3}H-TCP (Johnson *et al.*,1988) and MK-801 (Wong *et al.*,1987). This correlation provides useful support as to the validity of using brain sections as opposed to synaptic membranes or brain homogenates, a fact further enhanced by displacement studies and discussed later in the chapter.

The single population of binding sites indicated by the linear scatchard analysis of the saturation data (Fig 2.4) supports the findings of Jones *et al.*,(1989). However recently the possible existence of two strychnine-insensitive glycine receptors has been proposed (White *et al.*,1989). The authors described the presence of a high and low affinity site similar to that suggested by

Monaghan *et al.*,(1988) for the NMDA receptor (discussed earlier). It would appear that the binding in brain sections is comparable to binding at the high affinity site, however, the idea that two receptors exist remains unsubstantiated.

Further evidence for binding to the strychnine-insensitive site is illustrated by the narrow pH dependency (Fig 2.1), maximal binding occurred between pH 7-7.4, a finding supported by Danysz *et al.*,(1990) and Kishimoto *et al.*,(1981). Whilst the decreases in specific binding observed with increased temperature, suggests it is unlikely that binding represents a simple sequestration or binding to an enzyme transport site (Hannuniemi and Oja,1981).

Displacement studies (Table 2.1) correspond well to those data already published for binding in homogenates (Danysz *et al.*, 1990; Monahan *et al.*,1989; Galli *et al.*,1988), and suggest the existence of a complex structure-activity relationship, similar to that previously reported for NMDA (Watkins *et al.*,1990) and PCP (Nadler *et al.*,1990). The rank order of potency : Glycine>D-serine>D-alanine>D-cycloserine>L-serine>L-alanine>L-cycloserine was similar to that obtained from membrane binding studies (Snell *et al.*,1988) and a number of other systems including the ability to potentiate NMDA induced currents in Xenopus oocytes (Kleckner and Dingledine,1988), enhance NMDA stimulated release of ^{3}H-noradrenaline (Ransom and Deschenes,1988), enhance ^{3}H-TCP (Johnson *et al.*,1988; Snell *et al.*,*1987*) and MK-801 (Wong *et al.*,1987; Reynolds *et al.*,1987) binding in membranes, contract the guinea pig ileum (Galli *et al.*,1988), reverse kynurenate inhibition of NMDA-evoked

depolarization in cortical wedge preparations (Fletcher *et al.*,1989; Kemp *et al.*,1988), spinal cord (Birch *et al.*,1988b), hippocampus (Watson *et al.*,1988) and patch clamp studies of cultured cells (Ascher *et al.*,1989).

Two firm conclusions can be drawn from the observed rank order of potency. Firstly the strychnine-insensitive glycine binding site displays distinct structural preference and secondly it can exhibit a degree of stereoselectivity. Both of these aspects will be discussed individually.

The stereospecificity (or stereoselectivity) of the strychnine-insensitive glycine site is typified by the affinity changes observed by D/L-serine (Fig 2.5). Conversion from the D-isomer to the corresponding L- formation produced a 100 fold reduction in affinity. This observation is consistent with the D-isomer being the favoured orientation of all amino acids (with the possible exception of valine) (Snell *et al.*,1988).

The absence of an asymmetric carbon atom within the glycine molecule suggests that the stereoselectivity occurs due to the "spatial" positioning of the functional group(s) away from the active binding site, an idea supported by Snell *et al.*,(1988), however it is not possible to correlate with the reports by these authors as to the potencies of D/L-valine. It is however fairly obvious that the actual molecular structure/composition either solely or in combination with the "spatial" orientation, is responsible for the relative affinities of the amino acids. It is particularly difficult to draw any firm conclusions concerning the

structural requirements of the glycine receptor given the relatively small range of compounds tested, nevertheless a number of points are worth mentioning.

The identity and position of the substituent on the α-carbon atom play an important role in determining the activity of the compound. Substitution of -H by -OH (serine) is less detrimental when compared to methyl (alanine), However cyclic derivative substitution (cycloserine) produces the greatest affinity reduction. The latter findings may relate simply to the presence of a cyclic ring within the molecule preventing suitable orientation of the "active part" of the molecule within the receptor cleft. A similar observation of lowered affinities with cyclic structures was reported by Snell and Johnson (1988) for cycloleucine. An alternative possibility is that the presence of a cyclic compound may be favourable with antagonist, as opposed to agonist activity, this concept will be discussed in detail later.

The inability of GABA to displace glycine binding may reflect the importance of chain length and the subsequent distance between the amino and carboxyl terminals. Increasing chain length (GABA possesses a chain length of 3 atoms) decreases affinity, a concept favoured by McDonald *et al.*,(1990) who reported the activity of β-alanine (2 atom chain length) as being between glycine and GABA. Similarly the actual presence of the carboxyl group appears essential, since thiol substitution (taurine) produces a complete loss of affinity, an observation possibly related to a need for charge balance within the molecule. A similar structure-activity relationship emerges when considering selective

and non-selective glycine antagonists. The IC_{50} values obtained for the known glycine antagonists 7-chlorokynurenic acid, HA-966, and the more general excitatory amino acid antagonists DNQX and CNQX compares favourably with reported literature values (Kemp *et al.*,1988; Birch *et al.*,1988a; Pellegrini-Giampietro *et al.*,1989; Dansyz *et al.*,1989a). Examination of the structures of these antagonists (detailed in Fig 1.2) reveals a common cyclic appearance, and a functional group at least 4 atoms from the α-carbon of the amino group. It is reasonable to assume that the presence of a cyclic component results in a fairly rigid structure, an attribute lacking in straight-chained amino acids. This rigidity may impose planar conformational restrictions on the molecule, a factor which may prove vital for antagonist characteristics. It is interesting to note that HA-966 displayed the lowest affinity of the antagonists tested. The absence of a benzene ring in this compound and thus the reduced conformational restriction may be reflected in the decreased potency. Although more recent reports (Singh *et al.*,1990b) suggest the presence of a chiral carbon atom is a significant factor in determining the activity of this molecule. Moreover the presence of an additional substituent appears to increase affinity, as illustrated by the marked alteration in potency following the addition of a chloride molecule to kynurenic acid (Kemp *et al.*,1988). Further evidence as to the importance of the identity and position of the substituent is provided by comparing the activity of the two quinoxaline derivatives, DNQX and CNQX. DNQX proved the most potent antagonist tested, whilst the activity of CNQX was 10 fold lower. Comparison of the two structures (Fig 1.2) reveals the only difference between the molecules is the identity of the substituent (NO_2 for

DNQX, CN for CNQX). Thus it is reasonable to assume that the NO_2 is a preferred substituent within the glycine binding site. The increased complexity, and highly polar nature of the cyano grouping may create unfavourable charge conditions resulting in decreased affinity. Positional importance is underlined by comparing the observed affinity of DNQX with the literature reports (Kessler *et al.*,1989a) for the higher affinity antagonist MNQX. The latter molecule shows a single positional change: the NO_2 molecule has been transferred to position 8 instead of the original position 7, producing a slight increase in affinity. This may be related to a more favourable electron distribution within the molecule or simply a more favourable spatial arrangement.

The number of substituents on a molecule may also be reflected in the affinity. The dichloro derivative of quinoxaline, DOQXA has been reported to display potent antagonistic properties (Kessler *et al.*,1989b). Similarly addition of either a second chloride (Baron *et al.*,1990) or an iodine molecule (Foster *et al.*,1990) to 7-chloro kynurenic acid results in an increase in affinity. The latter compounds have only recently been synthesised, however it is interesting to speculate whether the addition of a further halide atom would result in a further increase in affinity.

At the present speculation is all that is possible as to the structure of the strychnine-insensitive glycine receptor, however it is clear that the binding site is fairly large since it is able to accommodate large inflexible molecules such

as the quinoxalines. It remains to be seen whether advanced technology such as computer-assisted graphics (Manallack *et al.*,1990) can enable "designer" agonist and antagonist synthesis and therefore provide a deeper insight into an already complex receptor.

2.4.2: Regional distribution.

A high correlation exists between the anatomical distribution of the strychnine-insensitive glycine binding site in rat brain, and those previously reported by Bristow *et al.*,(1986). In addition comparison with the distribution patterns reported for the other NMDA ligands (see Introduction) provides further evidence for the well published concept of a close association between the strychnine-insensitive glycine site and the NMDA receptor. The close correlation can be extended to the monkey (Geddes *et al.*,1989) and more significantly the human (Jansen *et al.*,1989), underlying the involvement of glycine as a modulator of the physiological functions of the NMDA receptor.

The high levels of binding observed in the dentate gyrus and the dendritic layers of the CA1 radiatum and oriens are consistent with the suggestion that these areas represent the termination fields for the glutamatergic pathways of the hippocampus (Taxt and Storm-Matheisen,1984; White *et al.*,1977). Furthermore the CA1 region has been reported to be particularly susceptible to excitotoxic neuronal damage, as may occur during cerebral ischemia (Gill *et al.*,1988; Bowery *et al.*,1988); and to sclerotic changes in epilepsy (Rothman and Olney,1986; Leach *et al.*,1988). It is thus of interest to note that this area

contains high densities of glycine and NMDA receptors, furthering the idea that these two receptors play a key role in excitotoxicity (Patel *et al.*,1990; Garthwaite and Garthwaite,1989), a concept discussed in the introduction. In addition the high receptor density in the CA1 may reflect the involvement of NMDA receptors in memory and learning (Collingridge and Bliss,1987) and the induction of long term potentiation (Collingridge and Lester, 1989) within the hippocampus. Information obtained from lesion studies (Gundlach *et al.*,1986) implies that the glycine receptors may be associated with neurones, specifically intrinsic neurones and afferent terminals in the hippocampus.

The distribution patterns observed in cortical regions may reflect the involvement of the glycine receptor, in conjunction with NMDA, in a number of cortical and sensory pathways.

The distribution of ^{3}H-glycine sites in the cerebral cortex coincides with those reported for glutamate (Monaghan and Cotman, 1985) and MK-801 sites (Bowery *et al.*,1988), namely higher densities in the outer regions. The lower densities observed in deeper cortical regions may reflect the predominance of other EAA receptors eg: kainate, in these regions (Jansen *et al.*,1989). Furthermore the observed distribution pattern of ^{3}H-glycine binding compares favourably with the previously reported uptake of ^{3}H-glycine (Wilkin *et al.*,1981b; Taxt and Storm-Mathesen,1984). High densities of the uptake and binding sites can be found in the outer layers of the cerebral cortex, caudate putamen and nucleus accumbens. In contrast areas displaying a low receptor

density eg: globus pallidus and the habenula, also display a low density of uptake sites. These observations are consistent with the belief that these latter areas are generally regarded to possess a mainly GABAergic input (Price and Bowery,1988) and thus display an predominantly inhibitory role.

The intermediate binding densities of the lateral medial geniculate nucleus and external plexiform layer of the olfactory bulb might reflect the possible role of NMDA receptors in sensory function (Cotman *et al.*,1987). Furthermore the lateral geniculate nucleus is known to receive a glutamatergic input via NMDA receptors from the optic nerve in the cat (Kemp and Sillito, 1982). Presynaptic NMDA, kainate and glycine receptors in the olfactory cortex have been postulated to regulate neurotransmitter release in this region (Collins *et al.*,1986).

The entorhinal cortex is another brain region exhibiting synaptic transmission mediated purely through NMDA receptors (Watkins *et al.*,1986), however the observed level of binding in this region is lower than may have been expected. In contrast, a better correlation of receptor density and function occurs in the cingulate cortex. NMDA-induced late EPSPs modulation by glycine has been demonstrated in this region (Thompson *et al.*,1989). Moreover the cingulate cortex, together with the hippocampus and amygdala, have been associated with the emotional disorder that accompanies phencyclidine abuse (Gundlach *et al.*,1986).

The low binding densities observed in the brain stem is consistent with the predominance of strychnine-sensitive glycine binding sites reported in this region (Young and Snyder,1973; Zarbin *et al.*,1981).

The binding density pattern observed in the cerebellum will be discussed in Chapter 4.

CHAPTER 3 - AGONIST AND ANTAGONIST MODULATION OF ^{3}H-MK-801 BINDING.

Chapter 3.

3.1: Introduction.

The previous chapter outlined the close association between the strychnine-insensitive glycine receptor and the NMDA receptor. The complex and integrated nature of the NMDA receptor is reflected in the schematic representation (Fig 1.1). Moreover the presence of multibinding domains implies that the functionality of this receptor may be dependent on the activation of one or more of these sites. Indeed a wealth of biochemical and electrophysiological evidence supports the concept of inter-site modulation of NMDA receptor function (Johnson and Ascher,1987; Wong *et al.*,1986:1988; Ransom and Stec,1988).

The discovery that glycine could facilitate NMDA / glutamate induced depolarisations in cultured mouse neurons (Johnson and Ascher,1987) marked a fundamental advance in EAA research. Similar facilitation has been subsequently demonstrated in neurones of rat cerebral cortex (Bertolino *et al.*,1988), cerebellum (Bertolino *et al.*,1988) and hippocampus (Mayer *et al.*,1989). Furthermore, biochemical reports of glycine enhancement of NMDA induced sodium (Foster and Wong,1987) and Ca^{2+} influx in cultured neurons (Reynolds *et al.*,1987) and brain slice release of dopamine (Crawford and Roberts,1989), acetylcholine (Ransom and Deschenes,1989) and noradrenaline (Ransom and Deschenes,1988) have substantiated the modulatory role of glycine. In addition the discovery of a similar potentiation *in vivo* (Thompson *et al.*,1989) has confirmed the physiological significance of glycine in the intact

system. Several workers have reported the abolition of NMDA receptor activation in the absence of glycine (Kleckner and Dingledine,1988; D'Angelo *et al.*,1990). To date no evidence has been reported to support a glycine modulation of NMDA receptor mediated events in the absence of glutamate.

The ability of glutamate and other NMDA agonists to facilitate their actions by means of a specific cation channel was discussed earlier. Together with the ability of glycine to increase the frequency of channel opening, without effecting conductance or duration of open time (Johnson and Ascher,1987) . The cationic channel associated with the NMDA receptor is thought to contain at least two distinct binding sites. The first is a selective binding site for the cation magnesium, which has been demonstrated to be a non-competitive inhibitor of NMDA receptor function (MacDonald *et al.*,1982). The second is referred to as the dissociative anaesthetic or PCP binding site, reflecting the nature of the selective agents which interact at this site. The evidence for the existence and localisation of these sites was previously described in the introduction to this thesis. Biochemical investigation of the phencyclidine site has demonstrated the existence of a number of selective ligands. These include ^{3}H-PCP (Snell *et al.*,1987) and its thienyl derivative, ^{3}H-TCP (Loo *et al.*,1986), which displays a greater potency than the parent molecule. In addition the ligand , ^{3}H-MK-801 has been demonstrated to exhibit a high affinity for this receptor (Wong *et al.*,1986). This ligand is the most potent and selective available to date, and is of particular relevance to the current study.

MK-801 ((+)-5-methyl-10,11-dihydro-5H-dibenzo-[a,d]cyclohepten-5,10-imine maleate) was first reported as a potent anticonvulsant compound of novel structure, which in addition possessed anxiolytic and sympathomimetic activity (Clineschmidt *et al.*,1982). It is a rigid, planar three ring structure with a nitrogen bridge orientated perpendicular to the central ring (Fig 1.2). Electrophysiological studies have demonstrated MK-801 to be a selective and non-competitive antagonist of NMDA mediated depolarisations (Kemp *et al.*,1986). The non-competitive nature was reflected in the marked flattening of the NMDA-dose response curve in the *in vitro* rat cortical slice preparation. Moreover biochemical investigations have demonstrated a high affinity, saturable binding site for ^{3}H-MK-801 in rat cortical membranes (Wong *et al.*,1986). Only compounds which are active at the phencyclidine (PCP) binding site display any appreciable affinity for ^{3}H-MK-801 binding sites (Wong *et al.*,1986). A finding reflected in parallel electrophysiological studies (Wong *et al.*,1986; Kemp *et al.*,1986). In rat cortical slice preparations MK 801 produced a potent and selective blockade of NMDA depolarising responses (Wong *et al.*,1986). Moreover a similar, if somewhat weaker, blockade was observed for phencyclidine, (±)ketamine and (±)SKF 10047 (Wong *et al.*,1986: Kemp *et al.*,1986). It is now well established that MK-801 elicits its noncompetitive antagonism through an interaction at the PCP receptor. Evidence to support this idea arises from the correlation observed between NMDA antagonism potency and affinity for PCP sites (Lodge *et al.*,1987; Wong *et al.*,1986). Further indication of the interaction of ^{3}H-MK-801 at the phencyclidine receptor is reflected in the behavioural characteristics reported for this compound. MK-801

whilst displaying potent anticonvulsant, anxiolytic, sympathomimetic and neuroprotective properties, has also been observed to share the behavioural characteristics (Piercey *et al.*,1988; Koek *et al.*,1988) which have plagued phencyclidine since its conception some 30 years ago.

As previously mentioned glutamate and glycine activation of the NMDA receptor is manifested through an alteration in the permeability of the ion channel associated with the receptor complex. The degree of activation being reflected in the degree of channel opening, which in turn is reflected in alterations in the binding of ligands to sites localised in the channel. On this basis the stimulation of ^{3}H-MK-801 (or ^{3}H-TCP) binding by either amino acid is thought to provide a functional index of the coupling of this unique domain to the NMDA receptor ionophore (Snell *et al.*,1987; Reynolds and Miller,1987; Wong *et al.*,1987). The pioneering biochemical investigations into the potentiation effect induced by glycine have centred around the use of this concept. Glycine enhancement of ^{3}H-TCP and ^{3}H-MK-801 binding was reported almost simultaneously by two independent groups (Snell *et al.*,1987; Reynolds *et al.*,1987). These studies, on well washed rat cortical membranes, revealed that the glycine potentiation was manifested as an increase in the maximal response observed with NMDA. In addition the potentiation was abolished in the absence of either glutamate or NMDA. These observations presumably reflect the use dependency associated with binding to the channel site (Lodge *et al.*,1987). The pharmacological characteristics of this stimulation, which closely parallelled the previous electrophysiological findings of Johnson and

Ascher (1987), were rapidly confirmed by a number of other groups (Wong *et al.*, 1987; Bonhaus *et al.*, 1987). Furthermore Ransom and Stec (1988) described a mutual coupling between glycine, NMDA agonist and the polyamine, spermidine, all of which interact with the ionophore labelled by ^{3}H-MK-801. The polyamine was found to increase the ^{3}H-MK-801 binding by a mechanism that was enhanced by glutamate and glycine. These observations have been recently confirmed by Reynolds (1990).

As previously described the binding of ^{3}H-MK-801 to the NMDA receptor is dependent upon initial receptor activation (Wong *et al.*, 1987). In addition it has been proposed that NMDA receptor activation is glycine dependent (Kleckner and Dingledine, 1988). The primary aim of this present study was to evaluate whether this apparent interdependence of glutamate and glycine displays any regional variation. To this end quantitative autoradiography of ^{3}H-MK-801 binding to rat brain sections has been employed to investigate the effects of selective agonists (glutamate, glycine) and antagonists (CPP, 7Cl-KYN) on ^{3}H-MK-801 binding in a variety of brain regions.

3.2: Methods.

3.2.1: Light microscopic autoradiography.

Tissue preparation as previously described for ^{3}H-glycine binding (chapter 2). Ten micron cryostat sections of rat brain were preincubated in 50 mM Tris-HCl buffer (pH 7.4, 24°C) for 1 hour, unless otherwise stated, in an attempt to reduce the levels of endogenous mediators. The sections were subsequently dried and incubated for 20 minutes in fresh buffer (24^{0}C) containing 30nM ^{3}H-MK-801 (S.A. 53 Ci/mmol). Sections were rinsed (2 x 30 sec, 4 °C) in fresh buffer followed by a rapid "dip" wash in distilled H_2O (4°C), and dried in a stream of cool air. Non specific binding was defined by the addition of 100µM unlabelled MK-801 to the incubation buffer.

3.2.2: Autoradiogram generation.

The autoradiograms were generated by apposing the bound sections to a tritium sensitive film (LKB Ultrafilm) for periods of between 2 - 3 weeks at 20°C. Films were developed in D19 (Kodak Ltd) for 1-2 min, fixed in Unifix (Kodak Ltd) for 5-6 min and washed in H_2O (15 minutes).

3.2.3: Densitometric analysis.

The autoradiographical images were analyzed by means of a computerised routine on a Quantimet 970 image analyser (Cambridge Instruments). Regional densities were converted to the corresponding ligand concentration by reference to tritium microscales standards (Amersham International).

3.2.4: Antagonism studies.

General autoradiographical procedure as previously described (Chapter 2). The results for dose response studies were obtained by the addition of varying concentrations of CPP (.1-100μM) and 7Cl-KYN (1-1000μM) to the incubation buffer. The reversal studies were obtained by the addition of 10μM CPP in the presence or absence of 100μM glutamate or 100μM 7-ClKYN in the presence or absence of 100μM glycine. Autoradiograms were obtained as previously described and analyzed on a Quantimet 970 image analyser.

3.2.5: Potentiation studies.

General autoradiographic procedure as previously described. Slices were preincubated for 2 hours in dose optimisation experiments, and varying concentrations of glutamate (.01-10μM) and/or glycine (.1-100μM) were added to the incubation buffer.

In all remaining studies, sections were preincubated for periods of between 2-12 hours. 1μM glutamate and/or 10 μM glycine were added to the incubation buffer. Autoradiogram generation and analysis were performed as previously described in Chapter 2.

3.2.6: Statistical analysis.

All data were analyzed by means of a computerised routine (LBAS) for 1 way ANOVA ($p<0.05$). Routine devised by Dr R. Gee, The School of Pharmacy, London.

3.3: Results.

3.3.1: Distribution of MK-801 binding sites.

The heterogeneous nature of ^{3}H-MK-801 binding to rat brain sections is shown in Figs 3.1 and 3.2. Quantitative analysis revealed the areas of highest binding density to be in the CA1 Stratum Radiatum (651.1 ± 95.4 fmol/mg tissue: 100%) and molecular layer of the dentate gyrus (590.6 ± 86.2). In contrast the areas of lowest binding included the globus pallidus (136.7 ± 23.8), the cerebellar granular cell layer (76.2 ± 18.4) and the frontoparietal cortex (169.3 ± 37.6).

A distinct laminal distribution was observed in all the cortical regions measured. An outer to inner progression through the frontal cortex revealed a binding levels of 81% (of that observed in the CA1 stratum radiatum) in the outer layer and 46% in the inner layer. This pattern was repeated in the fronto parietal cortex (outer 58%, inner 26%) although the levels observed were found to be lower than in the frontal cortex. The occipital cortex also displayed heterogeneity between laminae (outer 71%, inner 55%) however the variation was less marked in this region.

A more homogeneous distribution of binding was observed throughout the olfactory bulb. Levels of intermediate binding were observed in the olfactory tubercle (49 %), the external plexiform layer (53%) and the medial (54% and lateral (52%) anterior olfactory nuclei.

Fig 3.1: Autoradiograms of ^{3}H-MK-801 binding to rat brain sections.

Sections were incubated with 30 nM ^{3}H-MK-801 and autoradiograms generated as described in Methods. Abbreviations as listed below.

(A = Anterior: B = Medial: C = Posterior). Figures correspond to plates 15, 34 and 37 of The Rat Brain by Paxinos and Watson (1982).

CPu Caudate Putamen FrCx Frontal cortex

CICx Cingulate cortex Par Parietal cortex

Acb Nucleus accumbens SMDG Dentate gyrus molecular layer

SGDG Dentate gyrus granule layer

CA1 SO Stratum oriens SLM Stratum lacunosum moleculare

SR Stratum radiatum SP Stratum pyramidale

VPM Ventral posterior medial thalamic nuclei

VPL Ventral posterior lateral thalamic nuclei

OcCx Occipital cortex

DLG Dorsal lateral geniculate nucleus

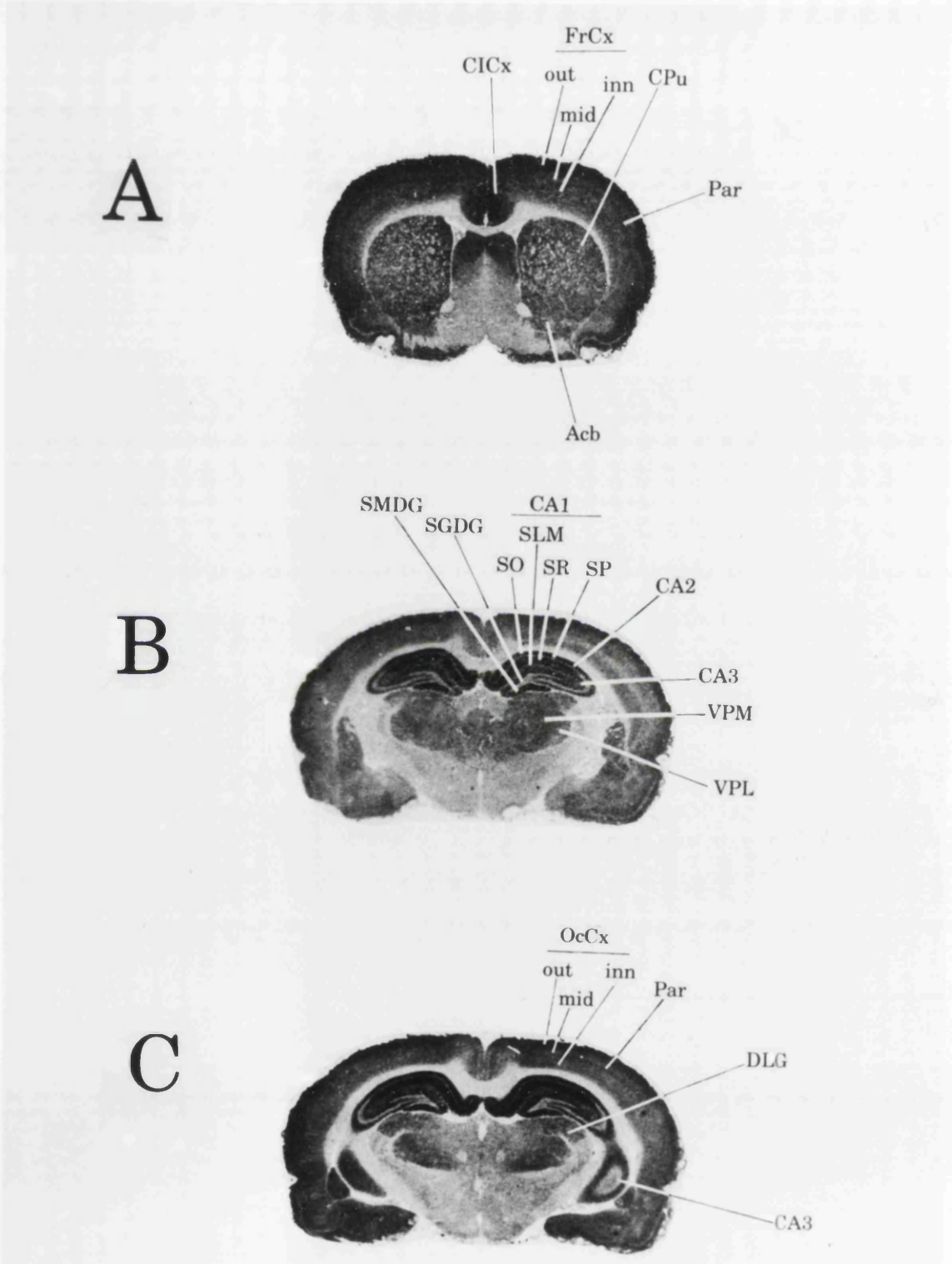
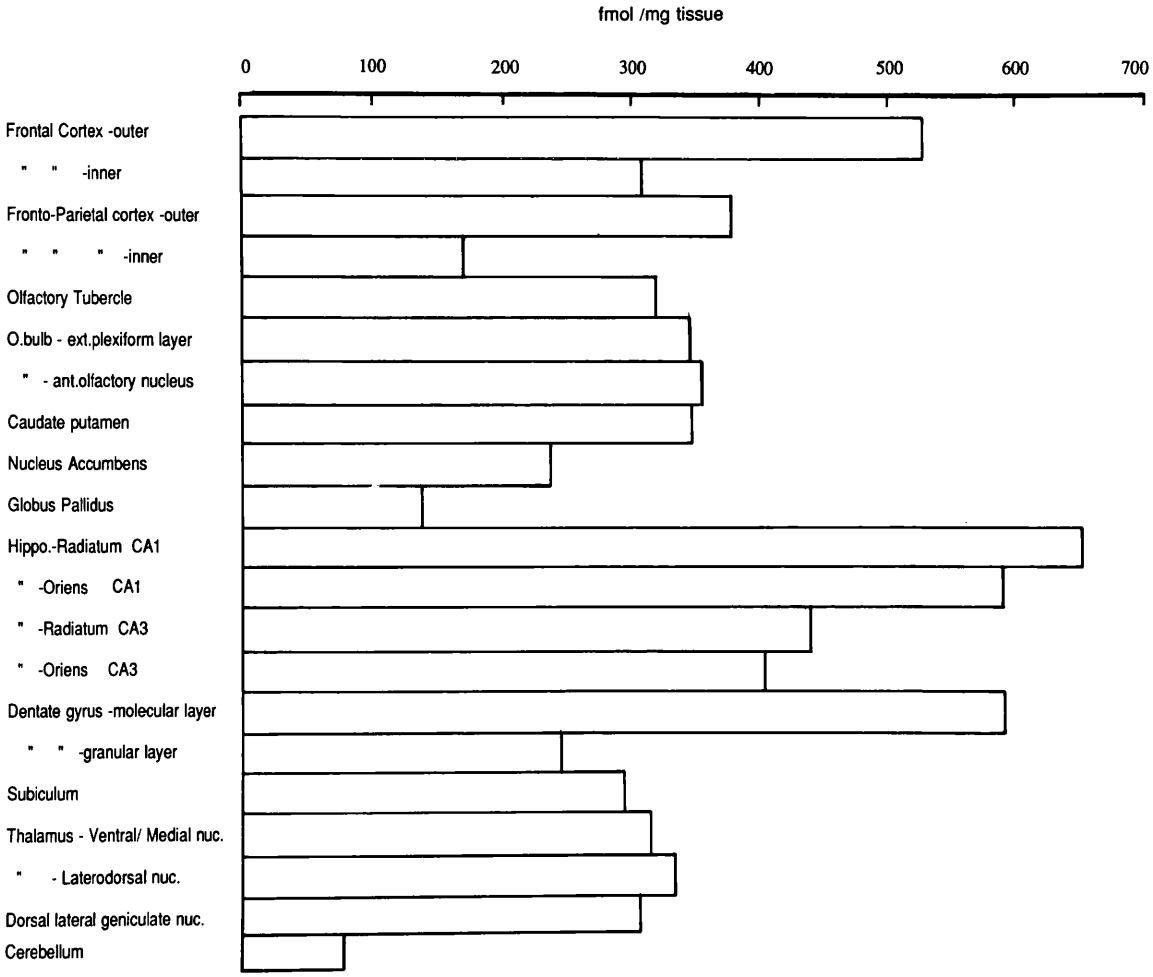

A
FrCx
CICx
out
inn
CPu
mid
Par
Acb
B
SMDG
SGDG
CA1
SLM
SO
SR
SP
CA2
CA3
VPM
VPL
C
OcCx
out
inn
Par
mid
DLG
CA3

Fig 3.2: Comparative regional densities of ^{3}H-MK-801 binding in rat brain sections.

Density values for each brain region from at least 3 sections each from 4 rats. Values were determined by densitometric analysis and calibrated by reference to tritium standards (Amersham plc) apposed to the same films.

REGIONAL DISTRIBUTION OF ^{3}H MK-801 BINDING SITES IN RAT BRAIN



In contrast the basal ganglia displayed a marked regional variation. The highest binding density was observed in the caudate putamen, corresponding to 54% of that in the CA1 Stratum radiatum with much lower degrees of binding in the nucleus accumbens (36%) and globus pallidus (21%).

An even distribution of intermediate levels of binding were observed in the diencephalic nuclei. Binding corresponding to 51% of that in CA1 were observed in the laterodorsal nuclei, with similar levels in the dorsal lateral geniculate nuclei (46%), ventral medial (48%) and ventral lateral (45%) nuclei.

The hippocampal formation displayed the highest binding densities in the brain. The stratum radiatum of the CA1 region was the most intensely labelled (651.1 ± 95.4), with somewhat lower binding observed in the stratum oriens (91%) and stratum pyramidale (70%). A similar distribution pattern was observed in the CA2 and CA3 regions, with the binding in the latter region being generally higher than that observed in the CA2. A distinct heterogeneous pattern of binding was observed in the dentate gyrus. The highest level of binding was localised in the molecular layer (91%) which contrasted with the much lower levels observed in the granular cell layer (38%). The subiculum (45%) and the habenula (39%) displayed similar levels of binding to that of the granule cell layer of the dentate gyrus.

The lowest level of binding of all the regions examined occurred in the granular layer of the cerebellum. The observed binding corresponded to only 12% of that

in the hippocampal CA1. Similar low levels of binding were observed in the brain stem (22%). The levels observed in the molecular regions of the cerebellum were too low to allow accurate determination.

3.3.2: Effect of non-competitive antagonists on the level of ^{3}H-MK-801 binding.

The ability of the glutamate antagonist CPP and the glycine antagonist, 7Cl-KYN to influence ^{3}H-MK801 binding to rat brain sections was investigated.

3.3.2.1: CPP.

An autoradiographical representation of the antagonism produced by CPP in a number of brain regions is illustrated in Fig 3.3. Quantitative analysis (Table 3.1) revealed a dose dependent inhibition of binding throughout all the regions studied. The specific binding densities observed in the presence of lower concentrations of CPP (.1 and 1μM) were found not to differ significantly ($p<0.05$) from those of control levels throughout all the regions studied. At higher concentrations (10μM) CPP produced a significant reduction in binding in all regions, which represented a minimum of 24% in all regions. The degree of inhibition ranged from 28% in the CA1 radiatum to a maximum of 51.9% in the ventral medial nuclei. Furthermore, with the exception of the granular layer of the dentate gyrus, the pyramidal cell layer of CA2 and CA3 and the stratum radiatum layer of CA2, the observed level of binding was found to be significantly lower than that observed in the presence of 1μM CPP. This dose dependent inhibition was repeated in the presence of 100 μM CPP. Analysis of densities observed in all regions revealed a significant decrease with respect

Fig 3.3: Autoradiographic representation of 7-Cl KYN and CPP inhibition of ^{3}H-MK-801 binding to rat parasagittal sections.

Slices were incubated with 30nM ^{3}H-MK-801 for 20 mins in the presence and absence of varying concentrations of 7-Cl KYN and CPP. Autoradiograms were generated as described in Methods.

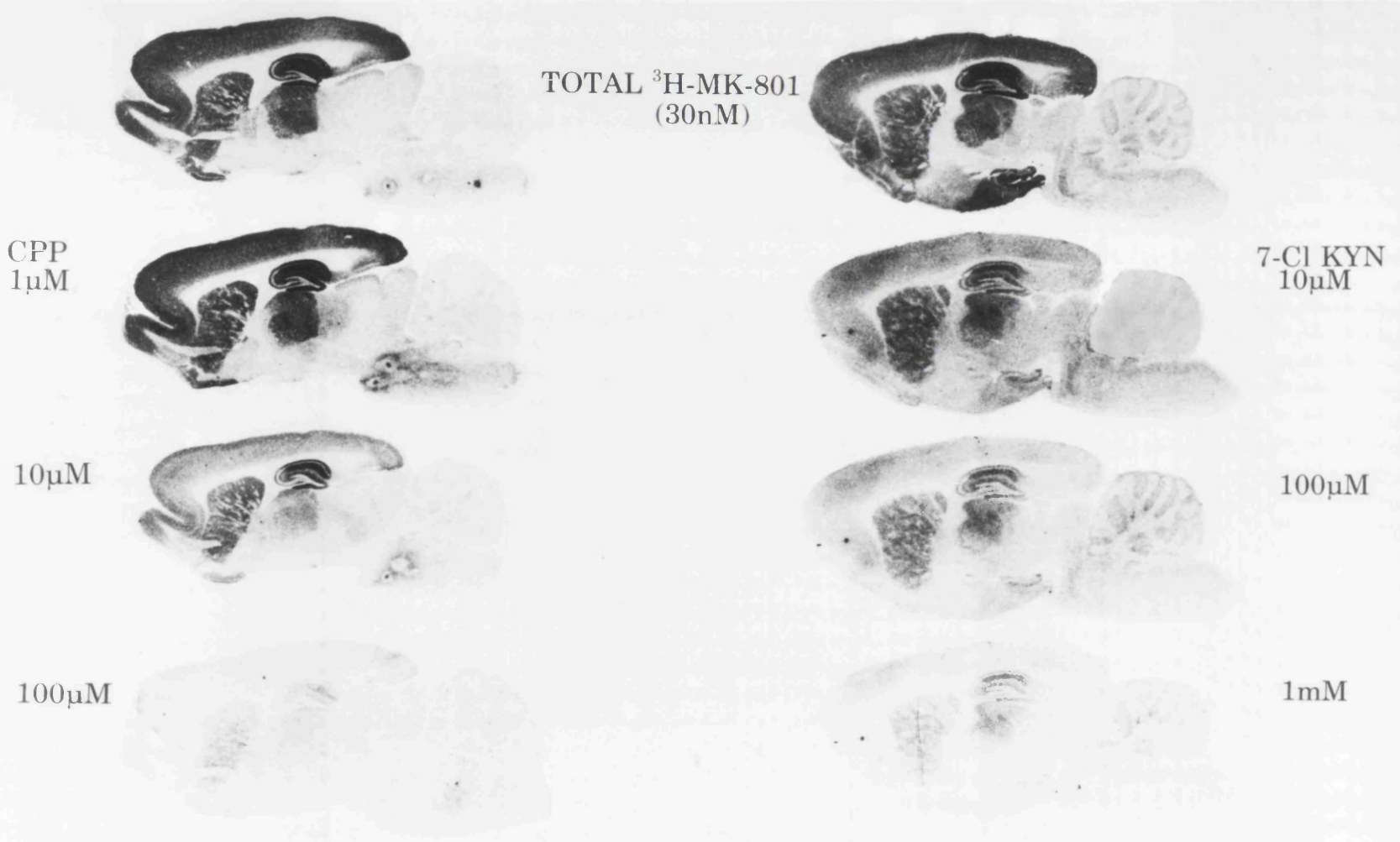

TOTAL ^{3}H-MK-801
(30nM)
CPP
1μM
10μM
100μM
7-Cl KYN
10μM
100μM
1mM

Table 3.1.: Inhibition of ^{3}H-MK-801 binding to parasagittal sections of rat brain by CPP.

Slices were incubated with 30nM ^{3}H-MK-801 for 20 min in the presence and absence of varying concentrations (.1-100μM) CPP. Non-specific binding was defined in the presence of 100μM unlabelled MK-801 and subtracted in all cases. Autoradiograms were generated as described in Methods. Quantitative analysis was performed by means of a computerised routine on a Quantimet 970 image analyzer. Density values were converted to corresponding ligand concentration by reference to tritium sensitive microscales (Amersham plc) apposed to the same film. Values represent mean density ± SEM (fmol/mg tissue) from 4 determinations in a variety of brain regions. Percentage values represent percentage of basal binding in each region.

* = $p < 0.05$: 1-way ANOVA.

REGION	CONTROL	CPP(.1μM)	%	CPP(1μM)	%	CPP(10μM)	%	CPP(100μM)	%
HIPPO CA1 OR	560 ± 62	588 ± 65	105	540 ± 48	96	391 ± 36*	70	119 ± 38*	21
PYR	403 ± 45	415 ± 43	103	369 ± 31	92	259 ± 31*	64	88 ± 12*	14
RAD	610 ± 61	616 ± 45	102	605 ± 45	99	439 ± 38*	72	144 ± 15*	24
CA2 OR	413 ± 55	387 ± 70	94	383 ± 40	93	226 ± 27*	55	57 ± 8*	14
PYR	238 ± 23	240 ± 37	101	211 ± 26	89	152 ± 20*	64	25 ± 7*	11
RAD	422 ± 38	443 ± 80	105	405 ± 48	96	260 ± 37*	62	62 ± 11*	15
CA3 OR	416 ± 55	407 ± 45	98	369 ± 45	89	225 ± 19*	54	66 ± 11*	16
PYR	213 ± 23	237 ± 20	111	217 ± 29	102	133 ± 27*	62	17 ± 11*	8
RAD	445 ± 55	475 ± 57	107	465 ± 45	104	285 ± 24*	64	72 ± 6*	16
DEN GYRUS MOL	542 ± 95	528 ± 60	97	537 ± 90	99	311 ± 18*	57	145 ± 7*	27
GRAN	228 ± 16	222 ± 21	98	193 ± 14	84	137 ± 19*	60	27 ± 5*	12
HABENULA	235 ± 28	244 ± 18	104	219 ± 22	93	143 ± 21*	61	56 ± 13*	24
SUBICULUM	262 ± 20	273 ± 13	105	219 ± 32	84	132 ± 15*	51	24 ± 8*	9
THALAMUS LD	294 ± 37	310 ± 72	106	302 ± 39	103	164 ± 38*	56	34 ± 19*	12
DLG	266 ± 13	232 ± 92	87	261 ± 39	98	160 ± 12*	60	44 ± 12*	17
VPM	314 ± 49	300 ± 55	96	270 ± 43	86	151 ± 19*	48	44 ± 12*	14
VPL	302 ± 28	309 ± 62	102	257 ± 29	85	166 ± 21*	55	36 ± 4*	12
C.PUTAMEN	347 ± 28	334 ± 37	96	315 ± 28	91	223 ± 25*	64	70 ± 10*	20
FR CX OUT	507 ± 26	533 ± 47	105	499 ± 55	99	345 ± 16*	68	74 ± 27*	15
MID	416 ± 35	423 ± 43	102	379 ± 43	91	237 ± 25*	57	49 ± 9*	12
INN	280 ± 26	304 ± 32	109	274 ± 31	98	177 ± 10*	63	22 ± 9*	8
OX CX OUT	434 ± 18	442 ± 27	102	426 ± 27	98	281 ± 11*	66	67 ± 19*	15
MID	393 ± 36	413 ± 46	105	363 ± 31	92	226 ± 24*	58	41 ± 15*	11
INN	338 ± 10	339 ± 18	100	296 ± 23	88	171 ± 13*	51	25 ± 12*	7

to control and to levels observed with 10μM. In excess of 70 % inhibition was observed throughout the regions studied, maximal inhibition (92.7%) was observed in the inner occipital cortex. The region which displayed the lowest degree of inhibition was the molecular layer of the dentate gyrus (73.2%).

3.3.2.2: Agonist modulation of CPP inhibition of ^{3}H-MK-801 binding

The ability of glutamate (100μM) to reverse the inhibitory actions of 10 μM CPP is illustrated autoradiogaphically in Fig 3.4. Quantitative analysis of the reversal in this and a number of other brain regions is shown in Table 3.2. The inhibitory effect of 10μM CPP was observed to be significant in all regions studies. The addition of 100μM glutamate to the incubation solution in the presence of 10μM CPP reversed the inhibition throughout the brain such that no significant difference from control binding was observed.

3.3.2.3: 7-Chloro kynurenic acid.

The dose dependent inhibition of ^{3}H-MK-801 binding produced by 7-ClKYN in a number of brain regions is shown in Table 3.3. Specific binding densities observed in the presence of 1μM 7-ClKYN were found not to differ significantly from those of control throughout the regions studied. A heterogeneous pattern of inhibition was observed with an increased concentration (10μM) of 7-ClKYN and this inhibition was significantly different from control in most regions studied. The degree of inhibition was lowest (21.7%) in the dorsal lateral geniculate nuclei. The inhibition in the dorsal lateral geniculate nucleus (21.4%) and the ventral posterior medial (21.4%) and lateral (22.5%) thalamic

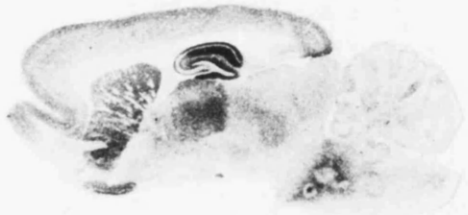
Fig 3.4: Autoradiographic representation of glutamate and glycine reversal of 7-Cl KYN and CPP inhibition of ^{3}H-MK-801 binding to rat parasagittal sections.

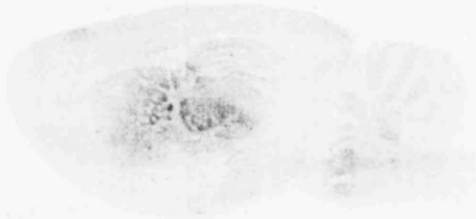
Slices were incubated with 30nM ^{3}H-MK-801 for 20 mins in the presence and absence of either 7-Cl KYN (10μM) ± 100μM glycine or CPP (10μM) ± 10μM glutamate. Autoradiograms were generated as described in Methods.

TOTAL ^{3}H-MK-801
(30nM)

TOTAL ^{3}H-MK-801
(30nM)

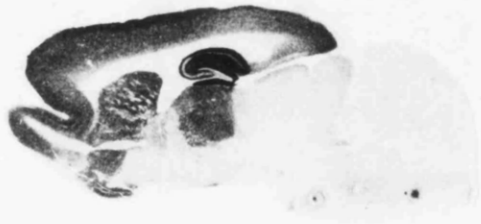
CPP (10μM)





7-Cl KYN(100μM)

CPP (10μM)
+ GLUTAMATE (10μM)



7-Cl KYN (100μM)
+ GLYCINE (100μM)

Table 3.2: Reversal of 7-Cl KYN and CPP inhibition of ^{3}H-MK-801 binding in rat brain sections by glycine and glutamate.

Rat parasagittal sections were incubated with 30nM ^{3}H-MK-801 for 20 mins in the presence or absence of 10μM 7-Cl KYN or 100μM CPP. Additional sections were incubated with 10μM 7-Cl KYN and 100μM glycine or 100μM CPP and 100μM glutamate. Non-specific binding was defined in the presence of 100μM unlabelled MK-801 and subtracted in all cases. Autoradiogram generation as described in Methods. Quantitative analysis by means of a computerised routine on a Quantimet 970 image analyzer. Density values were converted to corresponding ligand concentration by reference to tritium sensitive microscales (Amersham plc) apposed to the same film. Values represent Mean density ± SEM (fmol/mg tissue) from 4 determination for each brain region.

* = $p < 0.05$: 1-way ANOVA.

REGION		CONTROL	7ClKYN(100μM)	%	7ClKYN\GLY(.1mM)	%	CPP(10μM)	%	CPP\GLUT(10μM)	%
HIPPO CA1	OR	591 ± 35	132 ± 19*	22	589 ± 22	100	415 ± 21*	70	547 ± 39	93
	PYR	422 ± 32	72 ± 16*	17	483 ± 27	115	271 ± 25*	64	379 ± 32	90
	RAD	651 ± 35	172 ± 14*	26	631 ± 47	97	471 ± 21*	72	618 ± 20	95
CA2	OR	434 ± 30	88 ± 20*	20	397 ± 17	92	239 ± 35*	55	421 ± 20	97
	PYR	245 ± 17	45 ± 5*	19	257 ± 11	105	157 ± 18*	64	225 ± 18	92
	RAD	459 ± 24	75 ± 2*	16	430 ± 28	94	280 ± 15*	61	444 ± 19	97
CA3	OR	403 ± 38	63 ± 15*	16	451 ± 20	112	267 ± 17*	66	362 ± 25	90
	PYR	252 ± 23	21 ± 4*	8	271 ± 32	108	153 ± 11*	61	218 ± 18	87
	RAD	498 ± 34	98 ± 18*	20	550 ± 28	111	321 ± 21*	65	455 ± 22	91
DEN GYRUS	MOL	591 ± 36	124 ± 32*	21	648 ± 21	110	383 ± 28*	65	568 ± 33	96
	GRAN	245 ± 14	48 ± 13*	19	287 ± 31	117	147 ± 10*	60	262 ± 21	107
HABENULA		256 ± 17	43 ± 16*	17	242 ± 17	94	151 ± 18*	60	256 ± 15	100
SUBICULUM		292 ± 20	36 ± 16*	12	322 ± 23	110	142 ± 12*	49	250 ± 11	86
THALAMUS	LD	331 ± 25	73 ± 17*	22	334 ± 16	101	180 ± 24*	54	298 ± 13	90
	DLG	303 ± 18	67 ± 17*	22	352 ± 11	116	181 ± 21*	60	284 ± 18	94
	VPM	315 ± 27	87 ± 11*	28	317 ± 14	101	177 ± 15*	56	269 ± 11	86
	VPL	294 ± 25	83 ± 17*	28	334 ± 11	114	192 ± 13*	65	269 ± 16	91
C.PUTAMEN		347 ± 19	73 ± 15*	21	380 ± 13	110	263 ± 24*	76	337 ± 19	97
FR CX	OUT	527 ± 22	84 ± 11*	16	540 ± 28	103	359 ± 29*	68	517 ± 28	98
	MID	426 ± 23	66 ± 13*	15	459 ± 12	108	242 ± 26*	57	415 ± 22	98
	INN	303 ± 20	44 ± 6*	15	323 ± 14	107	194 ± 12*	65	269 ± 18	89
OX CX	OUT	465 ± 27	96 ± 20*	21	517 ± 22	111	302 ± 26*	65	428 ± 20	92
	MID	421 ± 21	62 ± 9*	15	467 ± 16	111	240 ± 34*	57	387 ± 22	92
	INN	374 ± 16	44 ± 6*	12	399 ± 18	111	181 ± 14*	50	321 ± 26	89

Table 3.3.: Inhibition of ^{3}H-MK-801 binding to parasagittal sections of rat brain by 7ClKYN.

Slices were incubated with 30nM ^{3}H-MK-801 for 20 min in the presence and absence of varying concentrations (1-1000μM) 7ClKYN. Non-specific binding was defined in the presence of 100μM unlabelled MK-801 and subtracted in all cases. Autoradiogram generation as described in Methods. Quantitative analysis by means of a computerised routine on a Quantimet 970 image analyzer. Density values were converted to corresponding ligand concentrations by reference to tritium sensitive microscales (Amersham plc) apposed to the same film. Values represent mean density ± SEM (fmol/mg tissue) from 4 determination for each brain region. Percentage values represent increase of basal binding in each region.

* = $p < 0.05$: 1-way ANOVA.

REGION	CONTROL	7ClKYN(1μM)	%	7ClKYN(10μM)	%	7ClKYN(100μM)	%	7ClKYN(1mM)	%
HIPPO CA1 OR	623 ± 37	657 ± 35	106	407 ± 38*	66	146 ± 18*	23	41 ± 9*	7
PYR	503 ± 22	471 ± 35	94	284 ± 49*	57	88 ± 22*	18	21 ± 3*	4
RAD	690 ± 22	700 ± 27	102	502 ± 32*	73	183 ± 21*	27	59 ± 4*	9
CA2 OR	452 ± 18	460 ± 31	102	265 ± 32*	59	91 ± 19*	20	7 ± 1*	2
PYR	250 ± 11	256 ± 7	102	140 ± 36*	56	46 ± 6*	19	6 ± 3*	2
RAD	496 ± 15	513 ±28	103	329 ± 18*	66	77 ± 6*	15	22 ± 4*	4
CA3 OR	448 ± 11	396 ± 24	88	243 ± 29*	54	73 ± 11*	16	20 ± 3*	4
PYR	288 ± 27	268 ± 19	93	114 ± 11*	40	40 ± 20*	14	14 ± 2*	5
RAD	549 ± 14	547 ± 48	100	352 ± 13*	64	109 ± 23*	20	24 ± 4*	4
DEN GYRUS MOL	637 ± 18	645 ± 47	101	448 ± 15*	70	174 ± 12*	27	34 ± 6*	5
GRAN	261 ± 22	265 ± 38	101	155 ± 14*	59	53 ± 19*	20	15 ± 2*	6
HABENULA	276 ± 17	291 ± 30	105	178 ± 18*	65	28 ± 3*	11	26 ± 7*	9
SUBICULUM	321 ± 25	299 ± 30	93	225 ± 15*	57	20 ± 3*	7	30 ± 2*	9
THALAMUS LD	367 ± 18	333 ± 9	91	287 ± 20*	78	79 ± 14*	21	133 ± 4*	9
DLG	338 ± 14	341 ± 17	101	266 ± 37	79	77 ± 20*	23	25 ± 2*	8
VPM	351 ± 10	331 ± 6	94	276 ± 35	79	91 ± 19*	26	24 ± 2*	7
VPL	328 ± 13	311 ± 20	95	255 ± 43	78	93 ± 20*	28	28 ± 8*	9
C.PUTAMEN	375 ± 15	364 ± 10	97	252 ± 43*	67	80 ± 20*	21	17 ± 9*	4
FR CX OUT	545 ± 37	534 ± 50	98	294 ± 7*	54	86 ± 11*	16	17 ± 9*	3
MID	435 ± 35	432 ± 32	99	298 ± 25*	68	66 ±17*	15	12 ± 7*	4
INN	324 ± 29	302 ± 35	93	195 ± 36*	60	46 ± 4*	14	20 ± 4*	6
OX CX OUT	495 ± 48	485 ± 58	98	267 ± 7*	54	98 ± 11*	20	26 ± 2*	5
MID	447 ± 18	421 ± 37	94	271 ± 39*	61	66 ± 8*	15	20 ± 7*	5
INN	379 ± 16	357 ± 35	94	225 ± 34*	60	46 ± 7*	12	9 ± 3*	3

nuclei were not significantly different from control. At increased doses (100μM) all regions displayed a significant inhibition, which was maximal in the subiculum (93.9%), and lowest in the granule cell layer of the dentate gyrus (72.7%). An autoradiographical representation of the inhibition produced by varying concentrations of 7-ClKYn in shown is Fig 3.3.

3.3.2.4: Glycine modulation of 7-ClKYN inhibition.

The ability of glycine (100μM) to reverse the inhibition produced by 100μM 7-ClKYN acid is illustrated autoradiographically in Fig 3.4. A detailed quantitative analysis of this reversal is shown in Table 3.2. The levels of specific binding observed in the presence of the antagonist were significantly lower than for the controls throughout the regions studied. An inhibition of at least 73% was observed in all the regions studied. The binding densities observed in the presence of 100μM 7-ClKYN and 100μM glycine were not significantly different from the observed control levels.

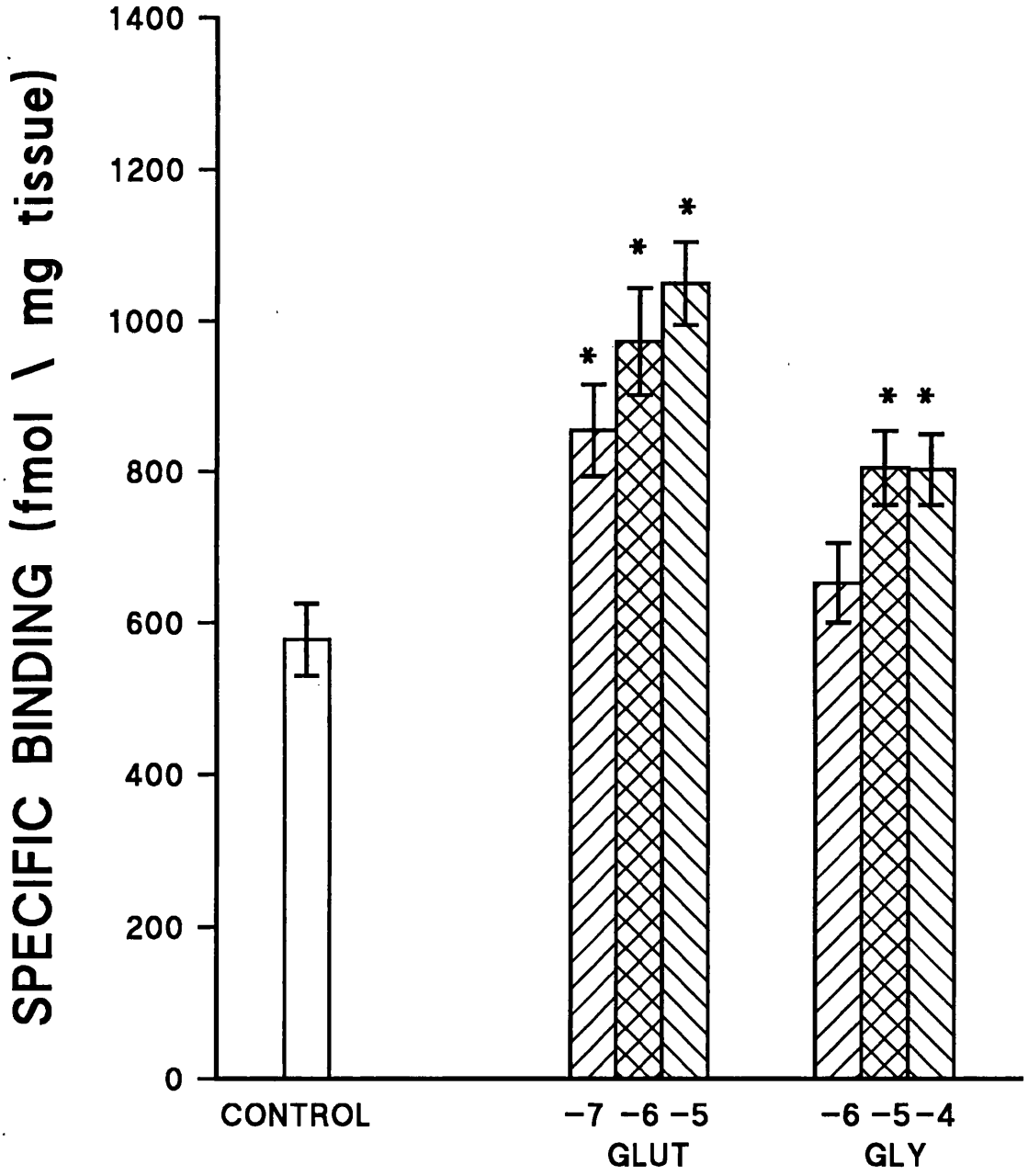
3.4: Modulation of ^{3}H-MK-801 binding by glutamate and glycine.

The dose dependent enhancement of ^{3}H-MK801 binding by glutamate and glycine in the CA1 Stratum radiatum is shown in Fig 3.5. The maximal stimulation of ^{3}H-MK-801 binding was observed with 1μM glutamate and 10μM glycine respectively. For this reason these two concentrations were used throughout the remainder of this study.

Fig 3.5: Dose dependent potentiation of ^{3}H-MK-801 by glutamate and glycine in CA1 stratum radiatum of the rat.

Slices were incubated with 30nM ^{3}H-MK-801 for 20 min in the presence and absence of varying concentrations of glutamate (.1-10μM) and glycine (1-100μM). Non-specific binding was defined in the presence of 100μM unlabelled MK-801 and subtracted in all cases. Autoradiograms were generated as described in Methods. Quantitative analysis was performed by means of a computerised routine on a Quantimet 970 image analyzer. Density values were converted to corresponding ligand concentration by reference to tritium sensitive microscales (Amersham plc) apposed to the same film. Values represent Mean density ± SEM (fmol/mg tissue) from 3 determinations.

* = $p < 0.05$: 1-way ANOVA.


SPECIFIC BINDING (fmol \ mg tissue)
1400
1200
1000
800
600
400
200
0
*
*
*
* *
CONTROL
-7 -6 -5
GLUT
-6 -5 -4
GLY

3.5: Glycine modulation of glutamate stimulated enhancement of ^{3}H-MK-801 binding in washed rat brain sections.

The effects of glutamate (1μM) and glycine (10 μM) either alone or in combination, on the binding in the CA1 stratum radiatum and three other brain regions is shown in Fig 3.6. Quantitative and autoradiographical analysis of the enhancement produced by these two amino acids in these and a number of additional brain regions is shown in Table 3.4 and Fig 3.7. The analysis of the observed basal binding densities in rat brain sections preincubated for 2 hours (T2) revealed the highest densities in the CA1 stratum radiatum and the molecular layer of the dentate gyrus. The addition of 10 μM glycine produced a significant enhancement of binding ($p<0.05$) in the majority of the regions studied . The enhancement was maximal in the dorsal lateral geniculate nucleus of the thalamus and corresponded to a 39% increase over control. No significant enhancement was observed throughout the CA2 region, or in the pyramidal cell layer of the CA1 and CA3 regions. In addition no significant enhancement was observed in the granular cell layer of the dentate gyrus. A similar pattern of enhancement was observed following the addition of 1μM glutamate. The observed enhancement was maximal in the outer layer of the frontal cortex, and corresponded to a 45% increase over control level. The co-addition of the two amino acids produced a further significant enhancement in all the regions studied. This effect was maximal in the outer regions of the frontal cortex (130% increase over basal). Furthermore the enhancement evoked by the two amino acids in combination was found to be significantly greater than the enhancement induced by either of the individual acids alone

Fig 3.6: Potentiation of ^{3}H-MK801 binding to parasagittal sections of rat brain by glutamate and glycine.

Slices were incubated with 30nM ^{3}H-MK801 for 20 min in the presence and absence of glutamate (⊠) (1μM) and glycine (⧄) (10μM) either alone or in combination. Non-specific binding was defined in the presence of 100μM unlabelled MK801 and subtracted in all cases. Autoradiograms were generated as described in Methods. Quantitative analysis was performed by means of a computerised routine on a Quantimet 970 image analyzer. Density values were converted to corresponding ligand concentration by reference to tritium sensitive microscales (Amersham plc) apposed to the same film. Values represent Mean density ± SEM (fmol/mg tissue) from 4 determinations in the CA1 stratum radiatum (CA1), dentate gyrus molecular layer (DG), the outer layer of the frontal cortex (FrCX) and the dorsal lateral thalamic nuclei (THAL).

* = $p < 0.05$: 1-way ANOVA.

control □ glycine ⧄

glutamate ⊠ glutamate / glycine ⧅

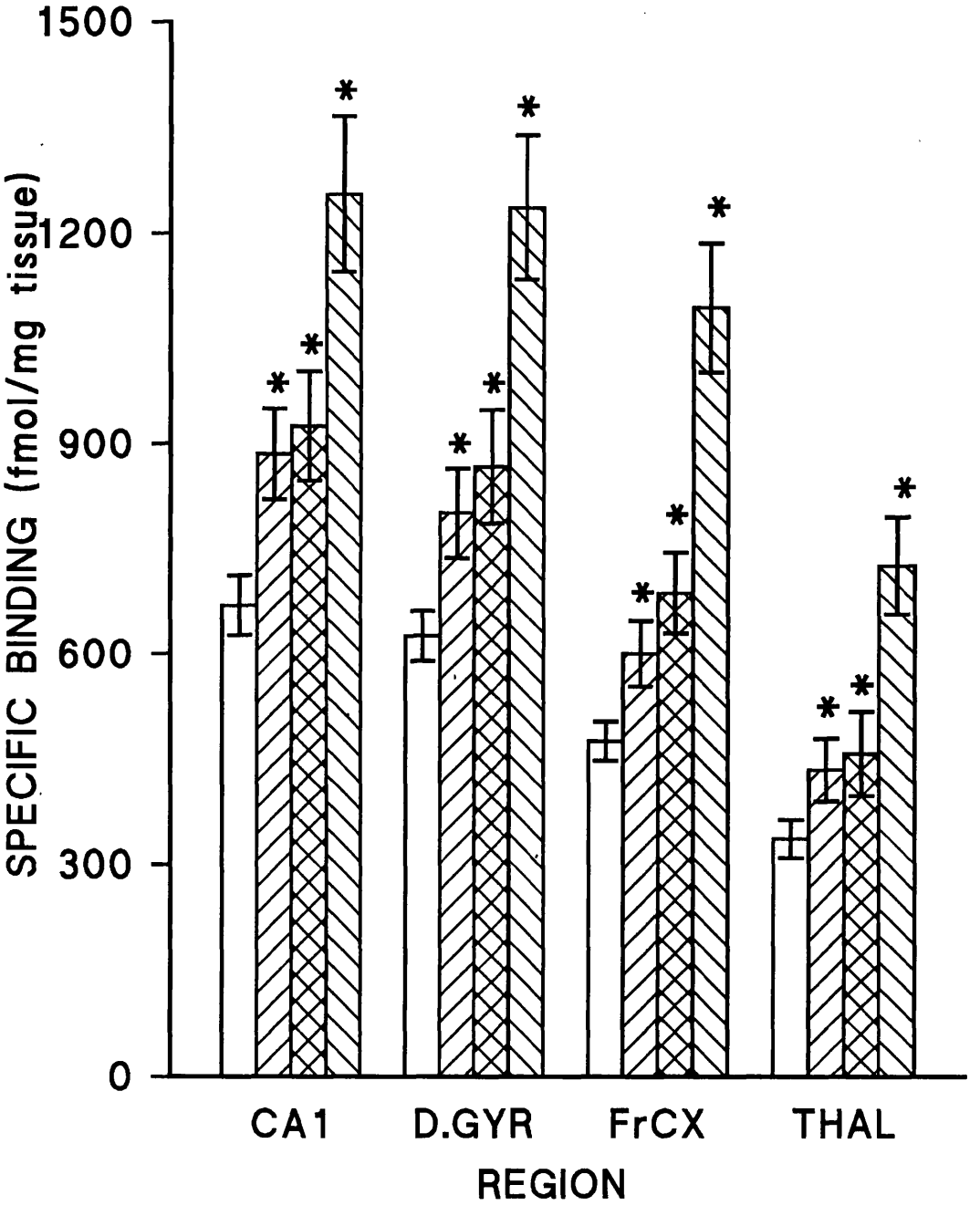

1500
1200
900
600
300
0
SPECIFIC BINDING (fmol/mg tissue)
CA1
D.GYR
FrCX
THAL
REGION

Table 3.4: Effect of 2 and 4 hour preincubation times on the potentiation of ^{3}H-MK-801 binding by glutamate and / or glycine in parasagittal sections of rat.

Slices were incubated with 30nM ^{3}H-MK-801 for 20 min in the presence and absence of glutamate (1µM) and / or glycine (10µM). Non-specific binding was defined in the presence of 100µM unlabelled MK-801 and subtracted in all cases. Autoradiogram generation as described in Methods. Quantitative analysis was performed by means of a computerised routine on a Quantimet 970 image analyzer. Density values were converted to corresponding ligand concentration by reference to tritium sensitive microscales (Amersham plc) apposed to the same film. Values represent Mean density ± SEM (fmol/mg tissue) from 4 determination for each brain region.

* = $p < 0.05$: 1-way ANOVA.

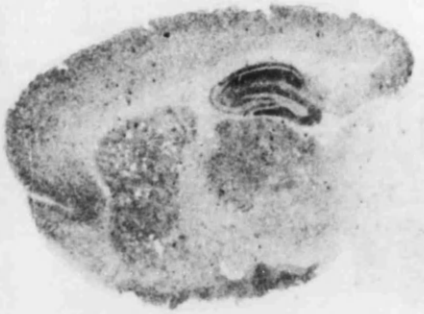
REGION		CONTROL	GLY(10μM)	GLUT(1μM)	GLUT/GLY	CONTROL	GLY(10μM)	GLUT(1μM)	GLUT\GLY
HIPPO CA1	OR	565 ± 31	729 ± 59*	763 ± 65*	1090 ± 90*	519 ± 46	659 ± 57*	768 ± 67*	1006 ± 122*
	PYR	261 ± 39	339 ± 52	365 ± 70*	587 ± 89*	297 ± 60	353 ± 41	404 ± 38*	636 ± 59*
	RAD	670 ± 43	885 ± 65*	925 ± 78*	1255 ± 110*	545 ± 80	719 ± 61*	786 ± 57*	1009 ± 82*
CA2	OR	420 ± 22	498 ± 50	539 ± 50	838 ± 79*	405 ± 47	513 ± 54	537 ± 48	792 ± 64*
	PYR	237 ± 18	257 ± 30	278 ± 37	451 ± 52*	202 ± 50	236 ± 32	269 ± 27	419 ± 38*
	RAD	440 ± 23	575 ± 62	633 ± 55	976 ± 102*	357 ± 68	453 ± 40	421 ± 42	600 ± 56*
CA3	OR	427 ± 30	528 ± 29*	575 ± 50*	830 ± 94*	405 ± 51	521 ± 37*	543 ± 39*	881 ± 73*
	PYR	254 ± 23	278 ± 14	294 ± 48	451 ± 52*	164 ± 86	197 ± 22	197 ± 71	356 ± 32*
	RAD	528 ± 30	659 ± 37*	686 ± 57*	967 ± 102*	435 ± 68	600 ± 72*	565 ± 41*	964 ± 85*
DEN GYRUS	MOL	626 ± 36	801 ± 63*	867 ± 81*	1236 ± 102*	455 ± 72	619 ± 42*	613 ± 50*	964 ± 98*
	GRAN	260 ± 25	289 ± 32	315 ± 22	470 ± 52*	218 ± 40	251 ± 30	259 ± 27	327 ± 43*
HABENULA		255 ± 20	355 ± 30*	366 ± 32*	535 ± 62*	337 ± 61	405 ± 36	390 ± 49	566 ± 41*
SUBICULUM		292 ± 19	395 ± 29*	365 ± 30*	558 ± 58*	312 ± 42	431 ± 51*	448 ± 39*	570 ± 56*
THALAMUS	LD	336 ± 22	437 ± 57*	457 ± 60*	726 ± 69*	296 ± 35	367 ± 39	462 ± 36*	628 ± 48*
	DLG	311 ± 26	434 ± 45*	445 ± 50*	714 ± 92*	285 ± 49	362 ± 37	432 ± 50*	665 ± 59*
	VPM	333 ± 28	461 ± 42*	463 ± 42*	717 ± 89*	279 ± 27	321 ± 35	424 ± 53*	623 ± 38*
	VPL	332 ± 26	458 ± 42*	462 ± 46*	713 ± 85*	268 ± 29	299 ± 36	419 ± 42*	600 ± 44*
C.PUTAMEN		359 ± 18	434 ± 32*	451 ± 48*	563 ± 60*	274 ± 47	323 ± 21	308 ± 33	529 ± 38*
FR CX	OUT	475 ± 28	600 ± 37*	687 ± 58*	1094 ± 92*	417 ± 23	528 ± 39*	617 ± 47*	877 ± 71*
	MID	367 ± 23	461 ± 29*	492 ± 37*	781 ± 82*	316 ± 35	386 ± 30*	430 ± 57*	619 ± 68*
	INN	267 ± 20	357 ± 30*	365 ± 32*	603 ± 93*	286 ± 22	356 ± 32*	385 ± 29*	504 ± 54*
OX CX	OUT	399 ± 27	516 ± 39*	539 ± 42*	889 ± 94*	361 ± 69	444 ± 40	533 ± 42	925 ± 107*
	MID	361 ± 27	460 ± 81*	461 ± 32*	719 ± 90*	316 ± 29	382 ± 32	396 ± 32	802 ± 84*
	INN	257 ± 18	353 ± 31*	358 ± 32*	490 ± 40*	284 ± 39	355 ± 29	352 ± 35	648 ± 90*

Fig 3.7: Autoradiographic representation of glutamate and glycine enhancement of ^{3}H-MK-801 binding in rat parasagittal sections.

Slices were preincubated for 2 hours at room temperature in Tris-citrate buffer and incubated with 30nM ^{3}H-MK-801 in the presence or absence of glycine (10μM) and / or glutamate (1μM). Autoradiograms were generated as described in Methods.

ENHANCEMENT OF ^{3}H-MK-801 BINDING BY GLUTAMATE AND GLYCINE.

TOTAL ^{3}H-MK-801 (30nM)



+ GLYCINE (10μM)

+ GLUTAMATE (1μM)

+ GLUTAMATE (1μM) /GLYCINE (10μM)

($p<0.05$). This observation was consistent for all the regions studied.

3.3.6: The effects of extended preincubation time on the glycine/glutamate enhancement of ^{3}H-MK-801 binding in rat brain.

The influence of varying the preincubation time from 4 (T4) to 12 (T12) hours on the enhancement of ^{3}H-MK-801 binding by glutamate and glycine in a variety of brain regions is shown in Tables 3.4 and 3.5.

3.3.6.1: 4 hour preincubation

The enhancement induced by 10 µM glycine displayed a greater regional variation than previously observed at T2. A significant enhancement in the level of binding was observed in the oriens and radiatum layers of CA1 and CA3, together with the molecular layer of the dentate gyrus, subiculum and all regions of the frontal cortex. The enhancement was observed to be maximal in the CA3 stratum radiatum and represented a 38% increase with respect to control. In contrast glutamate (1µM) elicited a more widespread enhancement of binding. In addition to the regions enhanced by glycine, glutamate evoked a significant enhancement in the pyramidal cell layer of CA1 and throughout the thalamic nuclei. The observed enhancement was maximal in the ventral posterior lateral nucleus, representing a 56% increase over basal. The co-addition of the two amino acids produced a universal significant enhancement of binding. The observed maximal being localised in the outer regions of the occipital cortex (156%).

Table 3.5: The effect of 8 and 12 hour preincubation times on the potentiation of ^{3}H-MK-801 binding by glutamate and / or glycine in parasagittal sections of rat.

Slices were incubated with 30nM ^{3}H-MK-801 for 20 min in the presence and absence of glutamate (1μM) and / or glycine (10μM). Non-specific binding was defined in the presence of 100μM unlabelled MK-801 and subtracted in all cases. Autoradiograms were generated as described in Methods. Quantitative analysis was performed by means of a computerised routine on a Quantimet 970 image analyzer. Density values were converted to corresponding ligand concentration by reference to tritium sensitive microscales (Amersham plc) apposed to the same film. Values represent Mean density ± SEM (fmol/mg tissue) from 4 determinations in a variety of brain regions.

* = $p < 0.05$: 1-way ANOVA.

REGION		CONTROL	GLY(10μM)	GLUT(1μM)	GLUT/GLY	CONTROL	GLY(10μM)	GLUT(1μM)	GLUT\GLY
HIPPO CA1	OR	523 ± 65	691 ± 58*	816 ± 64*	1157 ± 66*	458 ± 42*	504 ± 42	687 ± 53*	911 ± 56*
	PYR	225 ± 63	264 ± 36	444 ± 33	598 ± 115*	265 ± 32*	305 ± 24	429 ± 61*	591 ± 95*
	RAD	629 ± 51	731 ± 77	820 ± 89*	1399 ± 146*	527 ± 50*	717 ± 64*	815 ± 83*	1052 ± 92*
CA2	OR	348 ± 53	376 ± 76	464 ± 27	801 ± 97*	331 ± 48*	407 ± 51	444 ± 38	682 ± 52*
	PYR	184 ± 25	163 ± 47	251 ± 52	423 ± 45*	212 ± 29	283 ± 28	325 ± 30	433 ± 39*
	RAD	356 ± 30*	389 ± 38	410 ± 51	787 ± 54*	343 ± 48*	416 ± 38	412 ± 44	792 ± 50*
CA3	OR	307 ± 54*	417 ± 37*	462 ± 33*	936 ± 80*	346 ± 39*	381 ± 34	504 ± 50*	845 ± 78*
	PYR	195 ± 38	227 ± 35	331 ± 29	558 ± 43*	218 ± 22	254 ± 33	343 ± 30*	445 ± 48*
	RAD	367 ± 76*	534 ± 35*	598 ± 72*	1006 ± 124*	432 ± 52*	522 ± 47*	703 ± 68*	974 ± 103*
DEN GYRUS	MOL	463 ± 87*	560 ± 57	625 ± 50*	936 ± 52*	442 ± 58*	577 ± 43*	732 ± 70*	915 ± 111*
	GRAN	187 ± 37*	206 ± 44	325 ± 36	498 ± 64*	205 ± 21*	243 ± 30	365 ± 39*	508 ± 58*
HABENULA		242 ± 19	266 ± 39	342 ± 79	531 ± 17*	216 ± 17	289 ± 23	312 ± 64	542 ± 88*
SUBICULUM		243 ± 40	246 ± 48	318 ± 52	568 ± 91*	233 ± 19	326 ± 39	292 ± 29	652 ± 73*
THALAMUS	LD	303 ± 94	325 ± 44	402 ± 60*	662 ± 51*	247 ± 51*	292 ± 35	347 ± 47	686 ± 103*
	DLG	325 ± 95	282 ± 45	377 ± 72*	635 ± 68*	238 ± 41*	317 ± 41	312 ± 36	642 ± 94*
	VPM	305 ± 95	306 ± 50	400 ± 39*	682 ± 50*	242 ± 28*	321 ± 39	339 ± 39*	725 ± 67*
	VPL	274 ± 91	316 ± 64	409 ± 28*	628 ± 117*	240 ± 25*	313 ± 34	371 ± 40*	714 ± 70*
C.PUTAMEN		252 ± 43*	261 ± 21	294 ± 11	660 ± 75*	231 ± 71*	328 ± 32	317 ± 42	527 ± 50*
FR CX	OUT	415 ± 68	430 ± 35	463 ± 38	704 ± 39*	394 ± 41*	460 ± 40	493 ± 39*	725 ± 49*
	MID	309 ± 78	299 ± 27	360 ± 32	566 ± 64*	297 ± 44*	327 ± 32	402 ± 39*	636 ± 51*
	INN	227 ± 64	197 ± 29	295 ± 47	474 ± 51*	229 ± 49*	281 ± 28	322 ± 44*	608 ± 47*
OX CX	OUT	410 ± 36	400 ± 58	484 ± 78	847 ± 63*	313 ± 31*	377 ± 48	473 ± 58*	687 ± 61*
	MID	309 ± 92	295 ± 43	426 ± 28	703 ± 69*	270 ± 49*	334 ± 41	441 ± 37*	619 ± 60*
	INN	283 ± 48	273 ± 80	409 ± 51	597 ± 43*	203 ± 29*	223 ± 60	363 ± 41*	591 ± 52*

3.6.2: 8 hour preincubation.

The ability of glycine (10μM) to produce a significant enhancement in binding was restricted to 3 regions. Significant potentiation was observed in the stratum oriens layer of the CA1 and CA3 and in the stratum radiatum layer of CA3. The effect was maximal in the CA3 radiatum (46%). An enhancement was observed in all regions studied but was only significant in the above regions. The observed enhancement induced by 1μM glutamate was more widespread than that observed for glycine. Significant enhancements was observed in the oriens and radiatum layers of both CA1 and CA3, and in the dentate gyrus molecular layer and throughout the thalamic nuclei. Co-addition of the two amino acids produced a significant enhancement in all the regions studied with respect to that observed for the individual acids and to control. The greatest effect was observed in the stratum oriens of CA3 (205%).

3.6.3: 12 hour preincubation.

The enhancement by glycine was significant in the stratum radiatum of CA1 (36%) and CA3 (21%) together with the molecular layer of the dentate gyrus (31%). No other region displayed any significant enhancement in response to glycine. A significant enhancement was observed in all regions of the hippocampal CA1 and CA3, and in both cell layers of the dentate gyrus following the addition of 1μM glutamate. Significant enhancements was also observed in the ventral medial and lateral thalamic nuclei and frontal and occipital cortices where the greatest enhancements was observed in inner layers of the occipital cortex (78%). The enhancement observed following co-

addition was significant in all regions studied and greatest in the ventral medial thalamic nuclei (200%).

3.7: General comparison.

A statistical analysis of the control binding levels observed at all time points revealed a downward trend in the level of binding corresponding to an extension of the preincubation time. The control binding observed at T4 displayed no significant difference to that observed at T2 throughout all the regions studied. In contrast the control binding observed at T8 was found to be significantly lower than that of T2 in the caudate putamen, the middle and inner layers of the frontal cortex, the stratum radiatum of CA2 and CA3 together with the stratum oriens of CA3. In addition two other regions, the molecular and granular layers of the dentate gyrus also displayed significantly lower binding than observed at T2. The control binding at T12 was significantly lower than T2 in all the regions studied with the exception of the habenula, subiculum and the pyramidal cell layer of CA2 and CA3 and the granular layer of the dentate gyrus.

A similar statistical analysis of the observed enhancement induced by the coaddition of the amino acids at each preincubation times revealed no significant difference between the enhancement levels throughout all the regions studied.

3.4: Discussion.

The results presented in this chapter reflect the intrinsic and complex nature of the NMDA receptor. The modulation of receptor activation by selective agonists and antagonists acting at distinct sites, as reflected by ^{3}H-MK-801 binding, underlies the interdependence and co-operativity required for the efficient activation of the receptor.

3.4.1: Regional distribution.

The regional distribution of ^{3}H-MK-801 binding in supraspinal regions displayed a marked heterogeneity. The differential pattern of binding was not restricted to different regions of the CNS, but was also visible within individual regions eg hippocampus. The regional variation in binding levels concurs favourably with previously published observations in rat membrane studies (Wong *et al.*,1986: 1988) . Moreover an excellent correlation exists between the observed binding pattern and that previously described by Bowery *et al.*,(1988). In both studies a high degree of binding was observed in the stratum radiatum of the CA1 and the dentate gyrus molecular layer. In the CA1 the binding was localised over the dendritic layer of the radiatum and oriens, with a much lower level being observed in the pyramidal layer. This comparable distribution can be extended to include the autoradiographical distributions of other NMDA ligands including glutamate (Monaghan and Cotman,1985), CPP (Monaghan *et al.*,1988), APV (Monaghan *et al.*,1985), TCP (Loo *et al.*,1986) and glycine (Chapter 2).

The close association of the ionophore thought to contain the MK-801 binding site and the NMDA receptor is reflected in the reported influence of MK-801 on receptor functionality (Wong *et al.*, 1886:1987: Snell *et al.*, 1987). As discussed in chapter 2, for the strychnine-insensitive glycine receptor, the regional variation in receptor density can be correlated, at least in part, with the involvement of a particular region in a variety of neurological functions (as outlined below).

3.4.1.1: Hippocampus.

The hippocampal formation is known to play a vital role in a number of neurological functions mediated through NMDA receptors. These include memory and learning (Collingridge and Bliss,1987) and LTP (Collingridge and Lester,1989). Furthermore this area has been linked with a number of disorders including neurodegeneration (Gill *et al.*,1988) and epileptogenesis (Rothman and Olney,1988). As previously described (Introduction) NMDA receptors have been targeted for their involvement in all these processes. These receptors have been reported to participate in the synaptic activation of the hippocampal granule cells in the kindling model of epileptogenesis (Mody *et al.*,1987). Excess activation of these receptors initiates epileptiform activity which can be reduced by the selective competitive antagonists, APV and CPP (Mody *et al.*,1987). In addition, MK-801 has been reported to display potent anticonvulsant properties (Leander *et al.*,1988) which are consistent with the ability of this drug to evoke a noncompetitive blockade of receptor function (Wong *et al.*,1986).

The high density of binding sites observed in the hippocampus are consistent with the well documented neuroprotective nature of MK-801 (Gill *et al.*,1988: Bowery *et al.*,1988; Foster *et al.*,1988; Park *et al.*,1988) and of particular relevance in the hippocampus following the reported susceptibility of this region to neurodegenerative effects following ischaemic insult (Ito *et al.*,1975). MK-801 has been demonstrated to cause a significant reduction in the neuronal loss in rat hippocampus following neuronal damage induced by direct intracranial injection of NMDA and transient ischaemia (Foster *et al.*,1987). Furthermore this loss can be reduced even by post ischaemic administration of MK-801 up to 2 hours after the initial insult (Gill *et al.*,1988). Pretreatment with MK801 has also been reported to prevent the increase in calcium deposition which accompanies neuronal loss in the hippocampus (Gill *et al.*,1987; Bowery *et al.*,1988). ^{3}H-MK-801 binding has been suggested to represent an accurate marker for neuronal degeneration (Bowery *et al.*,1988). Further reports have suggested that MK-801 can enhance the recovery of the dentate gyrus granular cell layer and CA1 pyramidal cells following 5 or 10 min periods of anoxia (Kass *et al.*,1989; Aitkin *et al.*,1988). It may also be possible to relate the disruption of learning induced by MK-801 in the water maze test (Robinson *et al.*,1987) and the emotional disorganisation induced by PCP like drugs (Koek *et al.*,1988), with their interaction with MK-801 receptors in the hippocampal formation.

3.4.1.2: Olfactory bulb.

The sensory involvement of NMDA is reflected in the intermediate levels of binding observed in the lateral, medial geniculate nucleus and the external plexiform layer of the olfactory bulb (Cotman *et al.*,1987). Moreover MK-801 has been described to antagonise NMDA responses in the rat ventrobasal thalamus (a major supraspinal sensory relay nucleus) (Salt *et al.*,1988).

3.4.1.3: Cerebral cortex.

A laminae distribution pattern similar to that observed for the strychnine-insensitive glycine binding site (see Chapter 2) was observed throughout the cerebral cortical regions studied. The decrease in binding observed at deeper cortical regions may, as previously mentioned, indicate the predominance of other subtypes of EAA receptors in these regions (Johnson *et al.*,1989). The neuroprotective role of MK-801 is not confined to the hippocampus, the drug has been shown to antagonise NMDA induced neurotoxicity in cortical cell cultures (Rondouin *et al.*,1988). In addition the cortical receptors have been suggested to play a role in the characteristic behavioural responses associated with MK-801 and other phencyclidine like drugs. PCP has been demonstrated to mediate its behaviourial effects via an interaction with the dopaminergic system (Johnson,1983). In line with this concept MK801 has been reported to increase dopamine metabolite levels in the fronto parietal cortex and olfactory tubercle (Rao *et al.*,1990). In addition NMDA receptor modulation of the mesostriatal dopaminergic pathway has been reported (Carter *et al.*,1988). It is interesting to speculate whether the interaction of MK801 with this pathway

may occur via an interaction with cortical receptors.

The possible therapeutic potential of the non competitive EAA antagonists in Parkinsons disorder has been proposed following the reports that the antiparkinsonism drug memantine (Schneider *et al.*,1984) displayed a potent displacer activity of MK-801 binding in the human frontal cortex (Kornhuber *et al.*,1989). Electrophysiological conformation of the activity of memantine at the DA site has recently been reported by Bormann (1989). Moreover MK-801 has been demonstrated to attenuate Parkinson-like symptoms in several animal models of the disease (Carlsson and Carlsson,1989; Scmidt and Bubser, 1989).

3.4.1.4: Cerebellum.

The relatively low binding densities observed in the cerebellum are consistent with those previously described by Bowery *et al.*,(1988), together with the reported binding of ^{3}H-TCP in this region (Gundlach *et al.*,1986).

3.4.2: Agonism.

The use of so called "channel binding" ligands as markers of receptor activation has been used extensively to evaluate the mechanism of NMDA receptor activation (Snell *et al.*,1987; Wong *et al.*,1986). Furthermore the observed enhancement of ^{3}H-MK801 binding by selective agonists is consistent with previously published membrane studies (Snell *et al.*,1987; Reynolds *et al.*,1987; Wong *et al.*,1987).

The presence of endogenous amino acids in the tissue may provide an explanation for the significant enhancement of ^{3}H-MK801 binding elicited by glutamate and glycine in the majority of regions (at least at the earlier incubation times). The debate which surrounds the intrinsic concentrations of both glycine and glutamate was discussed in the Introduction. It is reasonable to assume that the enhancement induced by either amino acid alone is dependent upon the endogenous level of the other acid.

In contrast to the universal enhancement observed throughout this study following the co-addition of amino acids, a distinct regional pattern was observed in the enhancement induced by the individual acids. The glycine or glutamate potentiation observed at T2 were found to be significant in the majority of regions. However, this pattern displayed a marked decline at later incubation times. The apparent decrease in the regional ability to induce a significant response was consistent with the gradual reduction in levels of endogenous amino acids corresponding with an increase in the preincubation time, a concept analogous with the use of extensive washing in membrane studies (Loo *et al.*,1986; McKernan *et al.*,1989; Marvizon and Skolnick,1990). Evidence exists to demonstrate variations in the levels of glutamate and glycine throughout the CNS (Daly,1990; Liebschutz *et al.*,1977). Thus inter-regional variations in the levels of endogenous acids, a factor of particular relevance to glycine and glutamate when one reflects on the important metabolic role of these compounds may provide an explanation for the enhancement pattern observed in this study. The apparent regional variation

in this reduction provides a basis for speculation as to the possible correlation between the regional functionality and the levels of endogenous amino acids. It is interesting to observe the regions which retained the ability to elicit a significant enhancement correlated with the regions which have been demonstrated to play a prominent role in NMDA receptor function (eg hippocampus and dentate gyrus see earlier). However the situation is complicated by the results observed in the thalamic nuclei. This latter region was able to elicit a significant enhancement only following the addition of glutamate, an observation suggesting the presence of sufficient endogenous glycine. Whilst a functional role for the receptors in this region has been demonstrated (see earlier) no explanation can be provided for this apparent differential retention of one amino acid.

Additional evidence to support a reduction in the endogenous amino acid levels was provided by the significant decrease in the control binding observed at T12. The universal regional reduction serves to illustrate the use dependent nature of binding to the dissociative anaesthetic site previously reported by Lodge *et al.,(*1987). Moreover these observations are consistent with the binding of ^{3}H-MK801 to the activated state of the receptor (Kemp *et al.,*1986; Wong *et al.,*1986: 1988). The reduction in the endogenous levels of either amino acid leads to a decreased accessibility of the channel located binding site to selective ligands (Ransom and Stec,1988), a concept discussed later. The significant reduction in the basal binding may, in addition, provide an explanation as to the apparent increase in the number of regions which display

a significant enhancement in response to glutamate at this time point. A comparison of the observed density values at this and other time points revealed no significant variation throughout the regions concerned. It is reasonable to assume therefore, that the reduction in the control levels of binding, rather than an increase in the endogenous amino acids levels or an alteration in the receptor characteristics is responsible for the increased regional enhancement.

The inability of glutamate and glycine to produce a further significant enhancement following an extension in the preincubation time may, provide an insight as to the state of activation of the receptor. It is possible that the co-addition of glutamate and glycine at T2 raises the intrinsic concentration at the receptor sufficiently to allow maximum activation of the receptor (ie: all channels open). Moreover, the addition of the exogenous agonist at subsequent time points acts to replenish the endogenous amino acids removed following an extension of the preincubation time, with the resultant restoration of the receptor to its maximally activated state. The maximal nature of glycine enhancement of glutamate stimulated ^{3}H-MK 801 binding reported by a number of groups (Wong *et al.*,1987: Snell *et al.*,1987: Ransom and Stec 1988) may provide evidence to support this concept.

3.4.3: Mechanism of action.

The precise mechanism of glycine and glutamate potentiation of MK801 (Wong *et al.*,1987) or TCP (Snell *et al.*,1988) binding remains unclear. Early concepts

based on membrane binding studies (Wong *et al.*,1987; Benavides *et al.*,1988) suggested the enhancement was mediated via an alteration in the affinity (Kd) or the apparent receptor density (B_{max}) of MK801 and TCP. However subsequent analysis of the equilibrium binding of MK801 (Kloog *et al.*,1988a; Reynolds and Miller,1988) and TCP (Nadler *et al.*,1990; Johnson *et al.*,1988) revealed the rate at which equilibrium state was achieved (between 5 - 24 hours) was dependent upon the thoroughness of the washing procedure, the particular ligand used and the level of endogenous mediators. Thus the majority of the early studies employed conditions corresponding to between 50 - 70% of the equilibrium state and were not reflective of the true *in vivo* situation. More recent studies by Kloog *et al.*,(1988a) and Reynolds and Miller (1988) have demonstrated that glutamate and glycine markedly increase the rate of association and dissociation of the ligand to the receptor, whilst having no effect on the equilibrium constant. This concept may be translated as an increase in the fraction of time that the channel site is accessible to any selective ligand. These observations are consistent with those previously described by Johnson and Ascher (1987) that glycine could increase the frequency of channel opening. This idea has been expanded by Ransom and Stec (1988) who postulated that the length of time any one channel spends in the activated state was dependent upon the agonist concentration. At high concentrations the receptor is activated for a proportionally longer period of time than at lower concentrations. This extended activation results in an increase in the accessibility of the receptor to the selective ligand, a concept which, under non equilibrium conditions of binding may be manifested as an

apparent decrease in the affinity constant.

Initial kinetic analysis (Kloog *et al.*,1988a) described the enhancement as a pseudo-first order reaction although current understanding suggests the presence of a more complex situation. Javitt and Zukin (1989) have described a model based on near equilibrium binding which suggest that glycine acts to convert a glutamate-associated closed conformation of the channel (with a low affinity) into a high affinity open state. These authors have recently extended their theory (Javitt *et al.*,1990) to include the observation that glutamate facilitates the "fast" component of binding (that associated with binding to the activated form of the receptor complex). The modulation of ^{3}H-MK-801 binding by glycine and glutamate appears to be mediated via an increase in the channel opening (accessibility). Moreover biochemical (Ransom and Stec,1988) and electrophysiological (Johnson and Ascher,1987) data indicates that maximal effects are observed when both glycine and glutamate sites are occupied, although no allosteric changes are thought to occur. This mechanism by which sites regulate channel activation involves an independent, but mutually required action on channel conformation. This concept is supported by the data from the agonist potentiation and inhibition studies presented in this chapter.

3.4.4: Antagonism.

The ability of the selective $\overset{EAA}{\zeta}$ antagonists to elicit a concentration dependent inhibition of ^{3}H-MK-801 binding is consistent with previous observations in

membrane preparations (Lehmann *et al.*,1988; Kemp *et al.*,1987; Hood *et al.*,1990). 7ClKYN has been reported to attenuate NMDA receptor function by a potent and selective inhibitory action mediated through the strychnine insensitive glycine receptor (Kemp *et al.*,1987). Similarly CPP, a selective glutamate antagonist has been reported to inhibit NMDA receptor function by interaction with the agonist recognition site on the receptor complex (Lehmann *et al.*,1988). Electrophysiological (Kemp *et al.*,1987) and biochemical (Monaghan *et al.*,1988) evidence suggest that both these antagonists act in a competitive and reversible manner an observation reflected by the data obtained in this study. The lack of any apparent regional variation in the inhibition by 7ClKYN suggest that, at least in the regions studied, a homogeneous population of strychnine-insensitive glycine binding sites are present. These findings are in agreement with the data presented in Chapter 2. However, the inhibitory actions of 7ClKYN are complicated by the reported presence of the endogenous parent molecule, kynurenic acid throughout the CNS (Turski *et al.*,1988). Whilst kynurenic acid displays a lower affinity for the same receptor (Kessler *et al.*,1988:1989a; Watson *et al.*,1988), its presence cannot be ignored when evaluating the true intrinsic potency of 7ClKYN. Similarly the competitive nature of 7ClKYN maybe compromised by the varying concentrations of endogenous glycine competing for the same receptor (Kemp *et al.*,1988).

The lack of any significant variation in the inhibition of CPP suggests that at least in the regions studied that a homogeneous population of glutamate receptors are present. Moreover these results provide no evidence to support

the idea of a differential glutamate population (first postulated by Monaghan and colleagues (1988) see Introduction). A comparison of the inhibitory effect in the two so-called "antagonist preferring" regions of the thalamic nuclei and the inner layer of the cerebral cortex revealed no evidence of any differential antagonism. Whilst the inhibition in these regions was slightly greater than that observed in other regions the lack of any significant difference fails to provide any conclusive evidence to support this concept. It is possible that fluctuations in the inhibitory effects observed in these regions is as a consequence of variations in the concentration of the endogenous mediators as discussed earlier.

The competitive nature of these antagonists is illustrated by the ability of glycine and glutamate to reverse the inhibitory effects of the two antagonists. Furthermore the ability of two different antagonists, acting at discrete sites to reduce the binding of ^{3}H-MK-801 is consistent with the idea that both agonist sites must be occupied to allow adequate receptor activation (Kleckner and Dingledine,1988; Javitt *et al.*,1989:1990). This has been confirmed in membrane studies (Thomas *et al.*,1988; Sircar *et al.*,1989). In mechanistic terms, both antagonists are thought to decrease the rate of association and dissociation of ligand binding (a concept discussed earlier), thus decreasing the time that the receptor spends in the activated state (Bonhaus *et al.*,1989; Kloog *et al.*,1988:1990). This in turn will reduce the accessibility of the specific receptor to the ligand. The lack of any regional variation in either the inhibition or the reversal suggest that, at least within the regions studied, the

relationship between the sites is uniform.

The results presented in this chapter have confirmed the heterogeneous distribution of ^{3}H-MK-801 binding in rat brain sections. This binding pattern displayed an excellent correlation with the published distributions for other NMDA receptor ligands. In addition the results of quantitative autoradiography demonstrated that in washed slide mounted sections, ^{3}H-MK-801 binding is selectively regulated by both agonist and antagonists which act on the NMDA receptor complex. Overall these data provide good evidence that under non-equilibrium conditions, ^{3}H-MK-801 serves as a marker for the activated state of the NMDA receptor. Moreover this method may provide a means of investigating the state of receptor activation in a number of diseased states.

CHAPTER 4 - THE RELEASE OF GLYCINE FROM RAT CEREBELLUM AND HIPPOCAMPUS.

Chapter 4.

4.1: Introduction.

It is now well established that interneural communication in the vertebrate central nervous system (CNS) is predominantly a chemical phenomenon, and a wide variety substances have been proposed as central transmitters (Curtis and Johnston,1974; Fagg and Lane,1979).

Depolarisation of the presynaptic membrane, in response to neural stimulation, causes terminal vesicles to release a transmitter into the synaptic cleft. The released transmitter interacts with a specific receptor in the postsynaptic membrane to elicit a modification of cell activity. Furthermore there is a large body of evidence which supports the concept that neuronal neurotransmitter release is a calcium dependent process (Llinas and Heuser,1977).

The demonstration of synaptic release of a substance in response to neural stimulation, and the identification of a possible neurotransmitter role for a substance present major issues in modern neuroscience research. The majority of these investigations have involved the use of tissues slices, synaptosomal preparations, chopped tissue and more recently cultured systems. There are a number of advantages and disadvantages for all these systems, however a detailed evaluation of each system is beyond the scope of this thesis. The study presented in this chapter is based on the use of one of these techniques, that of the *in vitro* slice preparation.

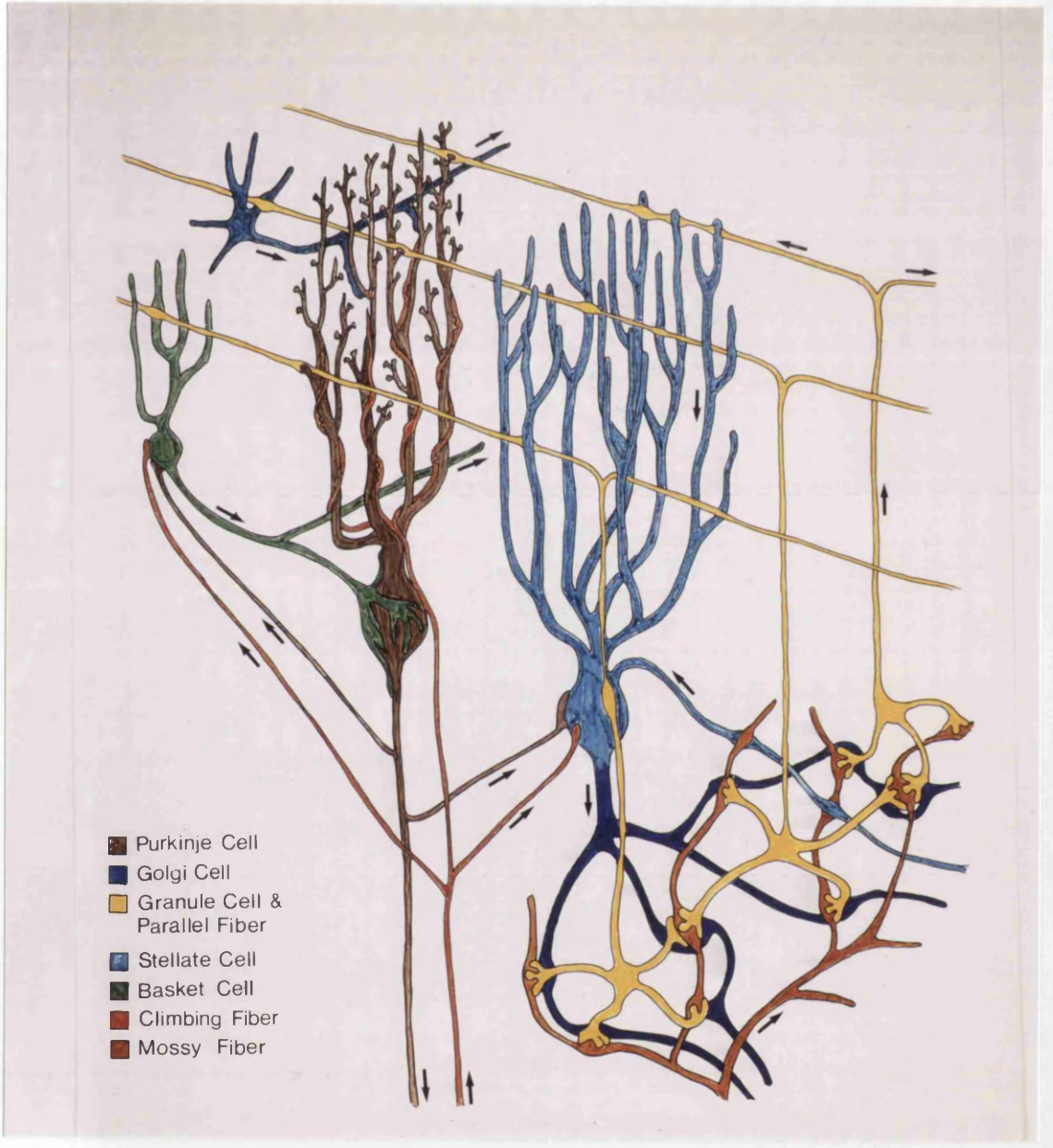
Tissue slice preparations are particularly useful for *in vitro* neurotransmitter release studies as the majority have been well characterised with respect to their metabolic, biosynthetic, transport and electrophysiological properties. In addition they allow investigation of the particular cell types involved in mediating both uptake and release since they retain much of the morphology and synaptic connections of the intact tissue. The release studies in this chapter revolve predominantly around the use of cerebellar tissue slices. However, by means of comparison, data obtained from hippocampal slices are also included. Before outlining the scientific basis for this study, the structure and neuronal circuitry of each of these regions is discussed.

4.1.1: Cerebellum.

The cerebellar cortex is divided by fissures into a number of lobes, each of which subdivide into a number of lobules. Each lobule, in turn, is divided into sublobules and folia (Ito,1984). The cerebellar cortex comprises 3 distinct layers, the molecular, Purkinje (or ganglionic) and granule cell layer. Moreover, the cortex contains only 5 major neuronal types (Purkinje, basket, stellate, Golgi and granule cells) and 2 major afferent terminals (mossy and climbing fibres). A schematic representation of the neuronal circuitry of the cerebellum is shown in Figure 4.1.

The granule cells are the most abundant neurons in the cerebellum, comprising of >90% of total population. They are located within the granular cell layer, just below the Purkinje cells. Their axons project up toward the surface of the

Fig 4.1: Schematic illustration of cerebellar synaptic circuitry. Figure modified from Llinas and Heuser (1977).


Purkinje Cell
Golgi Cell
Granule Cell &
Parallel Fiber
Stellate Cell
Basket Cell
Climbing Fiber
Mossy Fiber

cerebellum where they bifurcate to form T-shaped parallel fibres, whose terminals synapse in the dendritic trees of the inhibitory Purkinje, Golgi, stellate and basket cells. Furthermore the granule cells are the only intrinsic neurons to use an excitatory transmitter (glutamate).

The Purkinje cells are the largest neurons in the cerebellum and are located in a single row between the molecular and granule cell layers. The perpendicular orientation of the Purkinje cell dendrites, with regard to the parallel fibres, ensures multiple innervation within the molecular layer. The sole cerebellar output originates from the Purkinje cell axons, which project to the deep cerebellar nuclei and brain stem. In addition these axons possess collateral fibres which synapse on the soma of Golgi, basket and other Purkinje cells.

The inhibitory Golgi cell perikarya lie in the granular cell layer, slightly below the Purkinje cells and give rise to a highly branched, local axonal plexus. The Golgi cell axons synapse on granule cell dendrites and form part of the specialised cerebellar glomerulus which also includes the afferent mossy fibres.

The stellate and basket cell bodies are located entirely within the molecular layer, where both function as inhibitory interneurons. Stellate cell dendrites extend throughout the molecular layer, although their axons synapse solely on Purkinje cell dendrites. In contrast basket cell axons synapse with Purkinje cell bodies and dendrites.

There are 2 major afferent fibre inputs which project to the cerebellum. The mossy fibres relay excitatory potentials from their cell bodies located in the pontine nuclei, spinal cord and other regions. These fibres branch profusely throughout the granule cell layer and synapse in the cerebellar glomeruli with the granule cell dendrites and Golgi cell axons. In addition, a direct mossy fibre input onto the Golgi cell bodies, via the axosomatic synapses, *en marron*, has been described (Chan-Palay and Palay,1971).

The afferent input of the climbing fibre arises from the inferior olive which, in turn, receives proprioceptive input from the whole body. These excitatory fibres contact primarily with Purkinje cell dendrites, although collateral fibres also synapse on Golgi and basket cells. In general there is one climbing fibre contacting a single Purkinje cell.

A third afferent projection arises from the monoaminergic neurons in the locus coeruleus and raphe nucleus. These noradrenergic and 5HT inputs terminate within the cerebellum, predominantly on the somas and dendrites of the Purkinje cells.

4.1.2: Hippocampal formation.

The hippocampal formation is found on the posteromedial border of the cerebral hemispheres, extending from the rostromedially located septum to the ventrolateral located amygdaloid area. The formation divides into 3 major subfields, CA1, CA2 and CA3, each containing 4 distinct cellular bands, the

stratum oriens, stratum pyramidale, stratum radiatum and lacunosum moleculare. The principal neurons in this area are the pyramidal neurons, whose cell bodies are localised in the pyramidal cell layer. Basal pyramidal dendrites project upwards into the stratum oriens while lateral dendrites project into the radiatum and molecular layers. Located "around" the base of the hippocampus is the area dentata, which form an integral part of the hippocampal formation. This region has two main cell types, the granule and molecular cells. The former contains the principal neurons of the region and the granule cell dendrites (mossy fibres) project upwards into the stratum moleculare of CA3, through the more superficial molecular layer.

Perforant path fibres, originating in the entorhinal cortex, impose on the dendrites of the granule cell neurons (mossy fibres) within the molecular layer. In the outer third of the molecular layer lies the lateral perforant path, in the middle third the medial perforant path. The mossy fibres impose on the dendrites of the CA3 pyramidal cells, producing an excitatory input. In turn axon collaterals from the ipsi (Schaffer collaterals) and contralateral (Commisural) regions of the hippocampus provide the major excitatory input to the CA1 pyramidal cell dendrites.

4.1.3: Glycine and GABA in the cerebellum.

The role of glycine as a major inhibitory neurotransmitter in the spinal cord and brain stem is well established (Oja *et al.*, 1977; Aprison, 1990). However the majority of inhibitory synapses in other regions of the CNS utilise a second

inhibitory amino acid, GABA, as their major transmitter (Price and Bowery,1988). Recent evidence suggest that, in addition to a neurotransmitter function, glycine can modulate excitatory aminoacidergic synapses in the brain, via the activation of NMDA receptors (Johnson and Ascher,1987). The evidence to support the role of glycine as a modulator of NMDA receptor function was discussed in chapters 1 and 2.

In common with other brain regions the cerebellum is thought to employ GABA as the major neurotransmitter in all the inhibitory neurons (Golgi, basket and Purkinje cells) (Eccles *et al.*,1967; Curtis *et al.*,1970). However, the discovery that the cerebellum possessed high affinity glycine uptake sites (Wilson *et al.*,1976) suggested a possible role for glycine in this region. The localisation of these sites on inhibitory Golgi interneurons (Wilkin *et al.*,1981b) suggested that a single population of Golgi interneurons was capable of transporting both glycine and GABA. The apparent colocalisation of an excitatory and inhibitory amino acid in a single inhibitory neuronal population was confirmed immunocytochemically by Ottersen *et al.*,(1988). Using specific antibodies against glycine and GABA, they demonstrated that approx 70% of all Golgi cells labelled positively for both GABA and glycine-like immunoreactivity (LI). Of the remaining 30%, half were positive for glycine LI and the other half positive for GABA-LI.

4.1.4: Glycine release.

In addition to the specific uptake and colocalisation, Golgi-axon terminals have been demonstrated to release both ^{3}H-glycine and GABA (Morales and Tapia,1987). The release of glycine was reported to be calcium dependent consistent with the possible involvement of a neuronal mechanism. In parallel with the study presented in this chapter, the calcium dependency has been confirmed in a variety of release systems (Fletcher *et al.*,1989: Kingsbury *et al.*,1988: Holopainen and Kontro,1989). There is no evidence to support a similar mechanism in other brain regions, suggesting that neuronal release of glycine, a common inhibitory event in the spinal cord, may be unique to the cerebellum amongst supraspinal regions.

Previous researchers in this laboratory have developed an alternative experimental model, based on the use of Vibratome slices of brain tissue, to evaluate the neurotransmitter release in the CNS. The use of this model has provided a means for the evaluation of the nature of glycine release in an *in vitro* slice preparation. The data presented in this chapter represent an attempt to establish the presence of a neuronal release mechanism for glycine in the cerebellum. The calcium dependent nature of both exogenous and endogenous glycine release has been studied, in addition to the depolarising effects of various concentrations of potassium. Furthermore the possibility of a similar mechanism in another brain region, the hippocampus has been evaluated. The chapter also includes a pharmacological and autoradiographical determination of the possible postsynaptic site of action for the released

glycine, with particular reference to a possible excitatory role for glycine in the cerebellum. In addition the results of a preliminary study of agonist evoked glycine release in the cerebellum is presented.

4.2: METHODS.

4.2.1: Exogenous release

Male rats (SD strain, 200-300g) were sacrificed by decapitation and the brains rapidly removed. Cerebella tissue was dissected on ice, the hemispheres removed and the central vermis mounted sagitally on a perspex specimen block. The tissue was covered in agarose, to facilitate slice preparation, and immersed in ice-cold Krebs Henseleit (KH) (mM : NaCl 118, KCl 47, $NaHCO_3$ 25, KH_2PO_4 1.18, $MgSO_4$ 1.19, $CaCl_2$ 2.5, Glucose 11). 400µm brain slices were prepared using a Vibratome (Oxford Instruments) tissue sectioner. Following a 60 min preincubation in KH buffer (95% O_2 / 5% CO_2, 37 °C) the slices were transferred to fresh buffer containing 100nM ^{3}H-glycine (S.A.43.5 Ci/mmol) for 15 or 40 mins and subsequently transferred to a 1ml perfusion chamber (1 slice per chamber). Individual slices were perfused (0.6 ml min^{-1}) with KH buffer for 40 mins to allow for equilibration, and stimulated with KH buffer containing elevated concentrations of potassium for 5 mins in the presence and absence of calcium. Slices were subsequently washed for 20 mins after stimulation. Samples were collected each minute and the level of radioactivity in each assayed by scintillation spectrometry. Tissue slices were solubilized overnight in 400 µl of "Soluene" tissue solubiliser, neutralised by the addition of 4 mls of 1M HCl, and the level of activity remaining in the tissue estimated by scintillation spectrometry.

4.2.2: Hippocampal slices.

The experimental protocol was performed as described above. Due to the discrete nature of the hippocampal region, slices were prepared "in situ" from a coronal brain slice (5mm). The hippocampal region was dissected clear of adjoining tissue prior to loading with radiolabelled glycine.

4.2.3: Endogenous studies.

The tissue was prepared as described above under exogenous release. Fresh cut sections were transferred directly to the perfusion chamber without prior preincubation or loading. Samples were collected at 5 min intervals and assayed for glycine content by means of high performance liquid chromatography.

4.2.4: Agonist stimulated release.

The protocol for both the exogenous and endogenous release as previously described. Slices of rat cerebellum vermis were perfused with Mg^{2+} free KH buffer throughout. The slices were stimulated by perfusion with Mg^{2+} free KH buffer containing the appropriate agonist concentration in the presence of calcium. Exogenous and endogenous samples were collected and analysed as described above.

4.2.5: Receptor binding and autoradiography.

The methodology employed to investigate ^{3}H-glycine binding to sagittal sections of rat cerebellar vermis is described in Chapter 2.

4.2.6: Accumulation studies.

Slices of rat cerebellum were prepared as described above. Following a 60 min preincubation in KH buffer, slices were transferred to 25ml conical flasks, containing 10 mls of KH buffer and 100nM ^{3}H-glycine (S.A 43,5 Ci/mmol). Slices were loaded for a variety of times ranging from 5 to 60 minutes. Following loading each section was solubilised overnight in 400µl of "Soluene" tissue solubiliser, neutralised by the addition of 4 mls of 1M HCl, and the level of activity in each section estimated by scintillation spectrometry.

4.2.7: Statistical Analysis.

All data were analysed using the Mann-Whitney U-test for non-parametric data. Significance levels $p < 0.05$.

It must be noted that the identity of the released radiolabel was not established.

4.3: Results.

4.3.1: Receptor binding and autoradiography.

Compounds were tested for their ability to displace 100 nM ^{3}H-glycine from sagittal sections of the cerebellar vermis. The IC_{50} values obtained are shown in Table 4.1. The simple amino acids glycine and D-serine displayed similar potencies, in addition both the selective glycine antagonists, DNQX and 7-Cl KYN acid were equipotent. Strychnine failed to displace binding at concentrations up to 100 µM.

Autoradiographical analysis of binding (Fig 4.2) revealed a heterogeneous distribution of strychnine-insensitive binding sites. Subsequent densitometric analysis localised approximately 90% of the binding sites in the granule cell layer.

Scatchard analysis of glycine binding (Fig 4.3) was consistent with a single population of sites. The values for maximal binding (B_{MAX} 6.7 ± 1.3 pmol / mg tissue) and dissociation constant (K_D 271 ± 63 nM) were obtained from 4 independent studies.

4.3.2: Glycine accumulation in cerebellar slices.

The rate of ^{3}H-glycine accumulation in to sections of rat cerebellum is shown in Fig 4.4. Accumulation appeared linear for an initial 30 mins. At higher time points a decrease in the rate of accumulation was observed.

Table 4.1: Influence of glycine analogues and other compounds on ^{3}H-glycine binding to rat cerebellar sections.

Data represents mean IC_{50} ± SEM µM from at least 3 determinations. Slices were incubated with 100nM ^{3}H-glycine in the presence or absence of varying concentrations of displacer. Non-specific binding was determined in the presence of 100µM unlabelled glycine.

Fig 4.2: Representative autoradiograms of strychnine-insensitive ^{3}H-glycine binding to rat cerebellar sections.

Sections were incubated with 100 nM ^{3}H-glycine and autoradiograms generation as described in Methods. Gr-Cb = granular layer : Mol-Cb = molecular layer.

COMPOUND	IC_{50} ± SEM (uM)
GLYCINE	.56 ± .10
D-SERINE	.79 ± .19
7-Cl KYN	.96 ± .12
DNQX	.61 ± .17
STRYCHNINE	> 1000

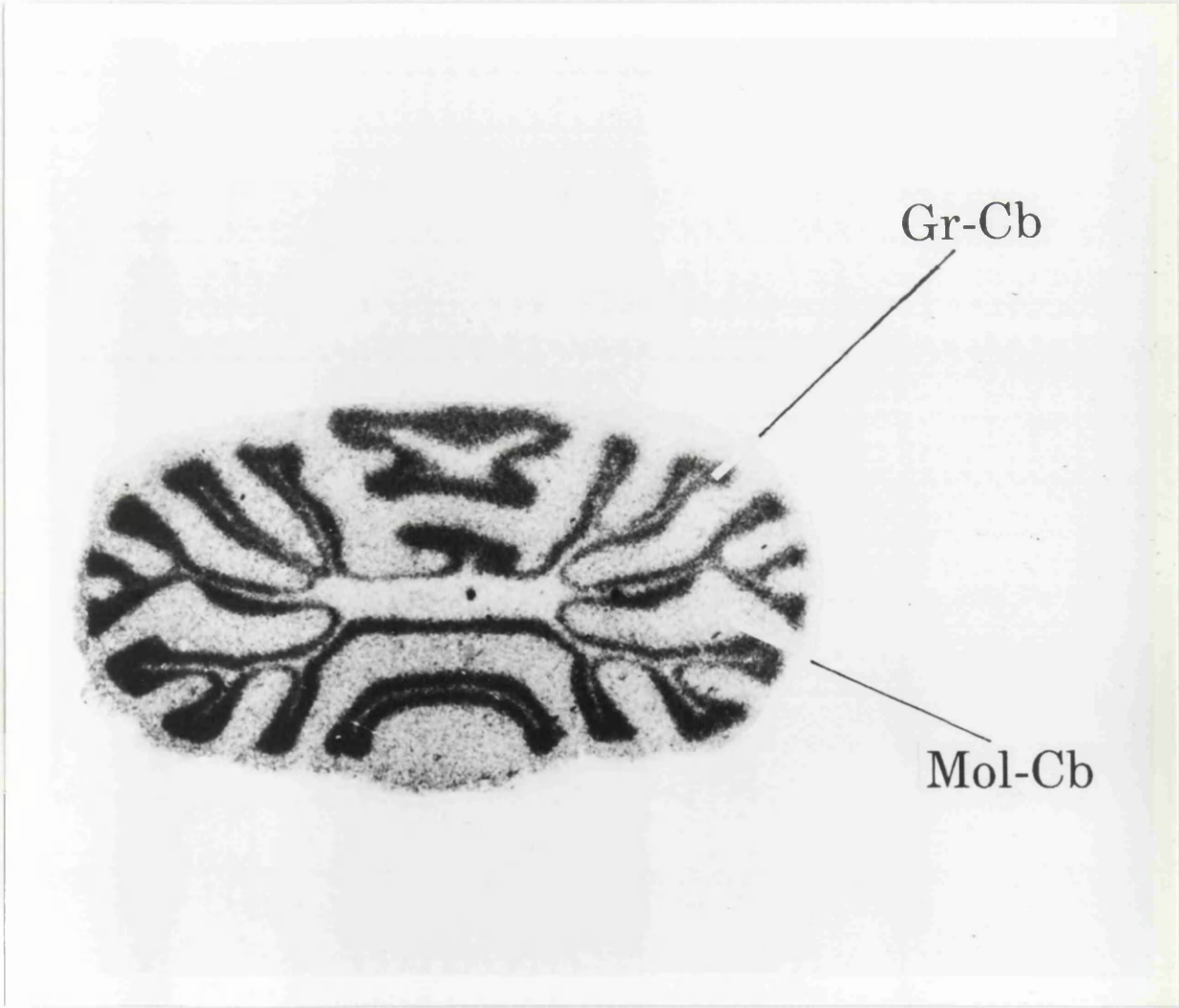


Fig 4.3: Scatchard analysis of ^{3}H-glycine binding to sections of rat cerebellum.

Slices were incubated with 100nM ^{3}H-glycine and from 5nM to 10µM glycine; non-specific binding was determined in the presence of 100µM unlabelled glycine. The graph represents the mean data of 4 separate determinations. The SEM was <10 % of the mean value in all cases. The data was analysed using a non-linear least squares curve fitting model. These data for cerebellar were best fitted by a single site model.

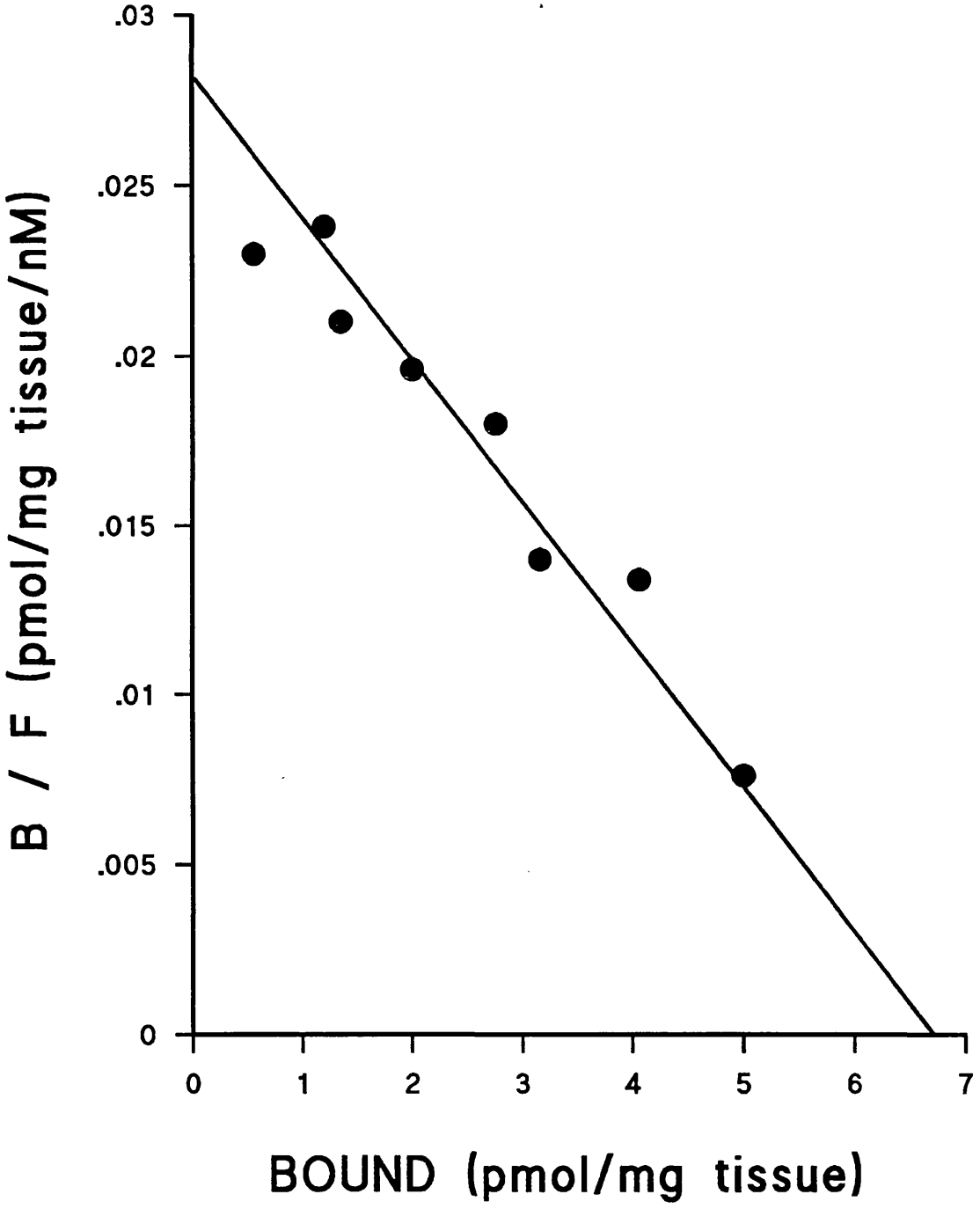
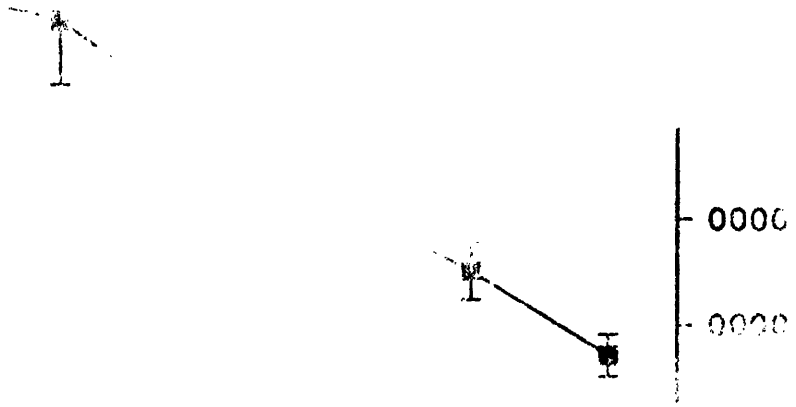
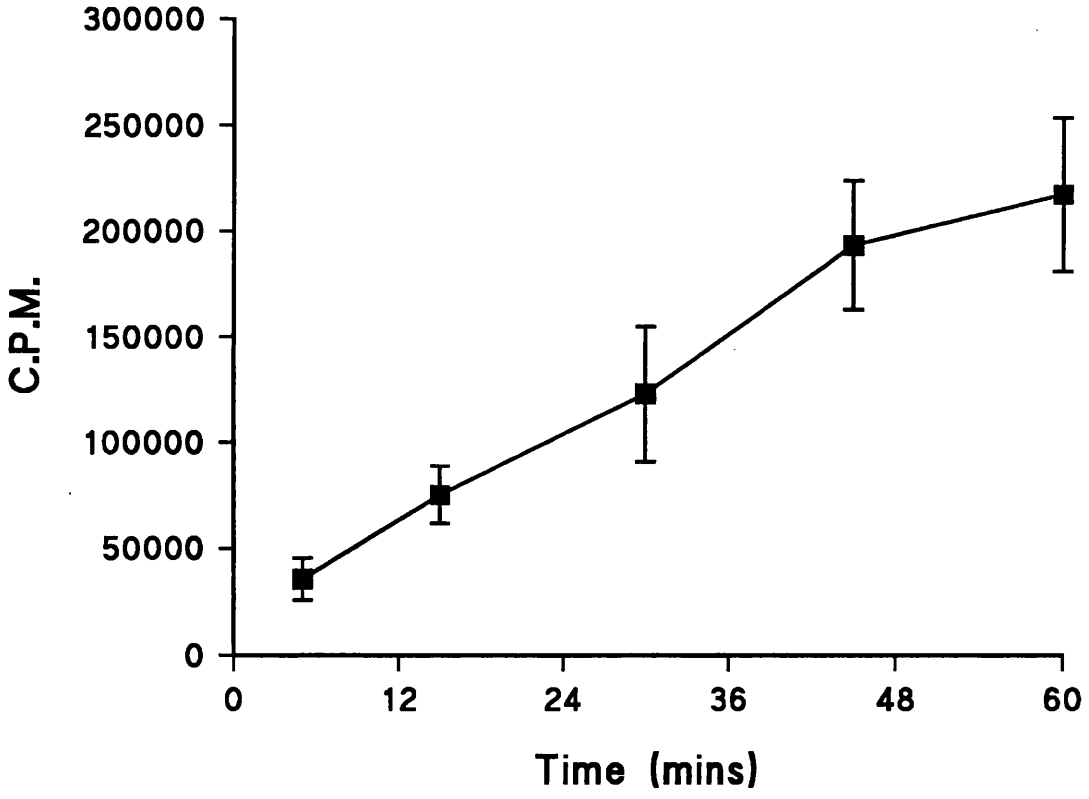

.03
.025
.02
.015
.01
.005
0
B / F (pmol/mg tissue/nM)
0
1
2
3
4
5
6
7
BOUND (pmol/mg tissue)

Fig 4.4 : Time course of ^{3}H-glycine accumulation by 400 µm Vibratome sections of cerebellum.

Slices were incubated with 100nM ^{3}H-glycine for 5 to 60 mins. Slices were solubilised overnight by the addition of 400µl of Soluene tissue solubiliser and the amount of radioactivity in each slice was estimated by liquid scintillation counting. Each point is the mean of triplicate determinations ± SEM.




300000
250000
200000
150000
100000
50000
0
C.P.M.
0
12
24
36
48
60
Time (mins)

4.3.3: Exogenous (radiolabelled) release.

The effect of K^+ depolarization on the release of ^{3}H-glycine from slices of rat cerebellum and hippocampus was investigated. In all experiments basal levels are represented as 100% and the peak response obtained during a 5 minute exposure to high K^+ is expressed as a % stimulation of basal.

The ability of 55 mM K^+ to stimulate release of ^{3}H-glycine from a cerebellar slice in a rapid and calcium dependent manner is illustrated in Fig 4.5 . The latter is a representative trace obtained from a single experiment. Quantitative data from a series of experiments are given in Table 4.2.

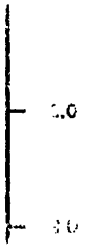
K^+ stimulated release of ^{3}H-glycine from preloaded (15 mins) cerebellar slices was dose-dependent in the presence of Ca^{2+} (Fig 4.6). A significant increase over basal was observed with all concentrations tested. Stimulation with 30 mM K^+ produced a 123.7 ± 3.3% increase over basal. This was significantly ($p < 0.05$) greater in the presence of 40 mM K^+ (138.6 ± 3.5%) and 55 mM K^+ (148.3 ± 2.53%). Slices preloaded for 40 minutes displayed similar significant dose related increases (Table 4.2).

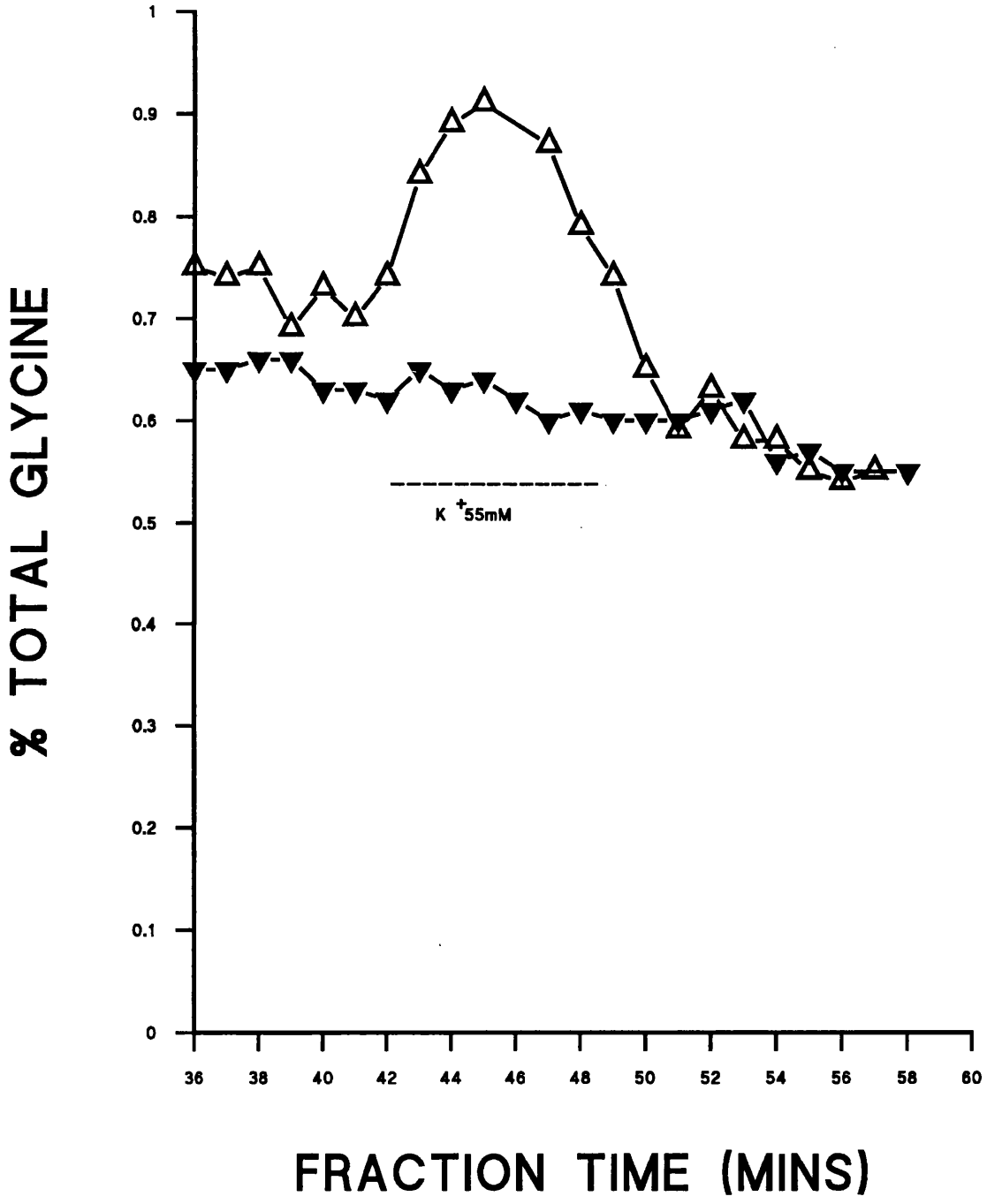
The ability of two concentrations of K^+ to release glycine from preloaded (40 mins) hippocampal slices is shown in Fig 4.7. A significant increase over basal was observed with both concentrations (K^+ 55 mM : 151.6 ± 13.1% 40 mM : 147.8 ± 8.8%). Furthermore omission of calcium from the superfusion medium had no significant effect on the stimulation at either dose (40 mM : 141.3 ±

Fig 4.5: Release of ^{3}H-glycine from 400µm Vibratome sections of rat cerebellum.

Slices were preincubated for 15 minutes in 100 nM ^{3}H-glycine, and superfused for 30 minutes to allow for the establishment of basal release. Slices were stimulated with 55 mM K^{+} for 5 minutes in the presence (▵) and absence (▾) of calcium (2.5mM). Top curve illustrates stimulation in the presence of Ca^{2+}: bottom curve in the absence of calcium. Data obtained from a single experiment. An increase over basal of 144% in the presence of calcium was observed (an equivalent to .3% of total radioactivity added).

(Total glycine = Σglycine released + total glycine remaining in tissue at end of experiment).




% TOTAL GLYCINE
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
K +55mM
36
38
40
42
44
46
48
50
52
54
56
58
60
FRACTION TIME (MINS)

EXOGENOUS			
K^+ (mM)	15 MINS	40 MINS	ENDOGENOUS
BASAL (2.5mM)	100 %	100 %	100 %
30	123.7 ± 3.3	130.0 ± 4.9	129.9 ± 6.3
40	138.6 ± 3.5	141.3 ± 4.2	137.2 ± 4.2
55	148.3 ± 4.5	153.5 ± 5.5	149.9 ± 4.4

Table 4.2. Potassium stimulated release of glycine from 400μm Vibratome sections of rat cerebellum.

Table shows quantitated analysis of glycine release following stimulation with varying concentrations of potassium. Exogenous (tritiated) data was obtained from sections loaded with ^{3}H-glycine for 15 or 40 mins, equilibration and stimulation as described in Methods. Samples were analysed by liquid scintillation counting. Values are Mean ± SEM from 3-5 experiments.

Data for endogenous release was obtained as described in Methods. Samples were analysed by means of High Performance Liquid Chromatography, and the area beneath each peak analysed by means of computer assisted analysis programme. Data are Mean ± SEM from 3 experiments.

Fig 4.6: The ability of various concentrations of K^+ to evoke the release of ^{3}H-glycine from Vibratome sections of rat cerebellum.

Slices were incubated with 100 nM ^{3}H-glycine for 15 mins, then superfused with KH buffer containing 2.5 mM Ca^{2+} for 40 minutes to establish a basal level of release. Slices were depolarised over a 5 minute period with either 30, 40 or 55 mM K^+. Peak ^{3}H-glycine release for each concentration of K^+ is expressed as a % stimulation over basal release and are the means ± SEM from at least 4 experiments. (□) represents stimulation in the presence of 2.5mM Ca^{2+} (▨) represents stimulation in the absence of Ca^{2+}.

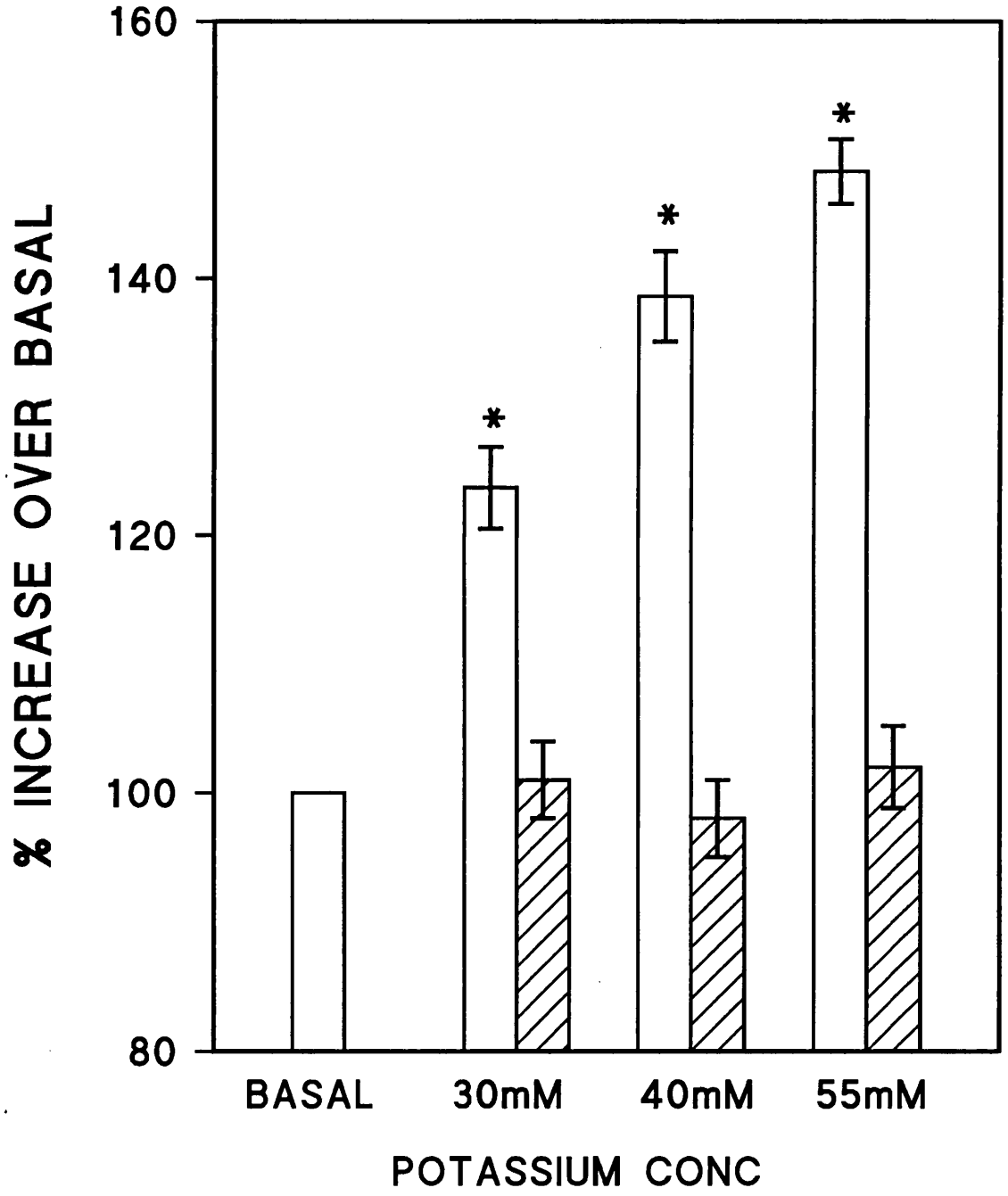
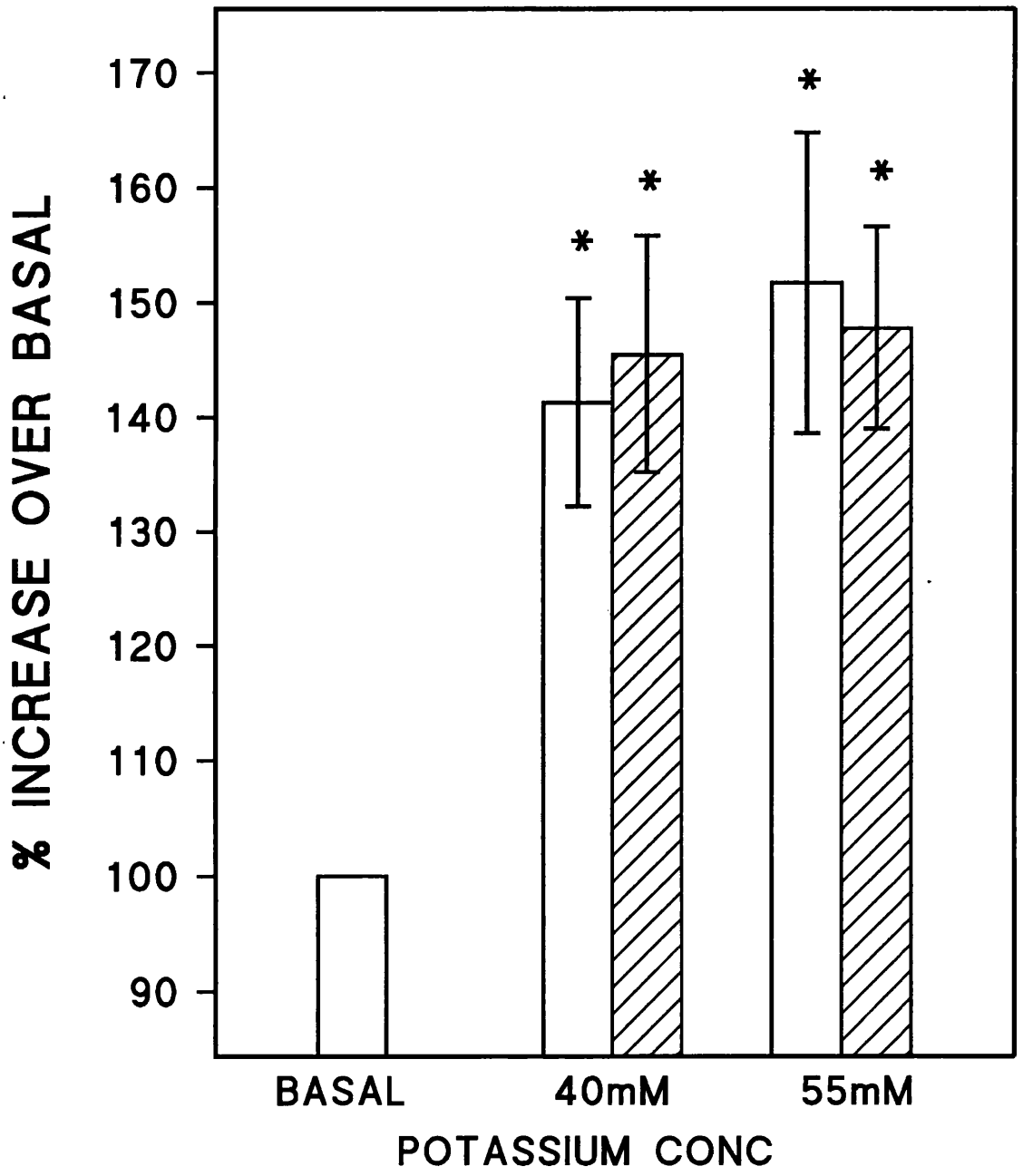

160
140
120
100
80
% INCREASE OVER BASAL
*
*
*
BASAL
30mM
40mM
55mM
POTASSIUM CONC

Fig 4.7: The ability of high concentrations of K^+ to evoke the release of ^{3}H-glycine from Vibratome sections of rat hippocampus.

Slices were incubated with 100 nM ^{3}H-glycine for 40 mins, then superfused with KH buffer containing 2.5 mM Ca^{2+} for 40 minutes to establish a basal level of release. Slices were depolarised over a 5 minute period with either 40 or 55 mM K^+. Peak ^{3}H-glycine release for each concentration of K^+ is expressed as a % stimulation over basal release and are the means ± SEM from at 3 experiments. (□) represents stimulation in the presence of 2.5mM Ca^{2+} (▨) represents stimulation in the absence of Ca^{2+}.


% INCREASE OVER BASAL
170
160
150
140
130
120
110
100
90
*
*
*
*
BASAL
40mM
55mM
POTASSIUM CONC

9.1% , 55 mM : 145.5 ± 10%).

4.3.4: K^+ stimulated release of endogenous glycine.

The ability of high K^+ to stimulate the release of endogenous glycine from cerebellar slices was investigated. A representative chromatogram of basal (bottom) and stimulated (55 mM K^+) (top) release of endogenous glycine is shown in Fig 4.8. The area beneath each peak was evaluated by a computer assisted programme. Stimulation was expressed as a % increase over the basal response in each case. Mean data obtained from a number of studies is shown in column 4 of Table 4.2.

The stimulation obtained with 30 mM K^+ (129.9 ± 6.3 %) was significantly increased by higher (40 mM (137.2 ± 4.3) and 55 mM (149.9 ± 4.4)) potassium concentrations. Stimulation in the absence of calcium was not significantly different from control in all cases.

4.3.5: Release of glycine by EAA agonists.

The ability of 3 EAA agonists glutamate, kainate and NMDA to evoke both exogenous (tritiated) and endogenous glycine release from cerebellar slice was investigated.

4.3.5.1: Exogenous.

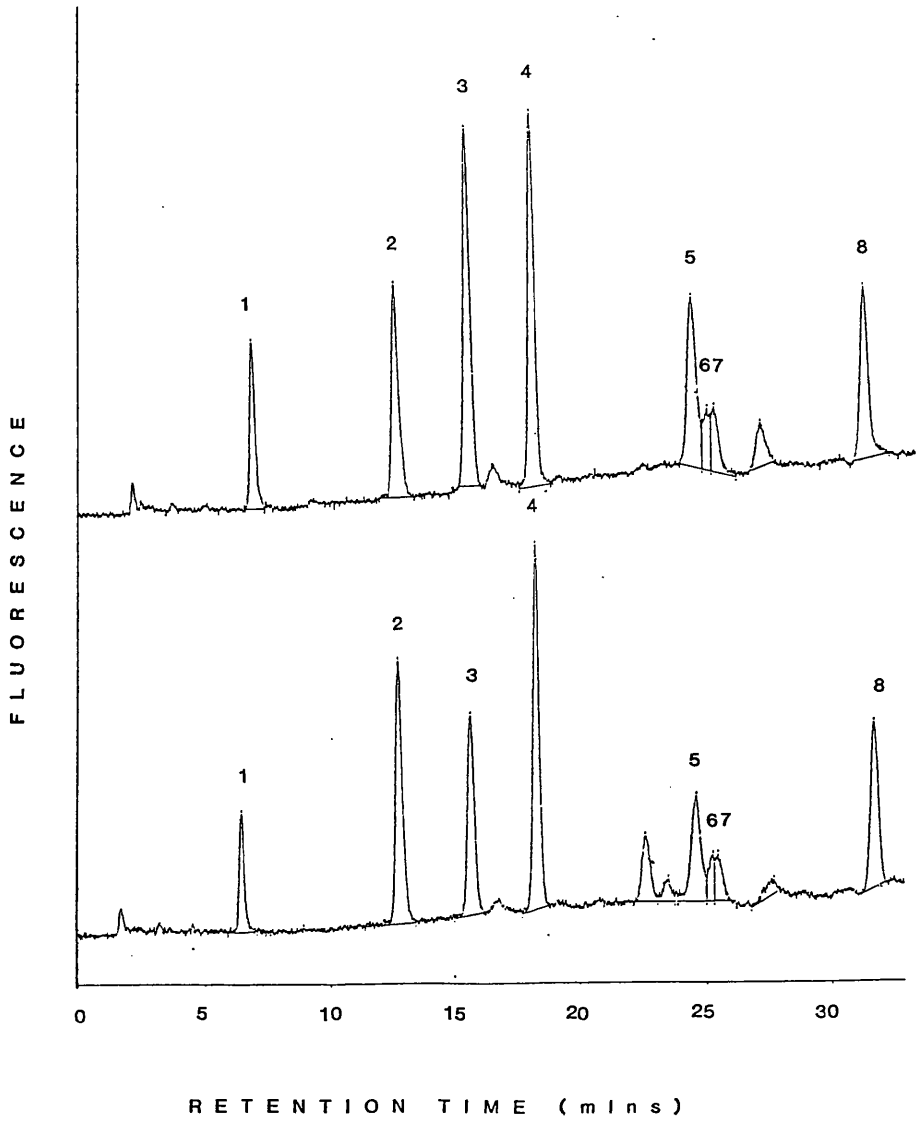
Glutamate and kainate (both 100μM) significantly stimulated the release of ^{3}H-glycine from cerebellar slices. Observed increases over basal of 292.6 ± 14.5%

Fig 4.8: Representative trace of potassium stimulated endogenous glycine release from 400µm Vibratome section of rat cerebellum.

Figure illustrates a sample trace of potassium stimulated release of endogenous glycine from 400µm Vibratome sections of rat cerebellum. The bottom trace represents the basal levels whilst the top trace represents stimulation in the presence of 55mM K^+ as described in Methods. The levels of all amino acids were analysed by means of a computer assisted, fluorescence-detected High Performance Liquid Chromatography following precolumn derivatisation by o-pthaldehyde thiol reagent. Separation was obtained with a gradient elution (sodium phosphate and methanol buffers). Peak determination and quantification was obtained by reference to known amino acid standards.

Numbers represent amino acids below. It must be noted that the author played no part in the HPLC or amino acid analysis. The specialised nature of this technique required all work to be undertaken by experienced graduates, to whom the author is extremely grateful.

1: Aspartate	2: Glutamate	3: Serine	4: Glutamine
5: Glycine	6: Threonine	7: Arginine	8: Taurine


FLUORESCENCE
1
2
3
4
5
67
8
4
1
2
3
5
67
8
0
5
10
15
20
25
30
RETENTION TIME (mins)

and 208.0 ± 12.8% respectively. Raising the concentration to 1mM produced a further significant increase in both cases (glutamate : 451.5 ± 12.3%, Kainate : 237.0 ± 17.7%). NMDA significantly stimulated release at both 100μM (124.5 ± 6.5%) and 1mM (146.6 ± 6.7%) but the effects were of a lower magnitude to those observed with either glutamate or kainate. Graphical representation of these data is shown in Fig 4.9.

4.3.5.2: Endogenous.

In addition the ability of glutamate and NMDA to stimulate endogenous glycine release was investigated. NMDA significantly stimulated release in a dose related manner (100μM : 142.9 ± 14.1%, 1mM :181.5 ± 19.2%). A similar dose effect was observed with glutamate (232 ± 16.4% : 302 ± 12.4%). In the presence of Mg^{2+} no stimulation with NMDA was observed. Graphical representation of these data is shown in Fig 4.10.

Fig 4.9: Ability of three excitatory amino acid agonists to evoke the release of ^{3}H-glycine from Vibratome sections of rat cerebellum.

Slices were incubated with 100 nM ^{3}H-glycine for 15 mins, then superfused with Mg^{2+} free KH buffer containing 2.5 mM Ca^{2+} for 40 minutes to establish a basal level of release. Slices were depolarised over a 5 minute period with KH buffer (Mg^{2+} free) containing varying amounts of agonists. Peak ^{3}H-glycine release for each concentration of agonist is expressed as a % stimulation over basal release and are the means ± SEM from 3 experiments.

(□) = glutamate: (▨) = kainate: (⊠) = NMDA

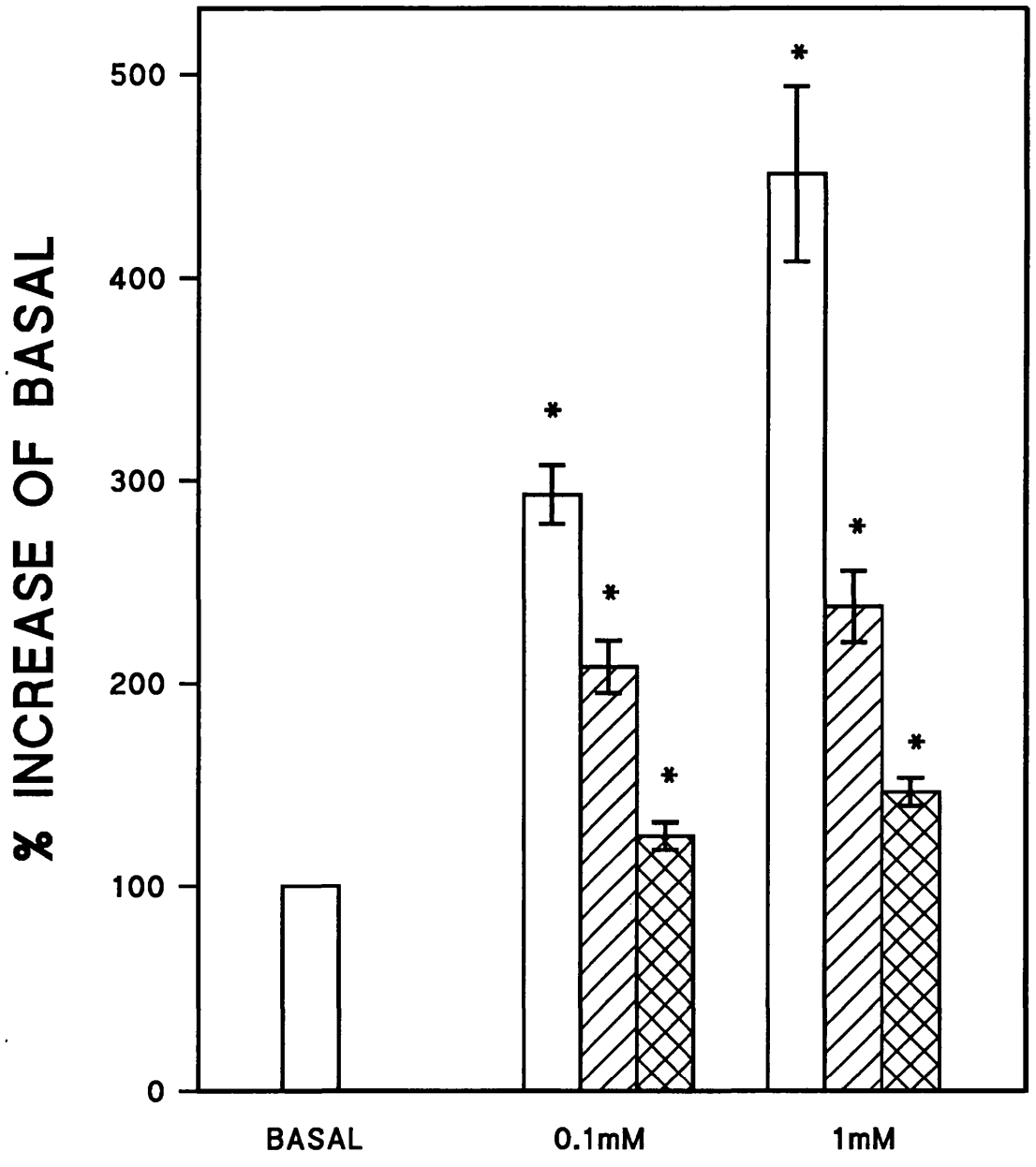
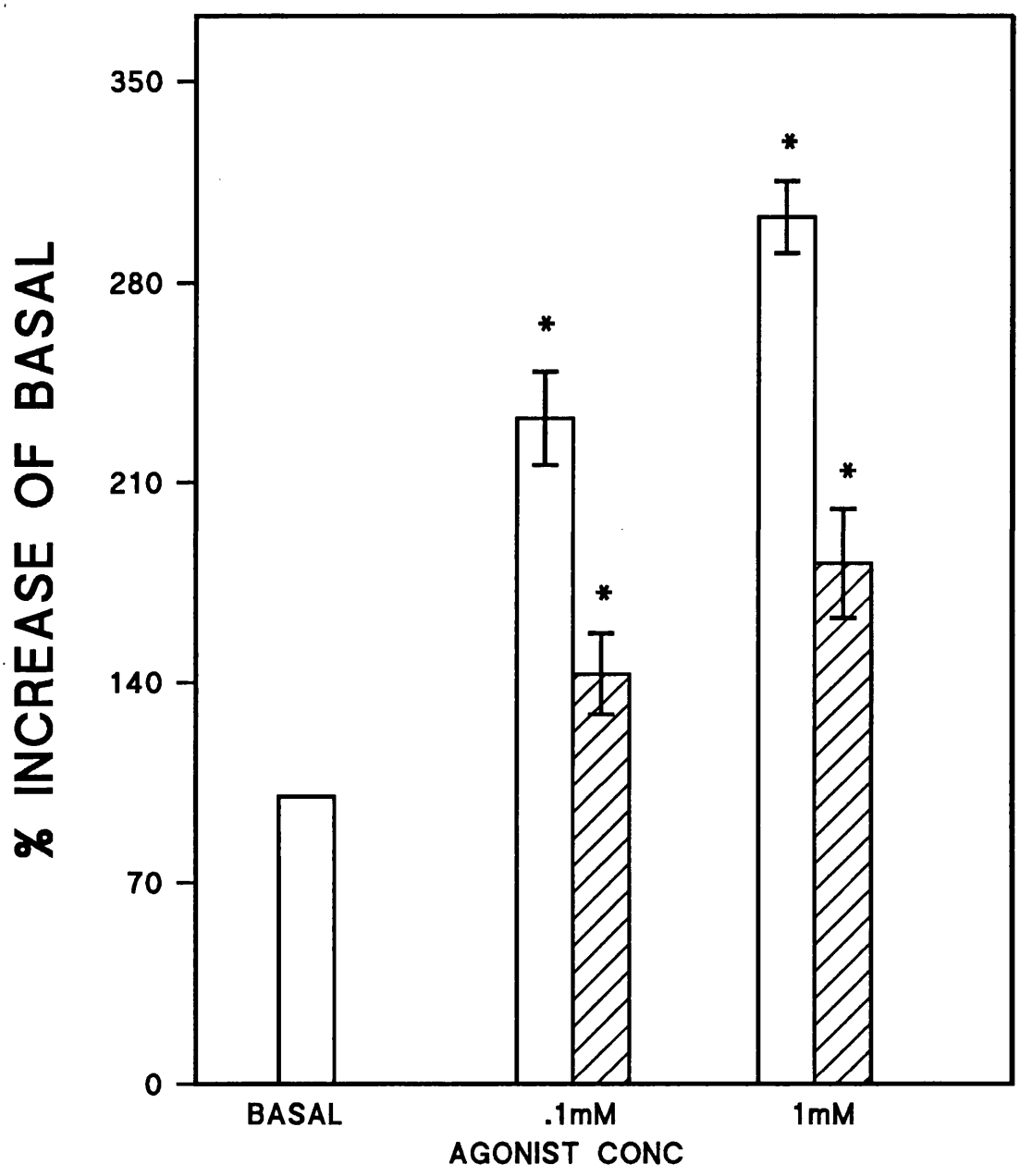

% INCREASE OF BASAL
500
400
300
200
100
0
*
*
*
*
*
*
BASAL
0.1mM
1mM

Fig 4.10: Ability of glutamate (□) and NMDA (◪) to evoke the release of endogenous glycine from Vibratome sections of rat cerebellum.

Slices were superfused with Mg^{2+} free KH buffer containing 2.5 mM Ca^{2+} for 40 minutes to establish a basal level of release. Slices were depolarised over a 5 minute period with KH buffer (Mg^{2+} free) containing varying amounts of agonists. Samples were analysed by means of HPLC. The area beneath each curve was analysed by a computerised routine, and the glycine release evoked for each concentration of agonists is expressed as a % stimulation over basal release. Values are the means ± SEM from 3 experiments.


% INCREASE OF BASAL
350
280
210
140
70
0
*
*
*
*
BASAL
.1mM
1mM
AGONIST CONC

4.4: Discussion.

The cerebellum has the ability to release glycine by means of a calcium dependent process. The findings in this chapter are in agreement with the previous observations in cerebellar glomerular particles (Morales and Tapia,1987). Similar observations have also been reported in cultured granule cells (Kingsbury *et al.*,1988; Holopainen and Kontro,1989). Accumulative evidence favours a neuronal basis for the release, although this remains to be established. By contrast glycine release from the hippocampus was clearly calcium independent. The latter may reflect a heterogeneous nature to glycine release in higher brain centres.

The dependency of the transmitter process on an influx of extracellular calcium into the presynaptic terminal is one of the major criteria to be fulfilled in transmitter release studies (Rubin,1970). The calcium dependent nature of exogenous glycine release from cerebellar slices is illustrated in Fig 4.5. As mentioned in the introduction to this chapter, there is a large body of evidence which suggests that demonstration of a calcium dependency reflects a neuronal involvement (Llinas and Heuser,1977). Our current understanding of transmitter release implies that the influx of extracellular calcium, through specific channels, leads to release of the transmitter substance from neuronal vesicular pools. Release from these pools has been reported to be maximal at calcium concentrations of between 2 - 3 mM (Levi *et al.*,1973; Morales and Tapia,1987). In keeping with this concept, the use of 2.5 mM calcium in this study may reflect release from presynaptic vesicular pools, however a number

of other points must be considered.

The presence of specific presynaptic neuronal pools highlights one of the major problems with the use of radiolabelled amino acid in release studies. Considering the widespread metabolic role of glycine, it is important to determine whether the exogenous amino acid enters the same pool as its endogenous counterpart. To this end, the calcium dependent nature of endogenous glycine release goes some way toward validating the exogenous data. Moreover the dose dependent nature of the exogenous and endogenous release (Table 4.2) suggest that in this particular model, exogenous release may indeed provide an accurate reflection of the endogenous situation.

The ability of elevated extracellular levels of potassium to release transmitter substances was first demonstrated by Mann *et al.,(*1939). Since then this procedure has been extensively used to induce putative transmitter release from a variety of preparations (Fagg and Lane,1979; Kingsbury *et al.,*1988; Morales and Tapia,1987). Due to its wide acceptance as a reliable and convenient means of stimulation, this method was adopted throughout this study. It has been suggested that the use of elevated potassium to cause depolarisation does not reflect the situation *in vivo* as well as that produced by electrical stimulation (Orrego,1979). Therefore a comparison of the two techniques is required but these experiments remain to be performed. Recent evidence indicates, however that both veratridine and potassium induce depolarisation of cultured astrocytes (Enkvist *et al.,*1988). The effectiveness of

veratridine in releasing transmitter substances agrees with its ability to collapse both electrical and concentrative components of the sodium electrochemical potential across the plasma membrane (Akerman and Nicholls, 1981), thus allowing a reversal of the uptake process. It was originally planned to use this compound as a means of investigating the neuronal origin of the release. However, in light of this publication it was considered that little benefit could be gained from its use.

Astrocytes are known to possess functional sodium channels (Bowman *et al.*,1984) which exhibit a similar sensitivity to veratridine as those in neurons (Nowak *et al.*,1987). However relatively little is known about the mechanism of potassium stimulated release from astrocytes. Depolarisation of the astrocytic cell membrane by potassium (Enkvist *et al.*,1988) has been attributed to an increase in potassium influx through voltage dependent potassium channels (Nowak *et al.*,1987, Jalonen and Holopainen,1989). It has been suggested that the increased extracellular potassium raises intracellular calcium levels by means of either voltage independent cation channels or by a mechanism mediated by a second messenger (Penner *et al.*,1988), leading to the release of calcium from intracellular stores. The involvement of the intracellular calcium stores may provide an explanation as to the observed calcium independent nature of neurotransmitter release (Terrian *et al.*,1988). The involvement of glial release may provide an explanation for the apparent variation in the release mechanism in the cerebellum and hippocampus.

Throughout investigations into the uptake and release of neurotransmitters, careful consideration must be given to the role of glial cells. To this end attempts were made to minimise the involvement of glial cells in this study. The ability of glial cells to transport glycine via specific carriers was first demonstrated by Wilkin and colleagues (1981). The authors reported that following a 15 min incubation, glial localisation of ^{3}H-glycine represented only 20% of that in neurons. Moreover an increased glial localisation was observed in experiments using longer incubations (G.P Wilkin, Personal communication). These findings are consistent with the differential rates of glycine uptake in cultured cells and astrocytes (Holopainen and Kontro,1989).

The concept of preferential uptake by different populations may provide an explanation as to the apparent non-linear nature of the accumulation curve (Fig 4.4). The apparent decrease in uptake observed at time points greater than 30 minutes, may reflect the increased involvement of glial uptake. Alternatively it may represent a saturation of the neuronal uptake mechanism. Table 4.2 displays data from experiments using cerebellar slices preloaded for 15 and 40 mins. The degree of stimulation was comparable at either time point, however a greater percentage of the accumulated radiolabel was released from slices loaded for 15 minutes. An observation consistent with the possible release of preferentially loaded radiolabel from a neuronal pool. An attempt to demonstrate this preferential release autoradiographically proved unsuccessful and it remains to be proved if such a system exists in the cerebellum.

As mentioned previously, the release of glycine from hippocampal slices displayed a calcium independent characteristic (Fig 4.7). Is it possible that the apparent variation in calcium dependency reflects a neuronal based release mechanism in the cerebellum whilst hippocampal release is glial based ? Evidence to support this concept comes from recent immunocytochemical studies (Storm-Mathisen,1990) which localised 90% of the GLY-LI in hippocampal glial cells. Similar immunocytochemical studies in the cerebellum isolated the Golgi axon terminals as the prime source of glycine (Ottersen *et al.*,1988). Furthermore this same group have recently demonstrated a calcium dependent depletion of GLY-LI from the Golgi axon terminals following potassium stimulation (Ottersen *et al.*,1990).

The role of glycine as a modulator of NMDA receptor function is well established (Chapter 1). Furthermore it has been suggested that glycine is a prerequisite for NMDA receptor activation (Kleckner and Dingledine,1988). Thus, the need for a neuronal release mechanism in the cerebellum may reflect the availability of the endogenous amino acid in this region. Evidence exists to suggest that the resting levels of endogenous glycine in higher brain centres are sufficient to saturate the strychnine-insensitive glycine receptor (Fletcher *et al.*,1989: 1990; Ascher *et al.*,1989; Kemp *et al.*,1988). In addition a glycine concentrations in excess of 1μM has been reported in the CSF (Ferraro and Hare,1985; Skilling *et al.*,1988). The recent demonstration of an *in vivo* glycine potentiation of NMDA receptors in rat thalamus (Salt,1989) and neocortex (Thomson *et al.*,1989) suggest, however, that regional variations in endogenous

glycine may be of functional significance.

The levels of endogenous glycine in the cerebellum have been reported to be lower than elsewhere in the brain (Morales and Tapia,1987). Furthermore electrophysiological studies of NMDA currents in cerebellar granule cells demonstrated that the level of endogenous glycine in the synaptic cleft was insufficient to allow NMDA receptor activation (D'Angelo *et al.*,1990). In contrast, the activities of two glycine precursor enzymes, serine hydroxymethyl transferase and glycine transaminase in the cerebellum compare favourably with areas where glycine is known to act as a neurotransmitter (Aprison and Nadi, 1978). These observation suggest that although the cerebellum displays the ability to synthesise glycine, a specific regulatory mechanism may control the release of this amino acid. Moreover the need for such a mechanism has been recently suggested by D'Angelo *et al.*,(1990).

A number of questions remain unanswered. Firstly, where is this mechanism based? Secondly, is the presence of such a process consistent with our current understanding of cerebellar synaptic processes, and finally what are the relevant sites of action?

As outlined in the introduction to this chapter, immunocytochemical (Ottersen *et al.*,1988) and autoradiographical (Wilkin *et al.*,1981) evidence implicate the Golgi axon terminals as the favoured site for this mechanism. However the concept is confused by the classical inhibitory role of these neurons (Eccles *et*

al.,1967). Is it possible that an inhibitory neuron could release an amino acid required for an excitatory function? The following is a hypothetical model which attempt to address this question.

The synaptic circuitry of the cerebellum is shown on page 174. The granule cells receive a simple monosynaptic glutamatergic input from the mossy fibres. In addition, a direct mossy fibre input onto the Golgi cell bodies has been described (Hamori and Szentagothai,1966). This latter connection occurs through the axosomatic synapses *en marron* (Chan-Palay and Palay,1974). These synapses may play a key role in coordinating the release of glycine from the Golgi cells in parallel to granule cell depolarisation. Excitatory volleys from the mossy fibre impose simultaneously on both the granule cell dendrites and courtesy of the *en marron* synapse on the Golgi cell bodies . Electrophysiological characterisation of this input (Eccles *et al.*,1966) revealed that the mossy fibre stimulation evokes a small diphasic wave followed by a second negative wave, approx .4 ms later. This sequence of field potential has been attributed to the incoming mossy fibre and the subsequent excitatory postsynaptic current in the granule and Golgi cells. Moreover the second negative wave has been demonstrated to possess a superimposed double spike potential, which in turn has been attributed to the results of impulses discharged first by Golgi cells and then , approx .5ms later by granule cells. This early firing of the Golgi cells may reflect the involvement of the extensive *en marron* synapses. It is possible that the premature discharge of the Golgi cells functions to release glycine and possibly GABA from the Golgi cell terminals. The release of glycine increases

the concentration in the synaptic cleft sufficiently to allow glutamate activation of NMDA receptors following granule cell depolarisation.

The above model assumes the presence of relevant receptors in the region. The most probable site of action for the released glycine is the strychnine insensitive glycine binding site associated with the NMDA complex (Chapter 1; Bristow *et al.*,1986). Previous autoradiographical investigation of the classical inhibitory glycine receptor (Zarbin *et al.*,1981) localised these sites in the molecular layer of the cerebellum. In contrast the strychnine-insensitive glycine receptor is concentrated in the internal granule layer (Fig 4.2, Chapter 2; Bristow *et al.*,1986), an area consistent with the presence of both the Golgi axon terminals and granule cell dendrites. A pharmacological characterisation of glycine binding in the cerebellum is shown in Fig 4.2 and Table 4.1. The values obtained for B_{max} and K_D are consistent with those in other brain regions (Chapter 2) and those reported in other preparations (Kishimoto *et al.*,1981; MacDonald *et al.*,1990). In addition the potency and selectivity of the agonist and antagonists tested were in agreement with the presence of a strychnine-insensitive site (Bristow *et al.*,1986; MacDonald *et al.*,1990). The functional significance of the NMDA receptor in the cerebellum has been questioned following reports of low affinity binding for glutamate (Monaghan *et al.*,1988), TCP (Maragos *et al.*,1988) and MK-801 (Bowery *et al.*,1988). However the relevance of these observations remains to be determined, especially following the electrophysiological demonstrations of functional receptors in the cerebellum (D'Angelo *et al.*,1990).

A major issue that remains unresolved is the involvement of GABA? The recent investigation of GLY-LI depletion following potassium stimulation also revealed a calcium dependent depletion of GABA-LI from the same terminals (Ottersen *et al.*,1990). This observation is consistent to the calcium dependent release of glycine and GABA from glomeruli particles (Morales and Tapia,1987) and in keeping with the idea that glycine and GABA were transported into the same neuronal population (Wilkin *et al.*,1981; Ottersen *et al.*,1988). The concept of co-localisation is not uncommon. A variety of neuronal populations have been demonstrated to colocalise and release neuropeptides and other transmitters (Hokfelt,1987). More recently a co-release of glutamate and taurine has been described in cultured cerebellar granule cells (Holopainen *et al.*, 1989). However the co-release of an inhibitory and excitatory amino acid from the same neuronal population is at the least paradoxical.

There is little doubt that the Golgi cell terminals are the source of the released amino acids, however questions as to the relevance of the co-release and the circumstances under which release occurs remain the subject of speculation. Reported observations (Morales and Tapia,1987) of a differential rate of release for glycine and GABA, permits speculation that the rapid release of glycine from Golgi cell terminals allows an initial activation of the NMDA receptor, whilst the slower co-release of GABA provides an inhibitory mechanism to close down the excited circuit. Whether the differential rate of release reported accurately reflects the situation *in vivo* remains to be seen, however this rather simplistic approach appears unlikely. Other aspects which

warrant investigation include the quantification of the co-release and a comparison of the receptor affinities for the two amino acids.

5.2: Agonist evoked release.

The use of EAA receptor agonists to induce depolarisation and subsequent transmitter release has been adopted in a number of studies (Gallo *et al.*,1987; 1990; Young *et al.*,1988). The effects are generally thought to be mediated via specific receptors, as opposed to interfering with the membrane carrier or the inhibition of a membrane bound protease (Zaczek *et al.*,1987; Robinson *et al.*,1986). The results shown in Fig 4.9 and 4.10 represent a preliminary study into the ability of agonist evoked glycine release. Time prevented a more in depth investigation however a number of points warrant consideration.

The ability of kainate, glutamate and NMDA to stimulate glycine release is consistent with the previous reports of a receptor mediated effect in the cerebellum (Holopainen and Kontro,1989). Non-NMDA receptors have also been localised on cerebellar astrocyte membranes (Levi and Gallo,1986) however to date no evidence to suggest the presence of NMDA receptors on these membranes.

The observed rank order of $\left\{\begin{array}{l}\text{effectiveness}\\ \downarrow\end{array}\right.$ (glutamate > kainate > NMDA) of release can be explained, in part, by the receptor specificity of each of the agonists. The stimulation by glutamate is consistent with the role as a mixed agonist (Watkins,1981) and its ability to mediate effects through any of the EAA

receptors. The ability of kainate to stimulate glycine release has been reported in cultured cerebellar granule cells (Holopainen and Kontro,1989). The reported stimulation was of a greater magnitude to that observed in this investigation, possibly reflecting the increased purity of a cultured system ? Moreover kainate hasan affinity for non-NMDA receptors (both kainate and AMPA) (Honore *et al.*,1982) , however it was not possible to determine the role of each receptor in this preparation.

One additional factor to consider when interpreting the results for both glutamate and kainate is the ability of these agonists to stimulate astrocytic glycine release (Holopainen and Kontro,1989). Although, as previously described, attempts were made to minimise the involvement of astrocytes, it is probable that a degree of the glutamate and kainate stimulation can be attributed to these cells.

The decreased stimulation of NMDA may reflect the relative receptor densities in the cerebellum. NMDA receptor density is lower in this region than in other parts of the brain (Monaghan *et al.*,1988; Monaghan and Cotman,1985) and in addition, it has been suggested that these receptors display a lower affinity to those elsewhere (Monaghan *et al.*,1988). The requirement of a Mg^{2+}free environment to observe an NMDA response is in line with the inhibitory role of this cation (Mayer and Westbrook,1987).

In relation to the hypothetical model described earlier, little is known about the receptors involved in the *en marron* synapse. The presence of non-NMDA receptors has been implicated (Garthwaite,1989), but whether NMDA receptors also have a functional significance remains unresolved.

It is clear that the role of glycine in the cerebellum is rather complex. Whilst the results in this chapter may provide a small insight into one possible role further evidence is required before any firm conclusions may be drawn.

CHAPTER 5 - FUTURE DIRECTIONS.

Chapter 5.

5.1: Future directions.

The results of the studies presented in this thesis have opened a number of possible avenues for future research. These data, in general, provide evidence to demonstrate the use of autoradiography as an alternative means of evaluating receptor interaction in discrete brain regions. In addition, the use of an autoradiographical technique, in tandem with specific lesion studies, permits the visualisation of changes in receptor density which may accompany periods of altered neurological activity.

It has previously been suggested that the binding of ^{3}H-MK-801 may provide an suitable marker for neuronal degeneration following periods of ischaemic damage (Gill *et al.*,1988; Bowery *et al.*,1988). Although evidence exists to demonstrate the alterations in ^{3}H-MK-801 binding following a period of ischaemia (Bowery *et al.*,1988) little is known about the other sites on the NMDA receptor complex. The autoradiographical procedure for the binding of ^{3}H-glycine to the strychnine-insensitive receptor (Chapter 2), may provide an alternative means of investigating the role of NMDA receptors in excitotoxic mechanisms within the CNS. Furthermore such a system may form the basis for a functional assay designed to test the therapeutic potential of selective agents at this site. The clinical profile of drugs acting at the glycine site may prove to be different from those of previously described NMDA antagonists (competitive and noncompetitive). Thus this site might provide an alternative therapeutic site of action in a variety of disease states.

The glycine / glutamate potentiation of ^{3}H-MK-801 binding (Chapter 3) occurs via an increase in the accessibility of the receptor to any selective ligand (Ransom and Stec,1988; Kloog *et al.*,1988; Javitt and Zukin,1988). This in turn may be manifested as an alteration in either the B_{max} or Kd (Snell *et al.*,1987; Wong *et al.*,1987). Thus an evaluation of the pharmacological changes which occur under the non equilibrium conditions employed in this assay may provide an alternative means of visualizing regional variations that may exist in the potentiating effects of the two agonists.

The autoradiographical demonstration of agonist and antagonist modulation of ^{3}H-MK-801 binding (Chapter 3) may prove a useful tool in evaluating such interactions in other brain regions. It remains to be seen whether the regional uniformity observed is consistent for other regions of the CNS. Two regions in particular which warrant investigation are the striatum and cerebellum. Glycine has been reported to potentiate NMDA receptor mediated quinolinic acid neurotoxicity in striatal neurones (Zhu *et al.*,1989). Furthermore reports exist to demonstrate an NMDA receptor modulation of the mesostriatal dopaminergic pathway (Carter *et al.*,1988). Thus it would be interesting to evaluate the modulation of binding in this region. It is further envisaged to extend the study to encompass the cerebellum. The low binding densities observed (Chapter 3) prevented an accurate determination of the agonists or antagonists interaction in this region. However the lack of information as to the binding of ^{3}H-MK801 in this region suggest that some benefit would be gained through optimisation of the existing assay to allow possible

visualisation of any receptor binding in this region. The need for a more indepth study is of particular relevance in light of the data presented in Chapter 4.

The Ca^{2+}-dependent nature of glycine (endogenous and exogenous) release from cerebellar slices (Chapter 4) suggests the presence of a neuronal release mechanism. However whether high K^+ stimulated release provides an accurate reflection of the *in vivo* situation remains to be tested. The use of electrically stimulated release may provide a means of evaluating this hypothesis. The latter has been shown to reflect closely the *in vivo* situation (Orrego,1979) thus a comparison of the release evoked by both systems may permit further speculation as to the possible mechanism of release present in this region.

The use of a high resolution coverslip autoradiographical technique may allow further investigation as to the possible source of the released glycine. A quantitative analysis of grain distribution of pre and post stimulated slices may provide some indication of the specific cell population involved.

The data obtained from the cerebellum has furthered the need to extend the comparison to encompass other brain regions. The release of ^{3}H-glycine from the hippocampal formation is described in Chapter 4, however an extension of this study to include an analysis of endogenous and electrical stimulated release is envisaged. Such data may permit a more detailed comparison of the mechanisms present in both regions.

Further experimentation is required to establish whether differences exist in the criteria for the release of glycine and GABA in the cerebellum. The use of dual labelled release studies may allow a comparison of the release characteristics of these two amino acids in the cerebellum. In addition a quantitative analysis of release (endogenous), together with a pharmacological analysis of the relevant receptor affinities and distribution, may provide an insight into the apparent need for excitatory and inhibitory amino acid co-release.

The preliminary investigation of agonist stimulated release (Chapter 4) yielded sufficient data to suggest a potential use of this system as a means of evaluating EAA receptor mediated release in the cerebellum. It is clear, however, that substantial optimisation and characterisation of the system is required. Initial experiments based on evaluating the calcium dependent nature of the release should allow an estimation of the neuronal involvement in the release. Similarly, a comparison of the Mg^{2+} dependency (a non-competitive NMDA antagonist) should allow the separation of the NMDA and non-NMDA receptor mediated components of the release (of particular relevance to the mixed agonists eg: glutamate). In addition an extended use of the potent and selective antagonists for each receptor may allow the complicated nature of receptor mediated glycine release in the cerebellum to be unravelled.

5.2 cGMP.

The postsynaptic effects of several classes of neurotransmitters appear to involve the transduction of an extraneuronal chemical signal into the stimulation of intraneuronal biochemical mechanisms. This transduction involves specific enzymic cascades with the resultant production of an intracellular second messenger. The existence of such interactions provides an alternative means of investigating complex receptor modulation or interaction.

The ability of glutamate to evoke large increases in cGMP levels within the CNS has been known for several years (Ferrendelli *et al.*, 1974; Mao *et al.*, 1974). This response is particularly marked in the cerebellum (Ferrendelli, 1978), a region which displays the highest levels of cGMP in the CNS (Ferrendelli *et al.*, 1970). Recent evidence has suggested that the NMDA receptor mediated enhancement of cGMP in the cerebellum may involve a specific cell to cell interaction (Garthwaite and Garthwaite, 1987). Furthermore a role for nitric oxide (NO) or EDRF (Endothelium Derived Relaxing Factor) as a "messenger molecule" mediating glutamate synaptic actions upon cGMP has been suggested (Garthwaite *et al.*, 1988; Bredt and Snyder, 1989). The role of NO as a widespread transducing molecule that augments intercellular communication with the stimulation of guanylate cyclase has been reported in a variety of cell types (Moncada *et al.*, 1989). Moreover the NMDA receptor mediated enhancement of cGMP levels *in vitro* have been reported to be inhibited by NMMA (N-mono-methyl-L-arginine) a compound which blocks the synthesis of NO (Garthwaite *et al.*, 1988; Bredt and Snyder, 1989; Knowles *et al.*, 1989). This

inhibition was reported to be reversed by the addition of excess arginine (Bredt and Snyder,1989). Moreover an NMDA receptor enhancement of guanylate cyclase activity *in vivo* (Danysz *et al.*,1989) have also been reported. Evidence exists to demonstrate a Ca^{2+} dependent release of NO (Garthwaite *et al.*,1988) and a Mg^{2+} inhibition of the NMDA receptor mediated cGMP enhancement (Garthwaite,1982). These data are consistent with the involvement of the ion channel associated with the NMDA receptor complex (Introduction). Furthermore it has been suggested that the Ca^{2+} influx through NMDA linked ion channels may act as the trigger for the formation of NO (Garthwaite *et al.*,1988).

The discovery of an interaction between glutamate and cGMP has prompted questions as to the focus of the cyclic nucleotides which are regulated by glutamate receptors. The enzyme guanylate cyclase has been demonstrated, immunocytochemically, in nearly all cell types (Zwiller *et al.*,1981; Nakane *et al.*,1983; Arino *et al.*,1982). However recent biochemical (Garthwaite and Garthwaite,1987) and immunohistochemical (de Vente *et al.*,1989) studies have suggested a granule cell - glia interaction as the most likely mechanism.

The ability of glycine to potentiate the glutamate stimulation of cGMP levels in the cerebellum was first demonstrated by Ferendelli *et al.*,(1974). However the significance of these data has only become apparent in recent years. More recently glycine has been demonstrated to induce cGMP accumulation in the cerebellum *in vivo* (Dansyz *et al.*,1989). As previously mentioned the

interaction of NMDA receptors and the cGMP second messenger system provides an alternative means of investigating the modulation of the NMDA receptor complex. To this end the data presented in the remainder of this chapter describes a pilot study designed to investigate glycine / NMDA interactions through measurement of cGMP. The primary objective of this study was to develop an assay model, based on adult tissue, which may provide a suitable basis for the assessment of novel therapeutic compounds at the various sites associated with the NMDA receptor complex. Data derived from studies involving immature and adult animals together with results obtained from primary neuronal cultures.

The dose dependent glutamate stimulation of cGMP levels observed in slices of 8 day old rat (Fig 5.1) concurs with previous reports (Garthwaite and Balazs, 1978; Foster and Roberts,1980). In animals of this age it has been reported that the glutamate response is mediated solely through NMDA receptors, moreover a good correlation has been reported between the period of maximal elevation of cGMP levels (8-12 day) and the developmental changes in the cerebellum (Garthwaite and Balazs,1978). However the failure to observe a glycine potentiation of the glutamate response may be due to problems in the experimental design. A comparison of the absolute cGMP concentrations observed in this study reveals a lower level of cGMP in these slices compared with published values (Garthwaite,1982; Foster and Roberts,1980; Garthwaite and Balazs,1978) . One possible explanation for this discrepancy may lie with the methodology employed in the slice preparation. Thus it is possible that any

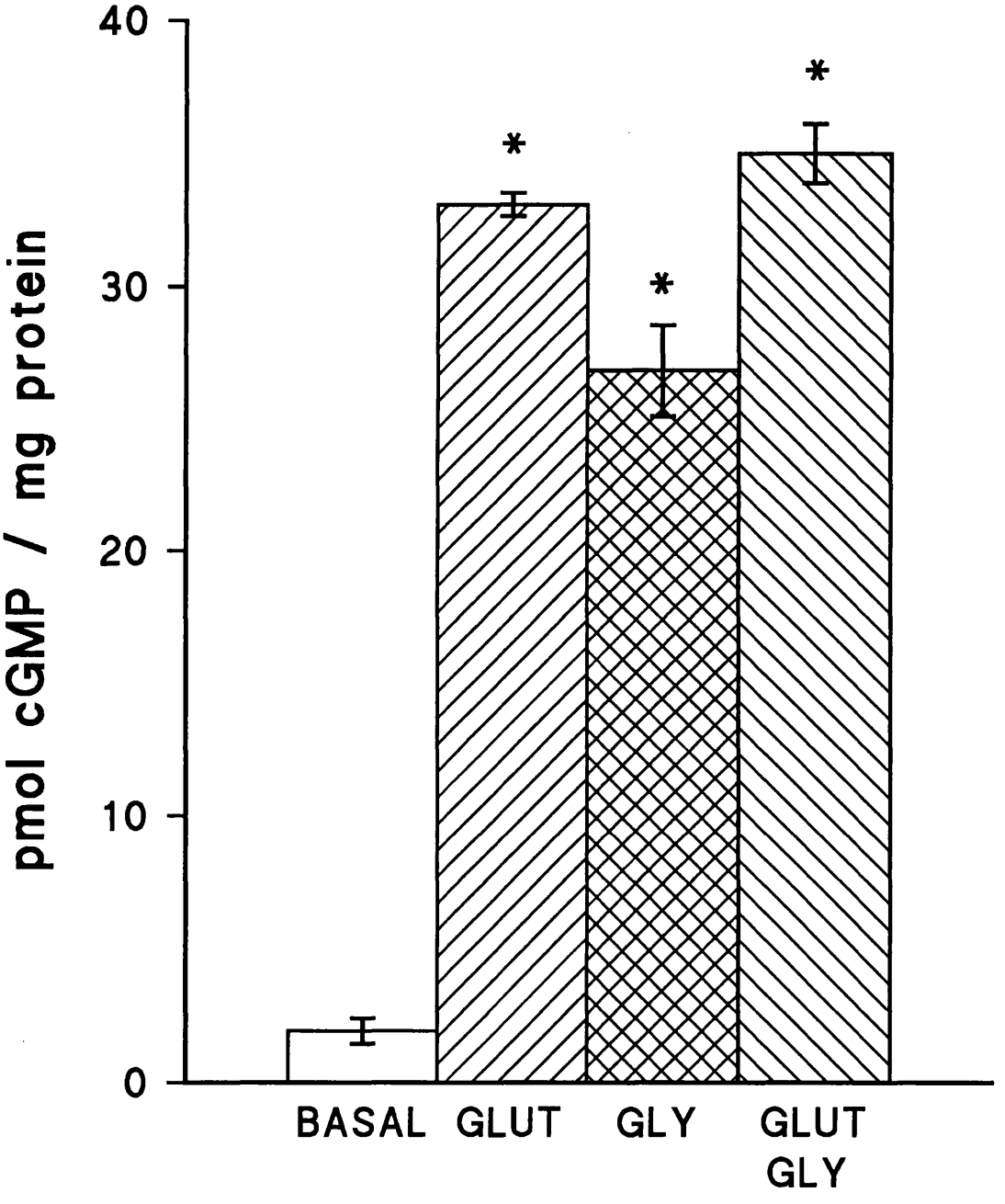
Figure 5.1. : The effect of glutamate and glycine on the levels of cGMP in slices of 8-day old rat.

Enhancement of basal (□) cGMP (pmoles/mg protein) in slices of 8-day rat cerebellum exposed to 3mM glutamate (▨), 10mM glycine (▩) and 3mM glutamate / 10mM glycine (▧). Each bar is the mean value obtained from 6-8 animals ± SEM. All values were compared to basal levels by means of Student t-test for unpaired data ($p<0.05$).

Tissue preparation.: Cross chopped slices (250 by 250µm) of 8-day Wistar rat cerebellum were prepared with a McIlwain tissue chopper and suspended in 50mls of Krebs-Henseleit solution, dispersed and made up to a final volume of 200mls. The slices were pre-incubated for 90 minutes at 37°C to allow cyclic GMP levels to equilibrate (buffer changed every 30 mins). Excess buffer was aspirated off to produce a tissue "slurry". 50µl aliquots of slices were transferred to small test tubes containing 400µl of Krebs buffer, and maintained at 37°C for 5 mins before addition of glutamate and glycine either alone or in combination. Amino acids were added in 50µl aliquots and the tissue incubated in a final volume of .5ml for 15 mins at 37°C before terminating the reaction in a boiling water bath (95°C for 3 mins). The samples were cooled and assayed for cGMP.

cGMP determination.: Cyclic GMP levels in the supernatent were measured using a saturation radioimmunoassay method of Brown *et al.*,(1971). 100µl of supernatent were transferred to eppindorf tubes containing 50µl of ^{3}H-cGMP (conc = 5nM, S.A. 15 Ci/mmol) and 50ul of antisera, vortexed and equilibrated for 120 mins at 4°C. Following incubation 1ml of ammonium sulphate (60% saturated) was added, tubes rapidly mixed and left for 10 mins at 4°C. Samples were centrifuged at 12,000g for 3 mins and the supernatent carefully aspirated off. The precipitate was resuspended in 100µl of distilled H_2O, and the amount of radiolabel present assayed by scintillation spectrometry. In each experiment a standard curve was established by the addition of 100µl of cGMP standards (conc range 5-80nM) dissolved in 50mM Tris buffer containing 4mM EDTA pH 7.4. Non-specific binding was determined by the addition of 100µl cGMP (10µM). Protein content of each sample estimated by method of Bradford.

Antisera.: γ-globulin fraction of rabbit serum: coded cG-c/2. Antisera was dissolved in .5ml of distilled H_2O and made up to 40 mls with 50mM Tris buffer (containing 4mM EDTA) following the addition of .3g of bovine γ-globulin.


40
30
20
10
0
pmol cGMP / mg protein
*
*
*
BASAL
GLUT
GLY
GLUT
GLY

glycine potentiation was masked by the poor quality of the tissue slices.

The problems associated with the production of viable tissue slices may, in part, provide an explanation for the poor responses observed in the adult rat or gerbil (Fig 5.2 and 5.3). It has previously been reported that the inability to observe large responses in the adult animal could be related to the poor slice preservation resulting from the use of an inappropriate technique (Garthwaite, 1982). Thus the poor responses observed from both species in this study concurs with this finding. It has been reported that the morphological preservation and cGMP responses in hand cut slices is superior (Garthwaite *et al.*,1979) however the use of such a technique in this study failed to provide any significant improvement. In addition evidence exists to demonstrate a decreased sensitivity to NMDA agonists in adult tissue when compared to that from immature animals. These factors may also warrant consideration.

The inconsistent data obtained with slice preparation prompted the search for an alternative basis for an assay. Fig 5.4 illustrates the glycine / NMDA enhancement of cGMP observed in cortical neuronal cultures. The glycine potentiation of the NMDA response is consistent with the modulatory role of this acid on the NMDA receptor complex (Johnson and Ascher,1987). Moreover these data do suggest a potential use of this assay model. It is envisaged that a further optimisation of the assay technique, together with the use of alternative culture systems (eg: cerebellum) will improve the magnitude of the response, thus permitting a further investigation of the receptor interaction

Fig 5.2.: Comparison of amino acid enhancement of cGMP levels in chopped and handcut slices of adult rat cerebellum.

The enhancement of basal (□) cGMP (pmoles/mg protein) in slices (chopped or handcut) of adult rat cerebellum exposed to 1mM glutamate (▨) and 10mM glycine (⊠). Each bar represents the mean value obtained from 6-8 animals ± SEM. Methodology as previously described for Fig 5.1.

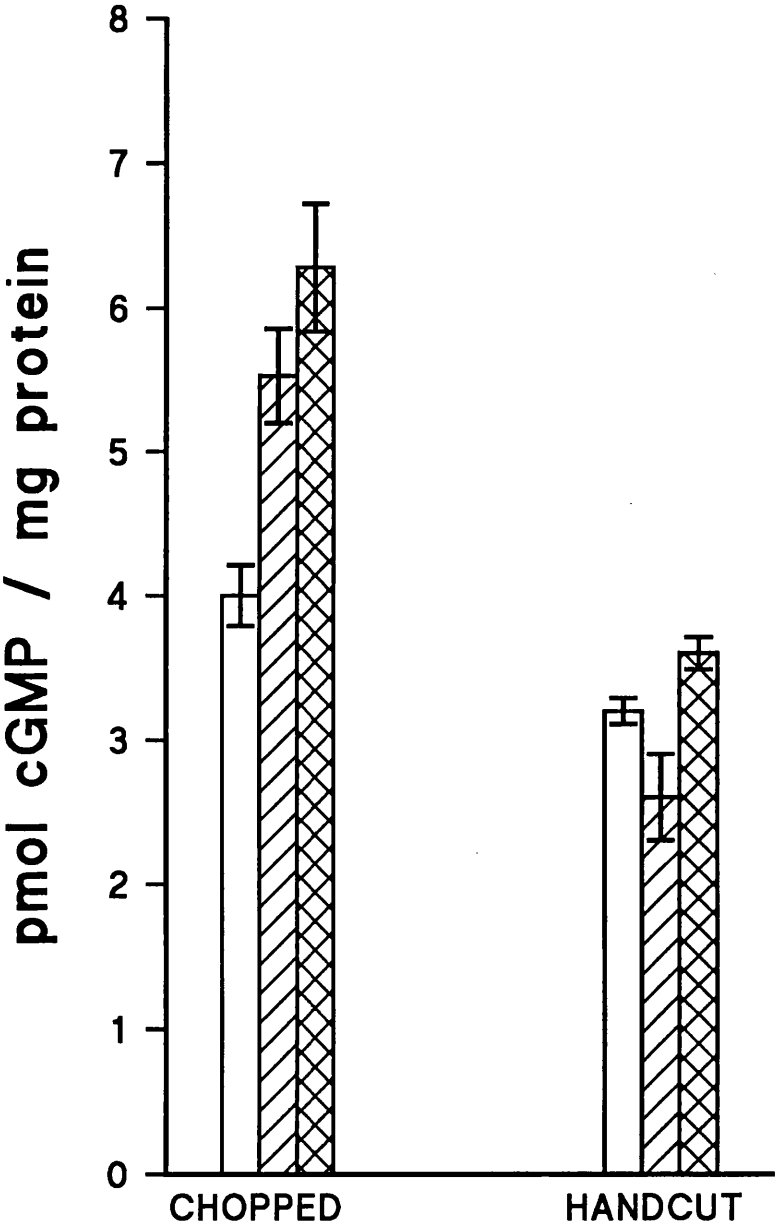

pmol cGMP / mg protein
8
7
6
5
4
3
2
1
0
CHOPPED
HANDCUT

Fig 5.3.: Comparison of amino acid enhancement of cGMP levels in chopped and handcut slices of adult gerbil cerebellum.

The enhancement of basal (□) cGMP (pmoles/mg protein) in slices (chopped or handcut) of adult gerbil cerebellum exposed to 1mM glutamate (▨), 10mM glycine (⊠) and 1mM glutamate/10mM glycine (▧). Each bar represents the mean value obtained from 6-8 animals ± SEM. Methodology as previously described for Fig 5.1.

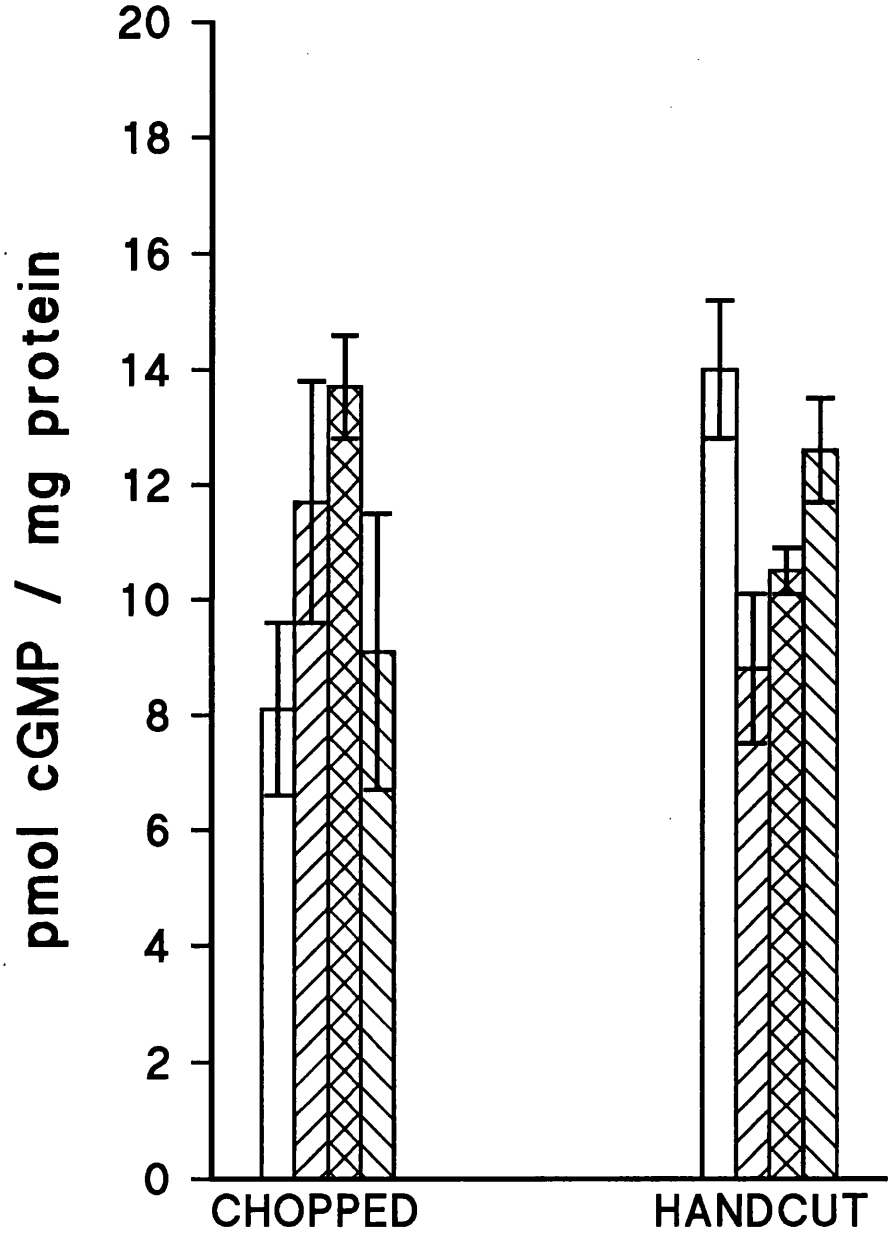
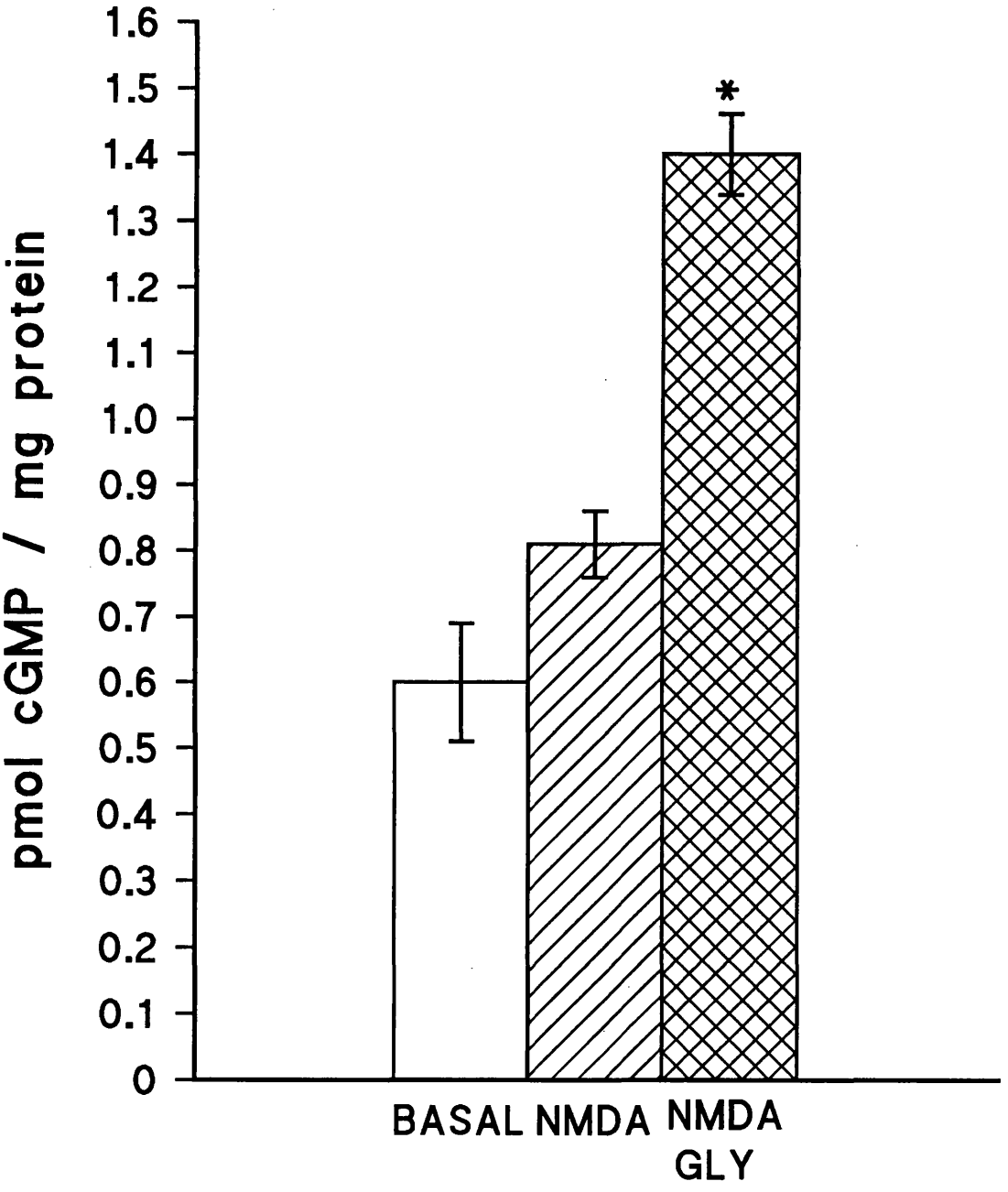

pmol cGMP / mg protein
20
18
16
14
12
10
8
6
4
2
0
CHOPPED
HANDCUT

Fig 5.4.: Enhancement of cGMP levels in rat cortical cell cultures by NMDA and glycine.

The enhancement of basal (□) cGMP (pmoles/mg protein) in cortical cell cultures exposed to 1mM NMDA (▨) and 1mM NMDA/100μM glycine (▩). Bar represent the mean of two separate determinations ± SEM.

Cortical cell cultures: Cortical cultures were provided by Miss C. Petty, Pharmacology department, School of Pharmacy. Dishes containing 9-day old cell cultures were washed twice with 1 ml of Mg^{2+}-free Locke's solution (154mM NaCl, 5mM, 5.6mM KCl, 3.6mM $NaHCO_3$, 2.3mM $CaCl_2$, 56mM D-glucose, 5mM Hepes, pH 7.4) prewarmed at 37°C and oxygenated with 95% CO_2 / 5% O_2. The dishes were preincubated in buffer for 5 mins at 37°C followed by a 1 min incubation with 1 ml of Mg^{2+} free Locke's solution containing an appropriate concentration of test drug. Cells were harvested in 0.5 ml of 0.4M perchloric acid at 4°C, and following neutralization with K_2CO_3 the cGMP levels in each sample was measured as previously described.


pmol cGMP / mg protein
1.6
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
BASAL
NMDA
NMDA
GLY

with the cGMP cascade. Unfortunately the availability of culture cells has limited progress such that the viability of the assay model remains to be tested.

CHAPTER 6 - REFERENCES.

Agrawal, S. G. and Evans, R. H. (1986). The primary afferent depolarizing action of kainate in the rat. *Br. J. Pharmacol.*, **87,** 345-355.

Aitkin, P. G., Balestrino, M. and Somjen, G. G. (1988). NMDA antagonists: lack of protective effect against hypoxic damage in CA1 region of hippocampal slices. *Neurosci. Lett.*, **89,** 187-192.

Akaike, N., Kawai, N. I., Kiskin, N. I., Kijuchko, E. M., Krishtal, O. A. and Tsyndrenko, A. (1987). Spider toxin blocks excitatory amino acid responses in isolated hippocampal pyramidal neurones. *Neurosci. Lett.*, **79,** 326-330.

Akerman, K. E. O. and Nicholls, D. G. (1981). Calcium transport by intact synaptosomes: the voltage dependent calcium channels and a re-evaluation of the role of sodium and calcium exchange. *Eur. J. Biochem.*, **117,** 491-497.

Alger, B. E. and Teyler, T. J. (1976). Long term and short term plasticity in the CA1, CA3 and dentate gyrus regions of the rat hippocampal slice. *Brain Res.*, **110,** 463-480.

Altshuler, R. A., Betz, H., Parakkal, M. H., Reeks, K. A. and Wenthold, R. J. (1986). Identification of glycinergic synapses in the cochlear nucleus through immunocytochemical localization of the post synaptic receptor. *Brain Res.*, **369,** 316-320.

Ambar, I., Kloog, Y. and Sokolovsky, M. (1988). Solubilization of rat brain phencyclidine receptors in an active binding form that is sensitive to N-methyl-D-aspartate ligands. *J. Neurochem.*, **51,** 133-140.

Andersen, P., Sundberg, S. H., Sveen, O., Swann, J. W. and Wigstrom, H. (1980). Possible mechanisms for long lasting potentiation of synaptic transmission in hippocampal slices from guinea pig. *J. Physiol.*, **302,** 463-482.

Anderson, W. W., Lewis, D.A., Swartzwelder, H. S. and Wilson, W. A. (1986). Magnesium free medium activates seizures-like events in the rat hippocampal slice. *Brain Res.*, **398,** 215-219.

Anderson, W. W., Swartzwelder, H. S. and Wilson, W. A. (1987). The NMDA receptor antagonist 2-amino-5-phosphovalerate blocks stimulus train induced epileptogenesis but not epileptiform bursting in rat hippocampal slice. *J. Neurophysiol.*, **57,** 1-21.

Andreasen, M., Lambert, J. D. C. and Jensen, M. S. (1989). Effects of new non-N-methyl-D-aspartate antagonists on synaptic transmission in the in vitro rat hippocampus. *J. Physiol.*, **414,** 317-336.

D'Angelo, E., Rossi, P. and Garthwaite, J. (1990). Dual-component NMDA currents at a single central synapse. *Nature,* **346,** 467-469.

Anson, J. and Collins, G. G. S. (1987). Possible presynaptic actions of 2-amino-4-phosphonobutyrate in rat olfactory cortex. *Br. J. Pharmacol.*, **91,** 753-761.

Aprison, M. H. (1990) The discovery of the neurotransmitter role of glycine. In "*Glycine Neurotransmission*", Ottersen, O. P. and Storm-Mathisen, J. (eds). John Wiley and Sons Ltd, New York, pp 1-25.

Aprison, M. H. and Daly, E. C. (1978). Biochemical aspects of transmission at inhibitory synapses; the role of glycine. In "*Advances in Neurochemistry*", Vol 3 Ch 5 Agranoff, B. W. and Aprison, M. H. (eds.), Plenum Press, New York, pp 203-294.

Aprison, M. H. and Nadi, N. S. (1978). Glycine : an inhibitor from the sacrum to the medulla. In "*Amino Acids as Chemical Transmitters*", Fonnum, F. (ed.), Plenum Press, New York, pp 531-570.

Aprison, M. H. and Werman, R. (1965). The distribution of glycine in cat spinal cord and roots. *Life Sci.,* **4,** 2075-2083.

Aram, J. A., Martin, D., Tomczyk, M., Zeman, S., Millar, J., Pohlar, G. and Lodge, D. (1989). Neocortical epileptogenesis in vitro: Studies with N-methyl-D-aspartate, phencyclidine, sigma and dextromethorphan receptor ligands. *J. Pharmacol. Exp. Ther.,* **248,** 320-328.

Arino, M. A., Lewicki, J. A., Brandwein, H. J. and Murad, F. (1982). Immunocytochemical localisation of guanylate cyclase within neurones of rat brain. *Proc. Natl. Acad. Sci.,* **79,** 1316-1320.

Ascher, P., Bregestovski, P. and Nowak, L. (1988). N-methyl-D-aspartate-activated channels of mouse central neurones in magnesium-free solutions. *J. Physiol. (Lond.),* **339,** 207-226.

Ascher, P., Henderson, G. and Johnson, J. W. (1989). Dual inhibitory actions of kynurenate on the NMDA-activated response of cultured mouse cortical neurones. *J. Physiol.,* **406,** 141P.

Ascher, P. and Johnson, J. W. (1989). The NMDA receptor, its channel and its modulation by glycine. In "*The NMDA Receptor*", Watkins, J. C. and Collingridge, G. L. (eds.), Oxford University Press, Oxford, pp 109-121.

Ascher, P. and Nowak, L. (1988a). The role of divalent cations in the N-methyl-D-aspartate responses of the mouse central neurones in culture. *J. Physiol. (Lond.),* **339,** 247-266.

Ascher, P. and Nowak, L. (1988b). Quisqualate and kainate-activated channels in mouse and central neurones in culture. *J. Physiol. (Lond.),* **399,** 227-246.

Ault, B., Evans, R. H., Francis, A. A., Oakes, D. J. and Watkins, L. C. (1980). Selective depression of excitatory amino acid induced depolarizations by magnesium ions in isolated spinal cord preparations. *J. Physiol.*, **307,** 413-428.

Baron, B. M., Stone, A. L., Siegal, B. W., Hurt, S.D., Dudley, M. W. and Palfreyman, M. G. (1990). NMDA receptor binding properties of 5,7-dichlorokynurenic acid, a radioligand glycine site antagonist. *Neurosci. Abstr.*, **16,** 430.12

Baudry, M., Evans, J. and Lynch, G. (1986). Excitatory amino acid inhibits stimulation of phosphatidylinositol metabolism by aminergic agonists in hippocampus. *Nature,* **319,** 329-331.

Beal, M. F., Kowall, N. W., Ellison, D. W., Mazurek, M. F. and Swartz, K. J. (1986). Replication of the neurochemical characteristics of Huntington's disease by quinolinic acid. *Nature*, **321**, 168-171.

Bear, M. F., Copper, L. N. and Ebner, F. E. (1987). A physiological basis for theory of synaptic modification. *Science,* **237,** 42-48.

Benavides, J., Rivy, J. P., Cater, C. and Scatton, B. (1988). Differential modulation of ^{3}H-TCP binding to the NMDA receptor by L-glutamate and glycine. *Eur. J. Pharmacol.,* **149,** 67-72.

Beneviste, H., Drejer, J., Schousbe, A. and Diemer, N. H. (1985). Elevation of the extracellular concentrations of glutamate and aspartate in rat hippocampus during transient cerebral ischaemia monitered by intracranial microdialysis. *J. Neurochem.,* **43,** 1369-1374.

Berridge, M. J. (1984). Inositol triphosphate and diacylglycerol as second messengers. *Biochem J.,* **228,** 345-360.

Bertolino, M., Vicini, S., Mazzetta, J. and Costa, E. (1988). Phencyclidine and glycine modulate NMDA-activated high conductance cationic channels by acting at different sites. *Neurosci. Lett.,* **84,** 351-355.

Betz, H. (1987). Biology and structure of the mammalian glycine receptor. *T. I. N. S.,* **10,** 113-117.

Betz, H. and Becker, C. M. (1988). The mammalian glycine receptor: Biology and structure of a neuronal chloride channel protein. *Neurochem. Int.,* **13,** 137-146.

Birch, P. J., Grossman, C. J. and Hayes, A. G. (1988a). 6,7-Dinitro-quinoxaline,2-3-dione and 6-nitro-7-cycno-quinoxaline-2,3-dione antagonise responses to NMDA in the rat spinal cord via action at the strychnine-insensitive glycine receptor. *Eur. J. Pharmacol.,* **156,** 177-18.

Birch, P. J., Grossman, C. J. and Hayes, A. G. (1988b). Kynurenic acid antagonists responses to NMDA via an action at the strychnine-insensitive glycine receptor. *Eur. J. Pharmacol.*, **154,** 85-87.

Biscoe, T. J., Davies, J., Dray, A., Evans, R. H., Francis, A. A., Martin, M. R. and Watkins, J. C. (1977). Depression of synaptic excitation and of amino acid induced excitatory responses of spinal neurones by D-α-aminoadipate, α,ε-diaminopimelic acid and HA-966. *Eur. J. Pharmacol.*, **45,** 315-316.

Biscoe, T. J., Evans, R. H., Headley, P M., Martin, M. R. and Watkins, J. C. (1976). Structure activity relationships of excitatory amino acids on frog and cat spinal neurones. *Br. J. Pharmacol.*, **58,** 373-382.

Bliss, T. V. P. and Gardner-Medwin, A. R. (1973). Long-lasting potentiation of synaptic transmission in the dentate area of the anaesthetised rabbit following stimulation of the perforant path. *J. Physiol.*, **232,** 331-356.

Bliss, T. V. P. and Lynch, M. A. (1988). Long term potentiation of synaptic transmission in the hippocampus: properties and mechanisms. *Neurol. Neurobiol.*, **35,** 3-72.

Boast, C. A., Gerhardt, S. C., Pastor, G., Lehmann, J., Etienne, P. E. and Liebman, J. M. (1988). The NMDA antagonists CGS 19755 and CPP reduce ischaemic brain damage in gerbils. *Brain Res.*, **442,** 345-348.

Bonhaus, D. W., Burge, B. C. and McNamara, J. O. (1987). Biochemical evidence that glycine allosterically regulates an NMDA receptor coupled ion channel. *Eur. J. Pharmacol.*, **142,** 489-490.

Bonhaus, D. W. and McNamara, J. O. (1988). NMDA receptor regulation of uncompetitive antagonist binding in rat membranes: kinetic analysis. *Mol. Pharmacol.*, **34,** 250-255.

Bonhaus, D. W., Yeh, G-C., Skaryak, L. and McNamara, J. O. (1989). Glycine regulation of NMDA receptor gated ion channel in hippocampal membranes. *Mol. Pharmacol.*, **36,** 273-279.

Bonta, I. L., De Vos, C. J., Grijsen, H., Hillen, E. L, Noach, E. L. and Sim, A. W. (1971). 1-hydroxy-3-amino-pyrollidone (HA-966). A new GABA-like compound with potential use in extrapyramidal diseases. *Br. J. Pharmacol.*, **43,** 514-535.

Bormann, J. (1989). Memantine is a potent blocker of NMDA receptor channels. *Eur. J. Pharmacol.*, **166,** 591-592.

Bosley, T. M., Woodhams, P. L., Gorden, R. D. and Balazs, R. (1983). Effect of anoxia on the stimulated release of amino acid transmitters in the cerebellum *in vitro*. *J. Neurochem.*, **40,** 189-201.

Bowery, N. G. (1987). Glycine binding sites and NMDA receptors in brain. *Nature,* **326,** 338.

Bowery, N. G., Hudson, A. L. and Price, G. W. (1987). GABAA and GABAB receptor site distribution in the rat central nervous system. *Neurosci.,* **20,** 365-383.

Bowery, N. G., Wong, E. H. F. and Hudson, A. L. (1988). Quantitative autoradiography of ^{3}H-MK-801 binding sites in mammalian brain. *Br. J. Pharmacol.,* **93,** 944-954.

Bowman, C. L., Kimelberg, H. K., Frangakis, M. V., Berwald-Netter, Y. and Edwards, C. (1984). Astrocytes in primary culture have chemically activated sodium channels. *J. Neurosci.,* **4,** 1527-1534.

Bredt, D. S. and Snyder, S. S. (1989). Nitric oxide mediates glutamate-linked enhancement of cGMP levels in the cerebellum. *Proc. Natl. Acad. Sci.,* **86,** 9030-9033.

Bridges, R. J., Hearn, T. J., Monaghan, D. T. and Cotman, C. W. (1986). A comparison of 2-amino-4-phosphonobutyric acid (AP4) receptors and [^{3}H]AP4 binding sites in the rat brain. *Brain Res.,* **375,** 204-209.

Bridges, R. J. Stevens, D. R., Kahle, J. S., Nunn, P. B., Kadri, M. and Cotman, C. W. (1989). Structure-function studies on N-oxayl-diamino-dicarboxylic acids and excitatory amino acid receptors: evidence that β-L-ODAP is a selective non-NMDA agonist. *J. Neurosci.,* **9,** 2073-2079.

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,** 33-307.

Burton, N. R., Smith, D. A. S. and Stone, T. W. (1988). A quantitative pharmacological analysis of some excitatory amino acid receptors in mouse neocortex in vivo. *Br. J., Pharmacol.,* **93,** 693-701.

Campochiaro, P. and Coyle, J. T. (1978). Ontogenic development of kainate neurotoxicity: correlates with glutamatergic innervation. *Proc. Natl. Acad. Sci.,* **75,** 2025-2029.

Carlsson, M. and Carlsson, A. (1989). The NMDA antagonist MK801 causes marked locomotor stimulation in monoamine depleted mice. *J. Neurol. Transm.,* **75,** 221-226.

Carter, C. J., L'Heureux, R. and Scatton, B. (1988). Differential control by N-methyl-D-aspartate and kainate of striatal dopamine release in vivo: a trans-striatal dialysis study. *J. Neurochem.*, **51,** 462-468.

Cha, J. H. J., Makowiec, R. L., Penney, J. B. and Young, A. B. (1990). L-3H-Glutamate labels the metabotropic excitatory amino acid receptor in rodent brain. *Neurosci. Lett.*, **113,** 78-83.

Chamberlin, N. L., Traub., R. D. and Dingledine, R. (1990). Role of EPSPs in initiation of spontaneous synchronized burst firing in rat hippocampal neurones bathed in high potassium. *J. Neurophysiol.*, **64,** 1000-1008.

Chan-Palay, V. and Palay, S. L. (1971). The synapse *en marron* between Golgi II neurons and mossy fiber in rat's cerebellar cortex. *Z. Anat. Entwickl. Gesch.*, **133,** 224-248.

Choi, D. W. (1987). Ionic dependence of glutamate toxicity. *J. Neurosci.*, **7,** 369-379.

Choi, D. W. (1988). Glutamate neurotoxicity and diseases of the nervous system. *Neuron,* **1,** 623-634.

Choi, D. W., Maulucci-Gedde, M. and Kriegstein, A. R. (1987). Glutamate neurotoxicity in cortical cell culture. *J. Neurosci.*, **7,** 357-368.

Charpak, S., Gahwiler, B. H., Do, K. Q. and Knopfel, T. (1990). Potassium conductances in hippocampal neurons blocked by excitatory amino acid transmitters. *Nature,* **347,** 765-767.

Clineschmidt, B. V., Martin, G. E., Bunting, P. R. and Papp, N. L. (1982). Anticonvulsant activity of MK 801, a substance with potent anticonvulsant, central sympathomimetic and apparent anxiolytic properties. *Drug Dev. Res.*, **2,** 123-134.

Coan, E. J. and Collingridge, G. L. (1985). Magnesium ions block an NMDA receptor mediated component of synaptic transmission in rat hippocampus. *Neurosci. Lett.*, **53,** 21-26.

Coan, E. J. and Collingridge, G. L. (1987a). Effects of phencyclidine, SKF 10,047 and related psychotomimetic agents on N-methyl-D-aspartate receptor mediated synaptic responses in rat hippocampal slices. *Br. J. Pharmacol.*, **91,** 547-556.

Coan, E. J. and Collingridge, G. L. (1987b). Characterization of an NMDA receptor component of synaptic transmission in rat hippocampal slices. *Neurosci.*, **22,** 1-8.

Coan, E. J., Saywood, W. and Collingridge, G. L. (1987). MK-801 blocks NMDA receptor mediated synaptic transmission and long term potentiation in rat hippocampal slices. *Neurosci. Lett.*, **80,** 111-114.

Collingridge, G. L. (1985). Long term potentiation in the hippocampus: mechanisms of initiation and modulation by neurotransmitters. *T. I. P. S.*, **6,** 407-411.

Collingridge, G. L. and Bliss, T. (1987). NMDA receptors -their role in long term potentiation. *T. I. N. S.*, **10,** 288-293.

Collingridge, G. L. and Davies, J. (1979). An evaluation of D-alpha-aminoadipate and D-(and DL-)alpha-aminosuberate as selective antagonists of excitatory amino acids in the substantia nigra and mesenchephalic reticular formation of the rat. *Neuropharmacol.*, **18,** 193-199.

Collingridge, G. L. and Davies, S. N. (1989). NMDA receptors and LTP in the hippocampus. In "*The NMDA Receptor*", Watkins, J. C. and Collingridge, G. L. (eds.), Oxford University Press, Oxford, pp 123-135.

Collingridge, G. L., Kehl, S. J. and McLennan, H. (1983). Excitatory amino acids in synaptic transmission in the Schaffer collateral-commissural pathway of the rat hippocampus. *J. Physiol.*, **334,** 33-46.

Collingridge, G. L. and Lester, R. A. J. (1989). Excitatory amino acid receptors in the vertebrate central nervous system. *Pharmacol. Reviews,* **40,** No.2, 145-210.

Collingridge, G. L., Reymann, K. G. and Davies, S. N. (1989). Involvement of protein kinase in two phases of LTP in rat hippocampal slices. *Soc. Neurosci. Abstr.*, **15,** 38.3.

Collingridge, G. L. and Singer, W. (1990). Excitatory amino acid receptors and synaptic plasticity. *T. I. P. S.*, **11,** 29-296.

Collins, G. G. S. (1982). Some effects of excitatory amino acid receptor antagonists on synaptic transmission in the rat olfactory cortex. *Brain Res.*, **244,** 311-318.

Collins, G. G. S., Anson, J. and Surtees, L. (1986). Presynaptic kainate and NMDA receptors regulate excitatory amino acid release in the olfactory cortex. *Brain Res.*, **265,** 157-159.

Contreras, P. C., Quirion, R. and O'Donohue, T. L. (1986). Autoradiographic distribution of phencyclidine receptors in the rat-brain using [^{3}H]1-(2-thienyl) cyclohexyl piperidine ([^{3}H]TCP). *Neurosci. Lett.*, **67,** 101-106.

Copani, A., Canonico, P. L. and Nicoletti, F. (1990). Beta-N-methylamino-L-

alanine (L-BMAA) is a potent agonist of metabotrophic glutamate receptors. *Eur. J. Pharmacol.,* **181,** 327-328.

Cotman, C. W., Flatman, J. A., Ganong, A. H. and Perkins, M. N. (1986). Effects of excitatory amino acid antagonists on evoked and spontaneous excitatory potentials in guinea-pig hippocampus. *J. Physiol.,* **378,** 403-415.

Cotman, C. W., Monaghan, D. T., Ottersen, O. P. and Storm-Mathiesen, J. (1987). Anatomical organisation of excitatory amino acids and their pathways. *T. I. N. S.,* **10,** 27-279.

Cowburn, R., Hardy, J., Roberts, P. and Briggs, R. (1988). Presynaptic and postsynaptic glutamergic function in Alzheimer's disease. *Neurosci. Lett.,* **86,** 109-113.

Crawford, M. and Roberts, P. J. (1989). 1-Hydroxy-3-aminopyrrolid-2-one (HA-966) and kynurenate antagonise N-methyl-D-aspartate induced enhancement of ^{3}H-dopamine release from striatal slices. *Biochem. Pharmacol.,* **38,** 4165-4168.

Crepal, V., Represa, A. and Ben-Ari, Y. (1988). Effect of ischemia and intra-amygdaloid kainate injection on the density of NMDA binding sites in the hippocampal CA1 region. *Eur. J. Pharmacol.,* **151,** 355-356.

Crooks, S. L., Robinson, M. B., Koerner, J. F. and Johnson, R. L. (1986). Cyclic analogues of 2-amino-4-phosphonobutanoic acid and their inhibition of hippocampal excitatory transmission and displacement of ^{3}H-APB binding. *J. Med. Chem.,* **29,** 1988-1995.

Cross, A. J., Slater, P., Simpson, M., Royston, C. and Deakin, J. F. W. (1987). Sodium dependent D-^{3}H-aspartate binding in cerebral cortex in patients with Alzheimer's and Parkinsons disorder. *Neurosci. Lett.,* **79,** 213-217.

Croucher, M. J., Collins, J. F. and Meldrum, B. S. (1982). Anticonvulsant actions of excitatory amino acid antagonists. *Science,* **216,** 899-901.

Cull-Candy, S. G., Howe, J. R. and Ogden, D. C. (1988). Noise and single channels activated by excitatory amino acids in rat cerebellar granule neurones. *J. Physiol.,* **400,** 189-222.

Cull-Candy, S. G. and Usowicz, M. M. (1987). Multiple-conductance channels activated by excitatory amino acids in cerebellar neurones. *Nature,* **325,** 525-528.

Curtis, D. R. (1963). The pharmacology of central and peripheral inhibition. *Pharmacol. Rev.,* **15,** 333-364.

Curtis, D. R. and Johnston, G. A. R. (1974). Amino acid transmitters in the

mammalian CNS. *Ergebn. Physiol.,* **69,** 94-188.

Curtis, D. R., Johnston, G. A. R., Game, C. J. A. and McCulloch, R. M. (1973). Antagonism of neuronal excitation by 1-hydroxy-3-amino-pyrrolidone-2. *Brain Res.,* **49,** 467-470.

Curtis, D. R., Phillis, J. W. and Watkins, J. C. (1960). The chemical excitation of spinal neurones by certain acidic amino acids. *J. Physiol.,* **150,** 656-682.

Curtis, D. R., Phillis, J. W. and Watkins, J. C. (1961). Actions of amino acids on the isolated hemisected spinal cord of the toad. *Br. J. Pharmacol.,* **16,** 262-283.

Curtis, D. R. and Watkins, J. C. (1963). Acidic amino acids with strong excitatory actions on mammalian neurones. *J. Physiol,* **166,** 1-14.

Daly, E. C. (1990). The biochemistry of glycinergic neurons. In "*Glycine Neurotransmission*", Ottersen, O. P. and Storm-Mathisen, J. (eds.), John Wiley and Sons, New York, pp 25-67.

Danysz, W., Fadda, E., Wroblewski, J. T. and Costa, E. (1989a). Different modes of action of 3-amino-1-hydroxy-2-pyrolidone (HA-996) and 7-chlorokynurenic acid in the modulation of N-methyl-D-aspartate sensitive glutamate receptors. *Mol. Pharmacol.,* **36,** 912-916.

Danysz, W., Fadda, E., Wroblewski, J. T. and Costa, E. (1990). ^{3}H-D-Serine labels strychnine-insensitive glycine recognition sites of rat central nervous system. *Life Sci.,* **46,** 155-164.

Danysz, W., Wroblewski, J. T., Brooker, G. and Costa, E. (1989b). Modulation of glutamate receptors by phencyclidine and glycine in the rat cerebellum: cGMP increase *in vivo*. *Brain Res.,* **479,** 270-276.

Davidoff, R. A., Shank, R. P., Graham, L. T., Aprison, M. H. and Werman, R. (1967). Is glycine a neurotransmitter? Association of glycine with spinal interneurones. *Nature,* **214,** 680-681.

Davies, C. H., Davies, S. N. and Collingridge, G. L. (1990). Paired-pulse depression of monosynaptic GABA-mediated inhibitory postsynaptic responses in rat hippocampus. *J. Physiol.,* **424,** 513-531.

Davies, J., Evans, R. H., Francis, A. A. and Watkins, J. C. (1979). Excitatory amino acid receptors and synaptic excitation in the mammalian central nervous system. *J. Physiol.,* **75,** 641-654.

Davies, J., Francis, A. A., Jones, A. W. and Watkins, J. C. (1981). 2-Amino-5-phosphonovalerate (2-APV), a potent and selective antagonist of amino acid-induced and synaptic excitation. *Neurosci. Lett.,* **21,** 77-81.

Davies, J. and Watkins, J. C. (1981). Differentiation of kainate and quisqualate receptors in the cat spinal cord by selective antagonism with g-D(and L) - glutamylglycine. *Brain Res.,* **206,** 172-177.

Davies, J. and Watkins, J. C. (1982). Action of D and L forms of 2-amino-5-phosphonovalerate and 2-amino-4 phosphonobutyrate in the cat spinal cord. *Brain Res.,* **235,** 378-386.

Davies, S. N., Lester, R. A. J. and Collingridge, G. L. (1988). Effects of pCB-PzDA and CNQX on synaptic responses in the rat hippocampal slice. *Neurosci. Lett.,* Suppl. **32,** S7.

Davies, S. N., Lester, R. A. J., Raymann, K. G. and Collingridge, G. (1989). Temporally distinct pre and post synaptic mechanisms maintain long term potentiation. *Nature,* **338,** 500-503.

Diamond, D. M., Dunwiddie, T. V. and Rose, G. M. (1988). Characterisation of hippocampal primed burst potentiation in vitro and in the awake rat. *J. Neurosci.,* **8,** 4079-4088.

Dingledine, R., McBain, C. J. and McNamara, J. O. (1990). Excitatory amino acid receptors in epilepsy. *T. I. P. S.,* **11,** 334-337.

Do, K. Q., Herrling, P. L., Streit, P., Turski, W. A. and Cuenod, M. (1986). In vitro release and electrophysiological effects in situ of homocysteic acid, an endogenous N-methyl-(D)-aspartic acid agonist, in the mammalian striatum. *J. Neurosci.,* **6,** 2226-2234.

Dragunow, M., Abraham, W. C., Marson, S. E. and Faull, R. L. M.(1988). LTP and c-fos is there a link? *Soc. Neurosci. Abstr.,* **14,** 565.

Drejer, J. and Honore, T. (1988). New quinoxalinediones show potent antagonism of quisqualate responses in cultured mouse cortical neurones. *Neurosci. Lett.,* **87,** 104-108.

Duchen, M. R., Burton, N. R. and Biscoe, T. J. (1985). An intracellular study of the interactions of N-methyl-DL-aspartate with ketamine in the mouse hippocampal slice. *Brain Res.,* **342,** 149-153.

Duggan, A. W. (1974). The differential sensitivity to L-glutamate and L-aspartate of spinal interneurones and Renshaw cells. *Exp. Brain Res.,* **19,** 522-528.

Eccles, J. C., Ito, M. and Szentagothai, J. (1967). *"The Cerebellum as a Neuronal Machine"*, Springer-Verlag, New York.

Eccles, J. C., Llinas, R. and Sasaki, K. (1966). The mossy fiber-granule cell relay in the cerebellum and its inhibition by Golgi cell. *Exp. Brain Res.*, **1,** 82-101.

Enkvist, M. O. E., Holopainen, I. and Akerman, K. E. O. (1988). The effect of potassium and glutamate on the membrane potential of suspensions of primary cultures of rat astrocytes as measured with a cyanine dye, DiS-C2-(5). *Brain Res.*, **462,** 67-75.

Errington, M. L., Lynch, M. A. and Bliss, T. V. P. (1987). Long term potentiation in the dentate gyrus: induction and increased glutamate release are blocked by D9-aminophosphonovalerate. *Neurosci.*, **20,** 279-284.

Evans, R. H., Francis, A. A., Jones, A. W., Smith, D. A. S. and Watkins, J. C. (1982). The effects of a series of phosphonic carboxylic amino acids on electrically evoked and excitant amino acid induced responses in isolated spinal cord preparations. *Br. J. Pharmacol.*, **75,** 65-75.

Evans, R. H., Francis, A. A. and Watkins, J. C. (1977). Differential antagonism by chlorpromazine and diazepam of frog motor neurone depolarization induced by glutamate-related amino acids. *Eur. J. Pharmacol.*, **44,** 325-330.

Evans, R. H., Francis, A.. A. and Watkins, J. C. (1978). Mg^{2++}-like selective antagonism of excitatory amino acid-induced responses by alpha-epsilon-diaminopimelic acid, D-alpha-aminoadipate and HA-966 in isolated spinal cord of frog and immature rat. *Brain Res.*, **148,** 536-542.

Fagg, G. E., Foster, A. C., Mena, E. E. and Cotman, C. W. (1982). Chloride and calcium ions reveal a pharmacologically distinct population of L-glutamate binding sites in synaptic membranes: correspondence between biochemical and electrophysiological data. *J. Neurosci.*, **2,** 958-965.

Fagg, G. E., Foster, A. C., Mena, E. E. and Cotman, C. W. (1983). Chloride and calcium ions separate L-glutamate receptor populations in synaptic membranes. *Eur. J. Pharmacol.*, **88,** 105-110.

Fagg, G. E. and Lane, J. D. (1979). The uptake and release of putative amino acid neurotransmitters. *Neurosci.*, **4,** 1015-1036.

Fagg, G. E. and Matus, A. (1984) Selective association of N-methyl-D-aspartate and quisqualate types of L-glutamate receptor with brain postsynaptic densities. *Proc. Natl. Acad. Sci. USA.*, **81,** 6876-6880.

Fagg, G. E., Olpe, H-R., Pozza, M. F., Baud, J., Steinmann, M., Schmutz, M., Portet, C., Baumann, P., Thedina, K., Bittiger, H., Allgeier, H., Heckendorn, R., Angst, C., Brundish, D. and Dingwall, J. G. (1990). CGP 37849 and CGP 39551: novel and potent competitive N-methyl-D-aspartate receptor antagonists with oral activity. *Br. J. Pharmaol.,* **99,** 791-797.

Fagg, G. E., Pozza, M. F., Olpe, H. R., Brugger, F., Bauman, P., Bittiger, H., Schmutz, M., Angst, C., Brundish, D., Allgeier, H., Heckendorn, R. and Dingwall, J. G. (1989). CGP 37849: Characterization of a novel and potent competitive N-methyl-D-aspartate (NMDA) receptor antagonist. *Br. J. Pharmacol.,* **97,** 582P.

DeFaudis, F. V., Orsensanz Munoz, L. M. and Fando, J. L. (1978). High affinity glycine binding sites in rat CNS: Regional variation and strychnine sensitivity. *Gen. Pharmacol.,* **9,** 171-176.

Ferendelli, J. A. (1978). Distribution and regulation of cyclic GMP in the central nervous system. *Adv. Cyclic Nucleotide Res.,* **9,** 453-464.

Ferendelli, J. A., Chang, M. M. and Kinscherf, D. A. (1974). Elevation of cyclic GMP levels in central nervous system by excitatory amino acids. *J. Neurochem.,* **22,** 535-540.

Ferendelli, J. A., Steiner, A. L., McDougal, D. B. and Kipnis, D. M. (1970). The effect of oxotremorine and atropine on cGMP and cAMP levels in mouse cerebral cortex and cerebellum. *Biochem. Biophys. Res. Comm.,* **41,** 1061-1067.

Ferkany, J. W., Kyle, D. J., Willets, J., Rzeszotarski, W. J. and Guzewska, M. E. (1989). Pharamcological profile of NPC12626, a novel competitive NMDA receptor antagonist. *J. Pharmacol. Exp. Ther.,* **250,** 100-109.

Ferraro, T. N. and Hare, T. A. (1985). Free and conjugated amino acids in human CSF: Influence of age and sex. *Brain Res.,* **338,** 53-60.

Flatman, J. A., Scwindt, P. C., Crill, W. E. and Srafstorm, C. E. (1983). Multiple actions of NMDA on cat neocortical neurones *in vitro*. *Brain Res.,* **266,** 169-173.

Fletcher, E. J., Beart, P. M. and Lodge, D. (1990). Involvement of glycine in excitatory neurotransmission. In "*Glycine Neurotransmission*", Ottersen, O. P. and Storm-Mathisen, J. (eds.), John Wiley and Sons Ltd, New York, pp 193-219

Fletcher, E. J., Jones, M. G. and Lodge, D. (1988). Strychnine resistant effects of glycine on central neurones. *Eur. J. Neurosci.,* **1,** 196-203.

Fletcher, E. J. and Lodge, D. (1988). Glycine reverses the antagonism of HA966 but not AP5 on rat cortical slices. *Eur. J. Pharmacol.,* **15,** 161-162.

Fletcher, E. J., Miller, J. D., Zeman, S. and Lodge, D. (1989). Non-competitive antagonism of N-methyl-D-aspartate by displacement of an endogenous glycine-like substance. *Eur. J. Neurosci.,* **1,** 196-2003.

Fletcher, A. E., Wilkin, G. P. and Bowery, N. G. (1989). A putative glycinergic cell in rat cerebellum which may modulate NMDA receptor function in granule cells. *Br. J. Pharamcol.,* **96,** 85P.

Forsythe, I. D and Clements, J. D. (1988). Glutamate autoreceptors reduce EPSC in cultured hippocampal neurones. *Soc. Neurosci.,* **14,** 791 (Abstr.)

Foster, A. C. and Fagg, G. E. (1984). Acidic amino acid binding sites in mammalian neuronal membranes: their characteristics and relationship to synaptic receptors. *Brain Res. Rev.,* **7,** 103-164.

Foster, A. C. and Fagg, G. E. (1987). Comparison of L-[^{3}H]glutamate, D-[^{3}H]aspartate, DL-[^{3}H]AP5 and [^{3}H]NMDA as ligands for NMDA receptors in crude postsynaptic densities from rat brain. *Eur. J. Pharmacol.,* **133,** 291-300.

Foster, A. C., Gill, R., Kemp, J. A. and Woodruff, G. N. (1987). Systemic administration of MK801 prevents NMDA induced neuronal degeneration in rat brain. *Neurosci. Lett.,* **76,** 307-311.

Foster, A. C., Gill, R. and Woodruff, G. N. (1988). Neuroprotective effects of MK801 *in vivo* : selectivity and evidence for delayed degeneration mediated by NMDA receptor activation. *J. Neurosci.,* **8,** 4745-4754.

Foster, A. C. and Kemp, J. A. (1989). HA-966 antagonises NMDA receptors through a selective interaction with the glycine modulatory site. *J. Neurosci.,* **9,** 2191-2196.

Foster, A. C., Kemp, J. A., Leeson, P. D., Grimwood, S., Marshall, G. R., Priestley, T., Carling, R. W. and Moore, K. W. (1990). Kynurenic acid analogues as potent and selective antagonists at the glycine site on the NMDA receptor. *Neurosci., Abstr.,* **16,** 430.13.

Foster, A. C., Mena, E. E., Monaghan, D. T. and Cotman, C. W. (1981). Synaptic localisation of kainic acid binding sites. *Nature,* **289,** 73-75.

Foster, A. C. and Roberts, P. J. (1978). High affinity L-[^{3}H]glutamate binding to postsynaptic receptor sites on rat cerebellar membranes. *J. Neurochem.,* **31,** 1467-1477.

Foster, A. C., Whetsell, W. O., Bird, E. D. and Schwartcz, R. (1985). Quinolinic acid phosphoribosyltransferase in human and rat brain: activity in Huntington's disease and in quinolinate-lesioned rat striatum. *Brain Res.,* **336,** 207-214.

Foster, A. C. and Wong, E. H. F.(1987). The novel anticonvulsant MK801 binds to the activated state of the NMDA receptor in rat brain. *Br. J. Pharmacol.*, **91,** 403-409.

Foster, G. A. and Roberts, P. J. (1980). Pharmacology of excitatory amino acid receptors mediating the stimualtion of rat cerebellar cyclic GMP levels *in vitro*. *Life Sci.*, **27,** 215-221.

Frostholm, A. and Rotter, A. (1985). Glycine receptor distribution in mouse CNS. Autoradiographic localisation of ^{3}H- strychnine binding sites. *Brain Res. Bull.*, **15,** 473-486.

Galli, A., Fenigli, S., Anichini, A. and Pizzighelli, L. (1988). Stereoselective inhibition of ^{3}H-glycine binding to cortical membranes of rat brain by amino acids. *Pharmacol. Res. Comms.*, **2,** No.5, 407-408.

Galli, A., Franchi-Micheli, S., Mori, F., Luzzi, S. and Zillette, L. (1990). Glycine-related amino acids stereoselectively affect N-methyl-D-aspartate receptor-mediated contractions of the Guinea Pig ileum: Comparison with the inhibition of strychnine-insensitive ^{3}H-glycine binding to rat cortical membranes. *Neuropharm.*, **29,** No.2, 145-150.

Gallo, V., Giovannini, C. and Levi, G.(1990). Modulation of non NMDA receptors in cultured cerebellar granule cells. *J. Neurochem.*, **54,** 1619-1625.

Gallo, V., Suergiu, R. and Levi, G. (1987). Glutamate receptor subtypes in cultured cerebellar neurons: modulation of glutamate and GABA release. *J. Neurochem.*, **49,** 1801-1809.

Ganong, A. H. and Cotman, C. W. (1982). Acidic amino acid antagonists of lateral perforant path synaptic transmission agonist-antagonists interactions in the dentate gyrus. *Neurosci. Lett.*, **34,** 195-200.

Ganong, A. H., Lanthorn, T. H. and Cotman, C. W. (1983). Kynurenic acid inhibits synaptic and acidic acid induced responses in the rat hippocampus and spinal cord. *Brain Res.*, **273,** 170-174.

Garthwaite, G. and Garthwaite, J. (1984). Differential sensitivity of rat cerebellar cells in vitro to the neurotoxic effects of excitatory amino acid analogues. *Neurosci. Lett.*, **48,** 361-367.

Garthwaite, G. and Garthwaite, J. (1986). In vitro neurotoxicity of excitatory amino analogues during cerebellar development. *Neurosci.*, **17,** 755-767.

Garthwaite, G. and Garthwaite, J. (1989a). Quisqualate neurotoxicity; a delayed, CNQX sensitive process triggered by a CNQX insensitive mechanism in young rat hippocampal slices. *Neurosci. Lett.*, **99,** 113-118.

Garthwaite, G. and Garthwaite, J. (1989b). Differential dependence on calcium of NMDA and quisqualate neurotoxicity in young rat hippocampal slices. *Neurosci. Lett.*, **97**, 316-322.

Garthwaite, G. and Garthwaite, J. (1989c). Neurotoxicity of excitatory amino acid receptor agonists in young rat hippocampal slices. *J. Neurosci. Methods*, **29**, 33-42.

Garthwaite, J. (1982). Excitatory amino acid receptors and guanosine 3'5'cyclic monophosphate in incubated slices of immature and adult rat cerebellum. *Neurosci.*, **7**, 2491-2497.

Garthwaite, J. (1989). NMDA receptors, neuronal development and neurodegeneration. In "*The NMDA Receptor*", Watkins, J. C. and Collingridge, G. L. (eds.), Oxford University Press, Oxford, pp 187-206.

Garthwaite, J. and Balazs, R. (1978). Supersensitivity to the cyclic GMP response to glutamate during cerebellar maturation. *Nature*, **275**, 328-329.

Garthwaite, J., Charles, S. L. and Chess-Williams, R. (1988). EDRF as an intracellular messenger in the brain; release following activation of NMDA receptors. *Nature*, **336**, 385-388.

Garthwaite, J. and Garthwaite, G. (1987). Cellular origins of cyclic GMP response to excitatory amino acid receptor agonists in rat cerebellum *in vitro*. *J. Neurochem.*, **48**, 29-39.

Garthwaite, J., Woodhams, P. L., Collins, M. J. and Balazs, R. (1979). On the preparation of brain slices: morphology and cyclic neucleotides. *Brain Res.*, **173**, 373-377.

Geddes, J. W., Cooper, S. M., Cotman, C. W., Patel, S. and Meldrum, B. S. (1989). N-methyl-D-aspartate receptors in the cortex and hippocampus of baboon (Papio Anubis and Papio Papio). *Neurosci.*, **32**, 39-47.

Gill, R., Foster, A. C. and Woodruff, G. N. (1987). Systemic administration of MK-801 protects against ischemia-induced hippocampal neurodegeneration in the gerbil. *J. Neurosci.*, **7**, 3343-3349.

Gill, R., Foster, A. C. and Woodruff, G. N. (1988). MK801 is neuroprotective in gerbils when administered during the post ischaemic period. *Neurosci.*, **25**, 847-855.

Godfrey, P. P., Wilkins, C. J., Tyler, W. and Watson, S. P. Stimulatory and inhibitory actions of excitatory amino acids on inositol phospholipid metabolism in rat cerebral cortex. *Br. J. Pharmacol.*, **95**, 131-138.

Greenamyre, J. T., Olsen, J. M. M., Penney, J. B. and Young, A. B. (1985a).

Autoradiographic characterization of NMDA, quisqualate and kainate sensitive binding sites. *J. Pharmacol. Exp. Ther.*, **233,** 254-263.

Greenamyre, J. T., Penney, A. M., D'Amato, C. J. and Young, A. B. (1987). Dementia of the Alzheimer's type: changes in hippocampal L-^{3}H-glutamate binding. *J. Neurochem.*, **48,** 543-551.

Greenamyre, J. T., Penney, J. B., Young, A. B., D'Amato, C. J., Hicks, S. P. and Shoulson, I. (1985b). Alterations in L-glutamate binding in Alzheimer's and Huntington's disease. *Science.*, **227,** 1496-1497.

Greeningloch, G., Rienitz, A., Schmitt, B., Methfessel, C., Zensen, M., Beyreuther, K., Grundelfinger, E. D. and Betz, H. (1987). The strychnine binding sub unit of the glycine receptor shows homology with the nicotinic acetylcholine receptor. *Nature,* **328,** 215-220.

Griffiths, T., Evans, M. C. and Meldrum, B. S. (1984). Temporal lobe epilepsy, excitotoxins and the mechanism of selective neuronal loss. In "*Excitotoxins*", Fuxe, K., Roberts, P. J. and Schwarcz, R. (eds.), Macmillan, London, pp 331-342.

Gundersen, C. B., Miledi, R. and Parker, I. (1984). Glutamate and kainate receptors by rat brain messenger RNA in X. oocytes. *Proc. R. Soc. Lond. Ser.,* **B21,** 127-143.

Gundlach, A. L., Largent, B. L. and Snyder, S. H. (1986). Phencyclidine (PCP) receptors: Autoradiographic localization in brain with the selective ligand ^{3}H-TCP. *Brain Res.,* **386,** 266-279.

Gustafsson, B., Wigstrom, H., Abraham, W. C. and Huang, Y. Y. (1987). Long term potentiation in the hippocampus using depolarizing current pulses as the conditioning stimulus to single volley synaptic potentials. *J. Neurosci.,* **7,** 774-780.

Guyent, P. G., Filtz, T. M. and Donaldson, S. R. (1987). Role of excitatory amino acids in rat vagal and sympathetic baroreflexes. *Brain Res.,* **407,** 272-284.

Hajos, F., Garthwaite, G. and Garthwaite, J. (1986). Reversible and irreversible neuronal damage caused by excitatory amino acid analogues in rat cerebellar slices. *Neurosci.,* **18,** 417-436.

Haldeman, S. and McLennan, H. (1972). The antagonist action of glutamic acid diethyl ester towards amino acid-induced and synaptic excitations of central neurones. *Brain Res.,* **45,** 393-400.

Hamori, J. and Szentagothai, J. (1966). Participation of Golgi neuron

processess in the cerebellar glomeruli: an electron microscopic study. *Exp. Brain Res.*, **2**, 35-48.

Hannuniemi, R. and Oja, S. S. (1981). Uptake of leucine, lysine, aspartic acid and glycine into isolated neurons and astrocytes. *Neurochem Res.*, **8**, 873-884.

Hardy, J. and Cowburn, R. (1987). Glutamate neurotxicity and Alzheimer's disease. *T. I. N. S.*, **10**, 406-407.

Harris, E. W. and Cotman, C. W. (1985). Effects of synaptic antagonists on perforant path paired pulse plasticity: differentiation of pre and postsynaptic antagonism. *Brain Res.*, **334**, 348-353.

Harris, E. W. and Cotman, C. W. (1986). LTP of guinea pig mossy fibre responses is not blocked by NMDA antagonists. *Neurosci. Lett.*, **70**, 132-137.

Harrison, N. L. and Simmons, M. A. (1985). Quantitative studies on some antagonists of N-methyl-D-aspartate in slices of rat cerebral cortex. *Br. J. Pharmacol.*, **84**, 381-391.

Hayashi, T. (1954). Effects of sodium glutamate on the nervous system. *Keio J. Med.*, **3**, 183-192.

Hearns, T, Ganong, A. H. and Cotman, C. W. (1986). Antagonism of lateral olfactory tract synaptic potentials in rat pyriform cortex slices. *Brain Res.*, **379**, 372-376.

Hebb, D. O. (1949). "*The Organisation of Behaviour*", John Wiley , New York,

Herrling, P. L., Aebischer, B., Frey, P., Olverman, H. J. and Watkins, J. C. (1989). NMDA-antagonist properties of the enantiomers of 3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP) and of its unsaturated analogue 3-(2-carboxypiperazin-4-yl)-1-propenyl-1-phosphonic acid (CPPene). *Soc. Neurosci. Abstr.*, **15**, 133.12.

Hokfelt, T. (1987). Neuronal communication through multiple coexisting messengers. In "*Synaptic Function*", Edleman, G. M., Gall, W. E. and Cowan, W. M. (eds.), John Wiley, New York, pp 179-211.

Holopainen, I. and Kontro, P. (1989). Uptake and release of glycine in cerebellar granule cells and astrocytes in primary culture: potassium stimulated release from granule cells is calcium dependent. *J. Neurosci. Res.*, **24**, 374-383.

Holopainen, I., Kontro, P. and Oja, S. S. (1989). Release of taurine from cerebellar granule cells and astrocytes: co-release with glutamate. *Neurosci.*, **29,** 425-432.

Honey, C. R., Miljkovic, Z. and MacDonald, J. F. (1985). Ketamine and phencyclidine cause a voltage-dependent block of responses to L-aspartic acid. *Neurosci. Lett.*, **61,** 135-139.

Honore, T., Davies, S. N., Drejer, J., Fletcher, E. J., Jacobsen, P., Lodge, D. and Neilsen, F. E. (1988). Quinoxalinediones; potent competitive non NMDA glutamate receptor antagonists. *Science,* **241,** 701-703.

Honore, T., Lauridsen, J. and Krogsgaard-Larsen, P. (1982). The binding of [^{3}H]AMPA, a structural analogue of glutamic acid, to rat brain membranes. *J. Neurochem.*, **38,** 173-178.

Honore, T. and Nielsen, M. (1985). Complex structure of quisqualate-sensitive glutamate receptors in rat cortex. *Neurosci. Lett.*, **54,** 27-32.

Hood, W. F., Sun, E. T., Compton, R. P. and Monahan, J. B. (1989a). 1-Amino-1-carboxylate (ACBC): A specific antagonist of the NMDA coupled glycine receptor. *Eur. J. Pharmacol.*, **161,** 281-282.

Hood, W. F., Compton, R. P. and Monahan, J. B. (1989b). D-Cycloserine: A ligand for NMDA coupled glycine receptor has partial agonist characteristics. *Neurosci. Lett.*, **98,** 91-95.

Hood, W. F., Compton, R. P. and Monahan, J. B. (1990). NMDA recognition site ligands modulate activity at the coupled glycine recognition site. *J. Neurochem.*, **54,** 1040-1046.

Hopkin, J. and Neal, M. J. (1971). Effect of electrical stimulation and high potassium on the flux of ^{14}C-glycine from slices of spinal cord. *Br. J. Pharmacol.*, **42,** 215-223.

Huettner, J. E. and Bean, B. P. (1988). Block of N-methyl-D-aspartate-activated current by the anticonvulsant MK-801: Selective binding to open channels. *Proc. Natl. Acad. Sci. USA.*, **85,** 1307-1311.

Iino, M., Ozawa, S. and Tsuzuki, K. (1990). Permeation of calcium through EAA receptor channels in cultured rat hippocampal neurones. *J. Physiol.*, **424,** 151-165.

Imaly, J. A. and Linn, S. (1988). DNA damage and oxygen radical toxicity. *Science,* **240,** 1302-1309.

Ishida, A. T. and Neyton, J. (1985). Quisqualate and L-glutamate inhibit retinal horizontal cell response to kainate. *Proc. Natl. Acac. Sci.,* **82,** 1837-1841.

Ito, M. (1984). "*The Cerebellum and Neural Control*", Raven Press, New York.

Ito, U., Spatz, J. T., Walker, M. and Klatzo, I. (1975). Experimental cerebral ischaemia in Mongolion gerbil. Light microscopic observations. *Acta. Neuropathol.,* **32,** 209-223.

Jahr, C. E. and Stevens, C. F. (1987). Glutamate activates multiple single channel conductances in hippocampal neurons. *Nature,* **325,** 522-525.

Jalonen, T. and Holopainen, I. (1989). Properties of single potassium channels in cultured primary astrocytes. *Brain Res.,* **484,** 177-183.

Jansen, K. L. R., Faull, R. L. M. and Dragunow, M (1989). Excitatory amino acid receptors in the human cerebral cortex. A quantitative autoradiographic study comparing the distributions of ^{3}H-TCP, ^{3}H-glycine, L-^{3}H-glutamate, ^{3}H-AMPA and ^{3}H-kainic acid binding sites. *Neurosci.,* **32,** 587-607.

Jarvis, M. F., Murphy, D. E. and Williams, M. (1987). Quantitative autoradiographic localization of NMDA receptors in rat brain using [^{3}H]CPP: comparison with [^{3}H]TCP binding sites. *Eur. J. Pharmacol.,* **141,** 149-152.

Javitt, D. C., Frusciante, M. J. and Zukin, S. R. (1990). Rat brain NMDA receptors require multiple molecules of agonist for activation. *Mol. Pharmacol.,* **37,** 603-607.

Javitt, D. C., Jotkowitz, A., Sircar, R. and Zukin, S. R. (1987). Non-competitive regulation of phencyclidine/sigma receptors by the NMDA receptor antagonist D-(α)-2-amino-5-phosphonovaleric acid. *Neurosci. Lett.,* **78,** 193-198.

Javitt, D. C. and Zukin, S. R. (1989). Biexponential kinetics of ^{3}H-MK801 binding: evidence for access to closed and open NMDA receptor channels. *Mol. Pharmacol.,* **35,** 387-393.

Joels, M., Van Veldhuizen, M., Urban, I. J. A. and DeKloet, E. R. (1987). N-acetyl-aspartylglutamate: binding sites and excitatory action in the dorsolateral septum of rats. *Brain Res.,* **403,** 192-197.

Johnson, J. W. and Ascher, P. (1987). Glycine potentiates the NMDA response in cultured mouse brain neurones. *Nature,* **325,** 529-531.

Johnson, J. W. and Ascher, P. (1990). N-methyl-D-aspartate-activated channels. *Biophys. J.,* **57,** 1085-1090.

Johnson, K. M. (1983). Phencyclidine: behavioral and biochemical evidence supporting a role for dopamine. *Fed. Proc.,* **42,** 2579-2583.

Johnson, K. M., Scann, A. I. and Snell, L. D. (1988). Equlibrium analysis of ^{3}H-TCP binding: effects of glycine, magnesium and NMDA agonists. *Eur. J. Pharmacol.,* **152,** 141-146.

Johnston, G. A. R., Curtis, D. R., Davies, J. and McCulloch, R. M. (1974). Spinal interneurone excitation by conformationally restricted analogues of L-glutamic acid. *Nature,* **248,** 804-805.

Johnston, G. A. R., Curtis, D. R., DeGroat, W. C. and Duggan, A. W. (1968). Central actions of ibotenic acid and muscimol. *Biochem. Pharmacol.,* **17,** 2488-2489.

Johnston, G. A. R. and Iversen, L. L. (1971). Glycine uptake in rat central nervous system slices and homogenates. Evidence for different uptake systems in spinal cord and cerbral cortex. *J. Neurochem.,* **18,** 1951-1961.

Johnston, M. V., McDonald, J. W., Hood, T., Sackellares, L., Garofalo, E., Mckeever, P. E., Troncoso, J. and Gilman, S. (1989). Elevated NMDA receptor and reduced GABA receptor binding in hippocampus (HIP) from patients with temporal lobe epilepsy (TLE). (1989). *Soc. Neurosci. Abs.,* **15,** 478.14.

Jones, A. W., Smith, D. A. S. and Watkins, J. C. (1984). Structure-activity relationships of dipeptide antagonists of excitatory amino acids. *Neurosci.,* **13,** 573-581.

Jones, M. G., Anis, N. A. and Lodge, D. (1989). Digger wasp toxin blocks excitation by quisqualate on rat central neurones. *Neurosci. Lett.,* **36,** S25.

Jones, S. M., Snell, L. D. and Johnson, K. M. (1989). Characterization of the binding of radioligands to the N-methyl-D-aspartate, phencyclidine and glycine receptors in Bully Coat membranes. *J. Pharmacol. Methods,* **21,** 161-168.

Kaplita, P. V. and Ferkany, J. W. (1990). Evidence for direct interactions between the NMDA and glycine recognition site in brain. *Eur. J. Pharmacol.,* **188,** 175-179.

Karbon, E. W., Patch, R. J., Pontoecorvo, M. J. and Ferkany, J. W. (1990). Ifenprodil potently interacts with ^{3}H-PPP-labelled σ binding sites in guinea pig brain membranes. *Eur. J. Pharmacol.,* **176,** 247-248.

Kass, I. S., Chambers, G. and Cottrell, J. E. (1989). The NMDA antagonist aminophosphonovaleric acid and MK801 reduce anoxic damage to dentate gyrus and CA1 pyramidal cells in the rat hippocampal slice. *Exp. Neurol.,* **103,** 116-122.

Katzman, R. (1986). Alzheimers disease. *T. I. N. S.*, **9**, 522-525.

Kauer, J. A., Malenka, R. C. and Nicoll, R. A. (1988). NMDA application potentiates synaptic transmission in the hippocampus. *Nature,* **334**, 250-252.

Kauer, J. A. and Nicoll, R. A. (1988). An APV resistent non-associative form of LTP in rat hippocampus. In "*Synaptic Plasticity in the Hippocampus*", Hass, H. L. and Buzsaki, G. (eds.), Springer-Verlag, Berlin, pp

Kawasaki, K., Traynelis., S. F. and Dingledine, R. (1990). Different responses of CA1 and CA3 regions to hypoxia in rat hippocampal slice. *J. Neurophysiol.,* **63**, 385-394.

Kemp, J. A., Foster, A. C., Leeson, P. D., Priestley, T., Tridgett, R. and Iversen, L. L. (1988). 7-Chlorokynurenic acid is a selective antagonist as the glycine modulatory site of the N-methyl-D-aspartate receptor complex. *Proc. Natl. Acad. Sci.,* **85**, 6547-6550.

Kemp, J. A., Foster, A. C. and Wong, E. H. F. (1987). Non-competitive antagonists of excitatory amino acid receptors. *T. I. N. S.,* **10**, 294-298.

Kemp, J. A., Priestley, T. and Woodruff, G. N. (1986). MK801, a novel orally active anticonvulsant is a potent non-competitive NMDA receptor antagonist. *Br. J. Pharmacol.,* **89**, 535P.

Kemp, J. A. and Sillito, A. W. (1982). The nature of the excitatory transmitter mediating X and Y cells' input to the cat dorsal lateral geniculate nucleus. *J. Physiol.,* **323**, 377-391.

Kessler, M., Terramani, T. , Lynch, G. and Baudry, M. (1989a). A glycine site associated with N-methyl-D-aspartatic acid receptors: Characterization and identification of a new class of antagonists. *J. Neurochem.,* **52**, 1319-1328.

Kessler, M., Baudry, M. and Lynch, G. (1989b). Quinoxaline derivatives are high-affinity antagonists of the NMDA receptor-associated glycine sites. *Brain Res.,* **489**, 377-382.

Kessler, M., Baudry, M., Terramani, T. and Lynch, G. (1987).Complex interactions between a glycine binding site and NMDA receptors. *Soc. Neurosci. Abstr.,* **13**, 209.18.

Kessler, M., Terramani, T., Lynch, G. and Baudry, M. (1989c). A glycine site associated with NMDA receptors: characterization and identification of a new class of antagonists. *J. Neurochem.,* **37**, 1015-1024.

Kilpatrick, G. J., Jones, B. J. and Tyers, M. B. (1988). Characterisation of [^{3}H]-GR65630 binding to 5-HT_3 receptors in homogenates of rat area postrema and vagus nerve. *Br. J. Pharmacol.*, **95,** 871P.

Kingsbury, A., Gallo, V. and Balazs, R. (1988). Stimulus-coupled release of amino acids from cerebellar granule cells in culture. *Brain Res.*, **448,** 46-52.

Kishimoto, H., Simon, J. R. and Aprison, M. H. (1981). Determination of the equilibriium dissociation constants and number of glycine binding sites in several areas of the rat central nervous system, using a sodium-independent system. *J. Neurochem.*, **37,** 1015-1024.

Kleckner, N. W. and Dingledine, R. (1988). Requirement for glycine in activation of NMDA-receptors expressed in Xenopus oocytes. *Science,* **241,** 835-837.

Kloog, Y. Haring, R. and Sokolovsky, M. (1988a). Kinetic characterisation of the phencyclidine NMDA receptor interaction. Evidence for a steric blockade of the channel. *Biochemistry,* **27,** 843-848.

Kloog, Y., Lamdani-Itkin, H. and Sokolovsky, M. (1990). The glycine site of the NMDA receptor channel : Differences between the binding of HA-966 and 7-Chlorokynurinic acid. *J. Neurochem.*, **54,** 1576-1583.

Kloog, Y., Nadler, V. and Sokolovsy, M. (1988b). Mode of binding of ^{3}H-MK801 to the NMDA receptor and its therapeutic implication. *F. E. B. S. Lett.*, **230,** 167-170.

Knowles, R. G., Palacios, M., Palmer, R. M. J. and Moncada, S. (1989). Formation of nitric oxide from L-arginine in the central nervous system: a transduction mechanism for the stimulation of the soluble guanylate cyclase. *Proc. Natl. Acad. Sci.*, **86,** 5159-5162.

Koek, W., Woods, J. H. and Winger, G. D. (1988). MK801 a proposed noncompetitve antagonist of excitatory amino acid neurotransmission produces phencyclidine like behavioral effect in pigeons, rats and Rhesus monkeys. *J. Pharmacol. Exp. Ther.*, **245,** 969-974.

Koerner, J. F. and Cotman, C. W. (1981). Micromolar L-2-amino-4-phosphonobutyric acid selectively inhibits perforant path synapses from lateral entorhinal cortex. *Brain Res.*, **216,** 192-198.

Koh, J. Y., Goldberg, M. P., Hartley, D. M. and Choi, D. W. (1990). Non-NMDA receptor-mediated neurotoxicity in cortical culture. *J, Neurosci.*, **10,** 693-705.

Kornhuber, J., Bormann, J., Retz, W, Hubers, M. and Riederer, P. (1989). Memantine displaces ^{3}H-MK801 at therapeutic concentrations in postmortem human frontal cortex. *Eur. J. Pharmacol.*, **166,** 589-590.

Krogsgaard-Larsen, P., Honore, T., Hansen, J. J., Curtis, D. R. and Lodge, D. (1980). New class of glutamate agonist structurally related to ibotenic acid. *Nature,* **284,** 64-66.

Kuhar, M. J., Murrin, L. C., Malouf, A. T. and Klemm, N. (1978). Dopamine receptor binding in vivo: the feasibility of autoradiographic studies. *Life Sci.,* **22,** 203-210.

Kuhar, M. J. and Yamamura, (1975). Light autoradiographic localization of cholinergic muscarinic receptors in rat brain by specific binding of a potent agonist. *Nature,* **253,** 560-561.

Kushner, L., Lerma, J., Zukin, R. S. and Bennett, M. V. L. (1988). Coexpression of N-methyl-D-aspartate and phencyclidine receptors in Xenopus oocytes injected with rat brain mRNA. *Proc. Natl. Acad. Sci. USA.,* **85,** 3250-3254.

Lanthorn, T. H., Ganong, A. H. and Cotman, C. W. (1984). 2-amino-4-phosphonobutyric acid selectively blocks mossy fibre CA3 responses in guinea pig but not rat hippocampus. *Brain Res.,* **290,** 174-178.

Lanthorn, T. H., Hood, W. F., Watson, G. B., Compton, R. P., Rader, R. K., Gaoni, Y. and Monahan, J. B. (1990). Cis-2,4-methanoglutamate is a potent and selective N-methyl-D-aspartate receptor agonist. *Eur. J. Pharmacol.,* **182,** 397-404.

Largent, B. L., Gundlach, A. L. and Snyder, S. H. (1986). Pharmacological and autoradiographic discrimination of sigma and phencyclidine receptor binding sites in brain with (+)-[^{3}H]SKF10,047, 9+0-[^{3}H]-3-[3-Hydroxyphenyl]-N-(1-propyl0piperidine and [^{3}H]-1-[1-(2-Thienyl)cyclohexyl]piperidine. *J. Pharmacol. Exp. Ther.,* **238,** 739-748.

Larson, A. A. and Betz, A. J. (1988). Glycine potentiates strychnine-induced convulsions: Role of NMDA receptors. *J. Neurosci.,* **8,** 3822-3826.

Larson, J. and Lynch, G. (1988). Role of N-methyl-D-aspartate receptors in the induction of synaptic potentiation by burst stimulation patterned after the hippocampal theta-rhythm. *Brain Res.,* **441,** 111-118.

Lazarewicz, J. W., Wroblewski, J. T., Palmer, M. E. and Costa, E. (1988). Activation of N-methyl-D-aspartate-sensitive glutamate receptors stimulates arachadonic acid release in primary cultures of cerebellar granule cells. *Neuropharmacol.,* **27,** 765-769.

Leach, M. J., Hollox, K. J., O'Donnel, R. A. and Miller, A. A. (1988).

Hippocampal NMDA/phencyclidine receptor binding sites are reduced following forebrain ischaemia in the gerbil. *Eur. J. Pharmacol.*, **152,** 189-192.

Leander, J. D., Rathbun, R. C. and Zimmerman, D. M. (1988). Anticonvulsant effects of phencyclidine-like drugs. relation to NMDA antagonism. *Brain Res.*, **454,** 368-372.

Lehmann, J., Hutchison, A. J., McPherson, S. E., Monadori, C., Schmutz, M., Sinton, C. M., Tsai, C., Murphy, D. E., Steel, D. J., Williams, M., Cheney, D. L. and Wood, P. L. (1988). CGS 19755, a selective and competitive N-methyl-D-aspartate excitatory amino acid antagonist. *J. Pharmacol. Exp. Ther.*, **246,** 65-75.

Lehmann, J., Schneider, J., McPherson, S., Murphy, D. E., Bernard, P., Tsai, C., Bennett, D. A., Pastor, G., Steel, D. J., Boehm, C., Cheney, D. L., Liebman, J. M., Williams, M. and Wood, P. L. (1987). CPP, a selective N-methyl-D-aspartate (NMDA)-type receptor antagonist: characterization in vitro and in vivo. *J. Pharmacol. Exp. Ther.*, **240,** 737-746.

Levi, G. (1984). Release of putative transmitter amino acids. In "*Handbook of Neurochemistry -vol. 6*" Lajitha, A. (ed.), Plenum Press, New York, pp 463-509.

Levi, G. and Gallo, V. (1986). Release studies related to the neurotransmitter role of glutamate in the cerebellum: an overview. *Neurochem. Res.*, **11** 1627-1642.

Levy, W. B., Redburn, D. A. and Cotman, C. W. (1973). Stimulus coupled secretion of γ aminobutyric acid from rat brain synaptosomes. *Science,* **181,** 676-678.

Liebschutz, J., Airoldi, L., Brownstein, M. J., Chinn, N. G. and Wurtman, R. J. (1977). Regional distribution of endogenous and parental glutamate, aspartate and glutamine in rat brian. *Biochem. Pharmacol.*, **26,** 443-446.

Linden, D. J., Womg, K. L., Sheu, F. S. and Routtenberg, A. (1988). NMDA receptor blockade prevents the increase in protein kinase C substrate phosphorylation produced by long term potentiation. *Brain Res.*, **458,** 142-146.

Llinas, R. R. and Heuser, J. E. (1977). Depolarisation - release coupling systems in neurons. *Neurosci. Res. Prog. Bull.*, **15,** 4.

Lodge, D., Anis, N. A. and Burton, N. R. (1982). Effects of optical isomers of ketamine on excitation of cat and rat spinal neurons by amino-acids and acetylcholine. *Neurosci. Lett.*, **29,** 281-286.

Lodge, D., Aram, J. A., Church, J., Davies, S. N., Martin, D., O'Shaughnessy, C. T. and Zeman, S. (1987). Excitatory amino acids and phencyclidine like drugs. In "*Excitatory Amino Acid Transmission*", Hicks, T. P., Lodge, D. and McLennan, H. (eds.), Alan Liss, New York, pp 83-90.

Lodge, D. and Johnson, K. M. (1990). Noncompetitive excitatory amino acid receptor antagonists. *T. I. P. S.* **11,** 81-86.

London, E. D. and Coyle, J. T. (1979). Specific binding of ^{3}H-kainic acid to receptor sites in rat brain. *Mol. Pharmacol.,* **15,** 492-505.

Loo, P., Braunwalder, A., Lehmann, J. and Williams, M. (1986). Radioligand binding to central phencyclidine recognition sites is dependent on excitatory amino acid receptor agonists. *Eur. J. Pharmacol.,* **123,** 467-468.

Lowe, D. A., Neijet, H. C. and Aeibischer, B. (1990). D-CPP-ENE (SDZ EAA 494), a potent and competitive NMDA antagoinist- effect on spontaneous activity and NMDA induced depolarizations in rat neocortical slice preparation, compared with other CPP derivatives and MK-801. *Neurosci. Lett.,* **113,** 315-321.

Lynch, G. and Baudry, M. (1984). The biochemistry of memory a new and specific hypothesis. *Science,* **224,** 1057-1063.

MacDonald, J. F., Miljkovic, Z. and Pennfeather, P. (1987). Use-dependent block of excitatory amino acid currents in cultured neurones by ketamine. *J. Neurophysiol.,* **58,** 251-265.

MacDonald, J. F. and Nowak, L. M. (1990). Mechanisms of blockade of excitatory amino acid receptor channels. *T. I. P. S.,* **11,** 167-172.

MacDonald, J. F., Porietis, A. V. and Wojtowicz, J. M. (1982). L-Aspartic acid induces a region of negative slope conductance in the current-voltage relationship of cultured spinal neurones. *Brain Res.,* **237,** 248-253.

Manallack, D. T., Wong, M. G., O'Shea, R. D. and Beart, P. M. (1989). Topography of the glycine site of the NMDA receptor. *Mol. Neuropharamcol.,* **1,** 7-15.

Mann, P. J. G., Tennenbaum, M. and Quastel, J. H. (1939). Acetylcholine metabolism in the CNS. The effects of potassium and other cations on acetylcholine liberation. *Biochemical J.,* **33,** 822-835.

Mantione, C. R., Demirgoren, S. and London, E. D. (1990). Polyamine binding site on synaptosomal membranes. *Neurosci. Abstr.,* **16,** 229.6.

Mao, C. C., Guidotti, A. and Costa, E. (1974). The regulation of cyclic guanosine monophosphate in rat cerebellum: possible involvement of putative amino acid neurotransmitters. *Brain Res.*, **79,** 510-514.

Maragos, W. F., Penney, J. B. and Young, A. B. (1988). Anatomic correlation of NMDA and ^{3}H-TCP-labelled receptors in rat brain. *J. Neurosci.*, **8,** 493-501.

Martin, D. and Lodge, D. (1970). Ketamine acts as a non-competitive N-methyl-D-aspartate antagonist on frog spinal cord in vitro. *Neuropharmcol.*, **24,** 999-1003.

Martin, D. and Lodge, D. (1987). Biphasic effect of quinolinate on frog spinal, but not rat cortical neurones: N-methyl-D-aspartate like depolarisations and a novel type of hyperpolaristion. *Neurosci. Lett.*, **75,** 175-180.

Marvizon, J. C. G., Lewin, A. H. and Skolnick, P. (1989). 1-Aminocyclopropane carboxylic acid: A potent and selective ligand for the glycine modulatory site of the N-methyl-D-aspartate receptor complex. *J. Neurochem.*, **52,** 992-994.

Marvizon, J. C. G. and Skolnick, P. (1988). ^{3}H-glycine binding is modulated by magnesium and other ligands of the NMDA receptor cation channel complex. *Eur. J. Pharmacol.*, **151,** 157-158.

Marvizon, J. C. and Skolnick, P. (1990). An endogenous modulator of N-methyl-D-aspartate receptor-coupled glycine receptors. *Eur. J. Pharmacol.*, **188,** 23-32.

Mayer, M. L., MacDermott, A. B., Westbrook, G. L., Smith, S. J. and Barker, J. L. (1987). Agonist- and voltage-gated calcium entry in cultured mouse spinal neurons under voltage clamp measured using arsenazo III. *J. Neurosci.*, **7,** 3230-3244.

Mayer, M. L. and Vyklicky, L. (1989). Concanavalin A selectively reduces desensitisation of mammalian neuronal quisqualate receptors. *Proc. Nat. Acad. Sci.*, **86,** 1411-1415.

Mayer, M. L., Vyklicky, L. and Clements, J. (1989). Regulation of NMDA receptor desensitisation in mouse hippocampal neurones by glycine. *Nature,* **338,** 425-428.

Mayer, M. L. and Westbrook, G. L. (1985). The action of N-methyl-D-aspartatic acid on mouse spinal neurones in culture. *J. Physiol.*, **361,** 65-90.

Mayer, M. L. and Westbrook, G. L. (1987). Permeation and block of N-methyl-D-aspartatic receptor channels by divalent cations in mouse cultured central neurones. *J. Physiol.*, **394,** 501-527.

Mayer, M. L., Westbrook, G. L. and Vyklicky, L. (1988). Sites of antagonist action on N-methyl-D-aspartic acid receptors studied using fluctuation analysis and a rapid perfusion technique. *J. Neurophysiol.*, **60,** 645-663.

McCulloch, R. M., Johnston, G. A. R., Game, C. J. A. and Curtis, D. R. (1974). The differential sensitivity of spinal interneurones and Renshaw cells to kainate and N-methyl-D-aspartate. *Exp. Brain Res.*, **21,** 515-518.

McDonald, J. W., Penney, J. B., Johnston, M. V. and Young, A. B. (1990). Characterization and regional distribution of strychnine-insensitive ^{3}H-glycine binding sites in rat brain by quantitative receptor autoradiography. *Neurosci.*, **35,** 653-668.

McKernan, R. M., Castro, S., Poat, J. A. and Wong, E. H. F. (1989). Solubilization of the N-methyl-D-aspartate receptor channel complex from rat and porcine brain. *J. Neurochem.*, **52,** 777-785.

McLennan, H., Huffman, R. D. and Marshall, K. C. (1968). Patterns of excitation of thalamic neurones by amino acids and by acetylcholine. *Nature,* **219,** 387-388.

McLennan, H. and Liu, J. R. (1982). The action of six antagonists of the excitatory amino-acids on neurons of the rat spinal-cord. *Exp. Brain Res.*, **45,** 151-156.

McLennan, H. and Lodge, D. (1979). The antagonism of amino acid-induced excitation of spinal neurones in the cat. *Brain Res.*, **169,** 83-90.

McNamara, J. O., Russell. R. D., Rigsbee, L. and Bonhaus, D. W. (1988). Anticonvulsant and antiepileptogenic actions of MK-801 in the kindling and electroshock models. *Neuropharmacol.*, **27,** 563-568.

Meldrum B. S. (1985). Excitatory amino acids and anoxic-ischaemic brain damage. *T. I. N. S.*, **8,** 47-48.

Meldrum, B. S., Croucher, M. J., Badman, G. and Collins, J. F. (1983a). Antiepileptic action of excitatory amino acid antagonists in the photosensitive baboon, Papio Papio. *Neurosci. Lett.*, **39** 101-104.

Meldrum, B. S., Croucher, M. J., Czuczwar, S. J., Collins, J. F., Curry, K., Joseph, M. and Stone, T. W. (1983b). A comparison of the anticonvulsant potency of (+/-) 2-amino-5-phosphono-pentanoic acid and 2-amino-7-phosphonoheptanoic acid. *Neuroscience.*, **9,** 925-930.

Meldrum, B. S. and Garthwaite, J. (1990). Excitatory amino acid neurotoxicity and neurogenerative disease. *T. I. P. S.*, **11,** 379-387.

Mena, E. E., Fagg, G. E. and Cotman, C. W. (1982). Chloride ions enhance L-

glutamate binding to rat synaptic membranes. *Brain Res.*, **243,** 378-381.

Mewett, K. N., Oakes, D. J., Olverman, H. J., Smith, D. A. S. and Watkins, J. C. (1983). Pharmacology of the excitatory actions of sulphonic and sulphinic acid amino acid. In "*CNS Receptors -from Molecular Pharmacology to Behaviour*", Mandel, P. and DeFeudis, F. V. (eds.), Raven Press, New York, pp 163-174.

Michaelis, E. K., Michaelis, M. L. and Boyarsky, L. L. (1974). High-affinity glutamate binding to brain synaptic membranes. *Biochem. Biophys. Acta.*, **367,** 338-348.

Michell, R. H. (1975). Inositol phospholipids and cell surface receptor function. *Biochem. Biophys. Acta.*, **415,** 81-147.

Mody, I. and Heinemann, U. (1987). NMDA receptors of dentate gyrus granule cells participate in synaptic transmission following kindling. *Nature,* **326,** 701-704.

Mody, I., Stanton, P. K. and Heinemann, U. (1987). Activation of N-methyl-D-aspartate receptors parallels changes in cellular and synaptic properties of dentate gyrus granule cells after kindling. *Neurophysiol.*, **59,** 1033-1054.

Monaghan, D. T., Bridges, R. J. and Cotman, C. W. (1989). The excitatory amino acid receptors. *Ann. Rev. Pharmacol. Toxicol.*, **29,** 365-402.

Monaghan, D. T. and Cotman, C. W. (1982). Distribution of ^{3}H-kainic acid binding sites in rat CNS as determined by autoradiography. *Brain Res.*, **252,** 91-100.

Monaghan, D. T. and Cotman, C. W. (1985). Distribution of N-methyl-D-aspartate-sensitive L-[^{3}H]-glutamate binding sites in rat brain. *J. Neurosci.*, **5,** 299-2919.

Monaghan, D. T., Holets, V. R., Toy, D. W. and Cotman, C. W. (1983). Anatomical distributions of four pharmacologically distinct ^{3}H-L-glutamate sites. *Nature,* **306,** 176-179.

Monaghan, D. T., Olverman, H. J., Nguyen, L., Watkins, J. C. and Cotman, C. W. (1988). Two classes of NMDA recognition sites: Differential distribution and differential regulation by glycine. *Proc. Natl. Acad. Sci. USA.*, **85,** 9836-9840.

Monaghan, D. T., Yao, D. and Cotman, C. W. (1984). Distribution of ^{3}H-AMPA binding sites in rat brain as determined by quantitative autoradiography. *Brain Res.*, **324,** 160-164.

Monaghan, D. T., Yao, D., Olverman, H. J., Watkins, J. C. and Cotman, C. W. (1984). Autoradiography of D-[^{3}H]2-amino-5-phosphonopentanoate binding-sites

in rat brain. *Neurosci. Lett.,* **52,** 253-258.

Monahan, J. B., Corpus, V. M., Hood, W. F., Thomas, J. W. and Compton, R. P. (1989). Characterization of ^{3}H-glycine recognition site as a modulatory site of the N-methyl-D-aspartate receptor complex. *J. Neurochem.,* **53,** No.2, 370-375.

Monahan, J. B., Hood, W. F., Compton, R. P., Cordi, A. A., Snyder, J. P., Pellicciari, R. and Natalini, B. (1990). Characterization of D-3,4-cyclopropylglutamates as N-methyl-D-aspartate receptor agonists. *Neurosci. Lett.,* **112,** 328-332.

Monahan, J. B., Hood, W. F., Michel, J. and Compton, R. P. (1988). Effect of guanine nucleotides on NMDA receptor ligand interactions. *Mol. Pharmacol.,* **34,** 111-116.

Monaghan, J. B. and Michel, J. (1987). Identification of an N-methyl-D-aspartate-specific L-[^{3}H]glutamate recognition site in synaptic plasma membranes. *J. Neurochem.,* **48,** 1699-1708.

Moncada, S. Palmer, R. M. J. and Higgs, E. A. (1989). Biosynthesis of nitric oxide from L-arginine. A pathway for the regulation of cell function and communication. *Biochem. Pharmacol.,* **38,** 1709-1715.

Morales, E. and Tapia, R. (1987). Neurotransmitters of the cerebellar glomeruli: uptake and release of labelled γ-aminobutyric acid, glycine, serotonin and choline in purified glomerulus fraction and in granular layer slices. *Brain Res.,* **420,** 11-21.

Morrisett, R. A., Chow, C., Nadler, J. V. and McNamara, J. O. (1989). Biochemical evidence for enhanced sensitivity to N-methyl-D-aspartate in the hippocampal formation of kindled rats. *Brain Res.,* **496,** 25-28.

Muller, D., Jolly, M. and Lynch, G. (1988). Contributions of quisqualate and NMDA receptors to the induction and expression of LTP. *Science,* **242,** 1694-1697.

Murphy, D. E., Schneider, J., Boehm, C., Lehmann, J. and Williams, M. (1987c). Binding of [^{3}H]3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid to rat brain membranes: a selective, high affinity ligand for N-methyl-D-aspartate receptors. *J. Pharmacol. Exp. Ther.,* **240,** 778-784.

Murphy, D. E., Snowhill, E. W. and Williams, M. (1987b). Characterization of quisqualate recognition sites in rat brain using DL-[^{3}H]alpha-amino-3-hydroxy-5-methylisoxazole-4-propionoic acid (AMPA) and a filtration assay. *Neurochem Res.*, **12,** 775-782.

Murphy, S. N. and Miller, R. J. (1988). A glutamate receptor regulates calcium mobilisation in hippocampal neurons. *Proc. Nat. Acad. Sci.*, **85,** 8737-8741.

Murphy, S. N., Thayer, S. A. and Miller, R. J. (1987a). The effects of excitatory amino acids on intracellular calcium in single mouse striatal neurons in vitro. *J. Neurosci.*, **7,** 4145-4158.

Nadler, V., Kloog, Y. and Sokolovsky, M. (1988). 1-Aminocyclopropane-1-carboxylic acid (ACC) mimics the effects of glycine on the NMDA receptor ion channel. *Eur. J. Pharmacol.*, **157,** 115-116.

Nadler, V., Kloog, Y. and Sokolovsky, M. (1990). Distinctive structural requirement for the binding of uncompetitive blockers (phencyclidine-like drugs) to the NMDA receptor. *Eur. J. Pharmacol., -Mol. Pharm. Sec.*, **188,** 97-104.

Nakagawa, Y., Saitoh, J., Ishihara, T., Ishida, M. and Shinozaki, H. (1990). (2S,3S,4S) alpha-(carboxycyclopropyl)glycine is a novel agonist of metabotropic glutamate receptors. *Eur. J. Pharmacol.*, **184,** 205-206.

Nakane, M., Ichikawa, M. and Deguchi, T. (1983). Light and electron microscopic demonstration of guanylate cyclase in rat brain. *Brain Res.*, **273,** 9-15.

Neal, M. J. (1971) The uptake of ^{14}C-glycine by slices of spinal cord. *J. Physiol.*, **215,** 103-117.

Neal, M. J. and Bowery, N. G. (1979). Differential effects of veratridine and potassium depolarisation on neuronal and glial GABA release. *Brain Res.*, **167,** 337-343.

Neal, M. J., Cunningham, J. R., James, T. A., Joseph, M. and Collins, J. F. (1981). The effect of 2-amino-4-phosphonobutyrate (APB) on acetylcholine release from rat retina: evidence of on-channel input to cholinergic amacrine cells. *Neurosci. Lett.*, **26,** 301-305.

Neher, E. and Steinbach, J. H. (1978). Local anaesthetics transiently block currents through single acetylcholine-receptor channels. *J. Physiol.*, **277,** 153-176.

Nicoletti, F. and Canonico, P. L. (1989). Glycine potentiates the stimulation of PI hydrolysis by excitatory amino acids in primary cultures of cerebellar neurones. *J. Neurochem.*, **53**, 724-727.

Nicoletti, F., Iadarola, M. J., Wroblewski, J. T. and Costa, E. (1986b). Excitatory amino acid recognition sites coupled with PI metabolism. Developmental changes and interaction with alpha adrenoceptors. *Proc. Natl. Acad. Sci.*, **83**, 1931-1935.

Nicoletti, F., Meek, J. F., Iadarola, M., Chang, D. M., Roth, B. L. and Costa, E. (1986a). Coupling of PI metabolism with excitatory amino acid recognition sites in rat hippocampus. *J. Neurochem.*, **46**, 40-46.

Niccoleti, F., Valerio, C., Pellegrino, C., Draggo, F., Scangnni, U. and Canonico, P. L. (1988). Spatial learning potentiates the stimulation of phosphoinositide hydrolysis by excitatory amino acids in rat hippocampus. *J. Neurochem.*, **51**, 725-729.

Nishizuka, Y. (1984). Turnover of PI lipids and signal transduction. *Science,* **225**, 1365-1370.

Nishizuka, Y. (1986). Studies and perspectives of protein kinase C. *Science,* **233**, 305-312.

Novelli, A., Nicoletti, F., Wroblewski, J. T., Alho, H., Costa, E. and Guidotti, A. (1987). Excitatory amino acid receptors coupled with guanylate cyclase in primary cultures of cerebellar granule cells. *J. Neurochem.*, **7**, 40-47.

Nowak, L., Ascher, P. and Berwald-Netter, Y. (1987). Ionic channels in mouse astrocytes in culture. *J. Neurosci. Res.*, **17**, 101-109.

Nowak, L., Bregestovski, P., Ascher, P., Herbet, A. and Prochiantz, Z. (1984). Magnesium gates glutamate-activated channels in mouse central neurones. *J. Nature,* **307**, 462-465.

Ogita, K. and Yoneda, Y. (1990). 6,7-Dichloroquinoxaline,2-3-dione is a competitive agonist specific to strychnine-insensitive ^{3}H-glycine binding sites on the N-methyl-D-aspartate receptor complex. *J. Neurochem.*, **54**, No.2, 699-702.

Oja, S. S., Kontro, P. and Lahdesmaki, P. (1977). Amino acids as inhibitory neurotransmitters. *Prog. Pharmacol.*, **1/3**, 1-119.

Oliver, M. W., Larson, J. and Lynch, G. (1990). Activation of the glycine site associated with the NMDA receptor is required for induction of LTP in neonatal hippocampus. *Int. J. Dev. Neurosci.*, **8**, 417-424.

Oliver, M. W., Shacklock, J. A., Kessler, M., Lynch, G. and Baimbridge, K. G.

(1990). The glycine site modulates NMDA-mediated changes of intracellular calcium in cultures of hipocampal neurones. *Neurosci. Lett.,* **114,** 197-202.

Olney, J. W. (1978). Neurotoxicity of excitatory amino acids. In "*Kanic Acid as a Tool in Neurobiology,*" Geer, E. G., Olney, J. W. and McGeer, P. L. (eds.), Raven Press, New York, pp 95-121.

Olney, J. W. (1979). Excitotoxic amino acids and Huntington's disease. In "*Advances of Neurology, Huntington's Disease -vol. 34*", Chase, T. N., Wexer, A. and Barbeau, A. (eds.), Raven Press, New York, pp 609-624.

Olney, J. W., Ho, O. L. and Rhee, V. (1971). Cytotoxic effects of acidic and sulphur containing amino acids on the infant mouse central nervous system. *Exp. Ther. Brain Res.,* **14,** 61-76.

Olney, J. W., Misra, C. H. nd Rhee, V. (1976). Brain and retinal damage from lathyrus excitotoxin, α-N-oxayl-L-α,β-diamino propionic acid. *Nature,* **264,** 659-661.

Olney, J. W., Price, M. T., Samson, L. and Labuyere, J. (1986). The role of specific ions in glutamate specificity. *Neurosci. Lett.,* **65,** 65-71.

Olsen, R. W. and Snowhill, E. W. (1983). [^{3}H]Bicuculline methochloride binding to low-affinity γ-aminobutyric acid receptor sites. *J. Neurochem.,* **41,** 1653-1663.

Olverman, H. J., Jones, A. W. and Watkins, J. C. (1984). L-glutamate has higher affinity than other amino-acids for [^{3}H]D-AP5 binding-sites in rat-brain membranes. *Nature,* **307,** 460-462.

Olverman, H. J., Monaghan, D. T., Cotman, C. W. and Watkins, J. C. (1986). [^{3}H]CPP, a new competitive ligand for NMDA receptors. *Eur. J. Pharmacol.* **131,** 161-162.

Orrego, F. (1979). Criteria for the identification of central neurotransmitters and their application to studies with some nerve tissue preparations *in vitro. Neuroscience,* **4,** 1037-1057.

Ottersen, O. P., Davanger, S. and Storm-Mathisen, J. (1987). Glycine-like immunoreactivity in the cerebellum of rat and Sengalese baboon, Papio Papio: a comparison with the distribution of GABA- like immunoreactivity and with ^{3}H-glycine and ^{3}H-GABA uptake. *Exp. Brain Res.,* **66,** 211-221.

Ottersen, O. P., Storm-Mathisen, J. and Laake, J. H.(1990). Cellular and subcellular localisation of glycine studied by quantitative electron microscopic immunocytochemistry. In "*Glycine Neurotransmission*", Ottersen, O. P. and Storm-Mathisen, J. (eds.), John Wiley and Sons Ltd, pp 281-303.

Ottersen, O. P., Storm-Mathisen, J. and Somogyi, P. (1988). Colocalisation of glycine like and GABA like immunoreactivities in Golgi cell terminals in the rat cerebellum. A postembedding light and electron microscopic study. *Brain Res.*, **450,** 342-353.

Palacios, J. M. and Kuhar, M. J. (1980). Beta-adrenergic receptor localization by light-microscopic autoradiography. *Science,* **208,** 1378-1380.

Palacios, J. M., Niehoff, D. L., and Kuhar, M. J. (1981). Receptor autoradiography with tritium-sensitive film: potential for computerised densitometry. *Neurosci., Lett.,* 111-116.

Palay, S. L. and Chan-Palay, V. (1974). *Cerebellar cortex.* Springer-Verlag, New York, Heidleberg, Berlin.

Palmer, E., Monaghan, D. T. and Cotman, C. W. (1988). Glutamate receptors and PI metabolism: stimulation via quisqualate receptors is inhibited by NMDA receptor activation. *Mol. Brain Res.,* **4,** 161-165.

Palmer, E., Monaghan, D. T. and Cotman, C. W. (1989). *Eur. J. Pharmacol.,* **166,** 585-587.

Parker, I. and Miledi, R. (1987). Inositol trisphosphate activates a voltage dependent calcium influx in xenopus oocytes. *Proc. R. Soc. London Ser.,* **B321,** 27-36.

Patel, J., Zinkard, ., Thompson, C., Keith, R. and Salama, A. (1990). Role of glycine in N-methyl-D-aspartate mediated neuronal cytotoxicity. *J. Neurochem.,* **54,** 849-854.

Pellegrini-Giampietro, D. E., Galli, A., Alesiani, M., Cherici, G. and Moroni, F. (1989). Quinoxalines interact with the glycine recognition site of NMDA receptors: Studies in guinea-pig myenteric plexus and in rat cortical membranes. *Br. J. Pharmacol.,* **98,** 1281-1286.

Penner, R., Mathews, G. and Neher, E. (1988). Regulation of calcium influx by second messengers in rat mast cells. *Nature,* **334,** 499-504.

Perequansky, M. and Grantyn, R. (1989). Separation of quisqualate and kainate selective glutamate receptors in culture neurons from the rat superior colliculus. *J. Neurosci.,* **9,** 70-80.

Pert, C. B., Kuhar, M. J. and Snyder, S. H. (1975). Autoradiographic

localization of the opiate receptor in rat brain. *Science,* **16,** 1849-1854.
Peters, S., Koh, J. and Choi, D. W. (1987). Zinc selectively blocks the action of N-methyl-D-aspartate on cortical neurons. *Science,* **236,** 589-593.

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., Simler, R., Grenningloh, G. and Betz, H. (1984). Monoclonal antibodies and peptide mapping reveal structural similarities between the subunits of the glycine receptor of the rat spinal cord. *Proc. Natl. Acad. Sci.,* **81,** 7224-7227.

Piercey, M. F., Hoffmann, W. E. and Kaczkofsky, P. (1988). Functional evidence for PCP-like effects of the anti stroke candidate MK801. *Psychopharmacology,* **96,** 561-562.

Polz-Tejera, G., Schmidt, J. and Karten, H. J. (1975). Autoradiographic localization of alpha-bungarotoxin binding sites in the central nervous system. *Nature,* **258,** 349-351.

Pratt, G. D. and Bowery, N. G. (1989) The 5-HT_3 receptor ligand ^{3}H-BRL 43694 binds to presynaptic sites in the nucleus tractus solitarias of the rat. *Neuropharmacology,* **28,** 1367-1376.

Price, G. W. and Bowery, N. G. (1988). $GABA_A$ and $GABA_B$ receptor site distribution: An overview. *ISI Atlas of Science: Pharmacology,* 136-140.

Probst, A., Cortes, R. and Palacios, J. M. (1986). The distribution of glycine receptors in the human brain. Light microscopic autoradiographic study using ^{3}H-strychnine. *Neurosci.,* **17,** No.1, 11-35.

Procter, A. W., Palmer, A. M., Francis, P. T., Lowe, S. L and Neary, D. (1988). Evidence of glutamergic denervation and possible abnormal metabolism in Alzheimer's disease. *J. Neurochem.,* **50,** 790-802.

Putney, J. W. (1987). Calcium mobilizing receptors. *T. I. P. S.,* **8,** 481-486.

Rakic, P. (1971). Neuron-glia relationship during granule cell migration in developing cerebellar cortex. A golgi and electronmicroscopic study in Macacus Rhesus. *J. Comp. Neurol.,* **141,** 283-312.

Ransom, R. W. and Deschenes, N. L. (1988). NMDA-induced hippocampal ^{3}H-noradrenaline release is modulated by glycine. *Eur. J. Pharmacol.,* **156,** 149-155.

Ransom, R. W. and Deschenes, N. L. (1989). Glycine modulation of NMDA evoked release of ^{3}H-acetylcholine and ^{3}H-dopamine from striatal slices. *Eur. J. Pharmacol.,* **96,** 323-328.

Ransom, R. W. and Stec, N. L. (1988). Cooperative modulation of ^{3}H-MK801 binding to NMDA receptor ion channel complex by glutamate, glycine and polyamines. *J. Neurochem.,* **51,** 830-836.

Rao, S. L. N. (1964). Chemical synthesis of N-α-oxayl-L-α,β-diaminopropionic acid and optical specificity in its neurotoxic action. *Biochemistry,* **14,** 5218-5220.

Rao, T. S., Kim, H. S., Lehmann, J., Martin, L. L. and Wood, P. L.(1990). Interactions of phencyclidine receptor agonist MK801 with dopaminergic system. Regional studies in the rat. *J. Neurochem.,* **54,** 1157-1162.

Rassendren, F. A., Lory, P., Pin, J. P. and Nargeot, J. (1990). Zinc has opposite effects on NMDA and non NMDA receptors expressed in Xenopus oocytes. *Neuron,* **4,** 733-740.

Recaseris, M., Varga, V., Nanopoulos, D., Sacidoun, f., Vincendon, G. and Benavides, J. (1982). Evidence for cysteine sulphate as a neurotransmitter. *Brain Res.,* **239,** 153-173.

Reyman, K. G. and Mathies, H. (1989). 2-amino-4-phosphonobutyrate selectively eliminates late phases of long term potentiation in rat hippocampus. *Neurosci. Lett.,* **98,** 166-171.

Reynolds, G. P., Pearson, S. J., Halket, J. and Sandler, M. (1988). Brain quinolinic acid in Huntington's Diseae. *J. Neurochem.,* **50,** 1959-1960.

Reynolds, I. J. (1990). Mechanisms underlying the interaction of polyamines with the NMDA receptor. *Neurosci. Abstr.,* **16,** 229.11.

Reynolds, I. J., Harris, K. M. and Miller, R. J. (1989). NMDA receptor antagonists that bind to the strychnine-insensitive glycine site and inhibits NMDA-induced Ca^{2+} fluxes and ^{3}H-GABA release. *Eur. J. Pharmacol. -Mol. Sect.,* **172,** 9-17.

Reynolds, I. J. and Miller, R. J. (1988). Multiple sites for the regulation of the NMDA receptor complex. *Mol. Pharmacol.,* **33,** 581-584.

Reynolds, I. J. and Miller, R. J. (1989). Ifenprodil a novel type of NMDA receptor antagonist: interaction with polyamines. *Mol. Pharmcol.*, **36,** 758-765.

Reynolds, I. J., Murphy, S. N. and Miller, R. J. (1987). ^{3}H-labelled MK-801 binding to the excitatory amino acid receptor complex from rat brain is enhanced by glycine. *Proc. Natl. Acad. Sci.*, **84,** 7744-7748.

Roa, T. S., Cler, J., Emmett, M. R., Mick, S., Iyenger, S., Contreras, P. C. and Wodd, P. L. (1989). Ifenprodil and SL 82.0715 antagonise N-methyl-D-aspartate (NMDA)-coupled glycine receptor responses in vivo. *Eur. J. Pharmacol.*, **168,** 129-131.

Robinson, G., Crooks, G., Shrinkman, P. and Gallagher, M. (1988). A behavioral effect of MK801 mimics a deficit associated with the hippocampal damage. *Soc. Neurosci. Abstr.*, **14,** 248.

Robinson, M. B., Blakley, R. D. and Coyle, J. T. (1986). Quisqualate selectivity inhibits a brain peptidase which cleaves N-acetyl-l-aspartyl glutamate *in vivo*. *Eur. J. Pharmacol.*, **130,** 345-347.

Rondouin, G., Drain, M-J., Chicheportiche, R., Kamenka, J-M. and Privat, A. (1988). Non-competitive antagonists of NMDA receptor protect cortical and hippocampal cell cultures against glutamate neurotoxicity. *Neurosci. Lett.*, **91,** 199-203.

Roth, L. J., Diab, I. M., Wantanabe, M. and Dinerstein, R. J. (1974). A correlative radioautographic, fluorescent and histochemical technique for cytopharmacology. *Mol. Pharmac.*, **10,** 986-998.

Rothman, S. M. and Olney, J. W. (1986). Glutamate and the pathology of hypoxic ischemic brain damage. *Ann. Neurol.*, **19,** 105-111.

Rothman, S. M. and Olney, J. W. (1987). Excitotoxicity and the NMDA receptor. *T. I. N. S.*, **10,** 299-302.

Rothman, S. M., Thruston, J. H. and Hauhart, R. E. (1987). Delayed neurotoxicity of excitatory amino acids in vitro. *Neurosci.*, **22,** 471-480.

Rubin, R. P. (1970). The role of calcium in the release of neurotransmitters substances and hormones. *Pharmacol. Rev.*, **22,** 389-428.

Sacaan, A. I. and Johnson, K. M. (1989). Spermine enhances binding to the glycine site associated with the NMDA receptor complex. *Mol. Pharmacol.*, **36,** 836-839.

Salt, T. E. (1989). Modulation of NMDA receptor-mediated responses by glycine and D-serine in the rat thalamus in vivo. *Brain Res.*, **481,** 403-406.

Salt, T. E., Wilson, D. G. and Prasas, S. K. (1988). Anatgonism of NMDA and synaptic responses of neurones in the rat ventrobasal thalamus by ketamine and MK801. *Br. J. Pharmacol.*, **94,** 443-448.

Sato, K., Morimoto, K. and Okamoto, M. (1988). Anticonvulsant action of a non-competitive antagonist of NMDA receptors (MK-801) in the kindling model of epilepsy. *Brain Res.*, **463,** 12-20.

Schneider, E. P-A., Fischer, R., Clements, F., Balzereit, E-W., Funfgeld, D. and Hasse, H. J. (1984). Effects of oral memantine on symptoms of Parkinson's disease. *Dtsch. Med. Wschr.*, **109,** 987-989.

Schoemaker, H., Allen, J. and Langer, S. Z. (1990). Binding of ^{3}H-ifenprodil, a novel NMDA antagonist, to polyamine-sensitive site in the rat cerebral cortex. *Eur. J. Pharmacol.*, **176,** 249-250.

Schoepp, D. D. and Johnson, B. G. (1988). Excitatory amino acid agonist-antagonist interactions at 2-amino-4-phosphonobutyric acid-sensitive quisqualate receptors coupled to phosphoinositide hydrolysis in slices of rat hippocampus. *J. Neurochem.*, **50,** 1605-1613.

Scmidt, B. H., Weiss, S., Sebben, M., Kemp, D.E., Bockert, J. and Sladeczek, F. (1987). Dual action of excitatory amino acid on the metabolism of PI in striatal neurones. *J. Pharmacol. Exp. Ther.*, **32,** 364-368.

Scmidt, W. J. and Bubser, M. (1989). Anti-cataleptic effect of the NMDA antagonist MK801. *Pharmacol. Biochem. Behav.*, **32,** 621-623.

Scwartzkroin, P. and Waster, K. (1975). Long lasting facilitation of synaptic potential following tetanization in the in vivo hippocampal slice. *Brain Res.*, **89,** 107-119.

Seragor, E., Kuhn, D., Vyklicky, L. and Mayer, M. L. (1989). Open channel block of NMDA receptor responses evoked by tricyclic antidepressants. *Neuron,* **2,** 1221-1227.

Sheardown, M. J., Drejer, J., Jensen, L. H., Stidsen, C. E. and Honore, T. (1989). A potent antagonist of the strychnine insensitive glycine receptor has anticonvulsant properties. *Eur. J. Pharmacol.*, **174,** 197-204.

Shinozaki, H. and Konishi, S. (1970). Actions of several anthelmintics and insecticides on rat cortical neurones. *Brain Res.*, **24,** 368-371.

Shinozaki, H., Shibuya, I. (1974). New potent excitatant, quisqualic acid-effects on crayfish neuromuscular junction. *Neuropharmacology,* **13,** 665-672.

Simon, J. R., Contrera, J. F. and Kuhar, M. J. (1976). Binding of [^{3}H]kainic acid, an analogue of L-glutamate to brain membranes. *J. Neurochem.*, **26,** 141-147.

Simon, R. P., Swan, J. H., Griffiths, T. and Meldrum, B. S. (1984). Blockade of NMDA receptors may protect against ischaemic damage in the brain. *Science,* **226,** 850-852.

Singh, L., Donald, A. E., Foster, A. C., Hutson, P. H., Iversen, L. L., Iversen, S. D., Kemp, J. A., Leeson, P. D., Marshall, G. R., Oles, R. J., Priestley, T., Thorn, L., Tricklebank, M. D., Vass, C. A. and Williams, B. J. (1990b). Enantiomers of HA-966 (3-amino-1-hydroxypyrrolode-2-one) exhibit distinct central nervous system effects: (+)-HA966 is a selective glycine/N-methyl-D-aspartate receptor antagonist, but (-)-HA966 is a potent γ-butyrolactone-like sedative. *Proc. Natl. Acad. Sci.,* **87,** 347-351.

Singh, L., Oles, R. J. and Tricklebank, M. D. (1990a). Modulation of seizure susceptibility in the mouse by the strychnine-insensitive glycine recognition site of the NMDA receptor/ion channel complex. *Br. J. Pharmacol.,* **99,** 285-288.

Sircar, R., Frusciante, M. J., Javitt, D. C. and Zukin, S. R. (1989). Glycine reverses 7chlorokynurenic acid induced inhibition of ^{3}H-MK801 binding. *Brain Res.,* **504,** 325-327.

Sircar, R. and Zukin, S. R. (1985). Quantitative localization of [^{3}H]TCP binding in rat brain by light microscopic autoradiography. *Brain Res.,* **344,** 142-145.

Skilling, S. R., Smullin, D. H., Betz, A. J. and Larson, A. A. (1988). Extracellular amino acid concentrations in the spinal cord of freely moving rats following veratridine and nociceptive stimulation. *J. Neurochem.,* **51** 127-132.

Sladeczek, F., Pin, J. P., Recasens, M., Bockaert, J. and Weiss, S. (1985). Glutamate stimulates inositol phosphate formation in striatal neurones. *Nature,* **317,** 717-719.

Slater, N. T., Stezler, A. and Galvan, M. (1985). Kindling-like stimulus patterns induce epileptiform discharges in the guinea pig in vitro hippocampus. *Neurosci. Lett.,* **60,** 25-31.

Slather, W., Johnson, J. W., Henderson, G. and Ascher, P. (1990). Glycine insensitive desensitization of NMDA responses in cultured mouse embryonic neurones. *Neuron,* **4,** 725-731.

Slaughter, M. M and Miller, R. F. (1981). 2-Amino-4-phosphonobutyric acid: a new pharamcological tool for retina research. *Nature,* **211,** 182-185.

Slevin, J. T., Collins, J. F. and Coyle, J. T. (1983). Analogue interactions with the brain receptor labelled by kainic acid. *Brain Res.*, **265,** 169-172.

Smith, S. J. (1987). Progress on LTP at hippocampal synapses. A post synaptic Ca trigger for memory storage. *T. I. N. S.*, **10,** 142-144.

Snell, L. D. and Johnson, K. M. (1988). Cycloleucine competitively antagonizes the strychnine-insensitive glycine receptor. *Eur. J. Pharmacol.*, **151,** 165-166.

Snell, L. D., Morter, R. S. and Johnson, K. M. (1987). Glycine potentiates N-methyl-D-aspartate induced ^{3}H-TCP binding to rat cortical membranes. *Neurosci. Lett.*, **83,** 313-317.

Snell, L. D., Morter, R. S. and Johnson, K. M. (1988). Structural requirements for activation of the glycine receptor that modulates the N-methyl-D-aspartate operated channel. *Eur. J. Pharmacol.*, **156,** 105-110.

Snodgrass, S. R. (1979). In vitro binding studies with ^{3}H-NMDA. *Soc. Neurosci.*, **5,** 572 (Abstr.).

Stone, T. W. and Perkins, M. N. (1981). Quinolinic acid: a potent endogenous excitant at amino acid receptors in CNS. *Eur. J. Pharmacol.*, **72,** 411-412.

Storm-Mathisen, J. (1990). Glycine in the hippocampus. *2nd Spring Hippocampal Reaearch Conference, Grand Cayman, April 21-27 1990.*

Stringer, J. L., Greenfield, L. J., Hackett, J. T. and Guyenet, P. G. (1983). Blockade of long term potentiation by phencyclidine and opiates in the hippocampus in vivo and in vitro. *Brain Res.*, **280,** 127-138.

Sugiyama, H., Ito, I. and Hirono, C. (1987). A new type of glutamate receptor linked to inositol phospholipid metabolism. *Nature,* **325,** 531-533.

Swartz, K.J., Martin, J. B. and Beal, M. F. (1988). Glycine potenatiates NMDA response mediated quinolinic neurotoxicity in rat striatum. *Soc. Neurosci. Abstr., 14,* 97.

Tam, S. W. and Cook, L. (1984). *Sigma* opiates and certain antipsychotic drugs mutually inhibit (+)-[^{3}H]SKF 10,047 and [^{3}H]haloperidol binding in guinea pig membranes. *Proc. Natl. Acad. Sci. USA.*, **81,** 5618-5621.

Tang, C. M., Dichter, M. and Morad, M. (1989). Quisqualate activates a rapidly inactivating high conductance ionic channel in hippocampal neurons. *Science.*, **243,** 1474-1477.

Tauck, D. and Ashbeck, G. A. (1990). Glycine synergistically potentiates the enhancement of LTP induced by a sulfhydril reducing agent. *Brain Res.*, **519,** 129-132.

Taxt, T. and Storm-Mathisen, J. (1984). Uptake of D-aspartate and L-glutamate in excitatory axon terminals in hipppocampus: autoradiographic and biochemical comparison with γ-aminobutyrate and other amino acids in normal rats and in rats with lesions. *Neurosci.*, **11,** 79-100.

Tebecis, A. K. and Dimaria, A. (1972). Strychnine sensitive inhibition in the medullary reticular formation: Evidence for glycine as an inhibitory neurotransmitter. *Brain Res.*, **40,** 373-383.

Terrian, D. M., Rea, M. A. and Dorman, R. V. (1988). Relationship between prostoglandin synthesis and release of acidic amino acid neurotransmitters. *Aviat. Space. Environ. Med.*, **59,** (11 Pt 2). A2-9.

Thomas, J. W., Hood, W. F., Monahan, J. B., Contreras, C. and O'Donohue, T. L. (1988). Glycine modulation of the phencyclidine binding site in mammalian brain. *Brain Res.*, **442,** 396-398.

Thompson, A. M., Walker, V. E. and Flynn, D. M. (1989). Glycine enhances NMDA-receptor mediated synaptic potentials in neocortical slices. *Nature,* **338,** 422-424.

Thomson, A. M., West, D. C. and Lodge, D. (1985). An N-methylaspartate receptor-mediated synapse in rat cerebral cortex: a site of action of ketamine. *Nature,* **313,** 479-481.

Traynelis, S. F. and Dingledine, R. (1988). Potassium-induced spontaneous electrographic seizures in the rat hippocampal slice. *J. Neurophysiol.*, **59,** 259-276.

Triller, A., Cluzeaud, 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.

Trussell, L., Thio, L. L., Zorumski, C. F. and Fishbach, G. D. (1988). Rapid desensitization of glutamate receptors in vertebrate central neurones. *Proc. Natl. Acad. Sci.*, **85,** 4562-4566.

Turski, W. A., Herrling, P. L. and Do, K. Q. (1987). Effects of L-cysteine-sulphinate and L-aspartate, mixed excitatory amino acid agonists, on the membrane potential of cat caudate neurons. *Brain Res.*, **414,** 330-338.

Turski, W. A., Nakamura, M., Todd, W., Carpenter, B. K., Whetsell, W. O. and Schwarcz, R. (1988). Identification and quantification of kynurenic acid in human brain tissue. *Brain Res.*, **454**, 164-169.

Usherwood, P. N. R. and Duce, I. R. (1985). Antagonism of glutamate receptor channel complexes by spider venom polypeptides. *Neurotox.*, **6**, 239-250.

de Vente, J., Bol, J. G. J. M. and Steinbush, H. W. M. (1989). Localisation of cGMP in the cerebellum of the adult rat: an immunohistochemical study. *Brain Res.*, **504**, 332-337.

Verdoon, T. A. and Dingledine, R. (1988). Antagonist pharmacology of excitatory receptors expressed in xenpus oocytes, *Soc. Neurosci.*, **14**, 1193. (Abstr.).

Vezzani, A., Serafini, R., Samanin, R. and Foster, A. C. (1990). Autoradiograhical analysis of excitatory amino acid binding sites in rat hippocampus during the development of hippocampal kindling. *Brain Res.*, **526**, 113-121.

Vignon, J., Chicheportiche, R., Chicheporte, M., Kamenka, J. M., Geniste, P. and Lazdunski, M. (1983).[^{3}]TCP: a new tool with high affinity for the PCP receptor in rat brain. *Brain Res.*, **280**, 194-197.

Vyklicky, L., Benveniste, M. and Mayer, M. L. (1990). Modulation of N-methyl-D-aspartic acid receptor desensitization by glycine in mouse cultured hippocampal neurones. *J. Physiol.* **428**, 313-331.

Vyklicky, L., Krusek, J., and Edwards, C. (1988). Differences in the pore sizes of the N-methyl-D-aspartate and kainate cation channels. *Neurosci. Lett.*, **89**, 313-318.

Vyklicky, L., Krusek, J., Vyklicky, L. and Vyskocil, F. (1986). Spider venom of Araneus opens and desensitizes glutamate channels in chick spinal cord neurones. *Neurosci. Lett.*, **68**, 227-231.

Walther, H., Lambert, J. D. C., Janis, R. S. G., Heineman, U. and Hanan, B. (1986). Epileptiform activity in combined slice and hippocampus, subiculum and entorhinal cortex during perfusion with low magnesium medium. *Neurosci. Lett.*, **69**, 156-161.

Waser. P. G. and Luthi, U. (1962). Uber die Fixiering von ^{14}C-curarin in der Endplatte. *Helv. Physiol. Acta.*, **20**, 237-251.

Watkins, J. C. (1962). The synthesis of some acidic amino acids possessing neuropharmacological activity. *J. Med. Pharm. Chem.*, **5**, 1187-1199.

Watkins, J. C. (1989). The NMDA receptor concept: origins and development.

In "*The NMDA Receptor*", Watkins, J. C. and Collingridge, G. L. (eds.), Oxford University Press, Oxford, pp 1-18.

Watkins, J. C., Korgsgaard-Larsen, P. and Honore, T. (1990). Structure-activity relationships in the development of excitatory amino acid receptor agonists and competitive antagonists. *T. I. P. S.*, **11**, 25-33.

Watson, G. B., Wood, W. F., Monahan, J. B. and Lantharn T. H. (1988). Kynurenate antagonises actions of N-methyl-D-aspartate through a glycine sensitive receptor. *Neurosci. Res. Commun.*, **2**, 169-166.

Weber, E., Sonders, M., Quarum, M., McLean, S., Pou, S. and Keana, J. F. W. (1986). 1,3,-Di(2-[5-^{3}H]tolyl)guanidine: A selective ligand that labels sigma type receptors for psychotomimetic opiates and antipsychotic drugs. *Proc. Natl. Acad. Sci. USA.*, **83**, 8784-8788.

Weiloch, T. (1985). Hypoglycemia-induced neuronal damage prevented by an NMDA antagonist. *Science,* **230**, 681-683.

Weiss, J. H., Koh, J. Y. and Choi, D. W. (1988). Neurotoxicity of β-N-methylamino-L-alanine (BMAA) and β-N-oxalylamino-L-alanine (BOAA) on cultured cortical neurones. *Brain Res.*, **497**, 64-71.

Weiss, S., Kemp, D. E., Bauce, L. and Tse, F. W. (1990). Kainate receptors coupled to the evoked release of [^{3}H]-gamma-aminobutyric acid from striatal neurons in primary culture: potentiation by lithium ions. *Mol. Pharmacol.*, **38**, 229-236.

Werman, R., Davidoff, R. A. and Aprison, M. H. (1968). Inhibitory action of glycine on spinal neurones in the cat. *J. Neurophysiol.*, **31**, 81-95.

Westbrook, G. L. and Mayer, M. L. (1987). Micromolar concentrations of Zn^{2+} antagonize NMDA and GABA responses of hippocampal neurones. *Nature,* **328**, 640-643.

Westbrook, G. L. Mayer, M. L., Namboodiri, M. A. A. and Neale, J. H. (1986). High concentrations of N-acetylaspartylglutamate (NAAG) selectively activate NMDA receptors on mouse spinal cord neurons in cell culture. *J. Neurosci.*, **6**, 3385-3392.

Westerberg, E., Monaghan, D. T., Cotman, C. W. and Wieloch, T. (1987). Excitatory amino acid receptors and ischemic brain damage in the rat. *Neurosci. Lett.*, **73**, 119-124.

White, W. F., Brown, K. L., Frank, D. M. (1989). Glycine binding to rat cortex and spinal cord: Binding characteristics and pharmacology reveal distinct population of sites. *J. Neurochem.,* **53,** 503-512.

White, W. F., Nadler, J. V., Hamberger, A., Cotman, C. W. and Cummins, J. T. (1977). Glutamate as a transmitter of the hippocampal perforant path. *Nature,* **270,** 356-357.

Wilkin, G. P., Csillag, A., Balazs, R., Kingsbury, A. E., Wilson, J. E. and Johnson, A. L. (1981b). Localisation of high affinity ^{3}H-glycine transport sites in the cerebellar cortex. *Brain Res.,* **216,** 11-33.

Wilkin, G. P., Hudson, A. L., Hill, D. R. and Bowery, N. G. (1981a). Autoradiographic localisation of $GABA_B$ receptors in rat cerebellum. *Nature,* **294,** 584-587.

Williams, J. H. and Bliss, T. V. (1988). Induction but not maintenance of calcium-induced long-term potentiation in the dentate gyrus and area CA1 of the hippocampal slice is blocked by nordihydroguaiaretic acid. *Neurosc. Lett.,* **88,** 81-85.

Wilson, J. E., Wilkin, G. P. and Balazs, R. (1976). Metabolic properties of a purified preparation of large fragments of the cerebellar glomeruli: glucose metabolism and amino acid uptake. *J. Neurochem.,* **26,** 957-965.

Wolff, S. P., Garner, A. and Dean, R. T. (1986). Free radicals, lipids and protein degradation. *T. I. B. S.,* **11,** 27-31.

Wong, E. H. F., Castro, C., McKernan, R. M. and Poat, J. A. (1988). Solubilisation of the N-methyl-D-aspartate recptor from rat brain. *Br. J. Pharmacol.,* **94,** 322P.

Wong, E. H. F., Kemp, J. A., Priestley, T., Knight, A. R., Woodruff, G. N. and Iverson, L. L. (1986). The anticonvulsant MK-801 is a potent N-methyl-D-aspartate antagonist. *Proc. Natl. Acad. Sci. USA.,* **83,** 7104-7108.

Wong, E. H. F., Knight, A. R. and Ranson, R. (1987). Glycine modulates [^{3}H]-MK-801 binding to the NMDA receptor in rat brain. *Eur. J. Pharmacol.,* **142,** 487-488.

Woodhull, A. M. (1973). *J. Gen. Physiol.,* **61,** 687-708.

Woodruff, G. N., Foster, A. C., Gill, R., Kemp, J. A., Wong, E. H. F. and Iverson, L. L. (1987). The interaction between MK-801 and receptors for N-methyl-D-aspartate: functional consequences. *Neuropharmacology,* **26,** 903-909.

Wroblewski, J. T. and Danysz, W. (1989). Modulation of glutamate receptors: molecular mechanisms and functional implications. *Ann. Rev. Pharmacol. Toxicol.,* **29,** 441-474.

Yamamoto, C., Sawada, S. and Takada, S. (1983). Suppressing action of 2-amino-4-phosphonobutyric acid on mossy fibre induced excitation in the guinea pig hippocampus. *Exp. Brain Res.,* **51,** 128-134.

Yeh, G. C., Bonhaus, D. W. and McNamara, J. O. (1990). Evidence that zinc inhibits NMDA receptor-gated ion channel activation by non competeitive antagonism of glycine binding. *Mol. Pharmacol.,* **38,** 14-19.

Yeh, G. C., Bonhaus, D. W., Nadler, J. V. and McNamara, J. O. (1989). N-methyl-D-aspartate receptor plasticity in kindling: Quantitative and qualitative alterations in the N-methyl-D-aspartate receptor channel complex. *Proc. Natl. Acad. Sci.,* **86,** 8157-8160.

Young, A. B., Greenamyre, J. T., Hollingsworth, Z., Albin, R. and D'Amato, C. (1988a). NMDA receptor loses in putamen from patients with Huntington's disease. *Science,* **241,** 981-983.

Young, A. B. and Snyder, S. H. (1973). Strychnine binding site associated with glycine receptors of the central nervous system. *Proc. Natl. Acad. Sci. USA.,* **70,** 2832-2836.

Young, A. M. J., Crowder, J. M. and Bradford, H. F. (1988b). Potentiation by kainate of excitatory amino acid release in striatum: complementary *in vivo* and *in vitro* experiments. *J. Neurochem.,* **50,** 337-345.

Young, W. S. and Kuhar, M. J. (1979). Noradrenergic α-1 and α-2 receptors: autoradiographic visualization. *Eur. J. Pharmacol.,* **59,** 317-319.

Zaczek, R., Balm, M., Arlis, S., Drucker, H. and Coyle, J. T. (1987). Quisqualate-sensitive chloride-dependent transport of glutamate into rat brain synaptosomes. *J. Neurosci. Res.,* **18,** 425-431.

Zarbin, M. A., Wamsley, J. K. and Kuhar, M. J. (1981). Glycine receptor: light microscope autographic localization with ^{3}H-strychnine. *J. Neurosci.,* **1,** 532-547.

Zhu, S. G., McGeer, E. G. and McGeer, P. L. (1989). Effect of MK801, kynurenate, glycine, dextrophan and 4-acetylpyridine on striatal toxicity of quinolinate. *Brain Res.,* **481,** 356-360.

Zwiller, J., Ghandour, M. S., Revel, M. O. and Basset, P. (1981). Immunohistochemical localisation of guanylate cyclase in rat cerebellum. *Neurosci. Lett.,* **23,** 31-36.