CHARACTERISTICS OF ~~THE~~ NMDA RECEPTOR ~~COMPLEX~~ IN ~~MOUSE~~ BRAIN ~~UNDER PHYSIOLOGICAL AND PATHOLOGICAL CONDITIONS~~

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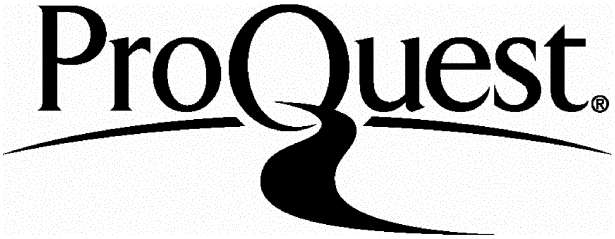

Abstract

In the present study permanent MCA-O in mouse was used to investigate the temporal progression of the focal infarction and to evaluate the extent of protection offered by antagonists acting at different sites of the NMDA receptor complex. The neuroprotective effect of various doses of dizocilpine was compared to the effect produced by CGP 37849, a competitive antagonist at the NMDA receptor complex and compound Z, a novel antagonist at the glycine site of the NMDA receptor complex. An 8-arm radial maze was also used to compare the effect of systemic administration of ligands for the glycine site with the effect produced by dizocilpine on spatial orientation.

Systemic administration of the glycine antagonist compound Z either before or after MCA-O resulted in a significant neuroprotective effect comparable to that obtained with dizocilpine. Also, compound Z and the partial agonist, HA-966, did not produce any impairment in radial maze performance, indicating a better side effect profile over dizocilpine.

If the NMDA receptor complex is to be a target for neuroprotective agents administered post-ischaemia, it is important to determine not only the time course of the functional integrity of the receptor but also the post-ischaemia period during which drugs can gain access to the receptors in the region of the infarct. Thus, we have studied the *ex vivo* distribution of ^{3}H-dizocilpine binding sites in mouse brain after

MCA-O and have compared this with the *in vitro* distribution of ^{3}H-dizocilpine binding sites at different times after MCA-O, using receptor autoradiography.

Although the population of NMDA receptors is maintained in the infarct region for a relatively long time, access to them *in vivo* appears to be sufficiently impaired within 2 or 4 hours of ischaemic insult.

Another important purpose of the present study was to verify whether it is possible to modulate the NMDA receptor complex under physiological conditions. Thus, the influence of modulators of NMDA receptor function on the *ex vivo* binding of ^{3}H-dizocilpine in normal mouse brain has been examined using receptor autoradiography to detect subtle changes in the regional levels of binding. Although significant changes in the levels of specific *ex vivo* ^{3}H-dizocilpine binding were produced by D-serine and D-cycloserine, it is suggested that *ex vivo* ^{3}H-dizocilpine binding does not represent a sensitive marker for modulation of the NMDA receptor complex *in vivo*.

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" Aver conosciuto voi resta probabilmente il risultato piu' bello che ho ottenuto in questi tre anni. Grazie."

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Abbreviations

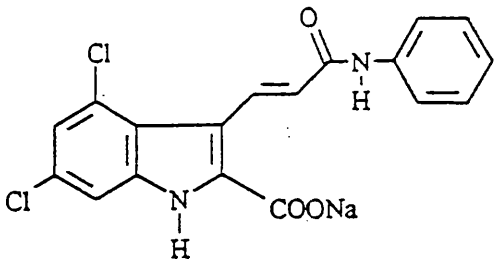
ACBC	aminocyclobutane carboxylic acid
ACCP	1-amino-1,3-dicarboxycyclopentane
ACEA 1021	5-nitro-6,7-dichloro-1,4-dihydro-2,3-quinoxalinediones
ACPC	aminocyclopropane carboxylic acid
AMPA	α-amino-3-hydroxy-5-methyl-isoxazole propanoic acid
AP4	2-amino-4-phosphonobutanoate
AP5	2-amino-5-phosphonopentanoate
AP7	2-amino-7-phosphonoheptanoate
CBF	cerebral blood flow
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-pentanoic acid
CGS 19755	1-cis-2-carboxypiperidine-4-yl-methyl-1-phosphonic acid
7-CKYNA	7-chlorokynurenic acid
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CNS	central nervous system
CPP	3-(-)-2-carboxypiperazine-4-yl-)propyl-1-phosphonic acid
CPP-ene	3-(-)-2-carboxypiperazine-4-yl)propenyl-1-phosphonic acid
CSF	cerebral spinal fluid
DAA	D-α-aminoadipate
DAP	D-aminopimelic acid
DNQX	6,7-dinitroquinoxaline-2,3-dione
EAA	excitatory amino acid

EPSP	excitatory post-synaptic potential
GABA	γ-aminobutyric acid
GTP	guanosine-3,5-triphosphate
GYKI	1-(4-aminophenyl)-4-methyl-7,8,methyenedioxy-5H-2,3- benzodiazepine
HA-966	3-amino-1-hydroxy-2-pyrrolidone
IP_3	inositol-1,4,5-triphosphate
IPSP	inhibitory post-synaptic potential
K_d	dissociation constant
KBP	kainate binding protein
L 687,414	R(+)-cis-β-methyl-3-amino-1-hydroxypyrrolid-2-one
LTP	long term potentiation
LY 233053	cis-4-(2H-tetrazol-5-yl)-methylpiperidine-2-carboxylic acid
MCA-O	middle cerebral artery occlusion
MK-801	(+)-5-methyl-10-11-dihydro-5H-dibenzo-(a,d)cyclohepten-5-10-imine maleate
MNQX	6,8,dinitroquinoxaline
mV	millivolt
NAAG	N-acetyl-aspartyl glutamate
NBQX	2,3-dihydroxy-6-nitro-sulphamoil-benzo(F)quinoxaline
NGF	nerve growth factor
NMDA	N-methyl-D-aspartate
NMLA	N-methyl-L-aspartate
NMMA	N-monomethyl-L-arginine
NPC 12626	2-amino-4,5-(1,2-cyclohexyl)-7-phosphonoheptanoic acid
NO	nitric oxide
NO_2	nitrogen oxide
ODC	ornithine decarboxylase

PCP	phencyclidine
PI	phosphoinositol
PKC	protein kinase C
SKF 10047	N-allylnormetazocine
TCP	N-(1-thienyl)-cyclohexyl-3,4-piperidine
1S,3R-ACPD	trans-1-amino-cyclopentyl-1,3-dicarboxylic acid
TRIS	2-amino-2-hydroxymethylpropane-1,3-diol
4-VO	4-vessel occlusion
2-VO	2-vessel occlusion
VSSC	voltage sensitive calcium channel

Compound Z = GV150526A

GV150526A is a structurally novel indole derivative with high affinity and high selectivity for the glycine site associated with the NMDA receptor complex. Its chemical structure is **((E)-3[(phenylcarbamoyl)-4,6-dichloroindole-2-carboxylic acid sodium salt)**:



Chemical structure of GV 150526A

CHAPTER 1: GENERAL INTRODUCTION

1.1. Excitatory amino acids

1.1.2. Historical developments

Since the pioneering studies in the 1950s on the effects exhibited by L-glutamate and L-aspartate on the central nervous system, the scientific world has experienced an exponential growth in excitatory amino acids (EAA) research. Most of this growth has occurred over the past decade. Up to ten years ago EAAs were regarded only as putative neurotransmitters and the pharmacology of EAA responses was largely unknown. Today, L-glutamate is accepted as the major excitatory neurotransmitter in the central nervous system, implicated in most of the primary physiological processes as well as in a number of neurodegenerative disorders in mammalian brain.

From the earliest electrophysiological studies, neurones were seen to display regional variations in sensitivity to a range of agonist amino acids (Curtis and Watkins, 1960, 1963; McLennan et al., 1968: Duggan, 1974; Johnston et al., 1974). Structure-activity studies showed that, for depolarising activity at EAA receptors, the basic amino group and acidic carboxyl group attached at the α-carbon need to be separated by a chain of 3 to 6 carbon atoms from a second acidic group (Curtis and Watkins, 1960). Data comparing agonist profiles allowed division of EAA receptors into subtypes defined by selective agonists: N-methyl-D-aspartate (NMDA), quisqualate and kainate (Watkins and Evans, 1981). Regional differences in potency between kainate and quisqualate indicated different sites of action for these two agonists (Watkins and Evans, 1981), although in the absence of selective antagonists to discriminate between the two receptor classes, electrophysiologists divided glutamate responses into NMDA and non-

NMDA types (Davies et al., 1982). The division into NMDA, kainate and quisqualate receptor subtypes was challenged by data showing quisqualate to inhibit ^{3}H-kainate binding activity (Unnerstall and Wamsley, 1983). This poor selectivity of quisqualate turned attention toward the more selective ibotenate derivative, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA; Krogsgaard-Larsen et al., 1980; 1984) which is now regarded as a selective ligand for an EAA receptor subtype with low affinity for kainate or NMDA sites (Honore' et al., 1982; Mayer and Viklicky, 1989). Current evidence suggests that at least five EAA receptor types exist, all with significantly distinct functions and defined by the selective ligands, NMDA, kainate and AMPA, which are ionotropic receptors (defined on the basis of their respective selective antagonists) and L-AP4 (2-amino-4-phosphonobutyrate) which was suggested from its antagonistic profile at excitatory synapses in brain and spinal cord (Foster and Fagg, 1984). More recently, glutamate has also been shown to activate metabotropic receptors (mGluRs) which are linked to G proteins. Work on mGluRs was previously hampered by the lack of specific agonists and antagonists; however, the recent identifications of such molecules has now made it possible to characterize more fully the specific roles these receptors play in brain function (Pin and Bockaert, 1995; Pin and Duvoisin, 1995).

1.1.2. Distribution

Glutamate is the most abundant free aminoacid in the central nervous system occurring as a charged molecule in mammalian brain tissue (Mora et al., 1984) and appearing to

be located predominantly in nerve endings, with a smaller glial pool (Aoki et al., 1987). Glutamate is specifically associated with synaptic vesicles in the target zones of excitatory afferents (Bramham et al., 1990). Thus, glutamate-like immunoreactivity is most abundant in hippocampal and cortical neurones, lateral septum and in the molecular layer of the cerebellum, in close proximity to its high affinity uptake sites (Wilkin et al., 1982; Storm-Mathisen et al., 1983; Schmidt and Wolf, 1988).

1.2.3. Release.

Ca^{++}-dependent glutamate release has been shown in neurones *in vivo* (Bliss et al., 1986; Butcher and Hamberger, 1987), in brain slices (Corradetti et al., 1984; Jarvie et al., 1990) and synaptosomes (Abdul-Ghani, 1985; Geula et al., 1988), in accordance with a neurotransmitter role. Accordingly, depolarisation or mobilisation of protein kinase C seem to trigger the opening of Ca^{++} channels and exocytosis of glutamate from vesicles (Kihara et al., 1989; Nicholls and Atwell, 1990). A second form of glutamate release, independent of Ca^{++}, has been demonstrated from non-vesicular sites and appears to use the same carrier as high-affinity EAA uptake (Koyama et al., 1989). Such reversal of uptake may be functional and has been considered to contribute to the pathology in perinatal ischaemia (Silverstein et al., 1986; Nicholls and Atwell, 1990).

1.1.4. Uptake.

Neurochemical and autoradiographic studies of the transport of exogenous glutamate into nerve terminals and glia show regional and pharmacological heterogeneity. For example, dihydrokynate inhibits EAA uptake in the striatum while D-(-)-α-aminoadipate (D-αAA) selectively antagonises uptake in the cerebellum (Ferkany and Coyle, 1986) where glial uptake predominates. High-affinity, Na^+-dependent transporters in neurones and glia throughout the CNS have Michaelis constants in the range of 1-50 μM (Nicholls and Atwell, 1990) and energy for the process is provided by a trans-membrane Na^+ gradient (Ferkany and Coyle, 1986). When uptake blockers are applied, or when energy supplies are compromised, as occurs during anoxia and ischaemia, uptake fails and glutamate could reach toxic concentrations (McBean and Roberts, 1985; Silverstein et al., 1986; Novelli et al., 1988, Henneberry et al., 1989). Under these conditions, ongoing efflux or "leakage" of glutamate can be detected by microdialysis (Bradford et al., 1987; Benveniste and Huttemeier, 1990).

Neurones and glia also possess further low-affinity uptake systems which recognise a large range of amino acids, and these may be important since their blockade potentiates responses to glutamate (Nicholls and Atwell, 1990). Considering the high levels of glutamate released and its cytotoxic potential (Olney et al., 1971), EAA uptake systems, therefore, might play an important role in protecting neurones from the neurodegenerative effects of this amino acid.

1.2. AMPA receptors

AMPA receptors activate channels which are permeable to Na^+ and K^+ but

impermeable to Ca^{++} (Ascher and Nowak, 1988b)

although subsequent findings have suggested the exisence of AMPA-operated Ca^{++} channels on interneurones (Ozawa, 1991). The kynurenate derivatives 6,7-dinitroquinoxaline-2,3-dione (DNQX) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) show some selectivity in antagonising AMPA responses over those to kainate and NMDA (Blake et al., 1988; Honore' et al., 1988), although these compounds also seem to interact competitively with the strychnine-insensitive glycine receptor (Birch et al., 1988a; Kessler et al., 1989). The antagonist 2,3-dihydroxy-6-nitro-sulphamoyl-benzo(F)quinoxaline (NBQX) has higher potency at AMPA receptors than CNQX and DNQX in electrophysiological and binding studies (Sheardown et al., 1990), and shows anticonvulsant activity in mice (Turski et al., 1992). The muscle relaxant, 1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5H-2,3-benzodiazepine (GYKI 52466) is also more selective for AMPA than kainate-mediated effects (Tarnawa et al., 1990). Binding studies clearly show separate high-affinty sites for kainate and AMPA, with further low-affinity AMPA sites being sensitive to kainate (Honore' et al., 1982; Krogsgaard-Larsen et al.,1984). Molecular biology has been used to isolate, clone and express a family of AMPA/kainate receptors (Wenthold et al., 1990; Gasic and Hollman, 1992)(see below). NMDA receptors and AMPA receptors occur in close proximity and they have been suggested to cooperate in producing epileptiform activity *in vitro* (Dingledine et al., 1990). However, while NMDA-induced seizures are blocked by antagonists at the NMDA receptor glycine site, AMPA seizures are blocked by DNQX but not NMDA antagonists (Schoepp et al., 1990b). The AMPA antagonists NBQX and GYKI 52466 are potent anticonvulsants (Chapman, 1991; Smith and Meldrum, 1992; Turski

et al., 1992) and NBQX also exhibits anxiolytic activity in rats and mice (Turski et al., 1992). Since AMPA is a neurotoxic EAA (Lewis et al., 1990), CNQX or DNQX have been used to block the neurotoxicity of glutamate *in vitro* (Frandsen et al., 1989). Similarly, the more specific AMPA antagonist, NBQX, appears to reduce cerebral infarction following ischaemia (Buchan et al., 1991), although this protection might partly arise from blockade of the glycine site associated with the NMDA receptor complex (Birch et al., 1988).

1.3. Kainate receptors

Kainate was originally found to mimic glutamate in depolarising cortical neurones (Shinozaki and Konishi, 1970) and its high potency at EAA receptors was accentuated by the lack of uptake mechanisms for kainate (Johnston et al., 1974). Systemic kainate produced remote brain lesions after generalised seizures, and injections of kainate could also produce discrete local lesions (Collins and Olney, 1982; Coyle, 1983). The first generation of agonists at the kainate receptor included domoate, kainate, 5-bromowillardine and acromelic acid (Davies et al., 1982). A range of kainate derivatives have been found to produce different effects *in vivo* and to possess different potencies in exciting spinal motoneurones and dorsal root fibers, which may be evidence for subtypes of the kainate receptor (Ishida and Shinozaki, 1991). Molecular cloning studies also provide evidence for kainate receptor heterogeneity (Egebjerg et al., 1991). Phenobarbitone seems to protect neurones from neurotoxicity produced by kainate rather than AMPA (Frandsen et al., 1990), and methohexitone and Ni+ preferentially

block kainate responses over those of AMPA (Lodge et al., 1991), although barbiturates do not generally discriminate between kainate and AMPA responses (Simmonds and Horne, 1988). A receptor responding to kainate but not AMPA has been cloned and expressed in *Xenopus* oocytes, and this receptor is coupled to a Ca^{++}-permeable ionophore (Egebjerg et al., 1991). Functional studies indicate Ca^{++} entry mediated by kainate receptors (Iino et al., 1990) and the action of Ni^{+} as a channel blocker is also consistent with Ca^{++} conductance arising from activation of kainate receptors (Lodge et al., 1991).

1.4. AP4 receptors

Synaptic depolarisation in the rat hippocampus, lateral olfactory tract, spinal cord and piriform cortex can be antagonised by AP4, presumably by a presynaptic action depressing glutamate release (Koerner and Cotman, 1981; Evans et al., 1982; Anson and Collins, 1987; Hasselmo and Bower, 1991). Glutamate and AP4 have been shown to inhibit voltage-dependent Ca^{++} channels, thus inhibiting further glutamate release and the consequent excitation of the hippocampal perforant path synapse (Forsythe and Clements, 1990; Abe et al., 1992). AP4 does not inhibit binding of ^{3}H-glutamate in the brain (Koerner and Cotman, 1981; Fagg and Lanthorn, 1985) and binding of ^{3}H-AP4 appears to be associated to presynaptic elements (Fagg and Matus, 1984) and to be attenuated by guanine nucleotides (indicating linkage to G proteins, Butcher et al., 1986). AP4 is a non-competitive antagonist of EAA-induced PI turnover (Schoepp et al., 1990a) and it has recently been shown to possess agonist properties at type IV

metabotropic receptors (Thomsen et al., 1992). Presynaptic metabotropic AP4 receptors which inhibit glutamate release may be the mechanism for neuroprotection by the metabotropic agonist 1S,3R-ACPD (Chiamulera et al., 1992).

1.5. Metabotropic receptors

Metabotropic EAA receptors are linked to G proteins and mediate atypical electrical responses with slow onset and oscillating wave-forms (Sladeczek et al., 1985; Nicoletti et al., 1986). These receptors participate in excitatory synaptic transmission, although they have also been found to block excitatory transmission by a presynaptic mechanism (Baskys, 1992). The "metabotropic" effect of glutamate is the increased hydrolysis of membrane phosphoinositides (PI) by phospholipase C to produce diacylglycerol and inositol-1,4,5-triphosphate (IP3) (Michell, 1975). IP3 produces an increase in the intracellular concentration of Ca^{++} by an action within the endoplasmic reticulum, while diacylglycerol activates protein kinase C (Berridge, 1987; Putney 1987). Agonists for EAA receptors coupled to PI turnover include glutamate, quisqualate, 1S,3R-1-amino-cyclopentyl-1,3-dicarboxylic acid (1S,3R-ACPD), AP4 and ibotenate. Depolarization and PI responses produced by 1S,3R-ACPD in rat cerebral cortex were competitively antagonised by (S)-4-carboxyphenylglycine and (RS)-α-methyl-4-carboxyphenylglycine (Eaton et al., 1993). Metabotropic EAA receptors allow the entry of Ca^{++} through channels sensitive to blockade by Cd^{++} and Zn^{++} but not other channel blockers (Guiramand et al., 1991b) and the localization of these receptors has been visualised by autoradiography, using a cocktail of agonist drugs to inhibit binding to the ionotropic receptors (Cha et al., 1990). Regional variations in the sensitivity of

metabotropic receptors to agonists and antagonists suggest heterogeneity within this receptor group (Recasens et al., 1987; Schoepp et al., 1990). The cloning of 8 genes coding for metabotropic glutamate receptors, of which several generate different mRNAs by alternative splicing, proves the validity of this suggestion. Because molecular cloning has preceded pharmacological characterization in the identification of novel metabotropic glutamate receptors, it has provided the basis for a simple nomenclature: mGluRs are numbered following the order in which their cDNAs have been cloned (mGluR1 to mGluR8). mGluRs possess seven putative transmembrane domains but have no sequence homology with any known G-protein-coupled receptor. Expression studies confirmed the existence of PLC-coupled and L-AP4-sensitive mGluRs, but also revealed mGluRs negatively coupled to adenylate cyclase. Several agonists have provided a way of pharmacologically discriminating receptors from the different groups and, recently, some competitive antagonists for mGluRs have been developed (Watkins and Collingridge, 1994). mGluRs have been shown to be involved in many brain functions including the induction of synaptic plasticity phenomena, long term potentiation and long term depression. They are also likely to play a role in modulating Glu-induced neurotoxicity (Pin and Bockaert, 1995)

1.6. NMDA receptors

Amongst the different classes of EAA receptors, the NMDA receptor complex is the best characterised. It is associated with a cation selective channel that gates K^+, Na^+ and Ca^{++} and comprises a number of distinct pharmacological domains through

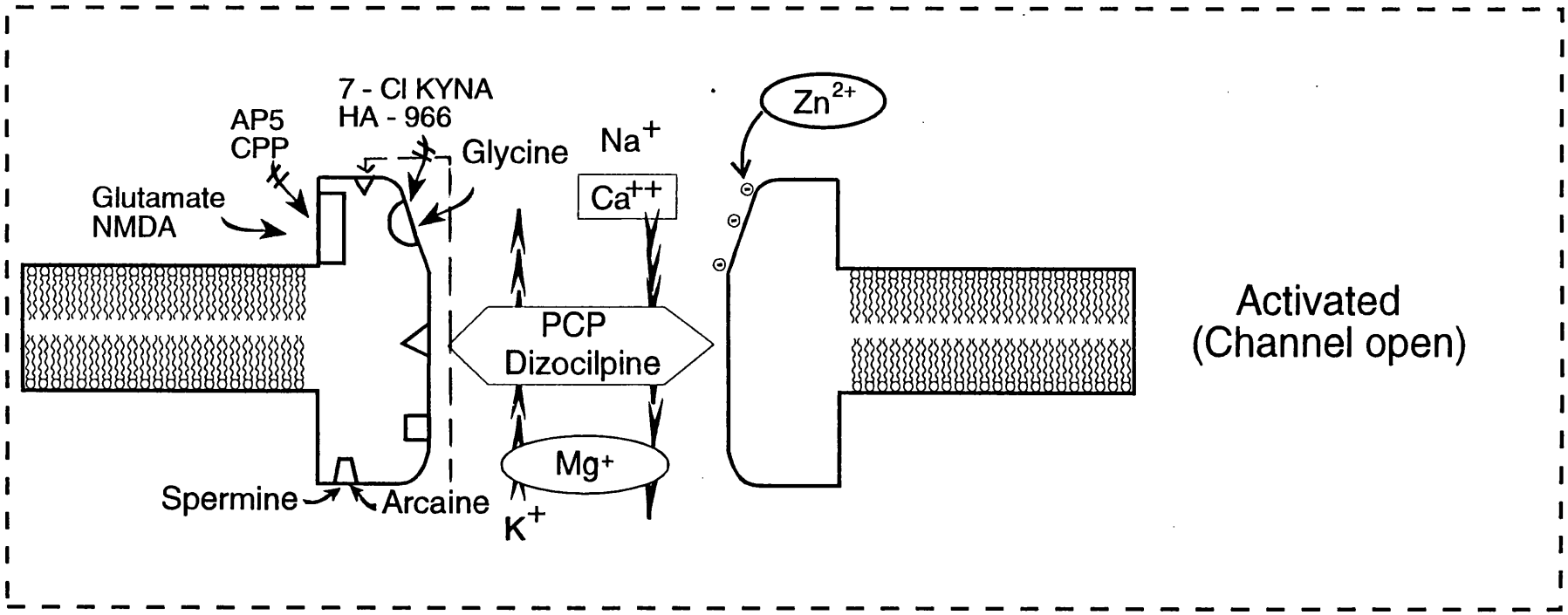
which its function can be modulated. In addition to the transmitter recognition site, the complex also has a strychnine-insensitive glycine site, a Mg^{++} recognition site, a regulatory site for zinc distinct from that of magnesium, a channel located site for phencyclidine (and related compounds) and a polyamine site. (Fig. 1.1).

1.6.1. Glutamate recognition site

Glutamate, NMDA, 1-amino-1,3-dicarboxycyclopentane (ACCP), and some sulphur-containing amino acids are agonists at the primary acceptor site and ibotenate, quinolinate and N-acetyl-aspartyl-glutamate (NAAG) are also weak agonists (Monahan and Michel, 1987; Monahan et al.,1990; Curtis et al., 1961; Johnston et al., 1968; Martin and Lodge, 1987). A number of selective competitive glutamate antagonists have been developed since the introduction of the early antagonists D-aminopimelic acid (DAP) and D-α-aminoadipate (DAA)(Evans et al., 1978; Collingridge and Davies, 1979). They include D-2-amino-5-phosphonovalerate (AP5), D-2-amino-7-phosphonovalerate (AP7) and the structurally rigid analogue of AP7, 3-(±)-2-carboxypiperazin-4-yl)propyl-1-phosphonate (CPP)(Davies et al., 1986; Lehman et al., 1987). The next generation of competitive NMDA antagonists include the CPP equivalent of AP5, CGS 19755 (1-cis-2-carboxypiperidine-4-yl-methyl-1-phosphonate, CPP-ene (3-(-)-2-carboxypiperazine-4-yl)propenyl-1-phosphonate), NPC 12626 2-amino-4,5-(1,2-cyclohexyl)-7-phosphonoheptanoic acid, CGP 37849 (DL-α-2-amino-4-methyl-5-phosphono-3-pentanoic acid, LY 233053 (Cis(±)-4-(2H-tetrazol-5-yl)-methylpiperidine-2-carboxylic acid (Ferkany et al., 1989; Lehman et al., 1988; Herrling et al., 1989; Fagg et al., 1990; Lowe et al., 1990).

Fig.1.1. Schematic illustration showing the various sites of allosteric interaction at the NMDA receptor channel complex

Explanation of the sites and mechanisms are provided in the text.


7 - Cl KYNA
HA - 966
AP5
CPP
Glycine
Na+
Zn2+
Glutamate
NMDA
Ca++
PCP
Dizocilpine
Mg+
Spermine
Arcaine
K+
Activated
(Channel open)

The ability of ^{3}H-glutamate to bind selectively to a site associated with the NMDA receptor has been questioned following evidence of interaction with other types of EAA receptors (Monahan and Michel, 1987; Foster and Fagg, 1984, 1987). ^{3}H-NMDA has also been of little use as an NMDA receptor ligand and radiolabelled antagonists such as ^{3}H-AP5, ^{3}H-CPP and ^{3}H-CGS 19755, in general, exhibit features comparable to ^{3}H-glutamate binding in terms of anatomical distribution and pharmacological specificity (Olverman et al., 1986; Monaghan et al., 1984, 1988). However, detailed analysis of the binding properties of ^{3}H-glutamate and ^{3}H-CPP has uncovered regionally distinct agonist- and antagonist-preferring binding sites (Monaghan et al., 1988). It is still unclear whether this is a reflection of two classes of NMDA recognition sites or two states of the same site.

1.6.2. Divalent cations

Ion channels associated with NMDA receptors have been shown to be selectively blocked by Mg^{++} in a voltage dependent manner (Ault et al., 1980; Mayer et al., 1984). Physiological concentrations of Mg^{++} in the extracellular fluid are well above those required to produce the block of NMDA receptor-mediated response. Thus, only when the membrane is depolarized (above -50 to -30mV) for prolonged periods do NMDA mediated responses become apparent, due to the reduction in the block produced by Mg^{++}. This confers a unique feature to the NMDA receptor activation, which is particularly important to fulfill specialized physiological roles such as induction of LTP (Collingridge and Bliss, 1987).

Electrophysiological and neurotoxic responses to NMDA are attenuated by Zn^{++} which has been shown to act both at voltage-dependent and -independent sites (Westbrook and Mayer, 1987). Tricyclic antidepressant such as imipramine produce weak antagonism of NMDA responses, possibly by acting at the same site as Zn^{++} (Reynolds and Miller, 1988). However, the voltage-sensitive feature of their blockade suggests that they might act simply as channel blockers (Seragor et al., 1989). The voltage-dependent site is suggested to lie within the ion channel. From binding kinetics, the voltage-independent site does not appear to be associated with thiol chelation but perhaps reflects an interaction with extracellular histidine residues (Legendre and Westbroke, 1990). This conclusion is supported by the observation that irreversible reaction of histidine residues with diethylpyrocarbonate appears to reduce the effectiveness of Zn^{++} inhibition of NMDA responses. Zn^{++} has no effect on NMDA-sensitive glutamate binding, but is able to reduce ion channel ligand binding by a decrease in association and dissociation rates (Wong et al., 1988; Yeh et al., 1990). Interestingly, Zn^{++} has been reported to inhibit NMDA receptors via a non-competitive interaction with the glycine site (Yeh et al., 1990). The fact that Zn^{++} inhibits glycine binding and glycine stimulation of ^{3}H-TCP binding, indicates a possible physiological regulation of the tonic excitatory influence of glycine by synaptically released Zn^{++} (Yeh et al., 1990). The ability of Zn^{++} to reduce NMDA-mediated neurotoxicity might constitute an important negative feed-back mechanism to control excessive synaptic excitation during disease states involving prolonged exposure to glutamate (Koh and Choi, 1988).

1.6.3. Dissociative anaesthetic binding site

Non-competitive antagonism of NMDA receptors by the dissociative anaesthetics phencyclidine and ketamine occurs via the ion channel associated with the receptor complex (Anis et al., 1983). The phencyclidine-like compounds 1-(1-(2-thienyl)-cyclohexyl)-piperidine (TCP) and (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]-cyclohepten-5,10-imine (MK801 or dizocilpine) selectively bind to the NMDA ionophore (Largent et al., 1986; Wong et al., 1986). Such molecules can only reach their site of action when the channel is activated, thus producing agonist- or use-dependent antagonism (Wong et al., 1986). Binding of ^{3}H-TCP and ^{3}H-dizocilpine is increased by NMDA receptor agonists and decreased by antagonists, showing that these ligands bind preferentially to the open, or activated ion channel (Foster and Wong, 1987; Woodruff et al., 1987; Huettner and Bean, 1988). These studies highlighted the possibility of utilising the binding of labeled non competitive antagonists that only bind to the activated state of the receptor as an assay for activation and blockade of the NMDA receptor complex. The sensitivity of the binding of these channel blockers to receptor activation and conformational change has allowed the characterization of other modulatory sites on the NMDA receptor complex.

1.6.4. Polyamine site

Endogenous polyamines such as spermine and spermidine affect many areas of cell biology, since they bind to DNA and RNA, promote membrane fusion for vesicular release and bind to and modify the function of many enzymes (Schuber, 1989). Spermine and spermidine increase the binding of ^{3}H-dizocilpine by a mechanism

enhanced by glutamate and glycine and blocked by NMDA antagonists (Ransom and Stec, 1988). The inability of spermine or spermidine to inhibit ^{3}H-glycine or ^{3}H-CPP binding argues for a distinct site of action. The neuroprotective agent, ifenprodil, and related phenylethanolamines, are suggested to be antagonists at a polyamine site associated with the NMDA receptor complex (Rao et al., 1989; Beart et al., 1991). Ifenprodil and its analogue SL 82.0715 have been shown to antagonize the NMDA-induced elevation of cGMP levels in rat cerebellar slices and to inhibit ^{3}H-CPP and ^{3}H-TCP binding in a non competitive manner (Carter et al., 1988). However, the complicated interaction between ifenprodil and the NMDA receptor complex is indicated by the biphasic displacement curve of ifenprodil in a ^{3}H-dizocilpine binding assay (Reynolds and Miller, 1989). Studies with radiolabeled ^{3}H-ifenprodil have shown this binding to be partially inhibited by some competitive NMDA antagonists and completely by the polyamines spermine and spermidine (Schoemaker et al., 1985). While the precise nature of the interaction between ifenprodil and the NMDA receptor complex remains unclear, studies on the action of endogenous polyamines suggest a possible mechanism for endogenous modulation of this receptor complex, possibly through a coupling with the enzyme ornithine decarboxylase (ODC, Koenig et al., 1989).

1.6.5. Glycine site

Glycine has long been established as an inhibitory neurotransmitter within the mammalian spinal cord, interacting at a receptor site selectively recognized by strychnine (Aprison and Werman, 1965; Tebecis and DiMaria, 1972). The post-synaptic

membrane hyperpolarisation produced by glycine has been regarded as a feedback mechanism employed by many inhibitory interneurones in the spinal cord to regulate inhibitory tone (Curtis, 1963). While receptors for GABA, the other major inhibitory aminoacid, were localised in the forebrain and cortex (Bowery et al., 1987), 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). Subsequently, receptor binding studies demonstrated the existence of a specific and high affinity glycine binding site in higher centres of rat brain (Kishimoto et al., 1981; Bristow et al., 1986). These sites are insensitive to strychnine, implicating the existence of at least two different glycine receptors. Electrophysiological studies described a close link between strychnine-insensitive glycine sites and NMDA receptors (Johnson and Ascher, 1987). Their investigations on patch-clamp cultured cells revealed that the endogenous amino acid glycine was capable of selectively potentiating NMDA responses in a concentration dependent manner (Johnson and Ascher, 1987). This effect was strychnine-insensitive and thus unrelated to the classical inhibitory glycine receptor. Single channel analysis showed that glycine potentiated the NMDA response by increasing the frequency of channel opening without affecting conductance or duration of open time (Johnson and Ascher, 1987). Parallel to these observations, in autoradiographic studies a nearly identical regional distribution between ^{3}H-glycine binding sites and NMDA-sensitive ^{3}H-glutamate binding site (Bowery et al., 1987; Bristow et al., 1986) was noticed. These studies had also shown that the D-isomers of serine and alanine had higher affinities for this glycine binding site than the L-isomers, with D-serine being only slightly less active than glycine itself. Since D-serine has very

low affinity for both the glycine uptake systems and the strychnine-sensitive glycine receptor, it became the agonist of choice in pharmacological studies (Johnson and Ascher, 1987; Kemp et al., 1988). From binding studies it is known that NMDA receptor ligands can regulate binding of the (radiolabelled) non-competitive NMDA antagonists,TCP and dizocilpine, by controlling their access to the receptor-coupled ion channel (Snell et al., 1987; Wong et al., 1987). On this basis it was demonstrated that NMDA receptor activation is regulated by glycine, but requires the presence of NMDA agonists (Bonhaus et al., 1989). Important data derived from electrophysiological studies in Xenopus oocytes indicated that glycine is essential for activation of NMDA receptors (Kleckner and Dingledine, 1988). This led to the suggestion that glycine may act as a coagonist at the NMDA receptor complex such that agonist binding at both glutamate- and glycine-recognition sites is required for channel activation.

Some selective antagonists at the strychnine-insensitive glycine site of the NMDA receptor complex were immediately reported. HA966, first designed as a GABA agonist, has been shown to act selectively at the glycine site by blocking glycine potentiation of NMDA responses, but with very little effect in the absence of added glycine (Kemp et al., 1988). It has been suggested that HA966 represents a low-efficacy partial agonist at the glycine site of the NMDA receptor complex (Foster and Kemp, 1989) and this was further substantiated by Kemp and Priestley (1991).

Kynurenic acid, a broad spectrum EAA receptor antagonist, has been shown to inhibit, competitively, strychnine-insensitive ^{3}H-glycine binding to rat brain membranes (Kessler et al., 1989) and part of its NMDA blocking effect in tissue slices could be reversed by the addition of glycine (Watson et al., 1988). The analogue 7-

chlorokynurenic acid has been shown to have a much higher selective affinity for the glycine site of the NMDA receptor complex, producing a complete block of NMDA responses on cortical neurones in tissue culture, including those elicited in the absence of added glycine (Kemp et al., 1988). In intact cortical tissue, the full antagonist 7-chlorokynurenic acid produced a reduction of NMDA responses, and this effect could be reversed by glycine and D-serine (Kemp et al., 1988). 5,7-dichlorokynurenic acid has been shown to be even more potent in this respect, both in cortical wedge brain slices and neonatal hemisected spinal cord of the rat (Leeson et al., 1991).

Over the past few years, the search to identify novel compounds acting at the glycine site has led to the development of a number of selective partial agonists and full antagonists and these will be discussed in detail in Chapter 4.

1.7. STRUCTURAL CHARACTERIZATION OF EAA RECEPTORS

1.7.1. Isolation and purification

Numerous biochemical, biophysical and, more recently, molecular biological techniques have been used to characterize diverse aspects of EAA receptor size, structure and function. Using both ionic and non-ionic detergents, EAA receptors have been solubilized from vertebrate CNS tissue. Binding studies on kainate receptors solubilized from *Xenopus* CNS revealed a similar agonist affinity in both solubilized and non-solubilized tissue (Henley and Barnard, 1989), suggesting that the kainate receptor configuration is little disturbed by isolation from its lipid matrix. The active purified receptor complex migrated on Sepharose gel filtration with a molecular weight of

570kDa (Hampson et al., 1989). AMPA binding sites have also been solubilized and partially purified from rat brain (Hunter et al., 1990) and they have been reported to migrate with a molecular weight of 425kDa on gel filtration chromatography. This high molecular weight, which approaches that of purified kainate receptors, suggests that these proteins are either very heavily glycosilated or form (or naturally exist in membrane as) dimeric pairs under solubilizing conditions (Hampson and Wenthold, 1988). With the exception of CNQX, agonists and antagonists bind with higher affinity to the solubilized AMPA protein than to membrane-bound receptor.

The NMDA receptor complex has been solubilized from rat and porcine brains by numerous workers (Ambar et al., 1988, McKernan et al., 1989 Ogita and Yoneda, 1990b). In these studies the solubilized receptor bound ^{3}H-dizocilpine, ^{3}H-TCP and other non-competitive antagonists with a similar rank order but, with the exception of SKF 10047, with a slightly lower affinity to that recorded in membrane-bound protein (although in several of these studies it was observed that high detergent concentrations inhibited the level of total binding). As with membrane-bound receptors, non-competitive binding could be modulated by AP5, glycine and polyamines. However, Zn^{2+} which is recognised to bind to a voltage-independent site, was 100 times more potent in inhibiting ^{3}H-dizocilpine binding to the solubilized receptor than to the membrane-bound form (McKernan et al., 1989). This inhibitory site for Zn^{++} may normally be masked by non-specific membrane-associated charges, or may simply be located in a part of the protein complex that is made more accessible by solubilization. In a different study, significant differences were noted in the modulation of ^{3}H-CPP binding by Mg^{++} or glycine and it was suggested that the agonist and antagonist

recognition sites might in fact be located on different but closely interacting receptor subunits (Cunningham and Michaelis, 1990). More recent investigations have suggested that the NMDA receptor complex comprises four different subunits of molecular weight 70kDa, 58kDa, 42kDa, 36kDa and all four proteins are required to be associated (Ly and Michaelis, 1991). The association does not appear to be covalent (disulphide-linked) and the 42 kDa subunit appears to be more loosely associated to the other receptor subunits. Using sucrose density gradient centrifugation and size exclusion chromatography of the solubilized and purified receptor, it has been shown that the strychnine-insensitive glycine binding site is located on the 42kDa protein (Michaelis, 1991).

The interrelationship between binding sites (and possibly receptor subunits) is complex. Using comparative autoradiography in rat brain it has been noted that, relative to ^{3}H-glutamate, ^{3}H-CPP binding was low in the striatum and septum and high in the thalamus and inner cerebral cortex (Monaghan et al., 1988). It has been suggested that the NMDA receptor might exist in either an agonist- or antagonist- preferring conformation or that these agonist- and antagonist- preferring regions might represent distinct receptor subtypes (Monaghan et al., 1988).

In general, in the interaction of glutamate, glycine and their antagonists a reciprocal relationship has been observed. That is, the glutamate antagonist CGS 19755 decreased the affinity of glycine agonists for their receptor. Conversely, the affinity of glycine antagonists was increased by the addition of CGS 19755 (Hood et al., 1990). Further support for the reciprocal binding relationship of glycine and competitive NMDA antagonists comes from evidence that glycine and D-serine inhibit the binding of ^{3}H-

CGS 19755 (Kaplita and Ferkany, 1990). A molecular model which fits the observed data suggests that the NMDA receptor has four independent amino acid binding sites, and two molecules of both glutamate and glycine are required to bind to the NMDA receptor for channel activation to occur (Benveniste and Mayer, 1991), although some criticisms of this model have been made.

The transmembrane spanning pore dimension for both NMDA and non-NMDA receptors has been investigated by comparing the permeabilities of different size cations (Viklicky et al., 1988). On the basis of the ionic selectivity of the NMDA receptor for Na^+ and Ca^{++} it has been suggested that the outer vestibule (and inner surface) of the NMDA receptor complex is lined with numerous anionic sites which stabilizes and "concentrates" divalent ions more than monovalent ions (Johnson and Ascher , 1990).

1.7.2. Cloning and sequencing EAA receptors

In 1989, a cDNA clone representing an EAA receptor channel was isolated from a library of rat forebrain cDNA (Hollman et al., 1989). Upon transcription and expression in *Xenopus* oocytes, the clone gave rise to a protein forming a functional receptor channel which possessed pharmacological and electrophysiological properties similar to the mammalian kainate receptor. Furthermore, the brain distribution of mRNA coding for this complex matched the distribution of kainate binding sites visualised by autoradiography (Gall et al., 1990; Keinanen et al., 1990). It was suggested that whilst the protein expressed in the oocytes may exist *in vivo* as a homo-oligomeric receptor-channel complex, it may also function as a subunit of hetero-oligomeric receptor-

channel complexes. In this way it would combine *in vivo* with other subunits to form native glutamate receptor-channel complexes with different characteristics (Hollman et al., 1989). Further probing has led to the identification of seven other mammalian non-NMDA subunits and two kainate binding proteins (KBPs)(Nakanishi and Masu, 1994). Based on amino acid sequence homology and pharmacological characteristics, the mammalian subunits have been grouped into three classes. The first group comprises the first four subunits discovered, GluR1 to GluR4 (Hollman et al., 1989; Nakanishi et al., 1990; Keinanen et al., 1990; Boulter et al., 1990). Amino acids sequence homology in the four conserved membrane spanning domains of these receptors is between 80 and 90%. The other subunits, GluR5-GluR7 (Bettler et al., 1990; Bettler et al., 1992) show 30- 40% sequence homology with GluR1 to GluR4 and so became members of a separate, second class of subunits (Egebjerg et al., 1991; Nakanishi and Masu, 1994). Kainate binding proteins KA-1 and KA-2 have been isolated from frog brain (Wada et al., 1989) and chick brain (Gregor et al., 1989), but did not form functional homomeric ion channels in transfected cells when expressed alone or in combination with subunits GluR1 to GluR4 (Werner et al., 1991). GluR5 and GluR6 are homomeric channels activated by kainate but not AMPA. However, when either is co-expressed with KA-2, greater responses are recorded and the heteromeric channels respond to both AMPA and Kainate (Egebjerg and Heineman, 1993). In summary, it appears that the close structural homology of subunits GluR1 to GluR4 parallels the similarities in their functional characteristics: they give rise to receptor-channel complexes with properties more similar to those of AMPA receptors, (i.e. activation by AMPA followed by rapid desensitisation and competitive antagonism by CNQX at submicromolar concentration;

Keinanen et al., 1990). On the other hand, kainate is bound with much higher affinity by GluR6 which is incapable of binding AMPA. All these studies have shown that the pharmacological selectivity for kainate or AMPA and the resulting electrophysiological characteristics of the stimulated channels are dependent on the homomeric or heteromeric protein composition of the individual channels.

A functional metabotropic glutamate receptor has also been cloned, sequenced and expressed in *Xenopus* oocytes (Masu et al., 1991). Although similar in size to the ionotropic AMPA/kainate receptors, the overall sequence homology with these receptors is rather low. Similar amino acid sequences have been observed in both adrenoceptors and muscarinic receptors. More recently, 7 further subtypes of G-protein coupled metabotropic receptors have been described (mGluR1-mGlur8; Abe et al., 1992; Thomsen et al., 1992; Okamoto et al., 1994). Of these, mGluR1 and mGluR5 are coupled to PI hydrolysis, mGluR2,3,4 and 6 have negative coupling to the cAMP cascade; however mGluR2 and mGluR3 interact with trans-ACPD, whereas mGluR4 and mGluR6 potently react with L-AP4 (Abe et al., 1992; Thomsen et al., 1992). Two new mGluR subtypes termed mGluR7 and mGluR8 have recently been characterized (Okamoto et al., 1994; Duvoisin et al. 1995).

Another recent development in the molecular biology of EAA receptors is the cloning of the NMDA receptor. In one report (Moriyoshi et al.,1991) a single protein which comprised 938 aminoacids residues was identified and cloned. Expression of this protein, named NMDAR1 or NR-1, in *Xenopus* oocytes provided a homomeric receptor displaying rather low current amplitudes but with all the pharmacological properties characteristics of the native NMDA receptor, including blockade by

dizocilpine, Mg^{++}, Zn^{++} and glycine antagonists. The amino terminus of the NMDAR1 (NR-1) sequence contains 56 glutamate and aspartate residues thus providing the high concentration of anionic sites necessary to facilitate Ca^{++} permeabilty through the receptor channel. Another group (Kumar et al., 1991) reported the cloning in E. coli of one NMDA receptor subunit from rat brain, the glutamate-binding protein (GBP) which, like NR-1, has 4 membrane-spanning domains. Four further NMDA receptor proteins (NR-2A, NR-2B, NR-2C and NR-2D) have close similarity to each other but only 20% identity with NR-1 (Monyer et al., 1992; Hollmann and Heinemann, 1994). Co-expression of any one of the four with NR-1 gave 100 times greater responses in *Xenopus* oocytes than channels composed of NR-1 only. In contrast to NR-1, none of the four other subunits (NR-2A to NR-2D) showed any receptor channel activity in a homomeric configuration or any heteromeric expression within the members of the NR-2 subunits (Ishii et al., 1993) The mouse genome has generated three further NMDA receptor subunits (ε1, ε2 and ε3) which have 11-18% sequence identity with NR-1 (Meguro et al., 1992; Kutsuwada et al., 1992). When co-expressed with NR-1, each of these subunits give functional NMDA receptor channels activated by glycine, and different sensitivities to AP5, dizocilpine and 7-chlorokynurenic acid (Kutsuwada et al., 1992). While ε1 has widespread distribution, ε2 is found mainly in the forebrain and ε3 in the cerebellum (Kutsuwada et al., 1992). These findings fit well with reports of NMDA receptors in different regions having slightly different pharmacology, binding characteristics and neurophysiology (Asher and Nowak, 1988b; Yoneda and Ogita 1991).

1.8. PHYSIOLOGICAL RELEVANCE OF EAA RECEPTOR ACTIVATION

Most types of central mammalian neurones can be depolarised by glutamate, and EAAs mediate neurotransmission at some 50% of central synapses (Curtis and Watkins, 1965; Johnston, 1976) and given the wide distribution of EAA-containing neurons throughout the CNS, it is not surprising that EAA transmitters have been, and continue to be, implicated in the control of a widening range of neural systems affecting almost every aspect of CNS function. From processing of sensory information, through learning and memory based on that information, to motor control of the response, EAA-mediated synaptic transmission is almost certainly involved at every stage.

1.8.1. Long term potentiation

Long-term potentiation (LTP) is the sustained facilitation of a neural pathway induced by tetanic stimulation of the afferent fibres (Bliss and Gardner-Medwin, 1973). In anesthetised rabbits, this amplification of synaptic events could be seen for up to 10 hours following induction and in chronically implanted animals, synaptic activity could remain enhanced for weeks. Additionally, LTP is specific to the synapses activated during tetany and there is a threshold of stimulus intensity which must be reached for activating a sufficient number of afferent fibres, before potentiation can occur (Andersen et al., 1980). The cooperative and associative properties of LTP have been suggested to represent an underlying mechanism for learning and memory (Collingridge and Bliss, 1987). Induction of LTP in most hippocampal pathways is dependent upon

NMDA receptor activation and this is demonstrated by the ability of AP5 to reversibly block LTP induction in the major excitatory pathways (Harris et al., 1984; Harris and Cotman, 1986; Errington et al., 1987). This feature was also displayed by the non-competitive NMDA antagonists PCP, ketamine, dizocilpine and 7-chlorokynurenic acid (Stringer et al., 1983; Coan and Collingridge, 1987b), whereas glycine has been reported to synergistically potentiate LTP in the hippocampus (Tauck and Ashbeck, 1990). For NMDA receptor channels to be opened, postsynaptic cells must be sufficiently depolarised to alleviate the voltage-dependent blockade by Mg^{++}. This explains the dependence of LTP induction on high-frequency stimulation (Collingridge, 1985). Maintenance of LTP, however, does not require perpetual activation of NMDA receptors since neither unpotentiated nor potentiated EPSPs are significantly affected by AP5 (Collingridge et al., 1983). Following the demonstration that LTP in CA1 neurons displays a delayed increase in sensitivity to AMPA agonists, it appears that full development of LTP is a slow postsynaptic process requiring activation of NMDA receptors (and kinase activity), possibly to produce some functional modification of AMPA receptors (Davies et al., 1989). It has also been demonstrated that, in LTP, postsynaptic induction leads to enhanced presynaptic glutamate release (Malgaroli and Tsien, 1992).

1.8.2. Production of nitric oxide

Receptor activation by kainate or NMDA can lead to the production and release of nitric oxide (NO), which stimulates soluble guanylate cyclase to produce cGMP (Bredt

and Snyder, 1990; Garthwaite et al., 1989; Kiedrowski et al., 1992). NO was previously known as endothelium-derived relaxing factor which mediates the relaxation of blood vessels and the cytotoxic actions of macrophages (Moncada et al., 1989; Bredt et al., 1990). At low concentrations, nitric oxide appears to act as a diffusible transmitter substance and may also behave as a retrogade messenger released by and acting on the same cell (Dawson et al., 1991). However, since NO and has a high rate of diffusion across the cell membrane, it is uncertain whether the stimulation of guanylate cyclase by NO occurs in the same cells where it is produced, or in a different cellular target. In cerebellar and hippocampal slices, as well as in cortical cultures, NMDA stimulates the activity of NO synthase, since the physiological stimulus for NO synthesis is a rise in cytosolic Ca^{++} levels (Bredt and Snyder, 1990; East and Garthwaite, 1991). This enzyme, which converts arginine to citrulline plus NO, depends on calmodulin and Ca^{++} and is expressed particularly in the cerebellum, olfactory bulb and dentate gyrus (Bredt et al., 1990). The NMDA receptor signal causes a protein kinase cascade initiated by cGMP, which could also be a mechanism for alterations to the phenotype of neurones, as occurs in cell maturation. L-N-monomethyl arginine blocks the synthesis of nitric oxide from arginine and inhibits the elevation of cGMP produced in response to NMDA activation (Gartwaite et al., 1989). Inhibitors of NO synthase inhibit LTP whereas sodium nitroprusside releases NO and facilitates synaptic activity (Böhme et al., 1991). A short-life local NO signal may also be involved in co-ordinating changes to cerebral blood flow, synapses and transmitter release (Edelman and Gally, 1992). Studies using NO synthase (NOS) inhibitors have provided initial evidence that NO is involved in the regulation of CBF. While there is agreement on the

participation of NO in the maintenance of resting CBF, there are still disagreements on the role of NO in other vascular responses of the cerebral circulation (Iadecola et al., 1994). Evidence has been presented favouring both a beneficial and a detrimental effect of NO in focal ischaemia (Schmidt et al., 1992). A redox-based mechanism for the neuroprotective and neurodestructive effects of NO and related nitroso compounds has been suggested (Lipton et al., 1993).

1.8.3. Alteration of cell proteins

EAA agonists rapidly and specifically increase levels of mRNA for neurotrophic factors and nerve growth factor (NGF) in rat hippocampus *in vivo* and *in vitro,* possibly linked to physical neuronal changes during LTP (Zafra et al., 1991). NMDA receptor activation in cultured cerebellar granule cells is followed by increased expression of mRNA for the proto-oncogene *c-fos* (Szekely et al., 1989). Several other examples where EAA receptors are activated also showed induction of *c-fos* expression in brain tissue: seizures, cerebral ischaemia and administration of kainate, NMDA or glutamate (Dragunow and Robertson, 1987; Popovici et al., 1988; Szekely et al., 1989). Altered transcription of functional or structural genes follows *c-fos* induction, in such a way that transitory second messenger signals are converted to long-term cellular changes (Morgan and Curran, 1991).

In additon to cellular maturation, communication between neurones and glia, LTP and learning, EAA receptors seem to be involved in the perception of pain, sight, smell, hearing and in the tonic and reflex control of the cardiovascular system. Although EAA

receptors participate widely in the normal function of the mammalian CNS, they also contribute to various pathological states.

1.9. RELEVANCE OF EAA RECEPTORS IN PATHOLOGICAL CONDITIONS

The view has been expressed that certain pathological conditions implicating EAA receptors can be regarded as extensions of physiological processes and, at present, much evidence concerns the role of NMDA receptors in CNS dysfunctions particularly with regard to epilepsy and neuronal degeneration.

1.9.1. Epilepsy

The involvement of EAA receptors in the pathogenesis of epilepsy has been strongly suggested since the discovery that the competitive NMDA receptor antagonists AP5 and AP7 are anticonvulsant in experimental models of epilepsy (Coutinho-Netto et al., 1981; Croucher et al., 1982). The hippocampal and neocortical slice preparations have since been used extensively to probe the mechanisms of epilepsy at the cellular level and it has been shown that the potency of a selection of NMDA antagonists to suppress evoked bursts in cerebral cortex correlated with their potency as blockers of NMDA-induced depolarisation (Aram et al., 1989). Induction of status epilepticus in slices by repeated trains of electrical stimuli is prevented by AP5 or the non-competitive NMDA antagonist dizocilpine (Stasheff et al., 1989). However, as with LTP, once seizure activity is induced, such antagonists shorten bursts but do not prevent their occurrence.

It has been suggested that non-NMDA receptors may be more important for the perpetuation of seizure activity once induced (Slater et al., 1985). *In vivo* models of epilepsy using genetically seizure-prone baboons and mice lend overall support to the involvement of NMDA receptors in epileptogenesis and development of full seizures, although there are substantial variations in the anticonvulsant potency of competitive NMDA antagonists between different models (Patel et al., 1990). EAA receptors appear to be abnormally clustered in the hippocampus of people suffering temporal lobe epilepsy (McDonald et al., 1989) and trials with dizocilpine have proved unsuitable because of many side effects (Chapman, 1991). Future drugs with different mechanisms of action may have more clinical efficacy. NBQX and GYKI 52466 are anticonvulsant in rodent and primate models of epilepsy, indicating that antagonism at AMPA or glycine receptors may be a more promising way of treating persistent epilepsies (Turski et al., 1992).

1.9.2. EAA neurotoxicity

The hypothesis that endogenous excitatory amino acids may play a role in the development of certain neurological disorders was first reported in 1957, following the observation that systemic injections of glutamate destroyed retinal cells in immature mice (Lucas and Newhouse, 1957). At the same time EAAs were being established as neurotransmitters with a direct physiological response in mammalian CNS neurones (Curtis and Watkins, 1959). Subsequently, Olney and collegues demonstrated that mice treated with glutamate at an early age presented neurotoxic lesions in the hypotalamus,

a brain region lacking a developed blood-brain barrier (Olney et al., 1969). The close correlation of the structure-activity relationship between the excitatory effects of EAAs on neurones and their neurotoxic potency was summarized by the "excitotoxic" hypothesis of cell death (Olney et al., 1971; Olney, 1983). This suggested that prolonged EAA-induced depolarization is responsible for pathological changes and neurotoxic effects. The neuroanatomical alterations caused by excitotoxins were characterized by swelling and degeneration of dendrites and cell bodies, which possess post-synaptic EAA receptors, whereas the axons were spared. The depolarising action of EAAs is mediated through different receptor subtypes and thus it was proposed that the neurotoxic effects of EAAs may also be mediated through pharmacologically distinct receptor subtypes (Schwarcz et al., 1984). However, recent evidence has challenged the current concept of the "excitotoxic" hypothesis suggesting that factors other than glutamate accumulation in the extracellular space play a crucial role in the neurotoxic process (Obrenovitch and Richards, 1994).

1.9.2.1. Mechanisms of excitotoxicity

The acute swelling of hippocampal neurones observed in response to NMDA application, has been proposed as the basis for cell injury (Rothman, 1984). Upon activation of NMDA receptors, both sodium and chloride enter the cell followed by water and, if the swelling is sufficiently high, lysis occurs (Rothman, 1985). Swelling, a pathophysiological characteristic of excitotoxin exposure, was also observed in other studies using murine cortical neurones, but an additional delayed neuronal injury was

noted (Choi et al., 1987; Choi, 1987). By manipulating the extracellular ion concentrations, it has been shown that in cells exposed to glutamate swelling is Na^{+}-dependent, whereas the delayed cell death is Ca^{++}-dependent and Na^{+}-independent (Choi, 1987; Choi et al., 1988). Although it has been reported that there is no real difference in the amount of Ca^{++} sequestered by vulnerable or resistant neurones, an increase in intracellular [Ca^{++}] appears to be the main mediator of neuronal injury (Siesjo, 1981; Siesjo and Wieloch, 1985; Hossman et al., 1985). Accumulation of intracellular Ca^{++} was suggested to be the mechanism of selective neuronal vulnerability in ischaemia, hypoglycemia and epilepsy and the removal of Ca^{++} during the excitotoxin exposure significantly reduced cell death (Murphy et al., 1988; Michaelis and Rothman., 1990).

Extracellular [Ca^{++}]is reported to be 1.3 mM whereas intracellular [Ca^{++}]appears to be less than 0.1 μM (Borle, 1981; Carafoli 1982). This concentration gradient for Ca^{++} across the cell membrane in physiological conditions is maintained by an ATP-dependent Ca^{++}/Na^{+} exchange mechanism (Nachshen et al., 1986). Ca^{++} enters neurones via voltage sensitive calcium channels (VSCCs) or agonist-dependent ion channels such as EAA receptors. An increase in extracellular [Ca^{++}] may therefore be produced by opening of a ligand gated channel (NMDA), by receptor-operated (metabotropic receptor) mobilization of intracellular [Ca^{++}] stores, or by receptor-mediated (AMPA/Kainate) depolarization and opening of VSCCs. Levels of intracellular [Ca^{++}] are normally closely regulated since they can influence a variety of cellular processes such as membrane permeability, transmitter release, post-synaptic

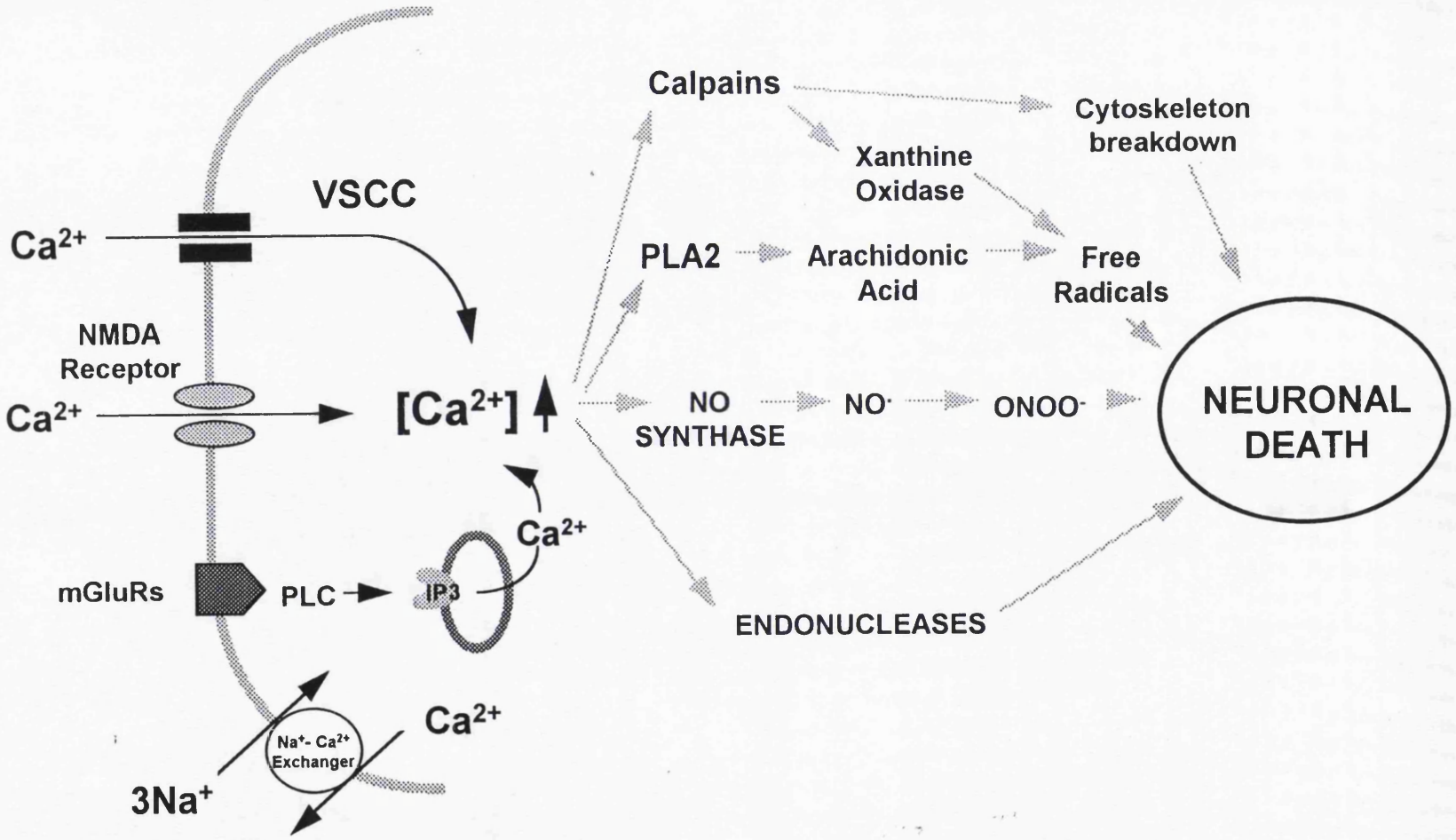
excitability, synaptyc plasticity, gene expression and modulation of the activity of numerous enzyme systems as well as cell energy metabolism (Siesjo, 1988;). Toxic levels of Ca^{++} induce severe perturbations in many cellular processes through proteolysis, lipolysis, disaggregation of cytoskeletal components and protein phosphorylation which ultimately result in cell death (Siesjo and Bengtsson, 1989). It is difficult to establish which Ca^{++}-activated intracellular processes are most directly involved in glutamate neurotoxicity (Fig. 1.2.).

1.9.2.1.1. Activation of phospholipase A2 and free radical formation

High intracellular Ca^{++} levels can activate phospholipase A2 which catalyzes the breakdown of membrane lipids releasing fatty acids and arachidonic acid. Arachidonic acid can undergo further metabolism by cyclo-oxygenase, thus producing oxygen radicals responsible for lipid peroxidation. Cortical neuronal damage can be partially attenuated by 21-amino steroids, which seem to act as free radical scavengers and lipid peroxidation inhibitors (Monyer et al., 1990). Another source of free radical formation is the enzymatic action of xanthine oxidase, which is generated from xanthine dehydrogenase by means of a Ca^{++}-activated protease (McCord, 1985).

A rise in cytosolic Ca^{++} level is also the physiological stimulus for nitric oxide (NO) synthesis. Toxicity of NO may involve NO itself or its combination with superoxide free radicals, which results in production of hydroxide free radical and NO2 free radicals which are more reactive and toxic than NO (Beckman et al., 1990).

Fig.1.2: MECHANISMS OF NEURONAL CELL DAMAGE RESULTING FROM INTRACELLULAR CALCIUM LOADING.



1.9.2.1.2. Activation of the protease calpain I

Neurones contain high levels of calpain I, a Ca^{++}-dependent protease which is strictly correlated to the biological process of transmembrane signalling. Thus, in physiological conditions calpain I may transduce brief rises in intraneuronal free Ca^{++} levels into lasting changes in neuronal structure and function.

During excitotoxic stimulation, it is involved in the degradation of several major neuronal structural proteins, including tubulin, microtubule-associated proteins, neurofilament polypeptides and spectrin, thereby contributing to cell disintegration (Siman and Noszek, 1988).

1.9.2.1.3. Activation of protein kinase C

Protein kinase C (PKC) is a Ca^{++}/phospholipid-dependent kinase, found in high concentrations in the CNS, which plays a pivotal role in signal transduction (Nishizuka, 1986). High levels of intracellular Ca^{++} along with diacylglycerol may alter Ca^{++} channels and phosphorilate other channels, which can result in disruption of protein synthesis (Connor et al., 1988; Siesjo and Wieloch, 1985).

1.10. EAA AND CEREBRAL ISCHAEMIA

Substantial evidence now exists to support a key role for glutamate and other EAA neurotransmitters in the processes leading to neuronal degeneration after ischaemic insult to the brain (Rothman and Olney, 1986; Choi, 1988). Normal, homeostatic mechanisms which serve to regulate the free extracellular concentration of these

aminoacids appear to be compromised during, and for a short time following, an infarct. The initial evidence for this came mainly from transient forebrain ischaemia studies in which a massive increase in the extracellular concentration of glutamate and aspartate was seen following ischaemia using the *in vivo* microdialysis technique (Benveniste et al., 1984). Early reports suggested that excessive glutamate accumulation following ischaemia was probably due to impaired neuronal and glial uptake systems (Drejer et al., 1985; Hagberg et al., 1987). More recent studies indicate that excessive release of glutamate due to a breakdown of the ionic gradients may play a role under ischaemic conditions (Takagi et al., 1993). The brain possesses efficient homeostatic mechanisms that keep ion concentration constant in CSF and brain. The functional basis for neuronal activity is provided by the ionic gradients across nerve cell membranes which are maintained by an interplay between ion channels, carriers and ATP-dependent pumps. Under ischaemic conditions, the breakdown of the energy dependent ionic pumps causes a rise in extracellular $[K^+]$ which will initiate glutamate release by depolarising the neurones which increases their firing rate and thus increases the vesicular release of glutamate (Hansen, 1985). Since this release is also ATP dependent, it may be inhibited in some cases of complete ischaemia in which there is a drastic reduction in energy levels. High extracellular $[K^+]$ may also promote the release of glutamate by reversal of the glutamate uptake carrier (Barbour et al., 1988). The high synaptic levels of glutamate will also induce arachidonic acid release (Dumuis et al., 1990) resulting in glutamate uptake inhibition (Barbour et al., 1989). The combined effect of these changes is sufficient to reduce glutamate uptake to less than 10% of normal (Attwell et al., 1989). The result of this combination of events is an accumulation of synaptic

glutamate at a concentration of 75-250 μM, which has been reported to be toxic (Choi, 1987) in cultured neurons.

Also, many of the histological consequences of ischaemia resemble those following excitotoxic lesions, since in both circumstances there is an axon-sparing lesion (Johansen et al., 1984). However, a number of works clearly show that high extracellular glutamate may not be the key to excitotoxicity in vivo (Novelli et al., 1988; Obrenovitch et al., 1994).

In parallel with the *in vivo* observations, the *in vitro* studies played a major part in determining the role of glutamate in hypoxic cell death. In 1982 it was found that slices of rat dentate gyrus were susceptible to 10 min of anoxia (Kass and Lipton, 1982) and it was than demonstrated that the presence of Mg^{++} enabled mature neurones in culture to withstand prolonged exposure to anoxia (Rothman and Samaie, 1985). The damage could also be attenuated by removal of Ca^{++} from the bathing medium. Other studies reported that exposure to only 50 μM glutamate for 5 min was enough to destroy large numbers of cultured cortical neurones (Choi, 1987a). Others were also able to demonstrate Ca^{++}-dependent degeneration caused by glutamate and other EAA agonists in hippocampal cultures (Rothman et al., 1987), cerebellar slices (Garthwaite and Garthwaite, 1986) and in neuronal cell cultures (Murphy and Miller, 1988). These *in vitro* studies along with the *in vivo* findings were able to establish a role for EAAs in ischaemia-induced neuronal degeneration, and were also able to demonstrate an important role for the NMDA receptor in mediating cell death.

Further evidence for an involvement of EAA in cerebral ischaemia comes from lesioning of the glutamatergic pathways projecting to the hippocampus, which has been shown to

prevent or ameliorate neuronal degeneration (Wieloch et al., 1985; Benveniste et al., 1989). These studies showed that destruction of the perforant path reduces damage to CA1 neurons induced by bilateral carotid occlusion and this protection could also be achieved by destruction of CA3 neurons (from which the excitatory Schaffer collaterals to CA1 originate). However, these beneficial effects may not be specific to glutamatergic deafferentiation since such lesions might produce neuroprotective effect simply by reducing synaptic activity or by releasing neurotrophic factors.

The main evidence for a link between excitotoxic mechanisms and neuronal degeneration after ischaemic insult is the neuroprotective action of EAA antagonists in ischaemia.

1.10.1 Neuroprotective properties of EAA antagonists in ischaemia

Using the bilateral carotid occlusion model of global ischaemia in rat, it was demonstrated that early morphological signs of damage to CA1 pyramidal neurons could be reduced by intra-hippocampal injection of the competitive NMDA antagonist AP7 (Simon et al., 1984). These observations were confirmed and extended by other groups following the development of competitive and non-competitive NMDA antagonists permeable to the blood-brain barrier which could be administered systemically. One of these, dizocilpine, a selective and potent non-competitive NMDA antagonist, has been extensively demonstrated to be neuroprotective in different models of global and focal ischaemia (Gill et al., 1987; 1988; Ozyurt et al., 1988). However, NMDA antagonists also provided some equivocal results since several groups have

been unable to demonstrate their neuroprotective effect in models of global ischaemia (Wieloch et al., 1989; Buchan and Pulsinelli, 1990). The use of different animal models and other methodological parameters might account for these inconsistencies and, at present, the consensus of opinion is that NMDA receptor antagonists prevent ischaemic neuronal damage in those models or brain regions in which energy levels are partially preserved whereas they are ineffective under conditions of total energy failure (McCulloch et al., 1991). Parallel studies using non-NMDA receptor antagonists, indicate that the AMPA receptor antagonist NBQX is effective against delayed neuronal degeneration following global ischaemia and less effective against focal ischaemic damage (Sheardown et al., 1990; Gill et al., 1992). These observations confirm that excitotoxic mechanisms are involved both in global and focal ischaemic damage.

1.11. ANIMAL MODELS OF CEREBRAL ISCHAEMIA

The understanding of physiological and pathological changes, together with the development of therapeutic approaches following cerebral ischaemia in humans, depends on the availability and reliability of animal models of cerebrovascular disorders. Some major problems exist with such experimental models, notably the high variability within animals and the parallelism with the human neuropathology which is not always appropriate. An obvious example is general anaesthesia, which is commonly used in animal models of cerebral ischaemia whereas, in humans, the ischaemic event usually occurs in conscious individuals. Anaesthesia might interact (in a synergistic or

antagonistic way) with an anti-ischaemic drug by modifying the cerebrovascular profile or the substrate requirements in discrete regions of the CNS (Ginsberg and Busto, 1989).

1.11.1. Animal models of global ischaemia

Under controlled physiological parameters, some of the many animal models developed over the years can give reproducible ischaemic damage which mimics the pathological changes seen following cardiac arrest in humans.

- Carotid occlusion in the Mongolian gerbil has been widely used because of the unusual cerebral circulation, which lacks connections between carotid and vertebrobasilar circulation. Bilateral carotid occlusion for a brief period results in complete forebrain ischaemia in all animals (Suzuki et al., 1983), whereas unilateral occlusion of a common carotid artery results in the development of ischaemic injury in 30-60% of animals (Mrsulja et al. 1975)

- The 4-vessel occlusion model in rats, 4-VO (Pulsinelli and Brierley, 1979) involves occlusion of both carotid and vertebral arteries but the major problem associated with this model is the enormous variability of local cerebral blood flow between different animals resulting in unpredictable ischaemic injury. This variation is probably a result of inter-animal variation in the collateral vascular supply.

- The 2-vessel occlusion model in the rat 2-VO (Smith et al., 1984) consists of bilateral carotid occlusion with systemic hypotension. One of the problems with this model is that systemic hypotension is often induced using drugs and the lowering of blood

pressure can take up to 5 min, with consequent variations in the degree of the ischaemia. It is important to remember that this is not a model of cardiac arrest but is a model of forebrain ischaemia and its clinical relevance has yet to be evaluated.

1.11.2.Animal models of focal ischaemia

The animal models of focal ischaemia involve permanent or, in a few cases, temporary occlusion of a major artery and they appear to be the most appropriate in relation to human stroke. Usually, the middle cerebral artery (MCA) is the vessel of choice since its occlusion produces biochemical and morphological features very similar to those of a brain infarction in a human cerebral hemisphere (Garcia, 1984). Middle cerebral artery occlusion (MCA-O) only affects part of the brain, producing a localized infarction of the territory supplied by the artery and allowing survival of the animals even though the vessel is permanently occluded. One major disadvantage of this model is the variability in the severity of the ischaemic damage which exists even when the physiological parameters are kept as constant as possible and this is probably explained by the variable types of collateral connections in individual animals. The MCA-O model has been used in large animals -cats, dogs, primates- for many years, mainly because these species show cardiovascular stability under anaesthesia. However, the most widely used standard models of focal ischaemia over the last years have been the rat models of MCA-O first developed by Tamura et al., (1981). Many of the methods used these days are similar to the original subtemporal approach for proximal occlusion of the MCA, although some modifications have been made (Shigeno et al., 1985; Bederson et al., 1986; Osborne et al., 1987). The use of rats has been favoured for several reasons

including the simplicity of the technique, the relative predictability of the lesion compared to higher species and the small body size (which allows the use of small doses of drug tested). More recently, a surgical procedure for MCA-O in mice has been described (Welsh et al. 1987) and the ischaemic lesion has been characterised (Gotti et al., 1990).

1.12. Project overview

Permanent MCA-O in mouse was used to investigate the temporal progression of the focal infarction and to evaluate the extent of protection offered by antagonists acting at different sites of the NMDA receptor complex, with particular regard to compound Z, a novel antagonist at the glycine site of the NMDA receptor complex. These data provide the basis for the study presented in Chapter 2.

If the NMDA receptor complex is to be a target for neuroprotective agents administered post-ischaemia, it is important to determine not only the time course of the viability of the receptor but also the period post-ischaemia during which drugs can gain access to the receptors in the region of the infarct. Thus, we have studied the *ex vivo* distribution of densities of ^{3}H-dizocilpine binding sites in mouse brain after MCA-O and have compared this with the *in vitro* ^{3}H-dizocilpine binding after MCA-O using receptor autoradiography. The aim was to provide a measure of the relationship between functional integrity and accessibility of the receptor post-ischaemia, and these data provide the basis for the study described in Chapter 3.

An important purpose of the present study was to verify whether it is possible to modulate the NMDA receptor complex under physiological conditions. In Chapter 4, the influence of modulators of NMDA receptor function on the *ex vivo* binding of ^{3}H-dizocilpine in mouse brain has been examined. Receptor autoradiography has been used to detect subtle changes in the regional levels of binding.

The effect produced by MCA-O in mice on the 8-arm radial maze has been examined to evaluate any possible disturbance on acquisition of a spatial orientation task. The radial maze has also been used to compare the effect of systemic administration of ligands for the glycine site with the effect produced by dizocilpine on spatial orientation. These data provide the basis for the study presented in Chapter 5.

CHAPTER 2: EFFECT OF NMDA RECEPTOR MODULATION IN THE MOUSE MIDDLE CEREBRAL ARTERY OCCLUSION (MCA-O) MODEL OF FOCAL ISCHAEMIA

2.1. INTRODUCTION

Several distinct lines of evidence have led to the now widely accepted hypothesis that EAA receptor mechanisms underlie the cerebral damage which follows brain ischaemia (see Chapter 1). Clinically, ischaemia is regarded as the cessation of blood flow in the circulation and it may be complete as a result of cardiac arrest or incomplete as in stroke. The human brain requires adequate supplies of oxygen and glucose as they provide the essential components of oxidative metabolism which in turn supplies, in the form of ATP, most of the energy requirements for synaptic transmission and maintenance of ionic gradients (Brierley and Graham, 1984; Garcia; 1984). According to the excitotoxic hypothesis, under ischaemic conditions the breakdown of energy-dependent homeostatic mechanisms leads to an increase in the extracellular level of EAA which, in turn, appears to be responsible for neuronal damage. The extent and pattern of neuronal degeneration following cerebral ischaemia depends on the duration and severity of the insult. In particular, the diffuse reduction of blood flow to the brain which occurs in global ischaemia as a result of cardiac arrest or hypotension, produces in a pattern of selective and delayed neuronal injury in specific areas of the brain (i.e. CA1). On the other hand, the human syndrome of stroke involves a rapid development of a focal neurologic deficit the origin of which can be traced to either the occlusion of a cerebral vessel, (usually arterial), or the spontaneous rupture of an intracranial artery with consequent cell damage within the area served by the occluded artery.

2.1.1. Neuropathology following global (transient forebrain) ischaemia.

Cerebral damage induced by global ischaemia affects selectively vulnerable neurones throughout the brain. In animal models of transient forebrain ischaemia the hippocampal CA1 neurones are most susceptible to ischaemic damage. By following the time course of CA1 neuronal damage in gerbils, it has been observed that these neurones died over a period of 24 hours and the expression "delayed neuronal degeneration" was introduced (Kirino et al., 1982). This phenomenon has been subsequently demonstrated in rat animal models (Pulsinelli et al., 1982; Smith et al., 1984) and humans following a cardiac arrest (Brierley and Graham, 1984). It represents an important indication that vulnerability of certain areas of the brain depends both on events occurring during and following ischaemia. In this respect, an important consideration related to the selective vulnerability observed after ischaemia, is the abundance of EAA receptors in the brain regions most susceptible to ischaemic damage (Meldrum, 1985).

2.1.2. Neuropathology following focal ischaemia

The normal blood flow to the brain is about 50 ml/100g tissue/min and, due to the inability of the brain to store oxygen or glucose, a blood flow below 10 ml/100g tissue/min has been demonstrated to produce irreversible brain damage (Brierley and Graham, 1984). The cerebral infarction produced by MCA-O comprises an area with no or very little blood flow and depleted energy levels, which is the core of the lesion where the tissue is irreversibly damaged. Surrounding this core there is an area, described as the penumbra, where blood flow and energy levels are partially maintained because of some residual collateral circulation (Astrup et al., 1981). The blood flow in

this area has been reported to be approximately 20% of normal (Tamura et al., 1981), which is the level at which synaptic transmission is abolished, although normal ionic homeostasis is maintained (Astrup et al., 1981). Therefore, the penumbra represents an area which can potentially become infarcted (if energy levels drop further) but, at the same time, might be responsive to therapeutic intervention. The histopathological changes seen following permanent MCA-O in rats, cats and monkeys have been described in detail by many investigators (Tamura et al., 1981; Tyson et al., 1984; Nedergaard, 1987; Obrenovitch et al., 1988). These changes in the mouse model of MCA-O are described in this chapter. An important difference with global ischaemia models is that no "delayed neuronal degeneration" is seen in focal ischaemia models, where the lesion is fully established within 3-4 hours (Nedergaard, 1987; Kaplan et al., 1990).

Although a vast selection of animal models have been developed, many of them are more suitable for exploring pathophysiological mechanisms rather than the evaluation of anti-ischaemic drug efficacy. Models of focal cerebral ischaemia have proved advantageous over the transient forebrain ischaemia in anti-ischaemic drug development with particular regard to NMDA antagonists. It has been suggested that NMDA antagonists are only beneficial in conditions in which energy levels are partially preserved, as in the penumbra following focal ischaemia, but not when the energy failure is complete as in global ischaemia (Wieloch et al., 1989; McCulloch et al., 1991). An explanation for the lack of neuroprotective effect of NMDA antagonists in animal models of global ischaemia could be the reverse of the Na^{+}/Ca^{++} pump due to the severe reduction of ATP levels (Siesjo and Bengtsson, 1989). In this case, intracellular

accumulation of Ca^{++} and the resulting delayed neuronal degeneration would not be prevented by NMDA receptor blockade. Alternatively, the contribution of NMDA receptors to neuronal degeneration in the global ischaemia models might be reduced by the drastic pH decrease. Support for this view comes from evidence that H^{+} reduced NMDA-mediated currents (Giffard et al., 1990; Traynelis and Cull-Candy, 1990) and that reduction of pH decreased glutamate neurotoxicity in cell culture studies (Giffard et al., 1990), although a reduction of pH is also observed in the penumbra of the focal lesion. The protective action of NMDA receptor antagonists can also be explained by inhibition of spreading depression (Obrenovitch, 1995).

2.2. NMDA ANTAGONISTS IN FOCAL ISCHAEMIA

2.2.1. Ion-channel blockers in focal ischaemia

Despite the controversial results obtained with ion-channel blockers in global ischaemia models, animal models of focal ischaemia have proved to be more consistently responsive to therapeutic intervention with this class of NMDA antagonists (see Chapter 1). For the most intensively studied ion-channel blocker, dizocilpine, there is general agreement on the neuroprotective effect when this NMDA antagonist is given prior to or shortly after the induction of focal ischaemia. Dizocilpine administered prior to permanent MCA-O in the cat at a dose of 5 mg/kg i.v., produced a 50% reduction in the volume of cortical infarction measured 6 hours later (Ozyurt et al., 1988). Similar efficacy for dizocilpine was demonstrated when administered up to 2 hours following MCA-O in the same model (Park et al., 1988), implicating a possible clinical application for such compounds, since therapy could be delayed following ischaemia. In the rat

MCA-O, dizocilpine (0.5 mg/kg i.v.) given 30 min prior to or after MCA-O gave the same extent (50%) of reduction in the volume of cortical damage (Park et al., 1988). These initial findings have been confirmed and extended by a number of studies using different timings of treatment or longer recovery times (i.e. Gill et al., 1991; Bielemberg and Beck, 1991), showing a similar range of protective effects (40-60%) for dizocilpine in the rat MCA-O model. The therapeutic window for treatment with dizocilpine in this model was reported to be up to 2 hours post-ischaemia (Hatfield et al., 1992).

Other ion-channels blockers were investigated as neuroprotective agents in the same model of focal ischaemia. Both TCP and PCP administered prior to or up to 3 hours post MCA-O, and with 48 hours recovery, were reported to reduce the volume of ischaemic damage (Duverger et al., 1987; Bielemberg and Beck, 1991). Studies with the morphinan ion-channel blockers such as dextromethorphan and its active metabolite dextrorphan have been performed using a model of transient focal ischaemia in rabbits. Both compounds produced a significant reduction in the volume of cortical damage when administered prior to or 1 hour after ischaemia (Steinberg et al., 1989).

The neuroprotective effects of Mg^{++} have been reported in a rat MCA-O model (Izumi et al., 1991), although the reduction of infarct size in this study could be due to an increased CBF to the penumbral areas by Mg^{++}, rather than NMDA receptor blockade. Therefore, compounds acting at the ion-channel site of the NMDA receptor complex are neuroprotective in focal ischaemia models and their therapeutic window appears to be up to 2-3 hours post insult.

2.2.2. Competitive NMDA antagonists in focal ischaemia

AP7 and CGP 19755 have been reported to be neuroprotective in the MCA-O model in rat (at the doses of 100 mg/kg i.v. and 10 mg/kg, respectively (Roman et al., 1989; Simon and Shiraishi, 1990), although some criticisms have been made on these studies. The most convincing evidence for the protective effects of competitive NMDA antagonists in focal ischaemia have come from studies using D-CPPene in the MCA-O model in cat (Bullock et al., 1990). In this model a dose-response neuroprotection was seen although this compound, when administered 1 hour following MCA-O, was not neuroprotective, probably due to its slow brain penetration. D-CPPene also produced some cardiovascular effects, an increased CBF and local changes of pH (Takizawa et al., 1991).

Antagonists acting at the transmitter recognition site of the NMDA receptor complex seem to be neuroprotective, although the pharmacokinetic profile appears to play an important role. As for the ion-channel blocking compounds, consistent results of neuroprotection were obtained in models of focal ischaemia whereas results obtained from global ischaemia models were dubious.

2.2.3. Compounds acting at the glycine site in focal ischaemia

Despite the diversity of potent glycine site ligands available, their *in vivo* activity appears to be compromised by the poor blood-brain barrier permeability and, at present, there are only a few reports concerning the neuroprotective effect of this class of compounds in animal models of focal ischaemia. The quinoxaline derivative ACEA 1021 has recently been found neuroprotective when administered (at 10 mg/kg followed by

continuous infusion of 7 mg/kg/hour i.v.) after MCA-O (Woodward et al., 1995; Warner et al., 1995).. When the dosing was started 60 min post MCA-O, a significant reduction of the volume of ischaemic damage (61%) was still observed. L-687,414 (3R-(+)-cis-4-methyl-HA966 has also been reported to be neuroprotective in a focal model of ischaemia in rat (Gill et al.,1991). HA-966 and 7-chlorokynurenic acid have been reported to be neuroprotective in a gerbil model of ischaemia (Patel et al., 1990). Part of the neuroprotection produced by the broad spectrum EAA antagonist kynurenic acid might also be due to an action at the glycine site of the NMDA receptor complex (Gill and Woodruff, 1990).

In the present study permanent MCA-O in mouse was used to investigate the temporal progression of the focal infarction and to evaluate the extent of protection offered by antagonists acting at different sites of the NMDA receptor complex. Dizocilpine was used as a positive control since its neuroprotective properties on this model have been previously reported (Gotti et al., 1990). The potential neuroprotective effect in the mouse model of various doses of CGP 37849, an orally active compound with competitive antagonist properties at the NMDA receptor complex, has been studied. A study protocol was also designed for testing the infarction reducing potency of compound Z, an antagonist at the glycine site of the NMDA receptor complex. Consideration of the best timing for evaluating neuronal morphological changes and drug neuroprotective potency in the mouse MCA-O model were also examined.

2.3. METHODS

Male CD1 mice (Charles River, U.K.) weighing 25-30 g were used. Animals were allowed food and water ad libitum except during the period they were removed from their cages for surgery and recovery (approximatively 90 min).

2.3.1. Middle cerebral artery occlusion in mice: surgical procedure

Mice were anaesthetized with chloral hydrate (400 mg/kg i.p.). A rectal temperature probe was inserted and core temperature was maintained within physiological limits by placing the animal on a heated operating table. Mice lying on their left side were placed under a dissecting microscope under low power magnification, a skin incision was made from the eye to the ear (right side). The underlying temporal muscle was deflected forward making an incision on the upper margin. The parotid gland and surrounding tissue were deflected downward. The middle cerebral artery (MCA) was visible through the temporal semi-translucent surface of the skull. Craniectomy was performed by drilling with a round burr (Ash 1-3). The inner layer of the skull was removed with fine forceps. The dura was carefully opened and the MCA was exposed. The artery was coagulated by bipolar diathermy (duration stimulus= 0.3 sec; amplitude 3) using bipolar forceps. Care was taken to avoid damage to the brain tissue in the area surrounding the MCA. The temporalis muscle and the parotid gland were replaced, the scalp incision was sutured. (Chiamulera et al., 1993, slightly modified). Animals were maintained in a warm environment (incubator 32°C) until recovery from

anaesthesia (about 1 h). Sham operated animals were treated in the same manner, except no MCA bipolar diathermy was performed. Generally, such manipulation did not cause any damage to the cerebral tissue. In some animals, a limited mechanical damage due to surgical manipulation was noted. After recovery, mice were housed with free access to water and food.

2.3.2. Evaluation of ischaemic damage

On day 3 after MCA-O, brains were carefully removed and frozen in isopentane pre-cooled in liquid nitrogen. Five coronal sections were taken at the following brain co-ordinates, according to the Stereotaxic Atlas of the Mouse Brain (Lehmann, 1974): A2100, A2750, A3250, A4150 and A4750 μm anterior to the interaural line. (Fig 2.1).

Sections were stained with methylene blue 0.1% or haematoxylin and eosin for image analysis and areas of brain damage were measured on an image analyzer (Quantimet 970, Cambridge Instruments). The area of ischaemic damage was expressed as mm^2 or as a percentage of the total area of the section.

12 μm coronal sections taken at the same brain co-ordinates were fixed with 4 % formaldehyde and stained with haematoxilin and eosin for microscopic examination. Individual variability in the ischaemic area was observed.

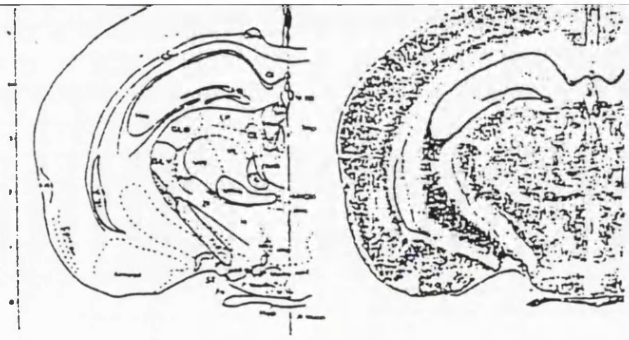
2.3.3. Experimental procedure

Treatments were performed using either of the following dose regimes:

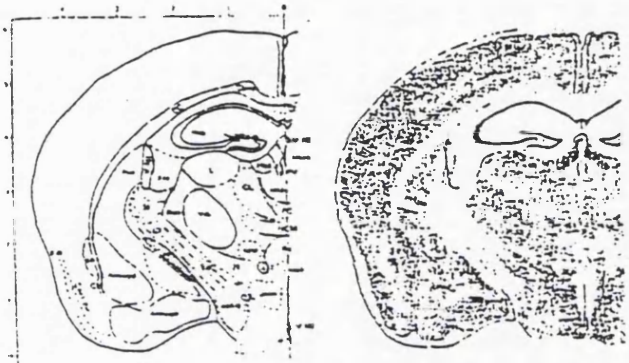
a. Single dose treatment post MCA-O. Dizocilpine (0.3, 1 or 3 mg/kg i.p.), CGP

Fig.2.1. Mouse brain co-ordinates.

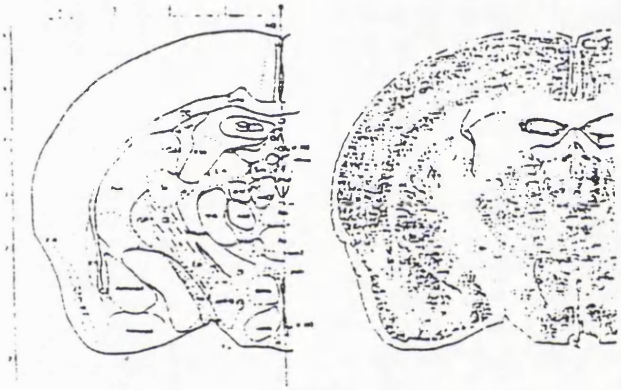
Illustration of coronal sections of mouse brain at 5 different brain co-ordinates (A2100, A2750, A3250, A4150 and A 4750) taken from the Stereotaxic Atlas of the Mouse Brain by Lehman (1974).



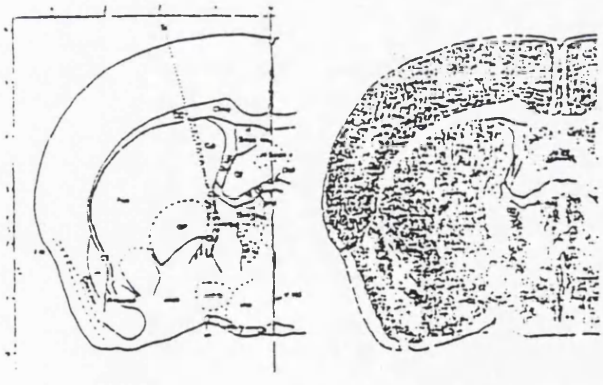
A 2.100



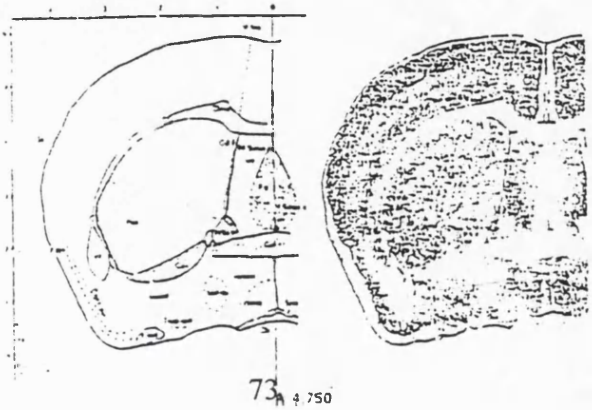
A 2.750



A 3.250



A 4.150



A 4.750

37849 (3 or 10 mg/kg i.p.), compound Z (10 mg/kg) or vehicle were administered 5 min, 30 min or 60 min after MCA-O. Behaviour was monitored by visual observation.

b. Repeated dose treatment post MCA-O. Dizocilpine (0.3 or 1mg/kg i.p.), CGP 37849 (10mg/kg i.p.) compound Z (10 mg/kg i.p.) or vehicle were administered 5 min, 3h, 6h, 24h and 36h after MCA-O. In some experiments the first dose was administered at either 30 min or 1h or 2h after the MCA-O and was followed by the same repeated dose regime (3h, 6h, 24h and 36h after the first dose).

c. Single dose treatment prior to MCA-O. Dizocilpine (0.3 or 1 mg/kg i.p), CGP 37849 (10 mg/kg i.p.), compound Z (3 or 10 mg/kg i.p.) or vehicle were administered 30 min prior to MCA-O.

2.4. RESULTS

2.4.1. Effect of MCA-O in mice

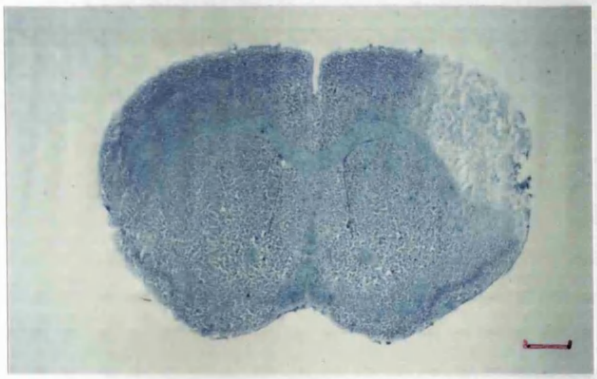
Blue ink was injected intravenously in some animals 1 hour after MCA-O to assess the brain area not perfused. Five minutes after the injection, mice were sacrificed and the brains removed and macroscopically observed. MCA-O caused a lack of Blue ink perfusion in a large area of the brain cortex. Macroscopic observation of the brains indicated that the temporal and parietal cortices were not perfused and this sometimes extended to the frontal cortex.

2.4.1.1. Histological changes

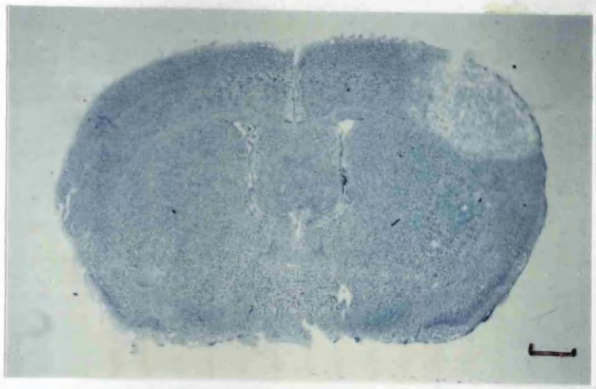
Histological staining of mouse brain sections from animals subjected to MCA-O revealed a large area of necrotic damage in the cerebral cortex. (Fig. 2.2) The area of infarction was delineated from the normal tissue and gliosis was always present in the infarct area. In evolving brain infarction the peripheral region around the infarcted area was characterized by the presence of many reactive astrocytes, oligodendrocytes and a proliferation of capillaries. In some animals, the infarcted area was infiltrated by phagocytes, microglial cells and rarely mononuclear and polymorphoeosinophilic cells. Neurones were absent in the central area of the lesion (the only neuronal cells within the infarct core, "ghost neurones", appeared with a marked pallor of the nucleus), whereas they were present in the peripheral region where they showed a wide variety of

Fig.2.2. Mouse coronal sections from animals subjected to MCA-O stained with methylene blue 0.1%

This figure shows an example of damage produced by MCA-O to the mouse brain and co-ordinates at which brain sections were taken in all experiments.



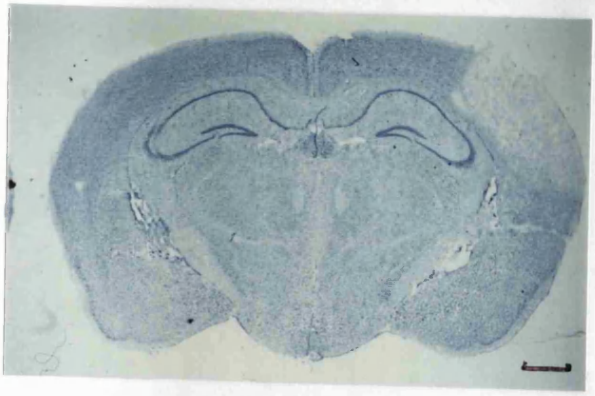
A4750
Scale bar= 1mm



A4150
Scale bar= 1 mm

A3250
Scale bar= 1 mm

A2750
Scale bar= 1 mm



A2100
Scale bar= 1 mm

structural changes that included pallor of the nucleus and cytoplasm, shrinkage of the perikaryon, nuclear pyknosis and cellular vacuolisation (Fig 2.3.).

The area (mean±s.e.m.) of the cortical infarct in mice at 3 days after MCA-O and sham operated mice is shown in Table 2.1. The maximal area of infarct occurred 1 day post-occlusion and measurement of the infarct area after 3 days in the different brains sections indicated comparable levels throughout. However, the area of damage was less after 4 and 5 days (Fig. 2.4).

2.4.2. Effect of dizocilpine on the ischaemic damage induced by MCA-O

The effect of dizocilpine on 3 days MCA-O-induced cerebral infarction is shown in Table 2.2. When administered as a single dose (i.p.) at various time points after MCA-O (30 min, 60 min or 120 min), dizocilpine (0.3mg/kg; 1mg/kg and 3mg/kg) failed to produce any neuroprotection. The mean values of the infarcted area for each section were not significantly different from control values (Table 2.2.). Only when dizocilpine was administered immediately after the MCA-O as a single dose (1 mg/kg) was there any reduction in the size of cerebral damage (Table 2.2. $p<0.05$ ANOVA).

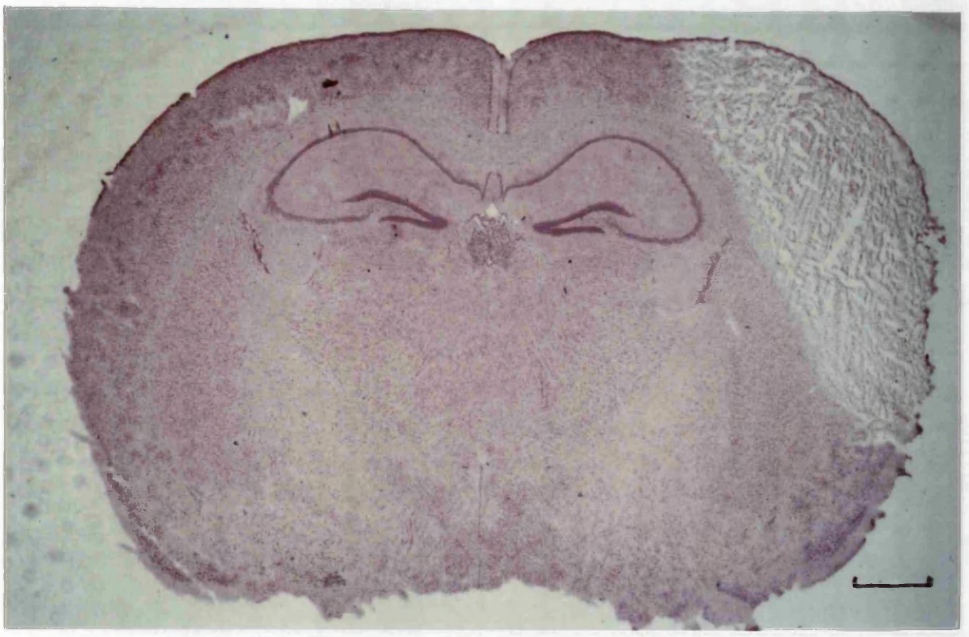
When dizocilpine (0.3mg/kg or 1mg/kg i.p.) was administered in consecutive doses 5min, 3h, 6h, 24h and 36h after MCA-O, a significant reduction in the infarcted area was obtained (Table 2.3.). Significant reduction of damage was obtained even if the start of dosing was delayed to 30 min post-ischaemia (Fig. 2.5). The qualitative appearance of the histological changes did not differ between untreated and treated

Fig. 2.3. Histological staining of mouse brain section 24 hours after MCA-O.

A. Mouse whole brain section stained with haematoxylin and eosin. Scale bar: 1mm.

B. Higher magnification of the section at boundary of infarct showing shrinkage and condensation of the perikaryon (dark neurons) and pallor of the neuron nucleoplasm (haematoxylin and eosin staining).
Scale bar: 25μm.

A



B

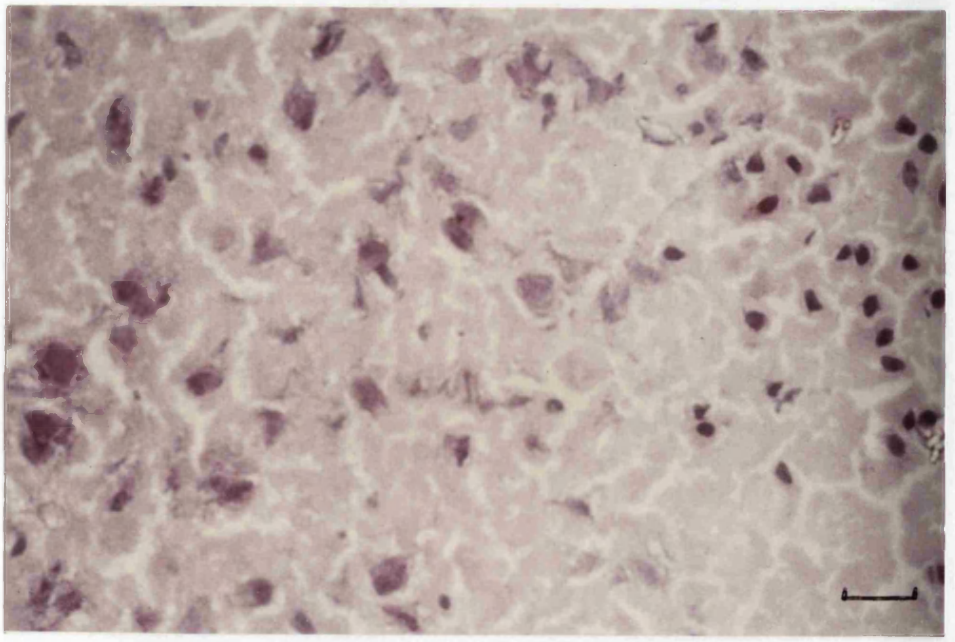


Table 2.1. Infarct area in mouse brain section 3 days after MCA-O

Section co-ordinates	A2100	A2750	A3250	A4150	A4750
Operated					
Mean mm^2	6.56	7.08	6.34	5.51	4.36
Mean %	15.58	15.39	13.84	11.15	8.28
s.e.m.	0.77	0.93	0.98	0.90	0.77
n	9	9	9	9	9
Sham					
Mean mm^2	0.22	0.37	0.44	0.46	0.31
Mean %	0.51	0.80	0.97	0.87	0.65
s.e.m.	0.33	0.53	0.71	0.77	0.49
n	6	6	6	6	6

Mean values are expressed as mm^2 and as a percentage of total area of brain section. Brain co-ordinates from Lehmann Atlas of mouse brain (1974).
$p<0.001$ vs operated, ANOVA.

Fig. 2.4. Mean area (± s.e.m.) of the cortical infarct determined up to 5 days in mice after MCA-O.

Values are expressed as mm^2 of brain damage. n=5 at each time point. Co ordinate of brain section: A3250. Maximum damage occurred by the first day and subsequently declined after 3 days.

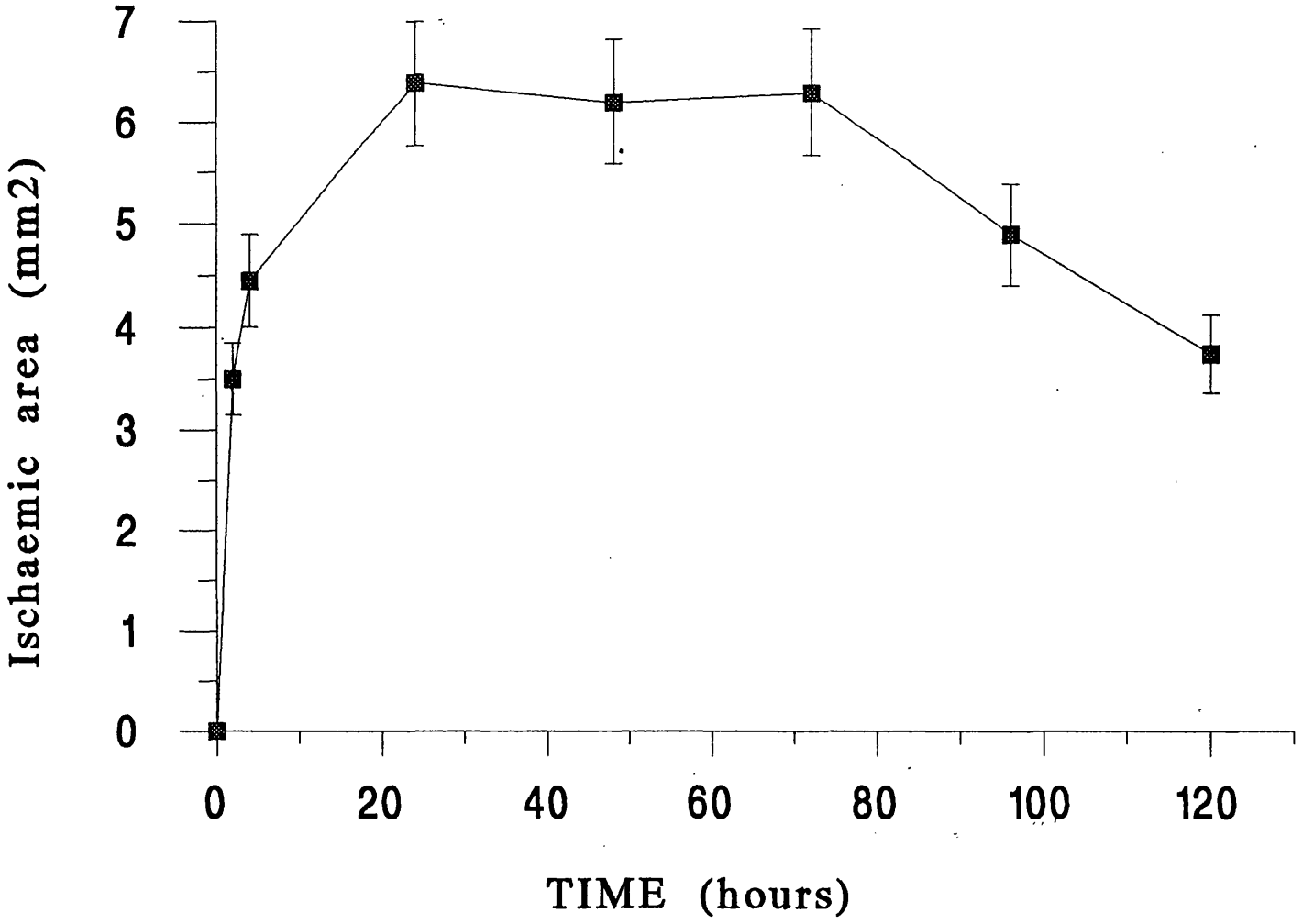

Ischaemic area (mm2)
0
1
2
3
4
5
6
7
0
20
40
60
80
100
120
TIME (hours)

Table 2.2. Effect of dizocilpine and CGP 37849 on infarct area in mouse brain sections 3 days after MCA-O. Single dose of drug administered intraperitoneally.

Mean values ± s.e.m. (control n=11, treatments n=3 or 4) represent area of infarct expressed as percentage of total area of brain section. For each treatment the time post-occlusion at which drugs were administered is indicated. Brain co-ordinates taken from the Atlas of the Mouse Brain (Lehmann, 1974).
* $p < 0.05$ ANOVA.

STEREOTAXIC COORDINATES		A2100	A2750	A3250	A4150	A4750
CONTROL (MCA-O alone)	Time of injection post occlusion	15.91±1.12	15.87±0.95	13.73±0.87	11.35±0.84	9.23±0.82
MCA-O plus:						
DIZOCILPINE 0.3 mg/kg	5 min	13.41±0.66	12.88±0.93	9.66±1.12	9.12±0.98	8.17±0.42
DIZOCILPINE 1 mg/kg	5 min	12.64±0.94	10.31±0.94*	8.34±0.78*	7.01±0.90*	5.62±0.59*
DIZOCILPINE 0.3 mg/kg	30 min	20.80±1.42	18.42±1.12	17.71±1.24	13.23±1.08	11.74±0.99
DIZOCILPINE 1 mg/kg	30 min	14.71±1.11	15.33±1.35	15.47±1.94	12.19±0.78	8.61±0.94
DIZOCILPINE 3 mg/kg	30 min	15.34±1.48	15.09±1.03	13.98±0.87	13.00±1.69	9.28±1.15
DIZOCILPINE 0.3 mg/kg	60 min	16.31±1.77	14.84±1.16	14.71±2.01	10.49±1.35	9.04±1.11
DIZOCILPINE 1 mg/kg	60 min	15.52±1.50	16.11±0.99	11.94±2.24	10.11±0.78	8.85±0.89
DIZOCILPINE 3 mg/kg	60 min	14.89±0.96	14.65±1.07	11.69±1.88	11.08±1.10	9.14±0.74
CGP 37849 3 mg/kg	5 min	14.11±1.12	13.24±0.99	11.36±0.78	10.11±0.85	8.12±0.88
CGP 37849 10 mg/kg	5 min	14.29±1.33	12.05±1.06	10.77±0.88	9.82±0.78	7.85±0.77
CGP 37849 3 mg/kg	30 min	18.11±1.56	14.91±1.26	10.19±2.00	9.56±1.31	7.84±0.92
CGP 37849 10 mg/kg	30 min	21.18±1.69	18.23±1.64	10.80±1.74	10.63±1.12	9.31±0.84
CGP 37849 3 mg/kg	60 min	14.31±0.79	10.62±1.08*	9.65±1.84	7.74±2.16	6.88±1.48
CGP 37849 10 mg/kg	60 min	17.84±1.52	15.56±0.98	9.58±1.96	9.66±1.76	7.23±1.66

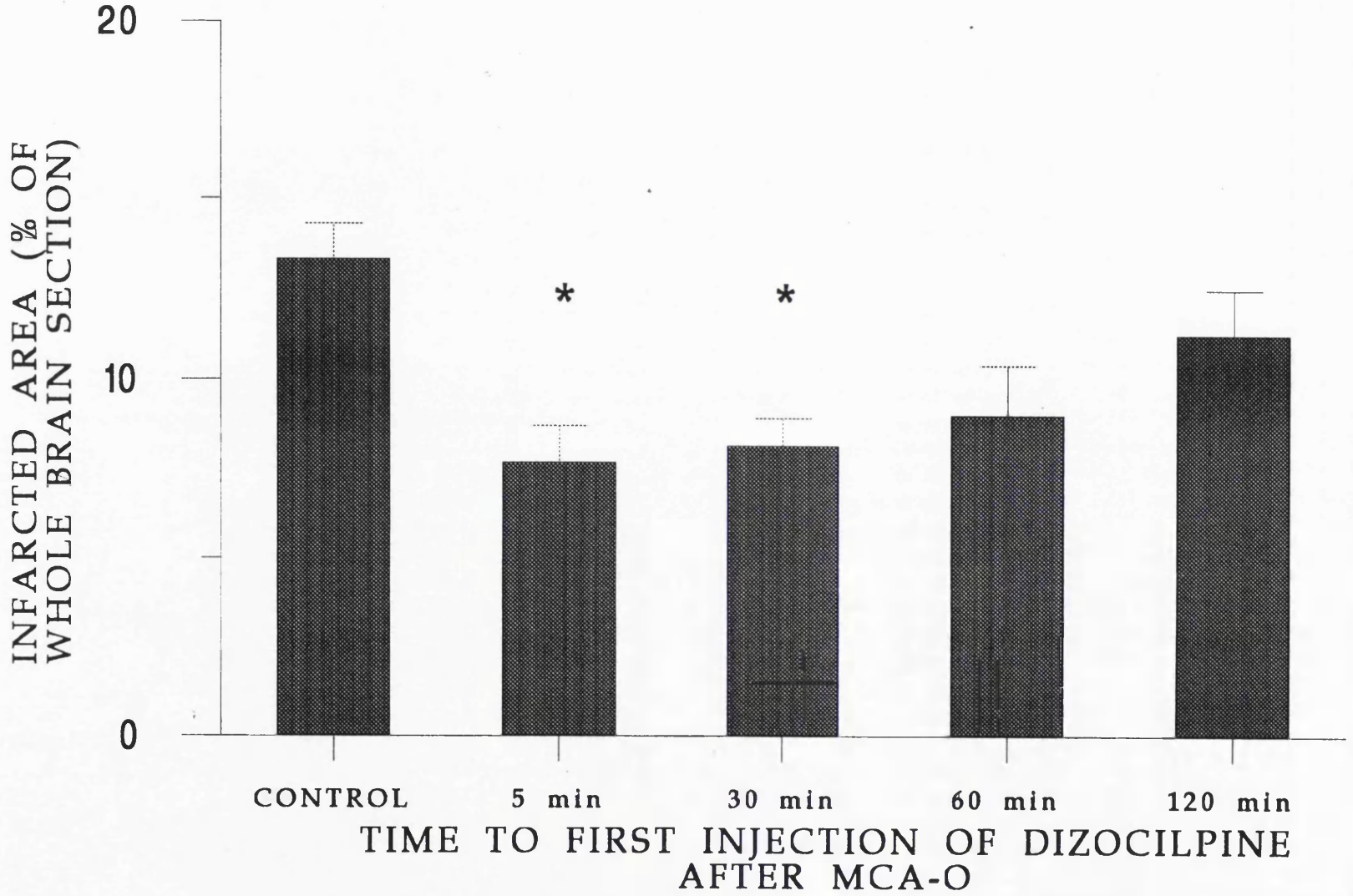
Table 2.3. Effect of dizocilpine and CGP 37849 on infarct area in mouse brain sections 3 days after MCA-O. Repeated doses of drug administered intraperitoneally.

Mean values ± s.e.m. (control n=7, treatments n=3 or 4) represent area of infarct expressed as percentage of total area of brain section. For each treatment the time post-occlusion at which the first dose of drug was administered is indicated. Subsequent doses were administered 3,6,24 and 36 hours after the first dose. Brain co-ordinates taken from the Atlas of the Mouse Brain (Lehmann, 1974).
* $p < 0.05$ ANOVA.(n=5).

STEREOTAXIC COORDINATES	Time to first dose after MCA-O	A2100	A2750	A3250	A4150	A4750
CONTROL (MCA-O alone)		15.03±0.72	14.79±0.89	12.55±1.11	12.02±1.32	8.74±0.89
MCA-O plus:						
DIZOCILPINE 0.3 mg/kg	5 min	10.03±0.89*	9.37±0.94*	7.71±1.02*	8.22±0.97*	6.29±1.88
DIZOCILPINE 1 mg/kg	5 min	12.11±1.15	11.51±1.88	7.41±0.98*	6.89±1.08*	7.00±1.33
DIZOCILPINE 0.3 mg/kg	30 min	10.68±0.87*	9.11±0.84*	8.16±0.77*	8.01±0.79*	7.03±1.06
DIZOCILPINE 0.3 mg/kg	60 min	11.74±1.45	9.73±0.86*	9.02±1.36	8.59±1.12	6.79±1.56
DIZOCILPINE 0.3 mg/kg	120 min	12.32±1.56	11.57±0.96*	11.23±1.24	9.53±0.89	8.61±0.90
CGP 37849 10 mg/kg	5 min	11.02±1.16	13.14±1.69	12.23±0.98	11.19±1.16	10.37±1.00
CGP 37849 10 mg/kg	30 min	13.80±1.07	10.56±0.98*	9.98±0.78*	8.13±1.24*	8.26±0.89
CGP 37849 10 mg/kg	60 min	14.37±1.59	13.96±1.11	12.26±0.68	9.68±1.03	8.82±0.96

Fig. 2.5. Neuroprotection afforded by dizocilpine treatment in MCA-O mice: influence of delay of dosing treatment post-occlusion.

Infarcted area was measured in control and treated (dizocilpine 0.3 mg/kg) mice 3 days after MCA-O. Values are expressed as % of the whole brain section. Time to first injection of dizocilpine after MCA-O is indicated. Area of damage was significantly lower in mice treated 5 and 30 min after MCA-O, whereas at 60 and 120 min the measured area was not significantly reduced.
* $p < 0.05$ ANOVA. $(n = 5)$.


20
10
0
INFARCTED AREA (% OF WHOLE BRAIN SECTION)
*
*
CONTROL
5 min
30 min
60 min
120 min
TIME TO FIRST INJECTION OF DIZOCILPINE AFTER MCA-O

animals, only the size of the infarcted area was reduced by the drug.

Dizocilpine (0.3 or 1 mg/kg i.p.) produced a significant protective effect when administered 30 min prior to MCA-O (Fig. 2.6. and Fig. 2.7).

2.4.3. Effect of CGP 37849 on the ischaemic damage induced by MCA-O

CGP 37849 (3mg/kg or 10mg/kg) produced no neuroprotection when administered as a single dose at any time point post-ischaemia (Table 2.2.).

The repeated dose-regime strategy also failed to produce any significant reduction in neuronal damage when administration was commenced 5 min after MCA-O but, surprisingly, a significant reduction in the size of the infarct was produced by repeated doses of CGP 37849 (10mg/kg) when the first administration was given 30 min after MCA-O (Table 2.3).

Pre-ischaemia treatment with a single dose CGP 37849 (10 mg/kg) appeared to produce a decrease in ischaemic damage, but this was not statistically significant (maximum effect in section A2100: 21.5% reduction, n=6)

2.4.4. Effect of compound Z on the ischaemic damage induced by MCA-O

Compound Z (3 or 10 mg/kg) administered prior to MCA-O resulted in a significant reduction in neuronal damage (Fig. 2.8., maximum effect in section A2100: 6.56 mm^2 ± 0.77 s.e.m (control, n=9), 2.62 mm^2 ± 0.51 s.e.m. (compound Z 3 mg/kg, n=8), 3.38 mm^2 ± 0.50 (compound Z 10 mg/kg, n=10).

When compound Z was administered as a single dose (10 mg/kg i.p.) at various time points after MCA-O, a reduction in the infarcted area was obtained up to 30 min post-

MCA-O, although it was statistically significant only in section A2100 (Table 2.4.).

When administered in consecutive doses, compound Z (10 mg/kg) produced a significant reduction of damage even if the start of dosing was delayed to 60 min post-ischaemia (Table 2.4.). Although a reduction of the area of infarct was observed when the first dose was administered 120 min post-ischaemia, the effect was not statistically significant (Table 2.4.).

Fig. 2.6. Effect of dizocilpine on infarct area in mouse brain sections 3 days after MCA-O. Single dose of drug (0.3 mg/kg i.p.) administered 30 min prior to MCA-O.

* $p < 0.05$ ANOVA.(n=5).

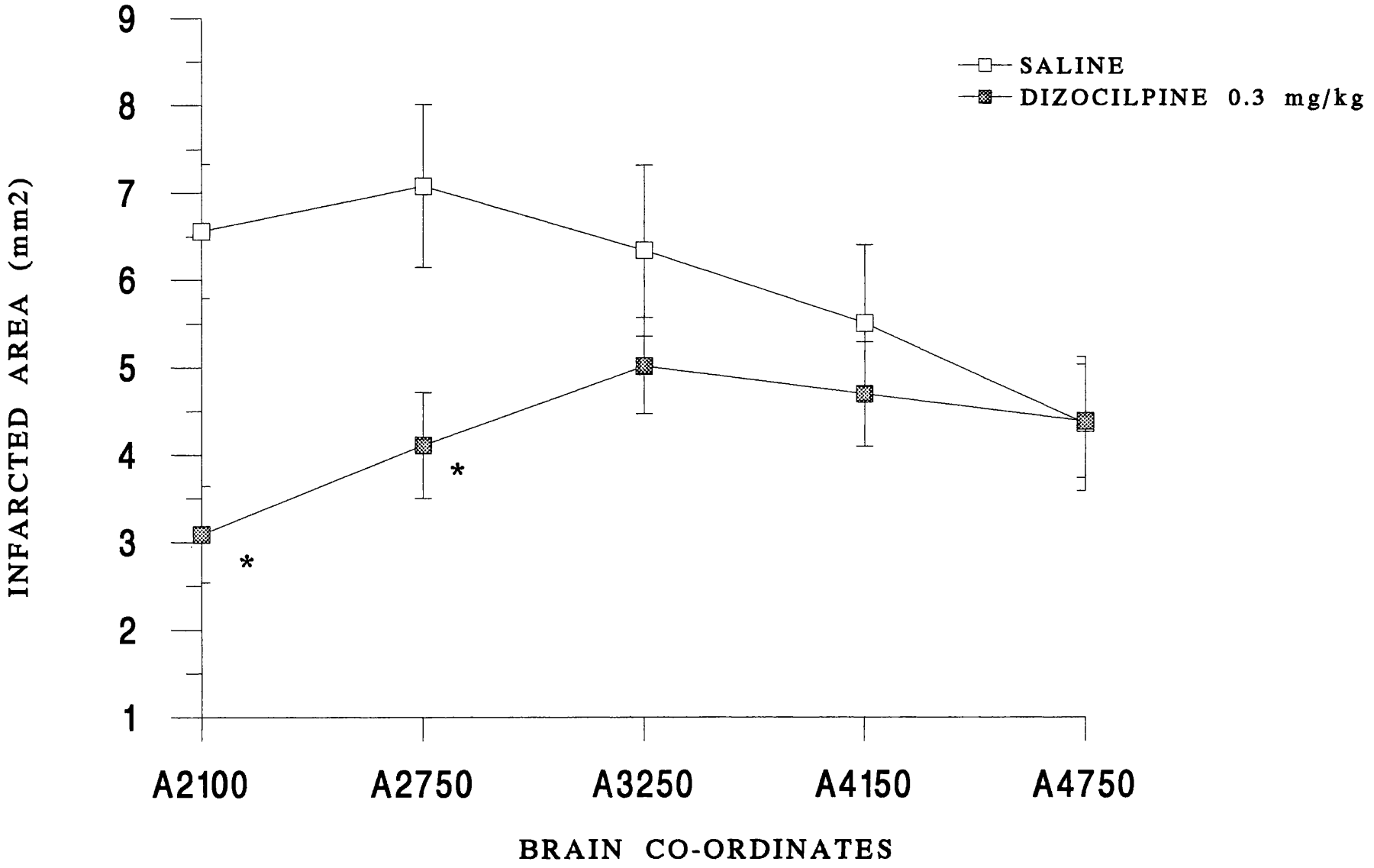

INFARCTED AREA (mm2)
9
8
7
6
5
4
3
2
1
SALINE
DIZOCILPINE 0.3 mg/kg
*
*
A2100
A2750
A3250
A4150
A4750
BRAIN CO-ORDINATES

Fig. 2.7. Effect of dizocilpine on infarct area in mouse brain sections 3 days after MCA-O. Single dose of drug (1 mg/kg i.p.) administered 30 min prior to MCA-O.

* $p < 0.05$ ANOVA. (n=5)

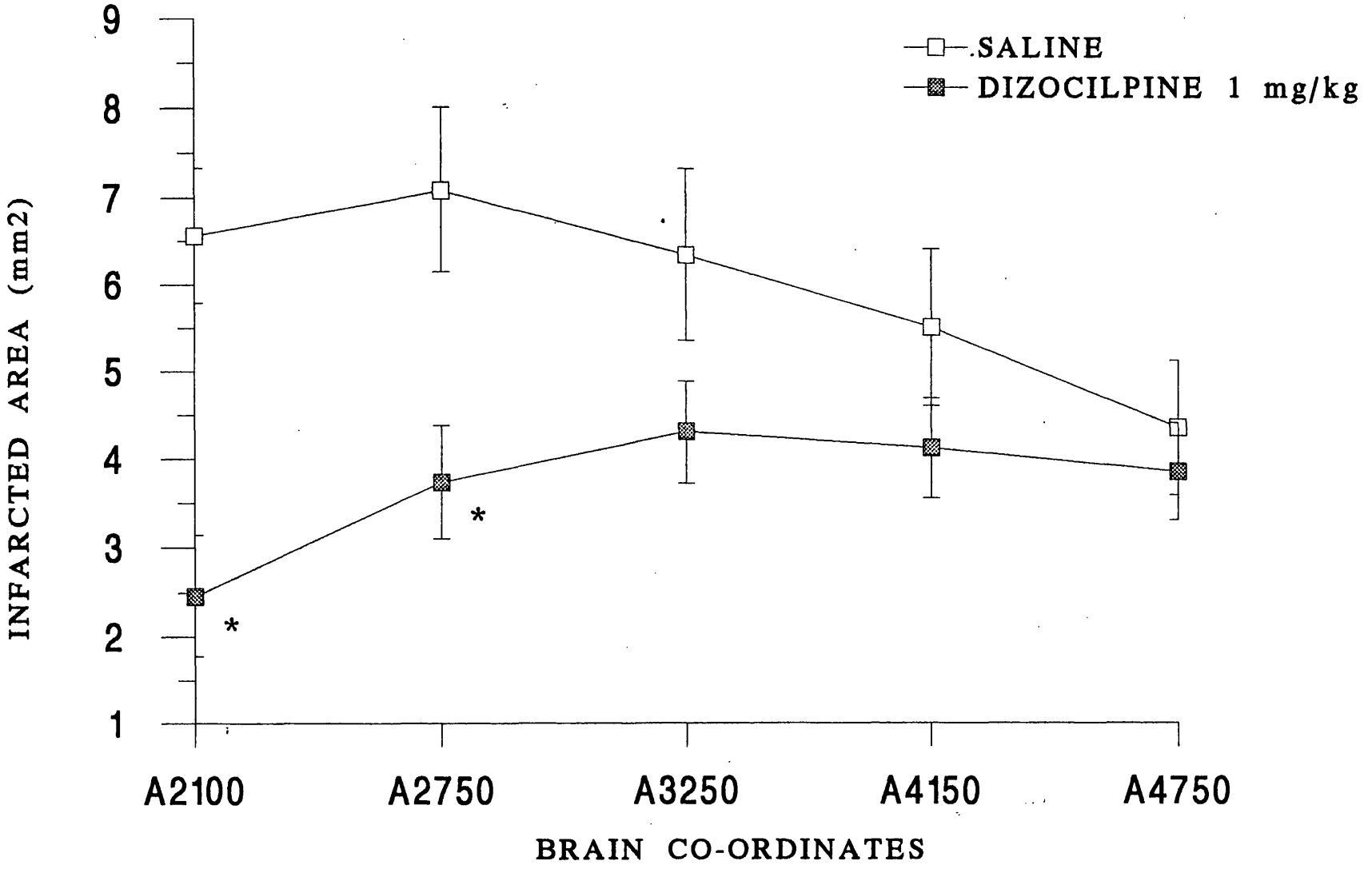
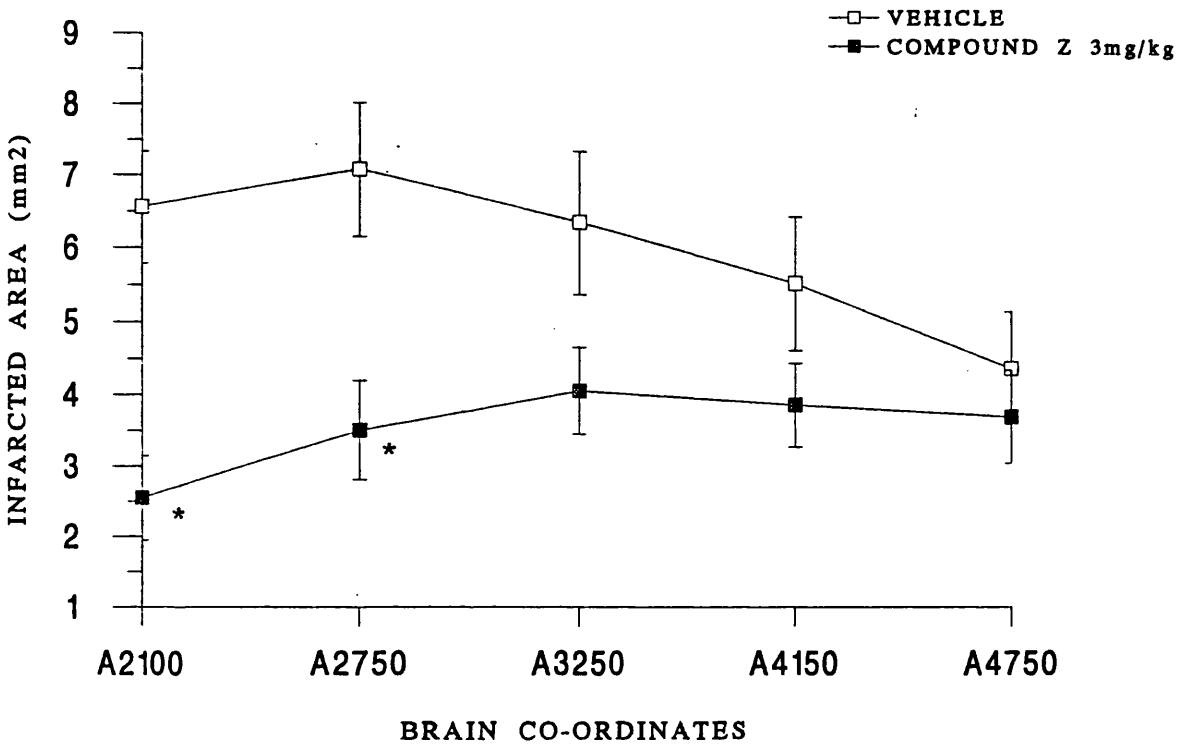

SALINE
DIZOCILPINE 1 mg/kg
INFARCTED AREA (mm2)
9
8
7
6
5
4
3
2
1
*
*
A2100
A2750
A3250
A4150
A4750
BRAIN CO-ORDINATES

Fig. 2.8. Effect of compound Z on infarct area in mouse brain sections 3 days after MCA-O. Single dose of drug (3 or10 mg/kg i.p.) administered 30 min prior to MCA-O.

* $p < 0.05$ ANOVA. (n=5)


VEHICLE
COMPOUND Z 3mg/kg
INFARCTED AREA (mm2)
9
8
7
6
5
4
3
2
1
*
*
A2100
A2750
A3250
A4150
A4750
BRAIN CO-ORDINATES

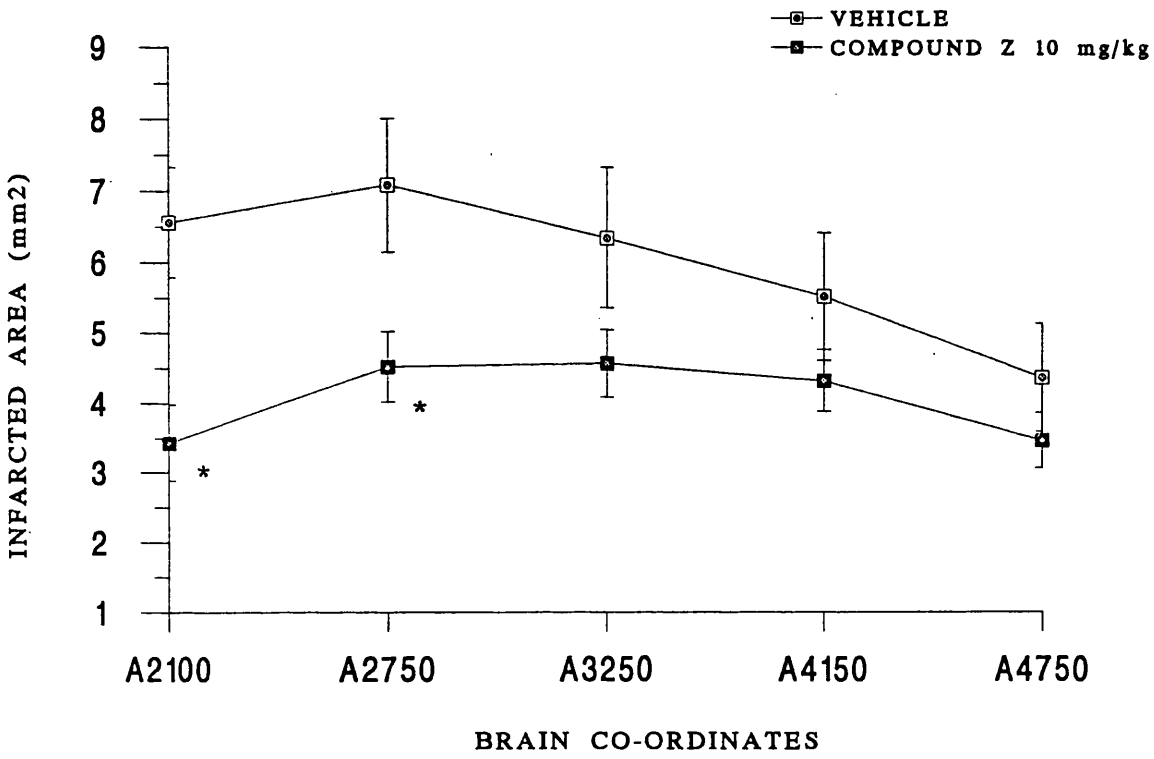

VEHICLE
COMPOUND Z 10 mg/kg
INFARCTED AREA (mm2)
9
8
7
6
5
4
3
2
1
*
*
A2100
A2750
A3250
A4150
A4750
BRAIN CO-ORDINATES

Table 2.4. Effect of compound Z on infarct area in mouse brain sections 3 days after MCA-O.

Mean values ± s.e.m. represent area of infarct expressed as percentage of total area of brain section. For each treatment the time post-occlusion at which drugs were administered is indicated. In the repeated doses treatment, compound Z was administered 3, 6, 24 and 36 hours after the first dose. Brain co-ordinates taken from the Atlas of the Mouse Brain (Lehmann, 1974).
* $p < 0.05$ ANOVA.(n=5).

Stereotaxic co-ordinates		A2100	A2750	A3250	A4150	A4750
Control (MCA-O alone)	Time of injection post-occlusion	15.91±1.12	15.87±0.95	13.73±0.87	11.35±0.84	9.23±0.82
Compound Z (Single dose 10mg/kg i.p.)	5 min	9.66±0.90 *	10.24±0.96 *	11.64±0.77	8.17±0.81 *	5.48±0.64 *
	30 min	10.38±0.91 *	13.64±0.78	12.08±0.86	10.51±0.70	8.44±0.70
	60 min	14.66±1.04	15.11±0.99	15.47±0.88	11.18±0.98	10.75±1.17
Control (MCA-O alone)	Time to first dose after MCA-O	15.03±0.72	14.79±0.89	12.55±1.11	12.02±1.32	8.74±0.89
Compound Z (Repeated doses 10 mg/kg i.p.)	5 min	9.76±0.94 *	9.42±0.76 *	8.40±0.81 *	6.64±1.20 *	6.84±1.11
	30 min	9.84±1.08 *	9.08±0.96 *	8.24±0.84 *	8.31±0.56 *	7.17±0.98
	60 min	10.50±0.89 *	11.44±0.85 *	11.66±1.18	9.33±0.99	8.18±0.86
	120 min	13.57±1.21	12.81±1.09	10.44±0.96	9.65±1.00	8.20±1.40

DISCUSSION

For the present study a model of focal ischaemia was chosen to investigate the neuroprotective effect of compounds acting at different sites of the NMDA receptor complex. The MCA-O in mouse offers, like the rat MCA-O model, the advantages of a simple technique. A practical advantage of this model, compared to the rat, is that the MCA can be identified under the semi-translucent skull allowing the size of the craniectomy to be kept to a minimum. Due to the small size of the animal, the technical procedure in the mouse is less sophisticated then in bigger animal species in terms of monitoring the pathophysiological parameters. This allows the use of a large number of animals but, at the same time, represents the main limitation of the mouse model since important information, such as cerebral blood flow, glucose use, blood pressure or brain temperature can-not be monitored continuously. However, the reproducibility of the brain lesion was confirmed in the present study and the qualitative and quantitative profile of the damage in operated mice, compared to sham operated animals, was well defined for testing the neuroprotective effect of drugs. Initial experiments were designed to validate the (ischaemia) model and as dizocilpine had been widely used as a neuroprotective agent in other models of focal ischaemia, it was the reference compound. Investigations have been made to determine the dose-response relationship and the therapeutic window using dizocilpine in this model. It was observed that dizocilpine failed to reduce the size of ischaemic damage when administered as a single dose treatment i.p. 30 min, 2 or 4 hours after MCA-O. By

contrast, dizocilpine did produce significant neuroprotection when a repeated dose regime (0.3 mg/kg/dose) was started at 30 min post MCA-O. Under these experimental conditions there was an apparent reduction in the area of ischaemic damage even when the start of multiple dosing was delayed for 1 or 2 hours following MCA-O, although it was statistically significant only at the level A2750. These observations indicate that both the concentration of drug and the time point of the initial application are crucial. The results are in agreement with other studies which showed a neuroprotective effect for dizocilpine up to 30 min or 1 hour following MCA-O in rat (Park et al., 1988; Hatfield et al., 1992), up to 2 hours after MCA-O in cat (Ozyurt et al., 1988), and also with data obtained in the mouse model using SL82.0715 (Gotti et al., 1990). In the present study, when dizocilpine was administered 5 min after MCA-O a significant neuroprotective effect was observed, irrespective of the single or multiple dosing strategy used.

It is well known that hypothermia produces a partial protection against ischaemia-induced neurodegeneration (Busto et al., 1989) and it was demonstrated that, in some cases, the neuroprotection produced by dizocilpine could be, at least in part, due to the hypothermic effect of this drug. (Buchan and Pulsinelli, 1990). Although important, hypothermia does not appear to compromise the positive results obtained in many studies with dizocilpine, in which the temperature had been strictly controlled. In the present study, body temperature was maintained within physiological limits during both surgery and recovery.

The plasma and CSF concentrations of dizocilpine required for neuroprotection from

quinolinate neurotoxicity (Willis et al., 1991) and from focal ischaemia (Gill et al., 1991) correlate with the known affinity of dizocilpine for the NMDA receptor complex, indicating that its neuroprotective effects are mediated by NMDA receptor blockade. Compounds acting at the ion-channel of the NMDA receptor complex are highly lipophilic molecules which can readily cross the blood-brain barrier and reach their target within minutes of administration. For this reason this class of compounds show a particular advantageous profile in the post-ischaemia period, giving a time window of up to 3 hours for therapeutic intervention. However, the behavioural profile shown by these compounds, generally regarded as PCP-like behaviour, has limited their progress into the clinic. This class of compounds also appears to produce morphological alterations (Olney et al., 1989b), cardiovascular effects (Lewis et al., 1990) and to affect learning and memory (Woods et al., 1991) and glucose utilization (Kurumaji et al., 1989; McCulloch et al., 1991).

CGP 37849 has been reported to be anticonvulsant when administered systemically by a competitive action at the NMDA receptor complex (Fagg et al., 1990). At a dose of 10 mg/kg, CGP 37849 has been reported to be effective *in vivo* by producing a reduction of the responses to iontophoretically administered NMDA by 90% in the hippocampus, (Fagg et al., 1990). In the present study, at the doses of 3-10 mg/kg, CGP 37849 produced no significant reduction in infarct size when administered as a single dose at any time point post-ischaemia. The lack of neuroprotection may be explained by the mechanism of action of the compound implying competition with excessive levels of extracellular glutamate following the ischaemic event, possibly indicating that under

ischaemic conditions higher doses are required. Since CGP 37849 has been reported to penetrate the blood-brain barrier slowly and to concentrate in the brain following peripheral administration (Fagg et al., 1990) it was decided to perform a repeated dose-regime treatment. A significant reduction in the size of the infarct was produced by repeated doses of CGP 37849 (10mg/kg) when the first administration was given 30min after MCA-O (Table 2.3.), although this result is difficult to explain in view of the lack of neuroprotection observed when the first administration was given 5 min after MCA-O.

Analogues of the early competitive NMDA antagonists (AP5 and AP7) show improved CNS permeability over the parent compounds and exhibit potent anticonvulsant effect following systemic administration. However, these compounds still penetrate the blood brain barrier relatively slowly. At neuroprotective doses competitive NMDA antagonists share some of the behavioural effects of the non-competitive ion-channel blockers, whereas these undesirable effects appear less evident at anticonvulsant doses (Woods et al., 1991).

It has been demonstrated that occupation of the glycine site of the NMDA receptor complex is a pre-requisite for receptor activation (Kleckner and Dingledine, 1988). The glycine recognition site represents, therefore, a potential mechanism by which the overexcitation of NMDA receptors that occurs during ischaemia can be controlled. Investigation of the neuroprotective effect of the glycine antagonist compound Z, administered as a single dose of 3 or 10 mg/kg i.p. 30 min before MCA-O, indicated a significant reduction of the infarcted area which was comparable to the reduction

produced by dizocilpine (0.3 or 1 mg/kg i.p.) at the same time before vessel occlusion. Of particular relevance is the reduction of infarct observed when compound Z was administered (i.p.) 60-120 min after the induction of ischaemia, indicating a better anti-ischaemic profile than dizocilpine in this model.

Compounds acting at the glycine site of the NMDA receptor complex generally show poor penetration across the blood-brain barrier. Good CNS bioavailability is exhibited by partial agonist such as HA-966, D-cycloserine and L-687,414 and this may be of therapeutic interest. Aside the variety of potent and selective full glycine antagonists available, in most cases their *in vivo* activity is very limited. Only recently, have some novel glycine antagonists been reported to exhibit anticonvulsant activity (L-701,252, ED50 4.1 mg/kg; L-695,902, ED50 12.5 mg/kg. Rowley et al., 1993) or anti-ischaemic effect (ACEA 1021) when administered systemically. Thus, the fact that systemic administration of compound Z produced significant neuroprotection in a model of focal ischaemia represents an interesting observation.

Under ischaemic conditions, a glycine site antagonist might prove more advantageous than a competitive NMDA antagonist which would have to compete with high levels of extracellular glutamate. The behavioural profile of glycine antagonists also appears to be improved over other NMDA antagonists acting at the glutamate or PCP recognition sites (see Chapter 5). In addition, while ion-channel blockers and competitive NMDA antagonists have been reported to impair memory and learning, D-cycloserine, a partial agonist at the glycine site, has been proposed as a memory enhancer (Monahan et al., 1989).

Many investigations have assessed histological outcome 3-6 hours after MCA-O and reduction of infarct size up to 24 hours after administration. However, it seems reasonable to measure infarct size also at later time points after MCA-O, since development of infarction could be only delayed by the drug treatment, without producing any significant effect on the final size of the damage at many hours after the vessel occlusion. For this reason, in the present study the extent of cerebral infarct and the potencies of various NMDA antagonists in suppressing the area of damage, were evaluated at 72 hours after MCA-O.

Initial investigations were focused on the histological profile and temporal development of the infarct. In the rat MCA-O model, the infarction is assumed to develop to its maximal size over the first 4 hours following MCA-O and does not change significantly in size for at least the next 6 days. By contrast, in the mouse MCA-O model the extent of the infarction was maximal at days 1-3 followed by a tendency to infarct reduction. It has recently been suggested that this might reflect an increase of tissue volume as a consequence of cerebral oedema in the early phase of the development of the infarct (day 1-3), which is no longer observed at later time points (day 7; Chiamulera et al., 1993). The oedema is suggested to be a consequence of endogenous proteins extravasation which is supposed to begin during the first few hours after MCA-O (Petito et al., 1979). For this reason, day 3 after MCA-O in this model may not be the best end-point for evaluation of neuroprotective drugs since the oedema process might determine a certain degree of unpredictability in the actual size of the lesion.

In many MCA-O studies, a greater significant protection by anti-ischaemic drugs has been found towards the extremities of the lesion which tend to have a greater

proportion of penumbra rather than core. In this study a fairly flat infarct size was observed throughout the different levels of mouse brain examined. The reason might be peculiar to this animal model and, consequently, to the brain co-ordinates we used. At the extremities of the lesion in mouse brain after MCA-O, the size of damage was very small and brain co-ordinates were therefore confined to A2100-A4750 to minimize errors.

CHAPTER 3: COMPARATIVE RECEPTOR AUTORADIOGRAPHY OF *EX VIVO* AND *IN VITRO* ^{3}H-DIZOCILPINE BINDING IN MOUSE BRAIN AFTER MIDDLE CEREBRAL ARTERY OCCLUSION

3.1. INTRODUCTION

3.1.1. Receptor autoradiography

Neurochemical methods involving the use of radioligand binding to membrane preparation have provided a useful way of clarifying the pharmacological characteristics of a certain receptor, generating valuable data on novel therapeutic agents, mechanisms of drug action and allosteric interaction within receptor complexes (e.g. Bonhaus et al., 1987). Radioligand binding techniques have also proved essential in studies with isolated receptor proteins (Michaelis et al., 1984; Boulter et al., 1990). However, the fact that receptors in disrupted membrane fractions may behave differently to those *in vivo*, and the insufficient information about their anatomical localisation provided by membrane binding assays, led to the development of the receptor autoradiography technique. Quantitative autoradiography of macroscopic specimens is now widely used for receptor studies and more recently it has been employed for measuring mRNA in tissue sections by *in situ* hybridization (Nunez et al., 1987). The major advantages of the technique are that it retains the morphology of the tissue allowing the resolution of discretely labelled structures and facilitates the detection of lower levels of radioactivity than liquid scintillation spectrometry (Rogers, 1979). Many initial technical problems have been solved by the development of ^{3}H-sensitive film (LKB Ultrofilm) in which an even emulsion consisting of silver bromide crystals suspended in gelatin is attached to a flat surface, and to which a number of sections can be apposed. Radioactive standards produce film images of varying optical density, and densitometry allows binding in different sections to be quantified and compared (Raimbow et al., 1982).

Early autoradiographic experiments performed binding studies *in vivo* by injecting animals with ^{3}H-ligands (Stumpf and Roth, 1966), to investigate the distribution of dopamine, opiate and cholinergic muscarinic receptors (Kuhar and Yamamura, 1975; Pert et al., 1975; Kuhar et al., 1978). Serious problems such as the metabolic degradation of radioligands led to rejection of this technique in favour of *in vitro* methods. Labelling tissue *in vitro* obviates such problems as the blood-brain barrier and metabolism by the liver. Experimental conditions can be tightly controlled by selecting ions, drugs, temperature and pH to prevent breakdown of the label, to optimise the signal-to-noise ratio, and to restrict binding from certain sites. However, *in vitro* assays do not take into consideration factors such as drug absorption, metabolism, penetration and distribution. In this regard, *ex vivo* autoradiography can be advantageous for studying receptor occupation *in vivo* and its correlation with functional effects. In *ex vivo* binding animals are injected with radiolabelled ligand and brain sections then cut for apposition to ^{3}H-sensitive film.

Agonist and antagonist radioligands for EAA receptors are often labelled with the hydrogen isotope tritium, mainly because tritiated drugs are chemically very similar to the unlabelled form and insufficient specific activity can be obtained with ^{14}Carbon. Amino acids can be regarded as very small drug molecules, to which the introduction of a foreign atom may destroy or reduce pharmacological activity as well as binding activity. Also, the long half-life (12.1 years) and the reduced health risk arising from tritium low energy radiation, are important factors to be considered. The most limiting disadvantage with using tritium is the time scale involved: exposure times of several

weeks, even months, are often required to obtain a suitable autoradiogram.

Since receptor proteins constitute a minority of the tissue in a brain slice, some of the radioactivity is expected to bind to other tissue components, particularly carrier proteins and enzymes. Radioligands may also bind to glass slides, or simply remain trapped in the section after washing. The sum total of these effects is defined as non-specific binding. This variable is accounted for by including slides with unlabelled drug to saturate the receptors. 100 times the IC_{50} is regarded as suitable (Burt, 1985). Any signal remaining when the receptor sites are saturated is assumed to represent unsaturable, non-specific binding. This value is subtracted from the other points to give estimates of label bound to receptors, the specific binding.

A further requirement is a method of separating the bound and free radioligand. When slide-mounted sections are used, the removal of free radioligand can be achieved with simple rinsing. The efficiency of this process depends on the use of thin tissue sections and several changes of fresh, cold washing buffer.

However, the most important requirement for autoradiographic experiments is a stable radioligand with selective high affinity for receptor systems. In theory, the ideal radioligand is the endogenous transmitter ligand. Radiolabelled AMPA, glutamate, aspartate, glycine, kainate, ifenprodil, dizocilpine, TCP, CPP, CNQX, CGS 19755, 5-7-dichlorokynurenic acid have all been used to study subtypes of EAA receptors (Honore' et al.1982, and 1989; Cross et al., 1986 and 1987; Wong et al., 1987; Murphy et al., 1988; Beart et al., 1991; Baron et al., 1991).

The development of quantitative autoradiographic binding assays for each of the EAA receptor subtypes has allowed the biochemical and pharmacological characterization of the glutamate receptor subtypes that correspond to the electrophysiologically defined receptors (Young and Fagg, 1990). Furthermore, the anatomical localization of each of the EAA receptors can now be assessed. In addition, receptor autoradiographic studies have also been used to determine kinetic and equilibrium saturation information about the pharmacological properties of the EAA receptor subtypes, both in normal and diseased tissue.

3.1.2. Rceptor autoradiography following MCA-O

As described in Chapter 2, the development of infarction, together with the detailed histological profile following ischaemia, have been characterized in various experimental models of cerebral stroke, and it appears that a severe reduction of CBF can lead to irreversible neuronal damage within a few hours (3-4) of the insult. Evidence indicates that over-activation of NMDA receptors is implicated in the sequence of cellular events leading to neuronal damage after ischaemia and this view is supported by the amelioration of ischaemic brain injury by NMDA receptor antagonists in several animal models (see Chapter 2). The NMDA receptor complex represents an important target for potential neuroprotective agents and, therefore, evaluation of the time course of the functional integrity of this receptor complex in the post-ischaemia period is crucial. In a model of global ischaemia in rat, no significant alteration in the NMDA receptor complex was detected in the hippocampus up to 24 hours after the ischaemic insult (Westerberg et al., 1987). In a model of global ischaemia in gerbil,

binding characteristics of the NMDA receptor complex appeared to be unchanged for at least 48 hours after the insult in the CA1 hippocampal region, even though a decrease in neuronal staining was observed at that time (Bowery et al., 1988). Although the neuroprotective effects of NMDA antagonists have been shown to be more reproducible in models of focal ischaemia (see Chapter 2), the time course of receptor viability in these animal models has been less studied. Experiments on synaptic membranes prepared from rat brain after focal ischaemia indicated that 12 hours after the ischaemic episode the NMDA receptor complex in the ischaemic area was not significantly altered (Dewar et al., 1989).

Binding of ^{3}H-dizocilpine to the NMDA receptor complex in mammalian brain tissue is agonist dependent, possibly reflecting the use-dependent functional antagonism produced by this non-competitive antagonist (see Chapter 1 and Chapter 4). ^{3}H-dizocilpine binding can therefore be regarded as an indicator of the activation state of the NMDA receptor complex and, consequently, it may represent a measure of the integrity and functionality of this receptor complex. For this purpose, in the present study we have utilized an *in vitro* receptor autoradiography technique in mouse brain sections and have assessed the distribution of ^{3}H-dizocilpine binding sites at different times after MCA-O. Also, if the NMDA receptor is to be a target for neuroprotective agents administered post-ischaemia, it is important to determine not only the time course of the viability of the receptor but also the period post-ischaemia during which drugs can gain access to the receptors in the region of the infarct. Thus, in the present study, we have studied the *ex vivo* distribution of densities of ^{3}H-dizocilpine binding sites in mouse brain after MCA-O and have compared this with the *in vitro* ^{3}H-

dizocilpine binding after MCA-O using receptor autoradiography. The aim was to provide a measure of the relationship between functional integrity and accessibility of the receptor post-ischaemia.

3.2. METHODS

3.2.1. Receptor autoradiography: experimental procedure

Male CD1 mice (20-30 g) were killed by decapitation. The brains were quickly removed, frozen in isopentane pre-cooled in liquid nitrogen and stored at -80 ^{o}C. Brains were allowed to equilibrate to the temperature of a Reichert-Jung cryostat (-20^{o}C) and 12 μm thick sections were cut and thaw-mounted onto glass microscope slides.

3.2.2. *In vitro* ^{3}H-dizocilpine autoradiography

Dried sections were preincubated at room temperature for 60 min in Tris-HCl (50 mM, pH 7.4) and allowed to air dry. After drying 100 μl of assay solution containing 40 nM ^{3}H-dizocilpine (53.1 Ci/mmol) with or without unlabelled drug were applied over each section. Sections were incubated for 20 min at room temperature, washed twice for 30 sec in Tris-HCl (50 mM, pH 7.4, 23^{o}C) and rinsed quickly in deionised water. Non-specific binding was determined in the presence of 100 μM unlabelled (+)-dizocilpine maleate salt in the incubation solution.

3.2.3. *In vitro* binding of ^{3}H-dizocilpine following MCA-O

Male CD1 mice were subjected to MCA-O using the same procedure described in Chapter 2 and they were sacrificed at different times after MCA-O. No radioactive tracer was administered to these animals prior to sacrifice. Each brain was removed rapidly and 12 μm coronal sections were taken at the following brain co-ordinates,

according to the Stereotaxic Atlas of the Mouse Brain (Lehmann,1974): A2100, A2750, A3250, A4150 and A4750 μm, and these sections were used for autoradiography. Experimental procedures for *in vitro* ^{3}H-dizocilpine binding were the same as described above.

3.2.4. *Ex vivo* binding of ^{3}H-dizocilpine

Male CD1 (20-30 g) were injected i.p with vehicle (saline 0.9 % w/v) or dizocilpine (1 mg/kg) dissolved in vehicle. Injections were administered in a volume of 100 μl. After 15 min mice were injected i.v. (tail vein) with 50 μCi of ^{3}H-dizocilpine in 50 μl saline and decapitated 10 min later. The brains were quickly removed and frozen in isopentane pre-cooled in liquid nitrogen. Cryostat sections were dried at room temperature and washed twice for 45 sec in Tris HCl buffer (pH 7.4, 23^{o}C), dip washed in double distilled water and air dried. Preliminary experiments determined that this length of rinse optimized the specific binding ratio. Background binding, obtained following injection of unlabelled dizocilpine 1 mg/kg, was subtracted in each brain region.

3.2.5. *Ex vivo* binding of ^{3}H-dizocilpine following MCA-O

At different times after MCA-O, animals were injected via the tail vein with 50 μCi ^{3}H-dizocilpine in 50 μl saline and decapitated 10 min later. In one group of animals, brains were removed 60 min after injection of radioligand. Without adding any further radioactivity, cryostat sections were subjected to the washing procedure for *ex vivo* ^{3}H-dizocilpine binding described above. Local densities of binding were determined in the same regions of interest as in the *in vitro* binding investigations by quantitive

densitometry.

3.2.6. Autoradiogram generation

At the end of each binding assay, autoradiograms were generated by exposing the dried sections to tritium-sensitive film (LKB Ultrofilm-^{3}H, Amersham) for about 4 weeks (exposure time depends on the amount of total binding present on the section). Some of the sections were placed in scintillation vials containing 10 ml Opti-phase (LKB) for direct quantitation of radioactivity by liquid scintillation spectrometry (LKB). High-level methacrylate strip ^{3}H standards (RPA 506 or RPA 507, Amersham) were exposed to the same film. After exposure, films were developed in Kodak D-19 for 1-2 minutes at room temperature, rinsed quickly in tap water, fixed in Kodak Unifix for 5 min and then washed in tap water for 10 minutes (23^{o}C).

3.2.7. Densitometric analysis of autoradiograms

Autoradiograms were analyzed with a computer-assisted video image based instrument (Quantimet 970, Cambridge instruments) and regional film densities were converted to the corresponding ligand concentration by reference to the tritium standards on the same film.

Abbreviation used are from Lehmann (1974) and Paxinos & Watson (1982)

3.2.8. Materials

Chemicals were obtained from the following sources: (+) ^{3}H-dizocilpine (specific activity 53.1 Ci/mmol) was custom-prepared by Amersham U.K.; (+) dizocilpine

maleate salt, RBI U.S.A.; all other reagents were of analytical grade.

3.3. RESULTS

The histological changes observed in mouse brain sections subjected to MCA-O, together with the quantification of the lesion are described in Chapter 2.

3.3.1. Distribution of ^{3}H-dizocilpine binding sites in mouse brain *in vitro*

The regional distribution of ^{3}H-dizocilpine binding sites in mouse brain sections was heterogeneous. The highest levels of specifically bound radioactivity were observed in the hippocampus (CA1 radiatum, CA2 radiatum and dentate gyrus) and frontal cortex as previously reported for rat (Bowery et al., 1988). Intermediate levels were observed in caudate putamen and thalamic nuclei and the lowest level was found in the cerebellum (Fig. 3.1. and Fig. 3.2.).

3.3.2. Distribution of ^{3}H-dizocilpine binding sites in mouse brain *ex vivo*

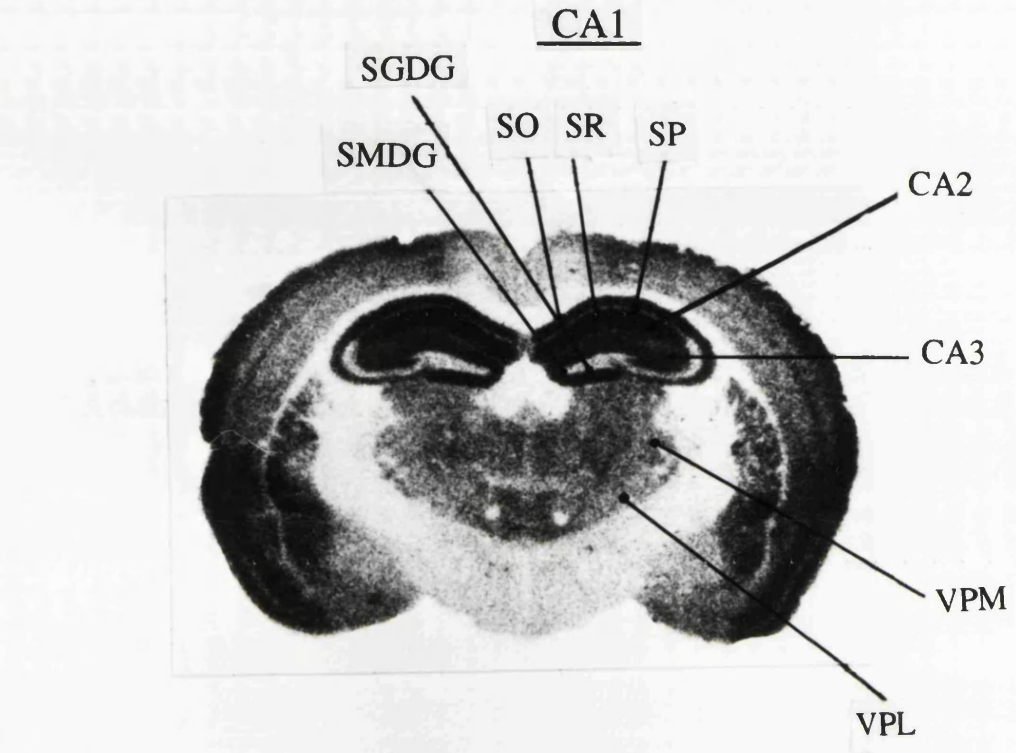
The topography of *ex vivo* binding of ^{3}H-dizocilpine in mouse brain is shown in Fig. 3.3. and Fig. 3.4. Highest densities of binding sites were detected in hippocampus and frontal cortex. Intermediate/low levels were found in caudate putamen and thalamus. The density of binding in the cerebellum was very low.

3.3.3. Comparison of the distribution of *in vitro* and *ex vivo* ^{3}H-dizocilpine binding sites

In order to determine whether there was any correlation between the *in vitro* and *ex vivo* distributions of ^{3}H-dizocilpine binding sites in mouse brain sections, the density of

Fig. 3.1. Receptor autoradiographs of ***in vitro*** ^{3}H-dizocilpine binding to mouse brain sections (coronal and parasagittal).

Sections were incubated with 40 nM ^{3}H-dizocilpine and autoradiographs generated as described in Methods.
Abbreviations are listed below:
CA1 SO Stratum oriens
SR Stratum radiatum
SP Stratum pyramidale
SGDG Dentate gyrus granule layer
SMDG Dentate gyrus molecular layer
VPM ventral posterior medial thalamic nucleus
VPL ventral posterior lateral thalamic nucleus
FrCx Frontal cortex
Cpu Caudate putamen
LD Laterodorsal thalamic nucleus
LP Lateral posterior thalamic nucleus


CA1
SGDG
SMDG
SO
SR
SP
CA2
CA3
VPM
VPL

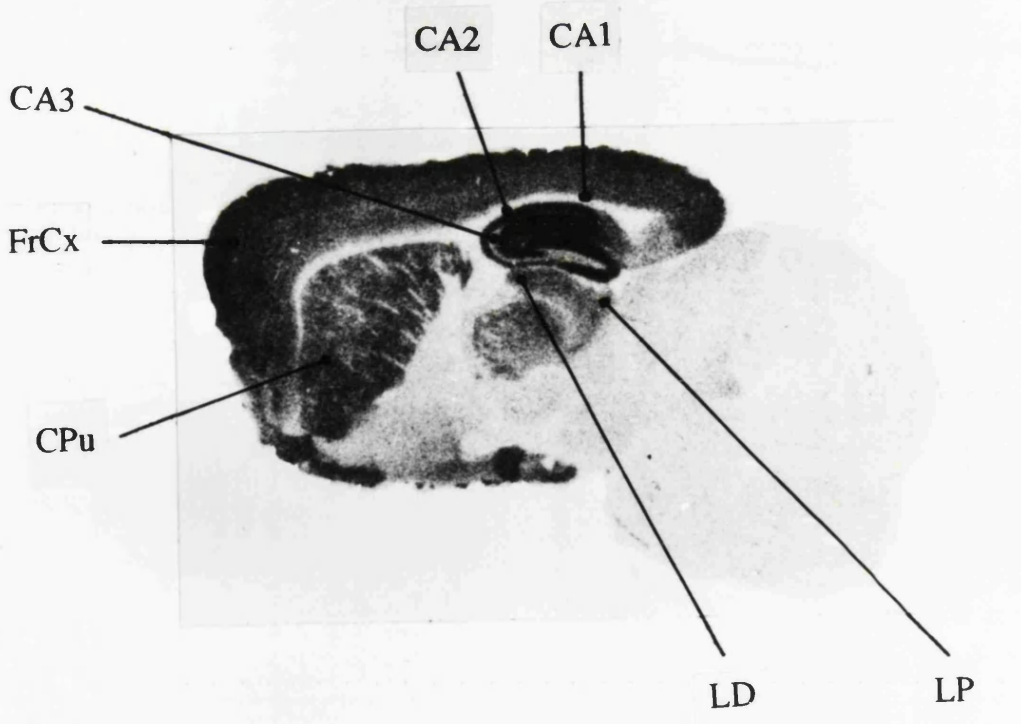

CA2
CA1
CA3
FrCx
CPu
LD
LP

Fig. 3.2. Comparative regional densities of *in vitro* ^{3}H-dizocilpine binding in mouse brain sections.

Density values from each brain region from at least 3 sections each from 3 mice. Values were determined by densitometric analysis and calibrated by reference to tritium standards apposed to the same films.

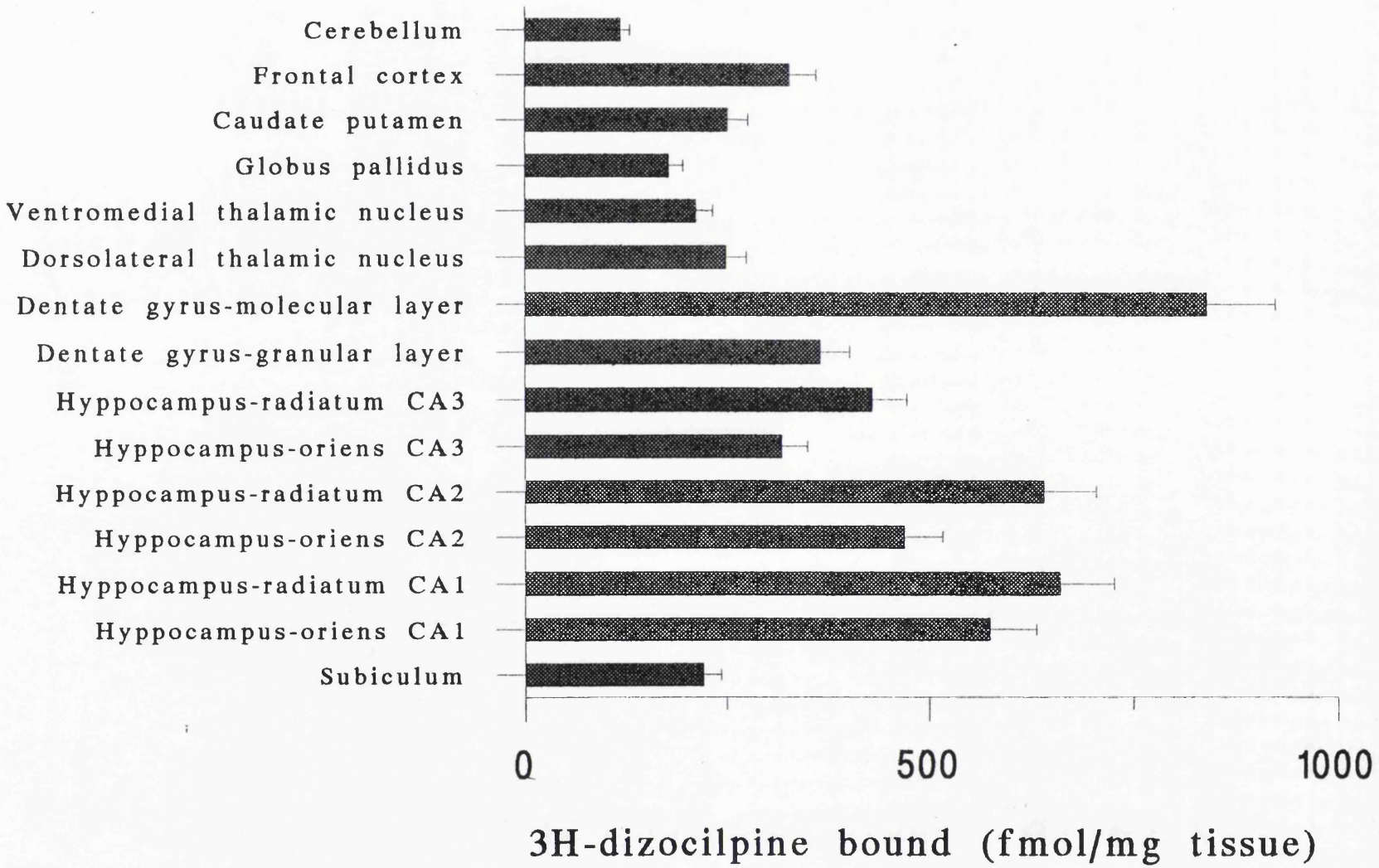
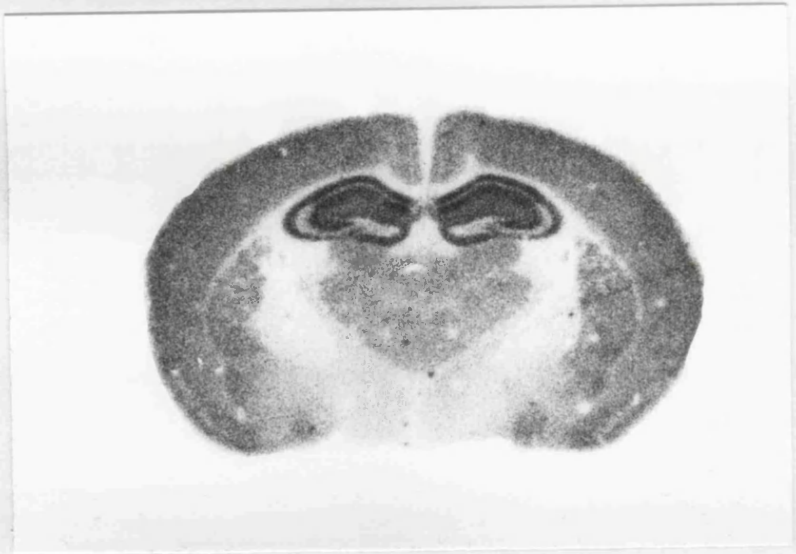

Cerebellum
Frontal cortex
Caudate putamen
Globus pallidus
Ventromedial thalamic nucleus
Dorsolateral thalamic nucleus
Dentate gyrus-molecular layer
Dentate gyrus-granular layer
Hyppocampus-radiatum CA3
Hyppocampus-oriens CA3
Hyppocampus-radiatum CA2
Hyppocampus-oriens CA2
Hyppocampus-radiatum CA1
Hyppocampus-oriens CA1
Subiculum
0
500
1000
3H-dizocilpine bound (fmol/mg tissue)

Fig. 3.3. Receptor autoradiographs of *ex vivo* ^{3}H-dizocilpine binding to mouse brain sections.

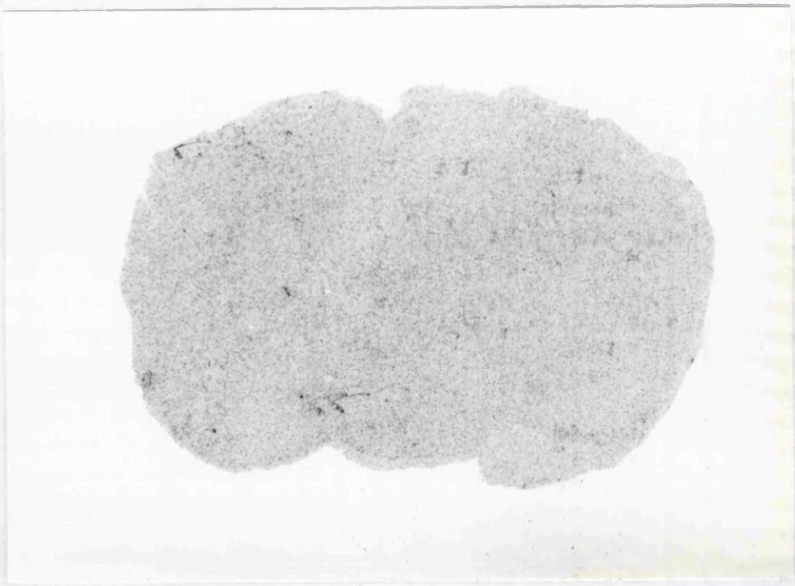
a. / c. "Total binding" to coronal and parasagittal sections obtained from animals injected i.p. with saline 15 min before i.v. injection of 3H-dizocilpine (50μCi/50μl) and decapitated 10 min later. The brains were quickly removed and autoradiographs generated as described in Methods.

b./ d. "Non specific binding" obtained from animals treated with unlabelled dizocilpine (1mg/kg i.p.) 15 min before i.v. injection of radioligand.

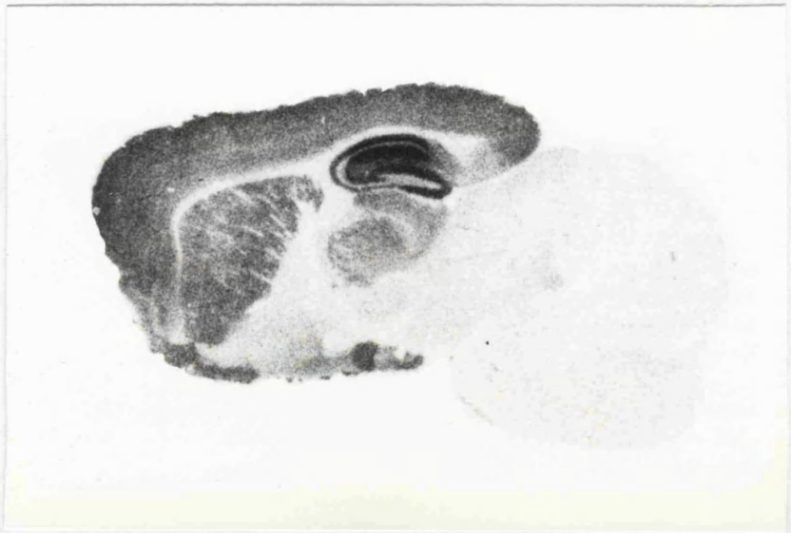
A



B



C



D

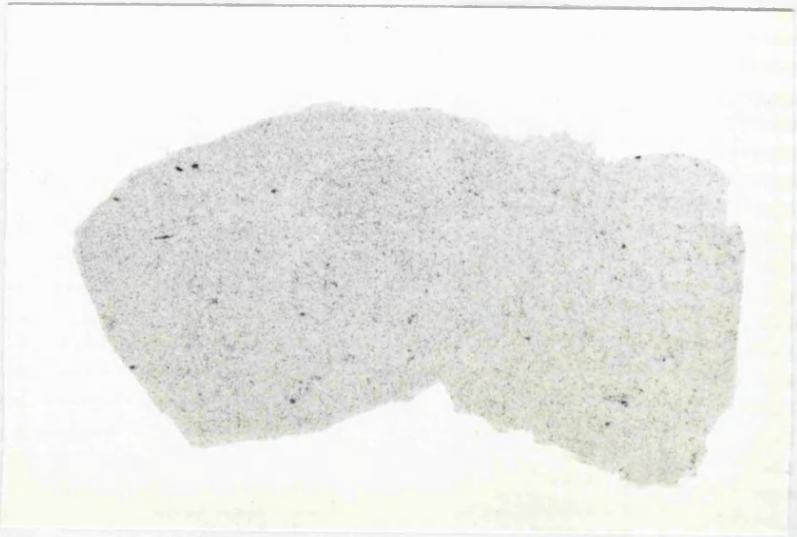


Fig. 3.4. Comparative regional densities of *ex vivo* ^{3}H-dizocilpine binding in mouse brain sections.

Density values from each brain region from at least 3 sections each from 3 mice. Background binding, obtained following i.p. injection of unlabelled dizocilpine (1 mg/kg), was subtracted in all cases.

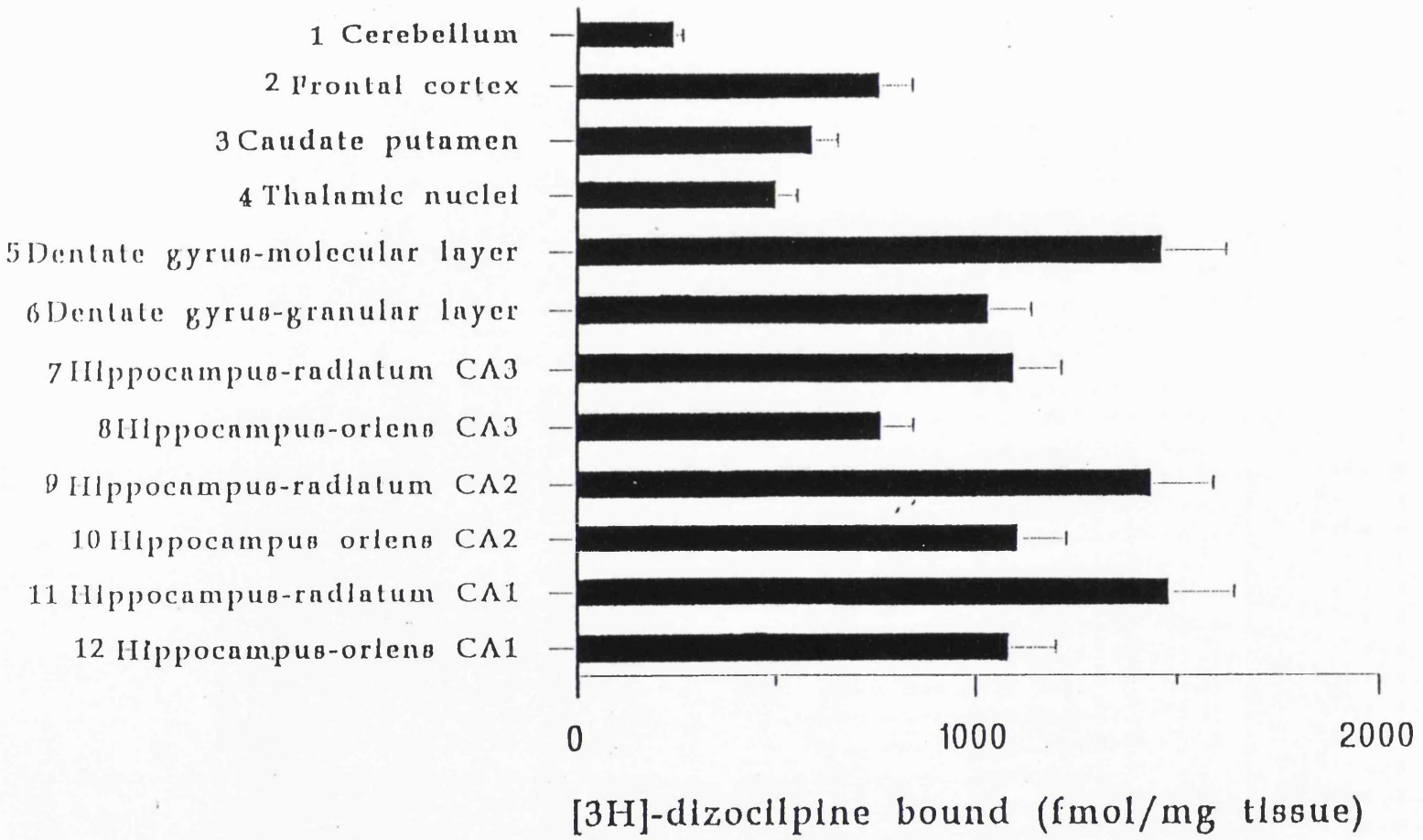
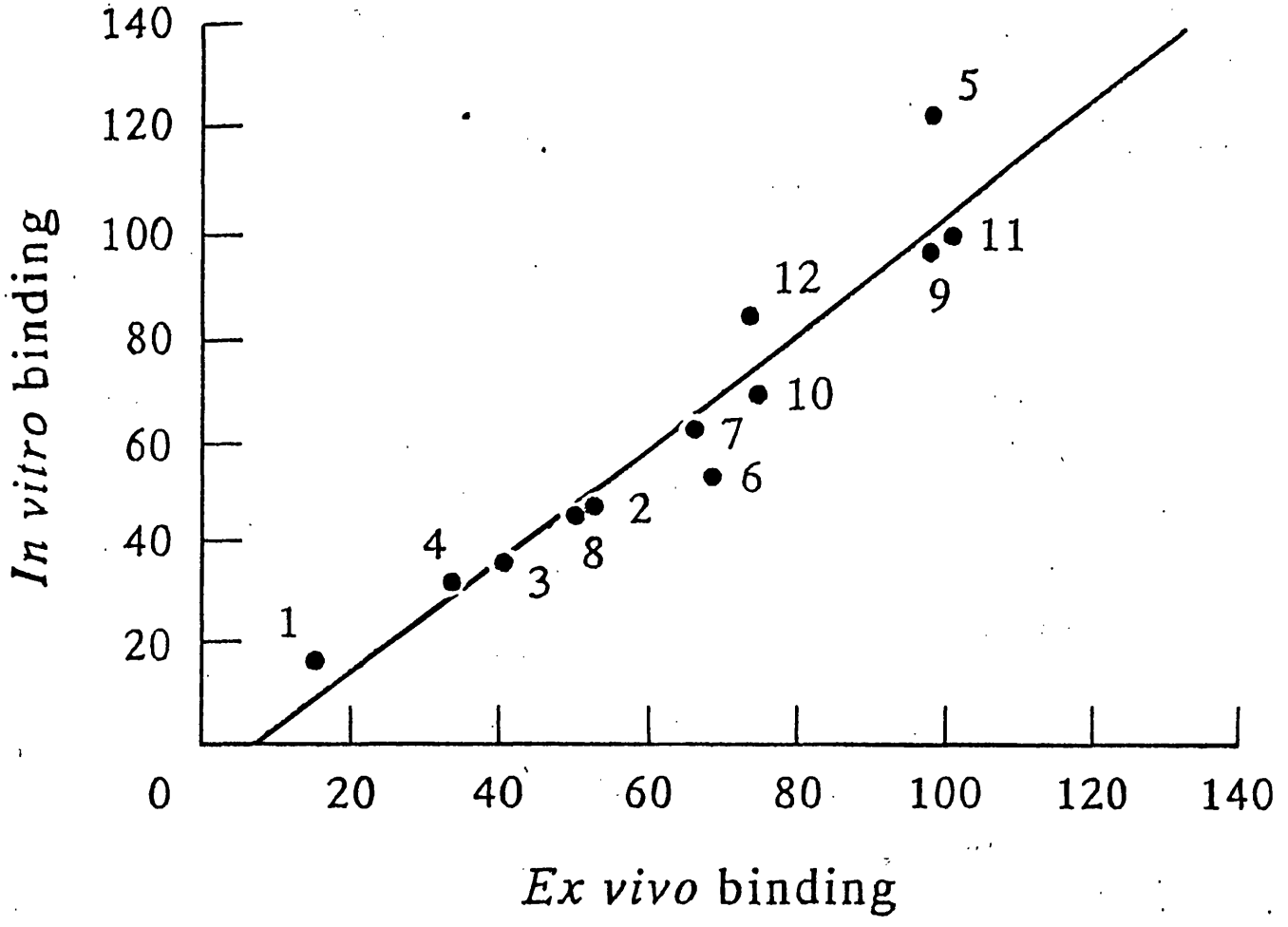

1 Cerebellum
2 Frontal cortex
3 Caudate putamen
4 Thalamic nuclei
5 Dentate gyrus-molecular layer
6 Dentate gyrus-granular layer
7 Hippocampus-radiatum CA3
8 Hippocampus-orlens CA3
9 Hippocampus-radiatum CA2
10 Hippocampus orlens CA2
11 Hippocampus-radiatum CA1
12 Hippocampus-orlens CA1
0
1000
2000
[3H]-dizocilpine bound (fmol/mg tissue)

Fig. 3.5. Correlation between *in vitro* and *ex vivo* ^{3}H-dizocilpine binding in mouse brain sections.

Numbers by each point refer to structures in Fig. 3.4. Density of binding in the CA1 radiatum was designated as 100%. Density of binding in other brain regions was expressed as a percentage of the CA1 radiatum value. For the *in vitro* binding unlabelled sections were incubated for 20 min in 40 nM ^{3}H-dizocilpine (53.1 Ci/ mmol) at 23°C and rinsed twice for 30 sec. Correlation coefficient: r= 0.956.


140
120
100
80
60
40
20
0
20
40
60
80
100
120
140
In vitro binding
Ex vivo binding
1
2
3
4
5
6
7
8
9
10
11
12

binding in the CA1 radiatum has been designated as 100%. The density in other brain regions was expressed as a % of the CA1 radiatum value. The relative densities of binding from a sample of 12 brain regions have been plotted as a correlelogram, giving a correlation coefficient for the regression line of 0.956 (Fig. 3.5.).

3.3.4.In vitro binding of ^{3}H-dizocilpine to mouse brain sections following MCA-O

^{3}H-dizocilpine binding *in vitro* showed no gross changes in regions surrounding the occluded MCA or elsewhere (ipsilateral or contralateral) in the first hours following MCA-O where there was histological evidence of infarction. An apparent decrease in ^{3}H-dizocilpine binding (-23.9 ± 8%, n=3) was observed in the ipsilateral cortex at 24 hours after MCA-O, but this was not statistically significant (Fig. 3.6.). However, a significant decrease in ^{3}H-dizocilpine binding did occur 2 and 3 days after MCA-O in the ipsilateral cortical region (55.2 ± 15% and 53.1 ±13% decrease respectively; n=3, $P<0.05$ ANOVA), compared to the contralateral cortex. Binding was further depressed after 5 (69.8 ± 8%, n=3) and 7 (85.3 ± 6%) days (Fig. 3.7.).

3.3.5.*Ex vivo* binding of ^{3}H-dizocilpine on mouse brain sections following MCA-O

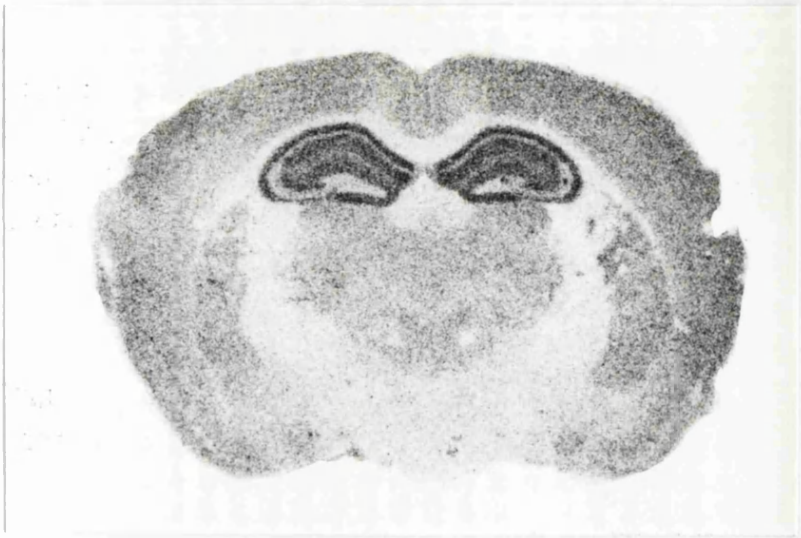
Following i.v. injection of ^{3}H-dizocilpine, the level of radioisotope in regions supplied by the occluded MCA was significantly reduced by 78.7 ± 4% within 2 hours of the ischaemic insult. The reduction of *ex vivo* ^{3}H-dizocilpine binding in the ischaemic area was significant at all times examined after the occlusion. Even after 2 or 4 hours binding was reduced by 75 % (Fig. 3.6. and Fig. 3.7.) However, when ^{3}H-dizocilpine was injected immediately after MCA-O (5 min), *ex vivo* binding in the ischaemic area was

Fig. 3.6. Receptor autoradiographs of ***in vitro*** and ***ex vivo*** ^{3}H-dizocilpine binding to mouse brain 24 hours after MCA-O.

a. *in vitro* ^{3}H-dizocilpine binding. No radioactive tracer was administered to the animals prior to sacrifice. Sections were incubated for 20 min at room temperature in the presence of 40 nM ^{3}H-dizocilpine, washed twice for 30 sec in Tris HCl (50 mM, pH 7.4) and rinsed quickly in deionised water.

b. *ex vivo* ^{3}H-dizocilpine binding. Animals were injected i.v. (tail vein) with 50 µCi ^{3}H-dizocilpine in 50 µl saline and decapitated 10 min later. Brain sections were washed twice for 45 sec in Tris HCl buffer and dip washed in deionised water.

A



B

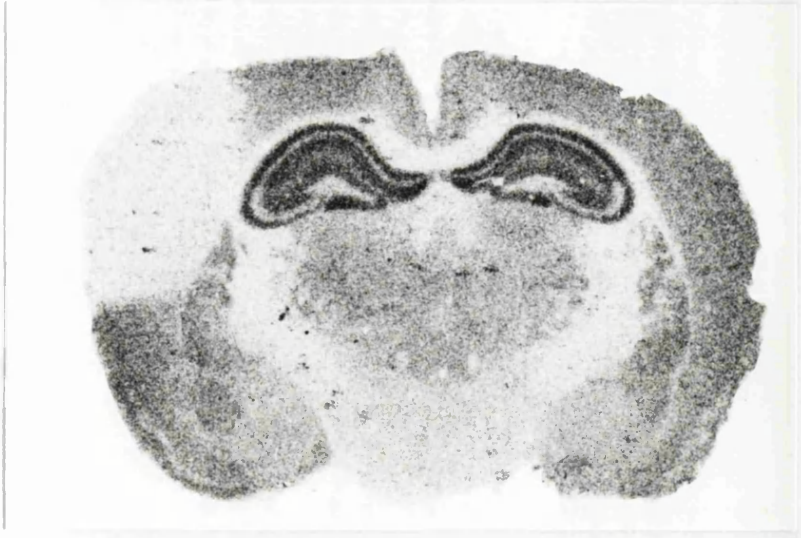


Fig. 3.7. Time course of the specific ^{3}H-dizocilpine binding *in vitro* and *ex vivo* in the ischaemic area of the mouse brain.

Mice were subjected to MCA-O using the same procedure described in Chapter 2 and they were sacrificed at different times after MCA-O. For the *in vitro* experiments no radioactive tracer was administered to those animals prior to sacrifice. For the ex vivo experiments animals were injected i.v. with 50μCi ^{3}H-dizocilpine and sacrificed 10 min later. Background level of binding was generated as described in Methods.

Each point represents the mean % ± s.e.m. from 3 brain sections (co-ordinate A2750) each from 3 mice.
*$p < 0.05$ ANOVA.

[3H]-DIZOCILPINE SPECIFIC BINDING (% OF CORRESPONDING CONTRALATERAL AREA)
0
50
100
HOURS AFTER OCCLUSION
0
10
20
30
40
50
60
70
80
EX VIVO
IN VITRO

not significantly different from that in the corresponding contralateral cortex whereas *ex vivo* binding at all subsequent times after ischaemia was always less than 30% of *in vitro* binding and was apparent within 2 hours of the ischaemic insult. By comparison, *in vitro* binding was maintained at control levels for at least 24 hours.

Animals in which brains were removed 60 min after the i.v. injection of ^{3}H-dizocilpine showed a density of binding less than 40% than in the equivalent areas 10 min after injection of the radioligand. Under these conditions, binding of ^{3}H-dizocilpine in the ischaemic area was not significantly different from the contralateral area up to 2 hours following MCA-O. Binding in the region of infarct was significantly reduced at all subsequent time points. Only at the earliest time point (5 min) after MCA-O was there an apparent increase in the level of *ex vivo* binding in the ischaemic area but this was significant only in one area (A4150) (Fig. 3.8; Table 3.1).

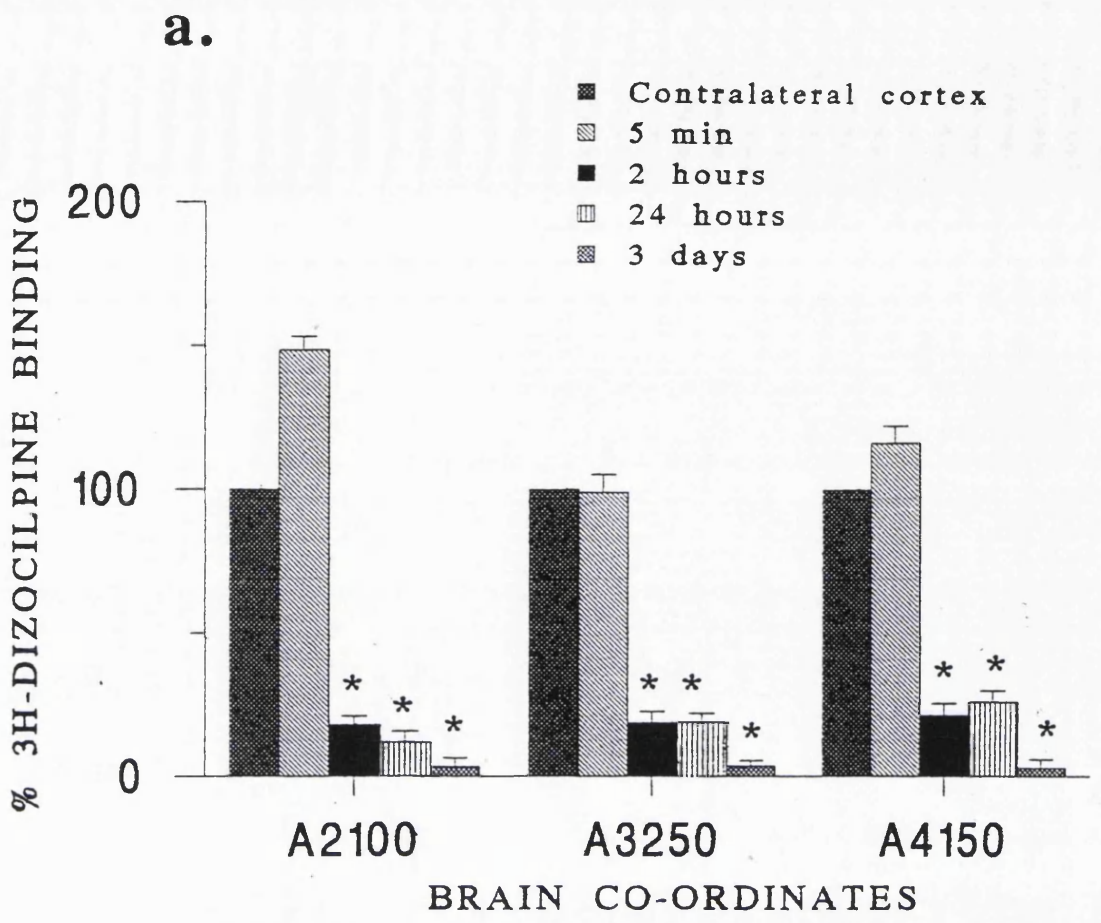
Fig. 3.8. % of specific *ex vivo* ^{3}H-dizocilpine binding in the ischaemic area of the mouse brain 10 min or 60 min after injection of radioligand.

a. % of specific binding *ex vivo* in the ischaemic area 10 min after i.v. injection of radioligand.

b. % of specific binding *ex vivo* in the ischaemic area 60 min after i.v. injection of radioligand.

Density of binding in the contralateral cortex was designated as 100%. Density of binding in the ischaemic area at different times after MCA-O was expressed as a percentage of the contralateral cortex value.

*$p<0.05$ ANOVA. (n=4)


a.
Contralateral cortex
5 min
2 hours
24 hours
3 days
% 3H-DIZOCILPINE BINDING
200
100
0
A2100
A3250
A4150
BRAIN CO-ORDINATES

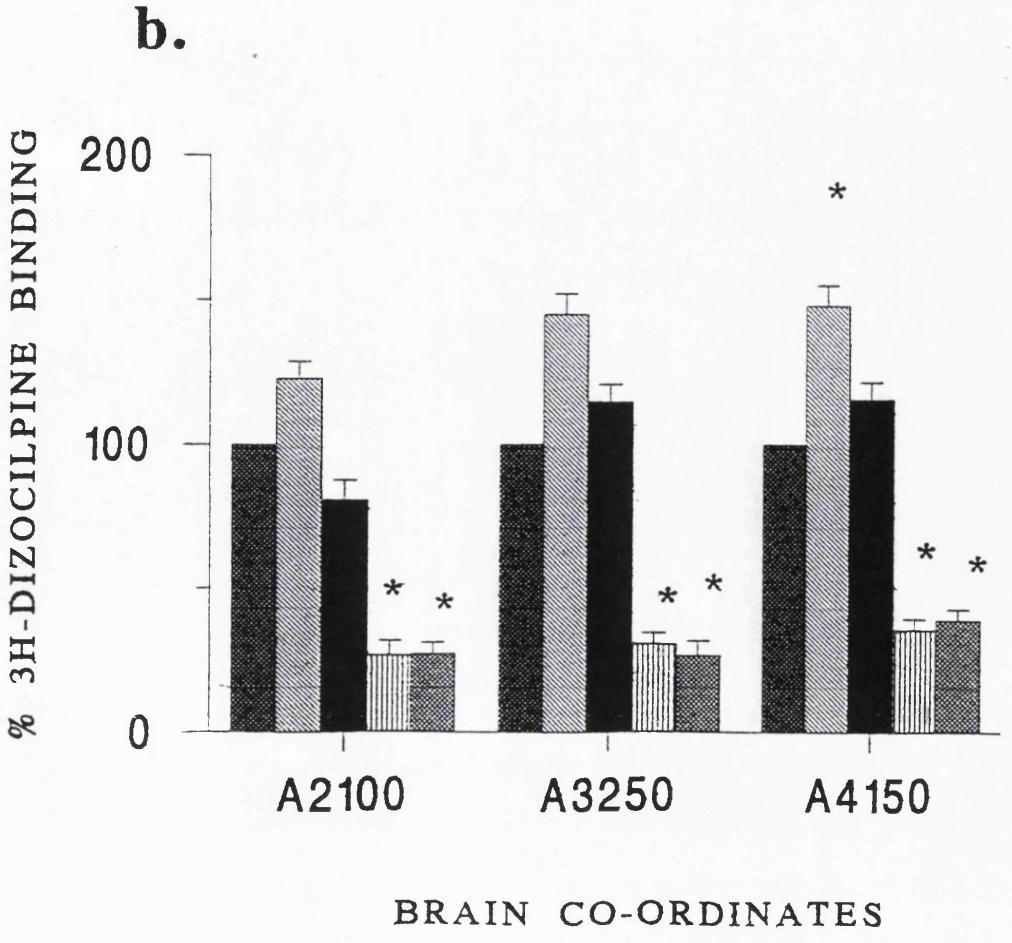

b.
% 3H-DIZOCILPINE BINDING
200
100
0
A2100
A3250
A4150
BRAIN CO-ORDINATES

Table 3.1. Specific ^{3}H-dizocilpine binding (fmol/mg tissue) *ex vivo* in the ischaemic area 10 min or 60 min after i.v. injection of radioligand.

	ex vivo binding 10 min			ex vivo binding 60 min		
	Contralateral cortex	Ischaemic cortex			Contralateral cortex	Ischaemic cortex
5 min	217.6	214.8		5 min	80.2	116.2
2 hours	209.6	39.1		2 hours	75.4	86.7
24 hours	236.0	44.5		24 hours	84.6	26.0
3 days	199.6	6.8		3 days	73.1	19.8

Density values were converted to corresponding ligand concentration by reference to tritium standards apposed to the same films. Values represent the mean fmol/mg tissue at different times after MCA-O in mouse brain section A3250 (Lehmann Atlas of the Mouse Brain, 1974).

3.4. DISCUSSION

In the present study we used the *in vitro* ^{3}H-dizocilpine binding autoradiographic technique as an indicator of the integrity and functionality of the NMDA receptor complex following an ischaemic insult. Evidence from these results suggests that 24 hours after ischaemia no significant change in the density of ^{3}H-dizocilpine binding occurred in the cortical region even though some neuronal damage was evident. One possible explanation is that damaged neurones fail to take up the histological stain but their membranes, which contain the ligand binding sites, still retain the capacity to bind ^{3}H-dizocilpine, and binding is significantly reduced only when the membranes are further degraded (at least 2 days of the ischaemic insult in our study). In this case, the presence of ^{3}H-dizocilpine binding sites would not necessarily indicate neuronal functionality. Evidence from a study on MCA-O in rat synaptic membranes indicate that 12 hours after ischaemia, a time at which neurones show histological damage, the potentiating effect of glutamate on ^{3}H-dizocilpine binding in the ischaemic area has been confirmed (Dewar et al, 1989). Thus, not only the capacity of binding but also the functionality of the receptor complex appears to be preserved. Binding is assumed to be located on neuronal cells rather than glia since no increase in binding, which would be expected due to the proliferation of glial cells following infarction, has been observed. On the other hand, while a reduction of the number of neurones occurs, the number of binding sites per individual neurone might increase in response to the ischaemic insult, thereby producing little or no apparent change in the overall number of sites. Nevertheless, our findings are in agreement with the general consensus about the

robust nature of the NMDA receptor complex.

The presence of ^{3}H-dizocilpine binding sites in the ischaemic area, and the ability of glutamate to enhance ^{3}H-dizocilpine binding in the same area many hours after MCA-O, does not necessarily mean that treatment with neuroprotective drugs at such time points will always prove advantageous. The neuroprotective effect of various NMDA antagonist, including dizocilpine, has only been demonstrated when drug treatment was started before or within 2-3 hours of the ischaemic insult.

In this study, dizocilpine administered as a single treatment i.p. 30 min, 2h or 4h after MCA-O (at doses of 0.3, 1 or 3 mg/kg) failed to protect from the infarction. Dizocilpine, as a single dose treatment, produced a significant neuroprotective effect only when administered 5 min after the MCA-O. The degree of neuroprotection shown by dizocilpine immediately (5 min) after MCA-O with the repeated dosing was similar to that seen with 1 mg/kg given as a single dose 5 min after MCA-O; this observation would indicate that the time point of the first initial application may be much more important than the plasma level of dizocilpine after the ischaemic insult. However, dizocilpine showed no neuroprotective effect in this study when administered as a single dose 30 min after MCA-O, whereas it produced a significant reduction of the ischaemic area when a repeated dose regime (0.3 mg/kg/dose) was started at the same time (30 min) after MCA-O. An apparent neuroprotective effect was observed also when the initial administration was delayed 1 or 2 hours following MCA-O. These data are in agreement with the therapeutic window for dizocilpine which is regarded by many investigators as 2-3 hours after the onset of focal ischaemia (Hatfield et al., 1992).

Since data from *in vitro* binding demonstrate that even when histological damage is present the functionality of the NMDA receptor complex is preserved, other critical factors preventing the efficacy of neuroprotective drugs must be involved in the first few hours after MCA-O. Thus, in the present study we have attempted to investigate further the period post-ischaemia during which drugs can gain access to the receptors in the region of the infarct. This was explored by assessing the *ex vivo* distribution of ^{3}H-dizocilpine in mouse brain after MCA-O using receptor autoradiography.

Evidence from *ex vivo* experiments in this study indicates that in the ischaemic area *ex vivo* ^{3}H-dizocilpine binding was reduced by 78% within 2h from MCA-O and at all subsequent times *ex vivo* binding was reduced by more than 75%. Only at 5 min after MCA-O was *ex vivo* binding not significantly different from control.

One obvious contributory factor is the reduction in cerebral blood flow which occurs, by definition, in the ischaemic area. CBF autoradiographs obtained after MCA-O in rat using ^{14}C-iodoantipyrine as the tracer are similar to the autoradiographs of ^{3}H-dizocilpine *ex vivo* binding (Tamura et al., 1981). It has been suggested that the uptake of ^{3}H-dizocilpine into ischaemic tissue is initially limited by the low CBF but, the subsequent massive increase in the concentration of extracellular glutamate occurring *in vivo* during ischaemia produces an increase in the level of radioligand binding in the ischaemic area (Wallace et al., 1992; McCulloch and Iversen, 1991). The level of ^{3}H-dizocilpine binding in the area of infarction was reported to be significantly higher than in the equivalent contralateral area at 60 min after the intravenous injection of radioligand (Wallace et al., 1992). In the present study, when *ex vivo* ^{3}H-dizocilpine binding was performed removing the brains 60 min after injection of radioligand, an

apparent increase was observed in the ischaemic area, although it was only statistically significant in section A4150. *Ex vivo* binding was then significantly reduced in the ischaemic area at all subsequent time points after MCA-O. Importantly, in our study cryostat sections were washed before apposing the ^{3}H-sensitive film but this step was essential to obtain any specific binding as well as a good correlation with the *in vitro* distribution of specific bound ^{3}H-dizocilpine. This, together with the different animal species used, might explain some of the discrepancies with the report by Wallace et al., 1992.

Thus, in the first few hours after MCA-O, the presence of high levels of extracellular glutamate occurring in the post-ischaemia period could account for the presence of similar densities of *ex vivo* ^{3}H-dizocilpine binding sites in the ischaemic and contralateral areas. This observation might explain the ability of dizocilpine to reduce ischaemic brain damage even when first given 2 hours after the onset of ischaemia. However, it should be noted that, in real terms (fmol/mg), densities of binding at 60 min after i.v. injection of radioligand were less than 40% of those in the equivalent areas 10 min after injection, even in the contralateral hemisphere. Taken together, these findings suggest that *ex vivo* ^{3}H-dizocilpine binding is indicative of a dynamic process strictly related both to the chemical characteristics of the tracer and to the evolution of ischaemia. This might be important from the therapeutic point of view, particularly in the design of dosing strategies. Accordingly, both the temporal progression of cerebral ischaemia with increased extracellular glutamate and the pharmacodynamic profile of dizocilpine might contribute to the neuroprotective effect of dizocilpine given 30 min after MCA-O as repeated doses but not as a single bolus dose. Further considerations

about the use of *ex vivo* ^{3}H-dizocilpine binding technique are discussed in Chapter 4.

An alternative explanation for the differences between the *in vitro a*nd *ex vivo* binding of ^{3}H-dizocilpine after MCA-O might be the presence of an injury-induced endogenous factor interfering with ^{3}H-dizocilpine binding, which could be washed out in the *in vitro* pre-incubation. In order to explore this hypothesis, some *in vitro* experiments were performed without any pre-incubation of the brain sections. No significant difference with the pre-incubated sections was observed in the regional distribution of *in vitro* ^{3}H-dizocilpine binding at all time points examined, indicating that endogenous factors induced by the ischaemic injury are unlikely to explain the difference.

In clinical trials, optimization of drug dosing and timing of drug treatment is required in order to provide drug concentrations which are appropriate for neuroprotection in the regions susceptible to ischaemic damage and, at the same time, minimize side effects on normal CNS function. The hydrophilic or lipophilic characteristics of the drug greatly influence its pharmacodynamic profile; thus, the different chemical properties of competitive and non-competitive NMDA antagonist appear particularly important in regions with low CBF. For highly hydrophilic competitive NMDA antagonists, the level in the CNS is not reflected by the plasma concentration, due to the very slow blood brain permeability. This could explain the poor anti-ischaemic efficacy of these molecules even though they are minimally influenced by the low blood CBF in the ischaemic regions. Some orally active competitive NMDA antagonists have recently been developed. However, their mechanism of action involving competition with very

high levels of extracellular glutamate following ischaemia, is presumably responsible for their general lack of neuroprotective effect.

By contrast, highly lipophilic non-competitive NMDA antagonists, such as dizocilpine, can enter into the CNS without any restriction by the blood-brain barrier and, consequently, their uptake into the brain is dictated mainly by the rate of delivery to the tissue. However, the undesirable side effects of dizocilpine and other ion-channel blockers in animals has certainly hindered their progress into the clinic. Systemic administration of these non-competitive NMDA antagonists in rodents elicits a distinct behavioural profile which is manifest as a characteristics motor syndrome consisting of lateral weaving, body rolling, increased locomotion and, at higher doses, ataxia (Koek et al., 1988; Tricklebank et al., 1989). Other disadvantages shown by this class of molecules include deficits in learning and memory (Woods et al., 1991), changes in glucose utilization and CBF (Kurumaji et al, 1989), cardiovascular effects (Lewis et al., 1988) and morphological alterations (Olney et al., 1989).

In conclusion these data suggest that although the population of NMDA receptors is maintained in the infarct region for many hours after MCA-O, this appears to be of little significance in vivo. Other critical factors, such as access impairment to the receptors *in vivo* within 2 hours of ischaemic insult, appear to be involved in the reduction of the neuroprotective activity of NMDA antagonists after this time.

CHAPTER 4: IN VIVO MODULATION OF THE NMDA RECEPTOR FUNCTION BY LIGANDS ACTING AT DIFFERENT SITES OF THE NMDA RECEPTOR COMPLEX IN NORMAL MICE

4.1. INTRODUCTION

Following the original observations of Johnson and Ascher (1987), the search for the role of the glycine site in modulating the NMDA receptor has been intensified. It is apparent from most studies that glycine site activation is needed for NMDA receptors to be functional, and omission of glycine from the extracellular medium prevents NMDA-induced currents (Kleckner and Dingledine, 1988) and the binding of TCP or dizocilpine to the NMDA receptor complex (Snell et al., 1987; Reynolds et al., 1987). Thus, the fact that glycine potentiated responses to a receptor expressed in *Xenopus* oocytes as well as in a solubilized preparation (McKernan et al., 1989), suggested that the glycine site is an integral part of the receptor protein. With regard to this latter observation, the recent cloning of a functional NMDA receptor subunit (see General Introduction., Moriyoshi et al., 1991) has demonstrated that both the glutamate and glycine recognition sites reside on the same protein. Speculation on the precise mode of glycine interaction with the NMDA receptor complex provided initial evidence that glycine might reduce NMDA receptor desensitization (Mayer et al., 1989). It was demonstrated that in low glycine concentrations, NMDA responses rose to an initial peak which than declined over 250ms and reached a much smaller steady state level. When the glycine concentration was increased (up to 3 μM) the peak response was potentiated and the response no longer showed any decline. 7-chlorokynurenic acid had the same effect as lowering the glycine concentration and increased the decline of NMDA responses (Vyklicky et al., 1990). A negative allosteric coupling between the glycine and glutamate recognition sites has also been proposed such that binding of an

agonist at the glutamate recognition site reduces the affinity of glycine for its own recognition site (Benveniste et al., 1990). However, these conclusions about desensitization and negative modulation were not supported by binding studies, which demonstrated enhancement of ^{3}H-glycine binding by glutamate and viceversa and suggested agonist and antagonist preferring states of the receptor (Monaghan et al., 1988). Patch clamp studies and kinetic analysis of the responses has led to the conclusion that binding of 2 glycine and 2 glutamate molecules are required to activate the NMDA receptor complex (Benveniste and Mayer, 1991).

4.1.1. Agonists

A combination of binding and electrophysiological studies have increased our knowledge of the structure-activity requirements for the glycine site. Only small substitutions are allowed on the parent glycine molecule: methyl (alanine), hydroxymethyl (serine) and cyclopropyl (ACCP) on the α-carbon are all effective agonists (Kleckner and Dingledine, 1989; Lodge and Johnson, 1990). Studies using expression of the NMDA receptor in *Xenopus* oocytes revealed an order of potency of ACCP> glycine> D-serine> D-alanine> cycloserine> L-serine> L-alanine (McBain et al., 1989) and similar profiles were reported for inhibition of ^{3}H-glycine binding (Snell et al., 1988; Monahan et al., 1989a). By contrast, larger substituents on the α-carbon reduced potency or resulted in partial agonists or antagonists.

4.1.2. Antagonists and partial agonists

One of the first compounds identified as an NMDA antagonist with affinity for the

glycine site was the pyrrolidone, HA-966. The antagonism produced by this compound in the rat cortical wedge preparation could be reversed by exogenous glycine or D-serine (Fletcher and Lodge, 1988). However, glycine was unable to potentiate responses to NMDA in this preparation (up to 1mM). From parallel studies, HA-966 was actually demonstrated to be a partial agonist at the glycine site, rather than a full antagonist, and this has been further substantiated by the findings of Foster and Kemp (1989) and Kemp and Priestley (1991).

It has been reported that the glycine site activity of HA-966 was exhibited by the R-(+) isomer, whereas the S-(-) isomer showed potent sedative properties. Modification of R-(+)-HA966 by the introduction of a methyl group (at position 4) resulted in the cis-β-methyl-HA966 (or L-687,414; Leeson et al., 1993). This compound is a glycine antagonist which is ten-fold more potent than R-(+)-HA966 but still with some weak agonist activity and, although intrinsically less potent than other more selective glycine antagonists, is able to cross the blood-brain barrier (Saywell et al., 1991).

This partial agonist property at the glycine site seems to be typical of other cyclic analogues of glycine such as 1-amino-1-carboxycyclobutane carboxylic acid (ACBC; Watson et al., 1989) and cycloleucine (Snell and Johnson, 1988). Another compound which shows partial agonist characteristics and is structurally related to HA-966 and ACBC is D-cycloserine (Watson et al., 1990).

Although kynurenic acid blocks NMDA receptor function by an action at the glycine site, it is described as a broad spectrum EAA antagonist since it also has affinity for the AMPA and kainate receptors (Evans et al., 1987). However, various halogen

substituted analogues of kynurenic acid have provided potent and selective glycine site antagonists. Unlike the parent compound, which is both weak and non selective for NMDA responses, the first analogue described, 7-chlorokynurenic acid, was demonstrated to be a potent, selective and full competitive NMDA antagonist acting at the glycine site (Kemp et al., 1988). Structure activity relationships of similar compounds revealed that 6,7- and 5,7-dichlorokynurenic acid and 6,7-dichloro-3-hydroxyquinoxaline-2-carboxylic acid have similar potencies, although only 5,7-dichlorokynurenic acid exhibits real selectivity for the glycine site (McNamara et al., 1990). Another potent compound identified from the kynurenic acid series was 7-chloro-5-iodokynurenic acid (Leeson et al., 1991), whereas thiokynurenates have been shown to be less active than the halogen substituted analogues (Moroni et al., 1991). Structural modification of the heterocyclic ring resulted in the development of further glycine antagonists such as 2-carboxyindoles (Huettner, 1989) and the 2-carboxytetrahydroquinolines (Leeson et al., 1991).

The quinoxaline derivatives such as CNQX and DNQX have also been shown to exhibit glycine antagonist properties, although they have a higher affinity for the AMPA and kainate receptor (Honore' et al., 1988; Birch et al., 1988). 6,8-dinitroquinoxalinedione (MNQX) is 30-fold more potent at the glycine site than the 6,7 analogue DNQX. 6,7-dichloroquinoxalic acid has also been shown to possess glycine antagonist characteristics (Kessler et al., 1989). Among the derivatives of quinoxaline-2,3-diones, certain N-hydroxy, N-amino and N-carboxymethyl substituted analogues were recently demonstrated to be potent glycine antagonists, e.g. ACEA-1021, with *in vivo* efficacy

after systemic administration (Cai et al., 1993; Woodward et al., 1995). The potential for development based on the quinoline moiety has recently produced other novel glycine antagonists such as L-695,902 (methyl-7-chloro-4-hydroxy-2(1H)-quinolone-3-carboxylate) and L-701,252 (3-(3-hydroxyphenyl)prop-2-ynyl-7-chloro-4-hydroxy-2(1H)-quinolone-3-carboxylate) (Rowley et al., 1993).

The identification of these various types of compounds interacting with the glycine site has enabled a detailed study of the interaction between this site and the NMDA receptor complex to be made, with particular regard to the *in vivo* significance. One question to answer was whether in intact preparations (such as slices *in vitro*) or in *in vivo* experiments, the glycine site is already maximally activated. Intraventricular and intracisternal administration of glycine have been shown to potentiate NMDA responses in elevating cerebellar cyclic GMP levels (Danisz et al., 1989) and the receptor agonist D-serine can potentiate NMDA responses after iontophoresis onto rat ventrobasal thalamic neurones (Salt, 1989). Direct intracerebellar injections of N-methyl-D-aspartate or D-serine were also reported to increase cerebellar cyclic GMP levels in a dose-dependent manner, *in vivo* in mouse (Wood et al.,1989) and the effect of D-serine was antagonized by competitive NMDA receptor antagonists, supporting an action at the NMDA-coupled glycine receptor. In the same study, the effect of D-serine was antagonized by D-cycloserine, a partial agonist at the glycine site of the NMDA receptor. However, the possibility of modulating NMDA receptor-mediated responses *in vivo* is still controversial.

An important purpose of the present study was to verify whether it is possible to modulate the NMDA receptor complex under physiological conditions.

In the present Chapter the influence of modulators of NMDA receptor function on the *ex vivo* binding of ^{3}H-dizocilpine in mouse brain has been examined. In particular, the effect of pre-treatment with D-serine, L-serine, D-cycloserine, 7-chlorokynurenic acid, ACBC (1-amino-cyclobutane-1-carboxylic acid), Kynurenic acid, cycloleucine, HA-966, compound Z, ifenprodil, NMDA, CGP 37849, and CNQX has been studied. Receptor autoradiography has been used to detect subtle changes in the regional levels of binding.

Also, we have utilized the *in vitro* quantitative autoradiography technique to further investigate the binding properties of ligands for the glycine site with particular respect to a novel glycine antagonist Z; in particular, we have determined the regional distribution of binding sites for ^{3}H-glycine in mouse brain slices using two selective glycine antagonists, 7-chlorokynurenic acid and compound Z, in order to determine the pattern of binding inhibition. Secondly, we have used an in vitro autoradiographic technique to examine the distribution of specific ^{3}H-5,7-dichlorokynurenic acid binding sites in mouse brain sections and have determined the inhibitory properties of glycine and compound Z on these binding sites. We have also measured, by an in vitro autoradiographic technique, the regional binding of ^{3}H-dizocilpine in relation to the inhibitory activity of compound Z, in mouse brain.

4.2. METHODS

4.2.1. *Ex vivo* binding of ^{3}H-dizocilpine

Male CD1 (20-30 g) were injected i.v. (tail vein) or i.p. with vehicle or test compounds dissolved in vehicle. All injections were administered in a volume of 100 µl. After 15 min mice were injected i.v. (tail vein) with 50 µCi of ^{3}H-dizocilpine in 50 µl saline and decapitated 10 min later. The brains were quickly removed and frozen. Cryostat sections were dried at room temperature and washed twice for 45 sec in Tris HCl buffer (pH 7.4, 23^{o}C), dipped washed in double distilled water and air dried. Preliminary experiments determined that this length of rinse optimized the specific binding ratio. Subsequent procedures were the same as described in Chapter 3 for preparation of autoradiograms.

4.2.2. ^{3}H-glycine and ^{3}H-5,7-dichlorokynurenic acid *in vitro* autoradiography to mouse brain sections.

Dried sections were preincubated in 50 mM Tris-citrate (pH 7.4) at 20^{o}C for 45 min to minimize any slide-to-slide and animal variability in the levels of endogenous ligands, and then dried at room temperature. Sections were then incubated in the same buffer (pH 7.4) containing 100 nM ^{3}H-glycine (18.7 Ci/mmol) or 100 nM ^{3}H-5,7-dichlorokynurenic acid (16.3 Ci/mmol) for 20 min at 4 ^{o}C (100 µl/section). The incubation was terminated by rapid aspiration of the radiolabelled solution followed by a rapid dip wash in ice cold buffer, a 10 sec rinse in fresh buffer (4^{o}C) and a final dip wash in deionised water (4^{o}C) to remove the excess of buffer salts before drying. In preliminary experiments this rinse procedure was found to optimize the ratio of specific to non-specific binding, whereas

longer rinse periods decreased the binding ratio. Non-specific binding was determined by including 1 mM unlabelled glycine and 1 mM 7-chlorokynurenic acid respectively in the incubation buffer.

The experimental procedures for ^{3}H-dizocilpine *in vitro* autoradiography to mouse brain sections as well as the procedure for autoradiograms generation and densitometric analysis of autoradiograms are described in Chapter 3.

Abbreviations used are from Lehmann (1974) and Paxinos & Watson (1982).

4.2.3. Materials

Chemicals were obtained from the following sources: ^{3}H-glycine (specific activity 18.7 Ci/mmol) and (+) ^{3}H-dizocilpine (specific activity 53.1 Ci/mmol), Amersham U.K.; ^{3}H-5,7-dichlorokynurenic acid (specific activity 16.3 Ci/mmol) Dupont products, Boston; (+) dizocilpine maleate salt, RBI U.S.A.; D- and L-serine, Sigma Chemical. All other reagents were of analytical grade.

4.3. RESULTS

4.3.1. *EX VIVO* AUTORADIOGRAPHY EXPERIMENTS

4.3.1.1 Effect of pre-treatment with ligands for the NMDA receptor complex on ^{3}H-dizocilpine binding *ex vivo*

The topography of *ex vivo* binding of dizocilpine in mouse brain has been reported in previous Chapter (3) and it was comparable to that obtained *in vitro* (Fig. 3.5. Chapter 3). Injection of D-serine (100 mg/kg i.p.) enhanced ^{3}H-dizocilpine binding ex vivo in many brain regions with the maximal effect in CA3 (22.8 ± 5.8% above basal at 100mg/kg $p<0.05$ ANOVA, n=5; Fig. 4.1. Tab. 4.1.). L-serine failed to increase binding in the same regions at the same dose (100mg/kg i.p.). D-cycloserine (100 mg/kg i.p.) produced a marginal although significant decrease particularly in CA1, CA3 and dentate gyrus (CA3, 88.7 ± 3.1% decrease $p<0.05$ ANOVA; n=6). The effect of 7-chlorokynurenic acid was variable with an apparent slight decrease in *ex vivo* ^{3}H-dizocilpine binding at 10 mg/kg. The NMDA antagonist, CGP 37849 had no effect up to 30 mg/kg. 1-aminocyclobutane-1-carboxylic acid (up to 100mg/kg), compound Z (10 mg/kg), kynurenic acid (up to 10mg/kg), cycloleucine (up to 100mg/kg), HA-966 (10 mg/kg), NMDA (up to 30 mg/kg), ifenprodil (10mg/kg) and CNQX (up to 80 mg/kg) also produced no change in the level of binding (Tab. 4.1.). In all cases the unlabelled compound was administered 15 min before ^{3}H-dizocilpine.

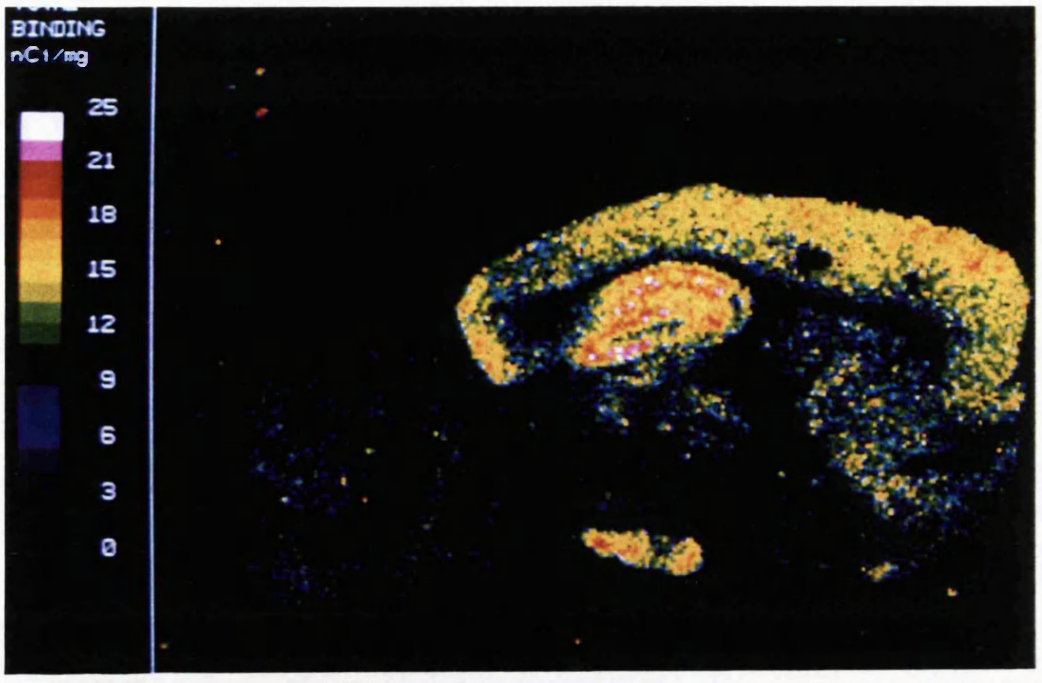
Fig. 4.1. Colour-coded receptor autoradiographs of ^{3}H-dizocilpine binding *ex vivo* to parasagittal sections of mouse brain.

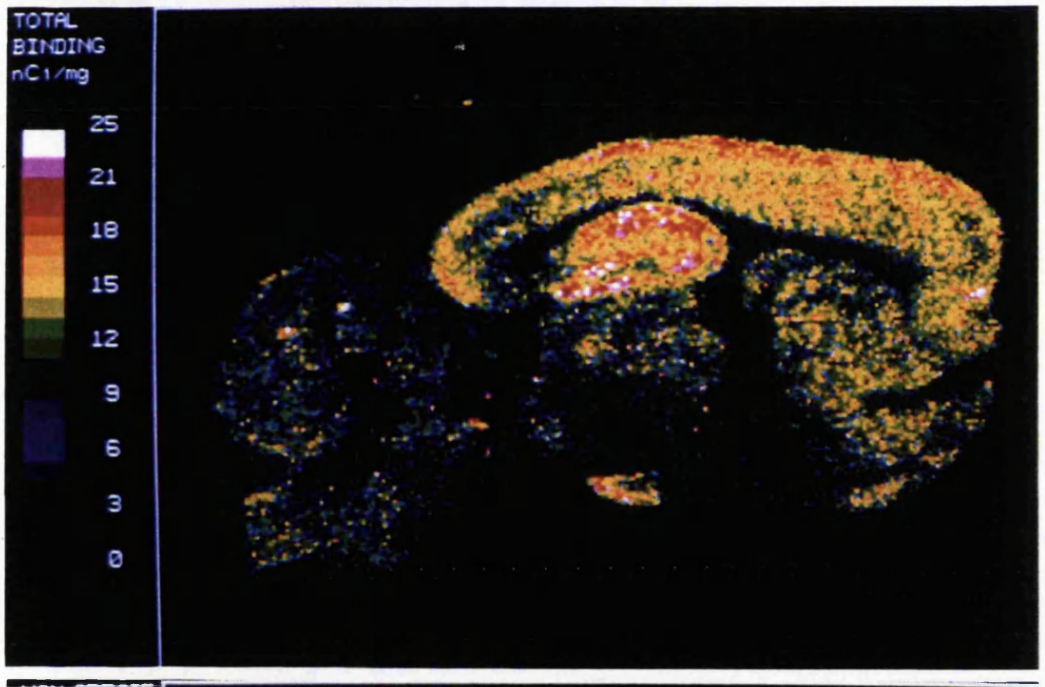
a. 3H-dizocilpine preceded (15 min) by saline i.p.

b. 3H-dizocilpine preceded (15 min) by D-serine (100mg/kg i.p.)

c. 3H-dizocilpine preceded (15 min) by unlabelled dizocilpine (1 mg/kg i.p.)

10 min after the injection of radioligand animals were sacrificed and the brains removed and processed as described in Methods.


BINDING
nCi/mg
25
21
18
15
12
9
6
3
0


TOTAL
BINDING
nCi/mg
25
21
18
15
12
9
6
3
0

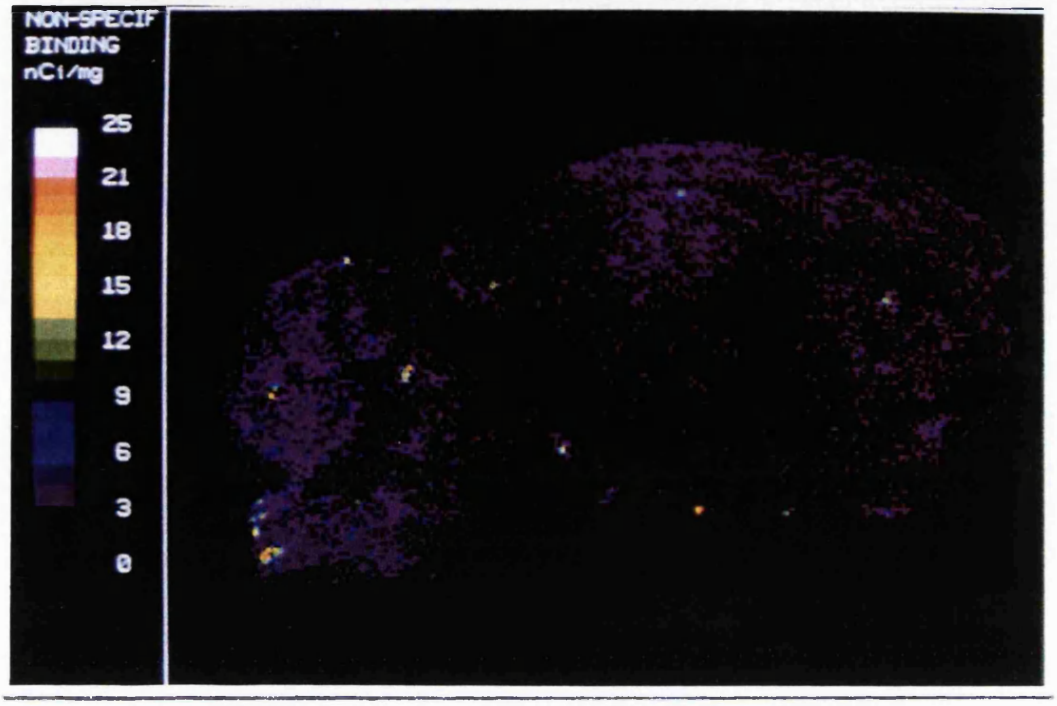

NON-SPECIF
BINDING
nCi/mg
25
21
18
15
12
9
6
3
0

Table 4.1. Compounds without effect on ^{3}H-dizocilpine binding *ex vivo* at the doses shown

LIGAND	Dose (mg\kg) i.p.	% Binding of basal (± s.e.m.)
NMDA	30	100.42 ± 2.1
CGP 37849	30	98.51 ± 4.4
Kynurenic acid	10	96.78 ± 3.7
1-aminocyclobutane-1-carboxylic acid	100	103.81 ± 1.8
Cycloleucine	100	102.26 ± 5.9
CNQX	80	99.05 ± 2.0
L-serine	100	104.81 ± 3.8
7-Chlorokynurenic acid	10	92.64 ± 5.6
HA-966	10	97.56 ± 3.5
Ifenprodil	10	106.28 ± 3.4
Compound z	10	97.22 ± 4.8

4.3.2. *IN VITRO* AUTORADIOGRAPHY EXPERIMENTS

4.3.2.1. Distribution of ^{3}H-5,7-dichlorokynurenic acid binding sites

Preliminary autoradiographical experiments have revealed a heterogeneous distribution of ^{3}H-5,7-dichlorokynurenic acid binding in mouse brain sections (Tab. 4.2.). The highest concentration of binding sites was found in the hippocampal formation. Levels of bound radioactivity were intermediate in caudate putamen and thalamus.

4.3.2.2. Inhibition of ^{3}H-5,7-dichlorokynurenic acid binding by glycine

In mouse brain, glycine (10μM, 100μM and 1mM) significantly inhibited ^{3}H-5,7-dichlorokynurenic acid binding in all regions examined (Tab. 4.3.). The effect of 10 μM glycine was, in general,maximal with no further displacement of binding at 100μM or 1mMglycine. Glycine failed to displace all the specifically bound radioligand in all regions.

4.3.2.3. Inhibition of ^{3}H-5,7-dichlorokynurenic acid binding by compound Z

In mouse brain compound Z, at 10uM, produced significant inhibition in many regions analyzed (Tab. 4.4.). Note the lack of inhibition of binding at the concentration of 1μM which was completely effective in displacing ^{3}H-glycine binding in all regions studied.

4.3.2.4. *In vitro* distribution of ^{3}H-glycine binding sites

Glycine binding to mouse brain sections exhibited a regional heterogeneity with the highest binding density present in the stratum radiatum of the CA1 hippocampal

Table 4.2. Distribution of ^{3}H-5,7-dichlorokynurenic acid binding sites in normal brain.

Entorhinal cortex	2877.4 ± 189
Frontal cortex	3208.3 ± 139
Cingulate cortex	3311.1 ± 266
Caudate putamen	2376.3 ± 236
Globus pallidus	2048.3 ± 135
Nucleus accumbens	2904.4 ± 216
Ventromedial thalamic nucleus	2889.5 ± 284
Dorsolateral thalamic nucleus	3084.6 ± 188
Amigdala	2607.2 ± 155
Hyppocampus radiatum CA1	3537.6 ± 252
Hyppocampus oriens CA1	3231.6 ± 392
Hyppocampus radiatum CA2	3314.1 ± 409
Hyppocampus oriens CA2	3375.2 ± 428
Hyppocampus radiatum CA3	3310.7 ± 302
Hyppocampus oriens CA3	2624.7 ± 284
Dentate gyrus molecular layer	3414.8 ± 497
Dentate gyrus granular layer	2437.9 ± 256
Subiculum	2081.0 ± 274
Cerebellum	1902.8 ± 144

Values represent the mean ± s.e.m. (n=4) of ^{3}H-5,7-dichlorokynurenic acid bound, fmol/mg tissue. Background binding was subtracted in all cases.

Tab.4.3. Effect of glycine on ^{3}H-5,7-dichlorokynurenic acid binding: regional distribution in mouse brain

	Specifically bound radiolabelled ligand in the presence of:			
Brain region	Control (fmol/mg)	Gly 10μM (fmol/mg)	Gly 100μM (fmol/mg)	Gly 1mM (fmol/mg)
CA1 or.	3231.6±392	973.2±336*	991.5±287*	1960.0±318*
CA1 rad.	3537.6±252	1892.0±215*	1524.5±292 *	1822.7±256*
CA2 or.	3375.2±428	1106.6±354*	1734.5±362*	1959.5±87*
CA2 rad.	3314.1±409	1625.9±297*	1611.1±288*	2035.3±122*
CA3 or.	2624.7±284	688.9±148 *	1901.3±310	1805.5±414
CA3 rad.	3310.7±302	1148.4±308*	1607.2±344*	2074.9±667*
SMDG	3414.8±497	2049.8±310	1375.6±211*	1670.9±214*
CPu	2376.3±236	870.1±313*	1690.4±123	865.0±141*
Thal nucl.	1962.2±287	327.2±81*	1077.6±109*	1309.3±129*
Fr.Cx.	3208.3±139	605.4±178*	758.5±271*	1405.5±492*
Cerebellum	1902.8±144	476.2±61*	532.8±74 *	714.7±155*

Values represent the mean and s.e.m. of 4 determinations for 3-5 animals
* $p < 0.05$ vs control, ANOVA.

Tab. 4.4. Effect of Compound Z on ^{3}H-5,7-dichlorokynurenic acid binding *in vitro*: regional distribution in mouse brain

	Specifically bound radiolabelled ligand in the presence of:		
Brain region	Control (fmol/mg)	Compound Z 1μM (fmol/mg)	Compound Z 10μM (fmol/mg)
CA1 or.	615.3±19.6	565.6±47.2	377.9±81.0*
CA1 rad.	679.7±28.5	636.8±68.1	365.6±36.2*
CA3 or.	351.3±38.6	488.3±64.4	465.6±39.9
CA3 rad.	507.3±80.9	541.7±20.2	427.6±23.9
SMDG	483.4±55.8	435.5±78.5	320.8±33.7
SGDG	238.6±63.8	205.5±19.2	133.1±41.1
DLG	179.4±49.7	215.9±25.7	147.9±55.8
VPL	296.3±67.5	255.2±47.8	184.7±33.1
CPu	363.8±77.9	360.3±15.5	230.0±42.3
FrCx ext.l.	292.0±79.7	409.8±60.7	192.6±40.7
FrCx med.l.	325.7±42.9	265.6±28.2	122.1±37.0*
FrCx int.l.	251.5±32.5	225.7±22.1	110.4±33.7*
Cerebellum	209.8±58.3	208.6±47.2	119.4±45.6

Values represent the mean and s.e.m. of 4 determinations for 3-5 animals.
* $p < 0.05$ vs control, ANOVA.

subregion (Fig. 4.2.). Essentially high levels of specific binding were observed in the hippocampal formation. Moderate to high levels of specific binding were detected in caudate putamen and frontal cortex. Cerebellum exhibited the lowest level of specific binding.

4.3.2.5. Inhibition of ^{3}H-glycine binding *in vitro* by compound Z

Compound Z, tested at the concentrations of 0.01μM and 0.1μM, inhibited ^{3}H-glycine binding in most of the brain areas examined (Tab. 4.5.; Fig. 4.3.). Compound Z, tested at concentration of 1μM, completely inhibited ^{3}H-glycine binding in almost every regions of mouse brain.

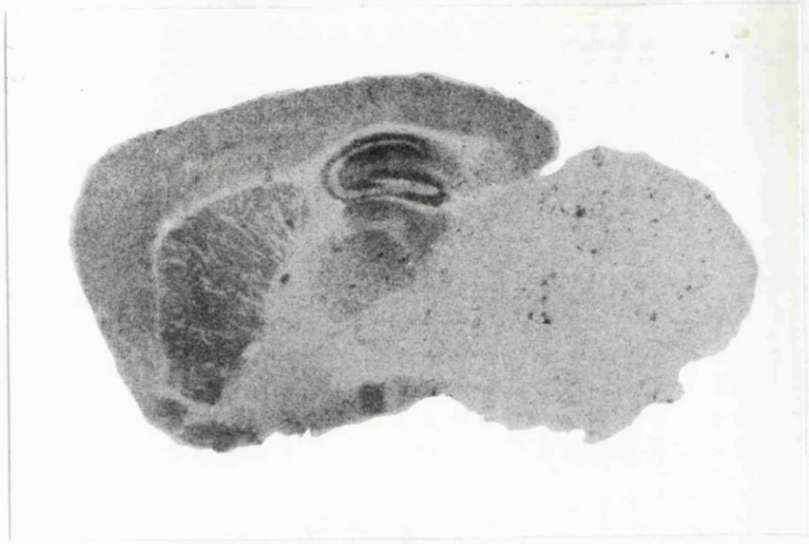
4.3.2.6. Inhibition of *in vitro* ^{3}H-dizocilpine binding by compound Z

The heterogeneous distribution of the regional binding sites of ^{3}H-dizocilpine in mouse brain sections determined by *in vitro* receptor autoradiography, is described in Chapter 3 (Fig. 3.2 ; Fig. 3.3). Compound Z, tested at concentrations of 0.1μM, 1μM and 10μM dose dependently inhibited ^{3}H-dizocilpine binding in all brain regions examined (Tab. 4.6.). The pattern of distribution of binding sites and the inhibitory activity of compound Z in each region of mouse brain are illustrated in Fig. 4.3. Clearly, ^{3}H-dizocilpine binding was inhibited by increasing concentrations of compound Z and, at the highest concentration tested (10μM), the inhibition was almost complete

Fig. 4.2. Receptor autoradiography of ^{3}H-glycine binding to mouse brain sections illustrating the regional distribution of binding sites.

a. Receptor autoradiographs of ^{3}H-glycine binding to parasagittal section of mouse brain. Sections were incubated with 100 nM ^{3}H-glycine and autoradiographs generated as described in Methods.

b. Comparative regional densities of ^{3}H-glycine in mouse brain sections. Density values for each brain region from at least 3 sections each from 3 mice.



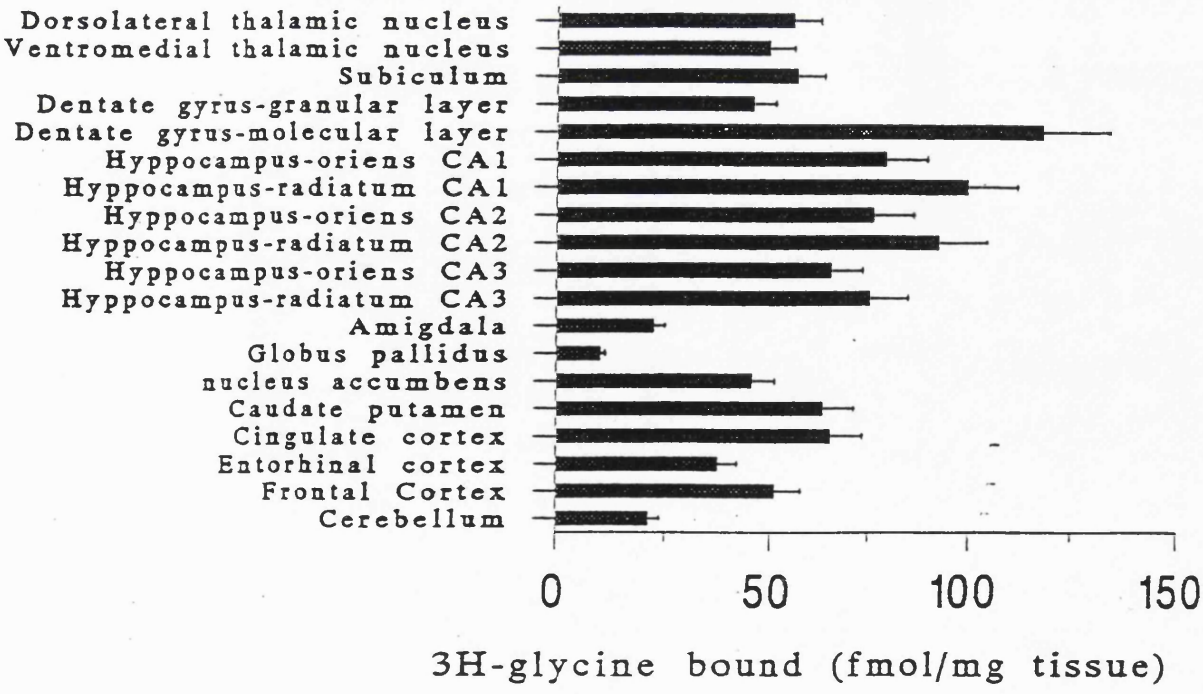
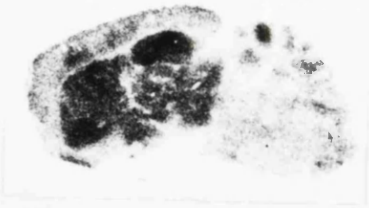
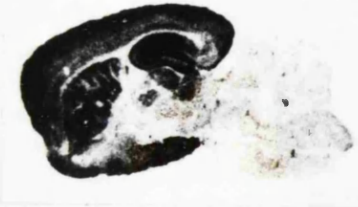

Dorsolateral thalamic nucleus
Ventromedial thalamic nucleus
Subiculum
Dentate gyrus-granular layer
Dentate gyrus-molecular layer
Hyppocampus-oriens CA1
Hyppocampus-radiatum CA1
Hyppocampus-oriens CA2
Hyppocampus-radiatum CA2
Hyppocampus-oriens CA3
Hyppocampus-radiatum CA3
Amigdala
Globus pallidus
nucleus accumbens
Caudate putamen
Cingulate cortex
Entorhinal cortex
Frontal Cortex
Cerebellum
0
50
100
150
3H-glycine bound (fmol/mg tissue)

Fig. 4.3. Inhibition of ^{3}H-glycine and ^{3}H-dizocilpine binding *in vitro* to mouse brain sections by compound z

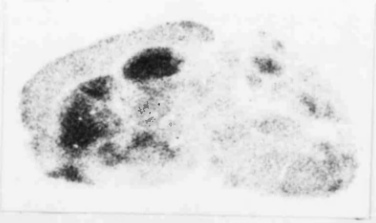
Total ^{3}H-glycine

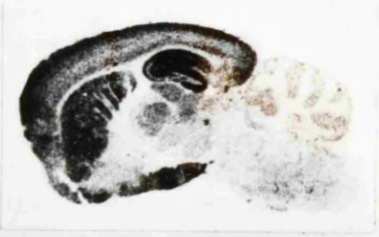




Total ^{3}H-dizocilpine

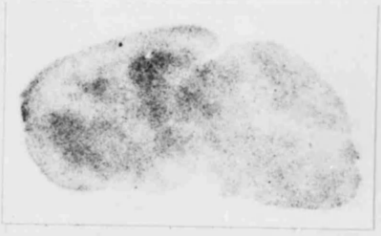
Compound Z
0.01 µM

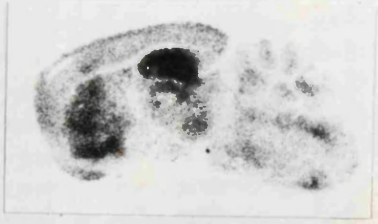




Compound Z
0.1 µM

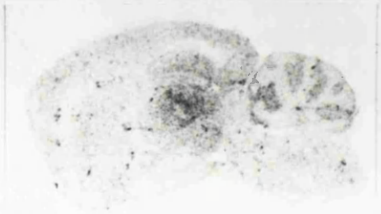
Compound Z
0.1 µM





Compound Z
1 µM

Compound Z
1 µM



Compound Z
10 µM

Tab. 4.5. Effect of Compound Z on ^{3}H-glycine binding *in vitro*: regional distribution in mouse brain

	Specifically bound radiolabelled ligand in the presence of:			
Brain region	Control (fmol/mg)	Compound Z 0.01μM (fmol/mg)	Compound Z 0.1μM (fmol/mg)	Compound Z 1μM (fmol/mg)
CA1 or.	163.1±18.7	76.5± 4.3*	37.2± 2.1*	0*
CA1 rad.	198.4±25.1	99.9±10.1*	39.1± 1.0*	11.1± 2.3*
CA3 or.	56.7± 6.7	30.6± 3.7	18.6± 2.7	0*
CA3 rad.	48.6±12.3	58.9± 4.2	27.8± 1.0*	3.1± 0.8*
SMDG	127.8±16.6	70.9± 9.1*	33.3± 1.6*	8.9± 1.2*
SGDG	85.5± 7.5	35.0± 2.7*	22.4± 1.6*	0*
DLG	49.7± 7.5	24.3± 2.1*	19.2± 1.0*	0*
VPL	37.9± 2.7	25.5± 2.1	17.0± 2.1*	0*
CPu	60.4± 6.2	29.7± 2.7*	16.6± 2.1*	8.1± 1.0 *
FrCx ext.l.	75.4± 5.9	33.0± 3.7*	20.7± 1.1*	0*
FrCx med.l.	54.5± 4.9	29.8± 1.6*	17.7± 2.7*	0*
FrCx int.l.	41.7± 8.5	12.1± 1.6*	17.0± 2.1*	0*
Cerebellum	20.7± 3.7	3.4± 1.0*	10.1± 1.9*	0*

Values represent the mean and s.e.m. of 4 determinations for 3-5 animals.
* $p < 0.05$ vs control, ANOVA.

Tab. 4.6. Effect of Compound Z on ^{3}H-dizocilpine binding *in vitro*: regional distribution in mouse brain

	Specifically bound radiolabelled ligand in the presence of:			
Brain region	Control (fmol/mg)	Compound Z 0.1μM (fmol/mg)	Compound Z 1μM (fmol/mg)	Compound Z 10μM (fmol/mg)
CA1 or.	251.8±18.9	65.5± 9.8*	22.2± 5.8*	11.9± 1.7*
CA1 rad.	348.3±23.9	100.9±27.1*	25.7± 8.7*	12.8± 1.1*
CA3 or.	158.7±20.5	60.8±19.9*	17.0± 5.6*	9.9± 0.9 *
CA3 rad.	175.7±43.5	50.6±19.4*	19.1± 6.6*	5.6± 0.7*
SMDG	252.5±15.0	77.3±10.5*	20.9± 9.2*	6.0± 1.5*
SGDG	68.9± 5.0	34.6± 8.7*	9.2± 4.7*	0*
DLG	49.7± 3.6	24.4± 6.7*	13.7± 3.2*	0*
VPL	96.2± 8.0	47.3± 6.8	19.6± 3.2*	4.5± 0.6*
CPu	123.1± 5.4	31.1± 8.7*	10.3± 2.2*	6.7± 1.7*
FrCx ext.l.	144.2±14.5	31.0± 5.6*	16.7± 5.8*	10.5± 1.1*
FrCx med.l.	93.8±13.2	26.4± 8.5*	12.6± 4.1*	6.4± 1.1*
FrCx int.l.	64.8± 7.7	30.6± 9.0*	9.9± 3.4*	5.6± 0.6*

Values represent the mean and s.e.m. of 4 determinations for 4-6 animals.
* $p < 0.05$ vs control, ANOVA.

4.4. DISCUSSION

The regional distribution of ^{3}H-5,7-dichlorokynurenic acid binding sites in mouse brain was found to be similar to the topography of specific binding sites for both ^{3}H-glycine and ^{3}H-dizocilpine, except for the low level of binding observed in the frontal cortex in one experiment and for a more homogeneous distribution of binding sites throughout the brain compared to the other ligands. The similarity between ^{3}H-glycine and ^{3}H-5,7-dichlorokynurenic acid binding distribution was consistent with the action of 7-chlorokynurenic acid as a glycine site antagonist. Furthermore, the finding that the distribution of the two binding sites are comparable with the distribution of ^{3}H-dizocilpine binding sites supports an association within the NMDA receptor complex. Glycine and compound Z showed a clear inhibitory effect on ^{3}H-5,7-dichlorokynurenic acid binding in most regions examined.

The regional distribution of ^{3}Hglycine binding site in the present study confirms data previously reported utilizing the same ligand (Bristow et al. 1986; Mc Donald et al.,1990). Qualitatively, the distribution of glycine, NMDA and TCP recognition sites is very similar suggesting that these sites are associated. Compound Z markedly inhibited ^{3}H-glycine binding at 0.01μM, and at 1μM the inhibition of binding was complete in the majority of brain regions. The high efficacy of compound Z as an inhibitor of ^{3}H-glycine binding, in addition to its inhibitory effect on both ^{3}H-dizocilpine binding and ^{3}H-5,7-dichlorokynurenic acid binding, are consistent with its action as a glycine site antagonist.

The regional distribution of ^{3}H-dizocilpine binding sites in the present study confirms data previously reported utilizing the same ligand (Bowery et al., 1988).

In the present study the inhibitory action of compound Z on ^{3}H-dizocilpine binding has been studied *in vitro* by means of receptor autoradiography, in order to see whether the anti-ischaemic activity that the compound exhibits *in vivo* (Chapter 2) correlates with an ability to inhibit ^{3}H-dizocilpine binding in specific areas of the brain. Compound Z inhibited ^{3}H-dizocilpine binding in all regions examined and this effect was concentration dependent (0.1μM-10μM). The inhibitory effect of compound Z is consistent with the hypothesis of allosteric negative modulation via the strychnine-insensitive glycine recognition site present on the NMDA receptor complex.

One purpose of the present study was to verify whether it is possible to modulate the NMDA receptor complex under physiological conditions. For this reason ^{3}H-dizocilpine binding in mouse brain was employed for an *ex vivo* study of the functional effects of different compounds on the NMDA receptor complex. An *ex vivo* technique could offer the advantage, over *in vitro* binding techniques, of studying the receptor state in the physiological environment and to allow investigation of the relationship between receptor occupancy and effect, either physiological, therapeutic or behavioural. For compounds being used as *ex vivo* ligands, certain criteria have to be fulfilled. Firstly, the ligand must have high affinity for the receptor in order to limit the competition for binding sites from endogenous compounds (and possibly a slow dissociation rate from the receptor). Secondly, the ligand must display high selectivity for the receptor under study. A compound may exhibit high affinity for one particular receptor but, if the relative

affinities for other receptor sites are also high, the interpretation of results is more complex. Also, it is important to employ ligands which are not metabolised in the brain and any metabolite formed outside of the brain not permeable to the blood-brain barrier, since this would compromise the exact analysis of what the levels of radioactivity reflect. ^{3}H-dizocilpine appears to possess these characteristics with ready penetration into the CNS after parental administration and high affinity and selectivity for the NMDA receptor complex. In addition, the very poor permeability of its metabolites into the CNS, make it potentially a good *ex vivo* marker for the NMDA receptor complex.

However, one disadvantage inherent in the technique is that measurement of bound radioactivity does not discriminate between specific binding, non-specific binding and residual blood radioactivity. Brains taken after injection of radioligand display high non-specific binding and a large proportion of bound radioactivity seemed not to be specifically associated with the ^{3}H-dizocilpine binding sites as it was not displaced by prior administration of unlabelled dizocilpine. On this basis, the utility of highly lipophilic agents, such as dizocilpine, as *ex vivo* ligands under physiological conditions has been challenged, suggesting that factors other than receptor density (i.e.lipophilicity of the compound) might cause differential distributions of radioactivity (Wallace et al., 1992; McCulloch et al., 1991). However, a specific component of binding sites with the same profile of the known distribution of the NMDA receptors could be detected with appropriate washing of the brain slices. In our study, after 2x45 s washing, there was a very good correlation between the *in vitro* distribution of specific ^{3}H-dizocilpine binding and ex *vivo* distribution of bound radioactivity. Common to all these studies, binding of ^{3}H-dizocilpine in cerebellum was very low compared to forebrain regions such as

hippocampus and cortex. This close correlation of ex *vivo* and *in vitro* data also supports the assumption that the radioactivity bound ex vivo is associated with dizocilpine rather than a product of metabolism of the drug. Under these experimental conditions, mice pre-injected with unlabelled dizocilpine (1 mg/kg) showed a marked and consistent decrease in bound radioactivity due to competition with the labelled drug for occupancy of its presumed PCP recognition site on the NMDA receptor complex. These observations are in agreement with previous studies on membrane homogenates showing that, after filtration and washing, 78% specific ^{3}H-dizocilpine *ex vivo* binding could be obtained (Price et al., 1988; Price et al., 1988).

Ex vivo ^{3}H-dizocilpine binding was used in the present study to investigate whether the strychnine-insensitive glycine recognition site associated with the NMDA receptor is or is not fully saturated *in vivo*. The increase in ^{3}H-dizocilpine binding *ex vivo* produced by pre-injection of D-serine is consistent with its known action as a glycine site agonist. Interestingly, D-serine pre-treatment increased or actually revealed ^{3}H-dizocilpine binding sites in brain areas where normally those sites are low or undetectable. Assuming that the effect of D-serine is mediated exclusively through an action at the specific recognition site, this finding would suggest a regional difference in the occupancy of the glycine site by the endogenous ligand *in vivo* and also that the glycine site associated with the NMDA receptor complex may not be fully saturated *in vivo*.

The physiological significance of glycine modulation of the NMDA receptor complex has been debated, since the concentration of glycine in cerebrospinal fluid appears to be in the low micromolar range which is sufficient to saturate the glycine binding site. Most initial

electrophysiological studies *in vivo* and in slices of adult tissue failed to show any consistent potentiating effect of glycine on NMDA responses (Fletcher and Lodge, 1988; Birch et al., 1988; Watson et al., 1988). However these studies showed that selective glycine antagonists did produce a non competitive block of NMDA responses in a glycine or D-serine reversible manner, confirming that the glycine site associated with NMDA receptors is present in normal adult tissue. The results of these studies also indicated that the glycine site was normally already maximally activated by the extracellular concentration of glycine present in these preparations. An alternative explanation of these findings is that NMDA evokes the release of glycine from neurones or glia thus increasing the glycine concentration to a level that would saturate the glycine site (Wong and Kemp, 1991). To add support to this theory L-glutamate and L-aspartate have been shown to release glycine from retina and hippocampal cultures (Yazulla et al., 1985; Shalaby et al., 1988). Furthermore, by changing the experimental conditions, such as applying NMDA to slices locally rather than in a bath-perfusion, potentiation of NMDA receptor-mediated responses have been observed (Thomson et al., 1989; Krebs et al., 1989). Controversy as to whether these glycine sites are fully saturated *in vivo* comes from uncertainty over the exact brain concentrations of glycine. Attempts at measuring the extracellular concentration of glycine *in vivo* have indicated levels as high as 1μM in cerebrospinal fluid (Ferraro and Hare, 1985; Skilling et al., 1988). Potentiation of NMDA responses in cultured neurons has been observed at glycine concentrations of 10nM (Johnson and Ascher, 1987) and the potentiatory effect appears to be near maximal at glycine concentrations of 1 to 3μM (Johnson and Ascher, 1987; Kemp et al., 1988). At an extracellular level of 1 μM, therefore, small fluctuations in concentration would

probably have little functional significance. On the other hand, convincing evidence exist to support the view that the glycine site may not always be fully saturated *in vivo.* Glycine potentiation of NMDA responses has been reported for NMDA-induced firing of rat thalamic neurones *in vivo* (Salt, 89) and other NMDA responses (Larson and Beitz, 1988; Singh et al., 1990). The glycine site appears to be functional *in vivo*, as glycine can be proconvulsive (Chapman, 1991) and glycine antagonists anticonvulsive (Smith and Meldrum, 1992).

Evidence for a positive modulation by D-serine on the *ex vivo* ^{3}H-dizocilpine binding was followed by data indicating a slight, though significant, inhibition of ^{3}H-dizocilpine binding produced by D-cycloserine. A possible explanation for this effect may be related to its partial agonist activity (Hood et al., 1989, Emmett et al., 1991) by producing glycine receptor antagonism in regions containing high levels of endogenous agonist. This is in accordance with the observation that D-cycloserine antagonizes the positive effect of D-serine in increasing cerebellar cGMP *in vivo* in mouse (Wood et al.,1989).

Following these initial observations, we thought that *ex vivo* ^{3}H-dizocilpine binding could represent a useful tool for the assessment of the specificity and potency of drugs interacting on the NMDA receptor complex, either at the glutamate recognition site or at allosteric modulatory sites such as the glycine site or polyamine site. This view was supported by previous studies showing a dose-dependent inhibition of specific *ex vivo* ^{3}H-dizocilpine binding produced by some non-competitive NMDA antagonists on mouse membrane homogenates (Price et al., 1988). These authors reported *ex vivo* ^{3}H-dizocilpine binding displacement using dizocilpine, TCP, etoxadrol and SKF 10,047, with

a rank order of potency consistent both with their affinity for the PCP site *in vitro* and with their ability to block NMDLA-induced seizures, indicating a direct relationship to the occupation of the ion-channel site.

The lack of effect of some of NMDA agonists and antagonists tested in the present study (N-methyl-D-aspartate, 7-chlorokynurenic acid, CNQX, Kynurenic acid, cycloleucine) on *ex vivo* ^{3}H-dizocilpine binding could have been due to their inability to readily penetrate the blood brain barrier, and thus have a low potency when applied systemically. In addition, although ACBC has a good permeability across the blood brain barrier, its lack of effect on the *ex vivo* binding of ^{3}H-dizocilpine was probably due to its short half life ($T^{1}/_{2}$ = 5 min) in mouse brain (Rao et al., 1990).

Thus, it was crucial to study the effect of various NMDA antagonists which do penetrate into the brain and could therefore inhibit *ex vivo* ^{3}H-dizocilpine binding by acting at one of the NMDA receptor complex sites.

The lack of effect of CGP 37849, ifenprodil, HA-966 and compound Z was, therefore, unexpected since they have been demonstrated to penetrate the blood brain barrier with relative ease. CGP 37849 is known to be a competitive NMDA antagonist with enhanced bioavailability, as indicated by its anticonvulsant activity following oral administration (Fagg et al., 1990). Ifenprodil appears to be a non-competitive NMDA receptor antagonist with activity both *in vitro* and *in vivo*, probably reflecting an interaction at the polyamine site of the NMDA receptor complex. *In vivo*, ifenprodil is a known neuroprotective drug which has been demonstrated to reduce the volume of ischaemic damage after systemic administration (Gotti et al., 1990). The anticonvulsant and

neuroprotective effects of HA-966 have also been shown *in vivo* (Vertanian and Taylor, 1991). Data from the MCA-O model in the present study (Chapter 2) demonstrated that compound Z is neuroprotective after systemic administration indicating its permeability across the blood-brain barrier.

In vitro, ifenprodil is a weak displacer of the NMDA channel ligand ^{3}H-TCP, although it does antagonise the glutamate-induced increase in ^{3}H-TCP binding (Carter et al., 1988)). Thus, although *ex vivo* ^{3}H-dizocilpine binding sites has the same distributions as those *in vitro*, the influence of ligands on ^{3}H-dizocilpine binding differs.

The use-dependence profile of the channel block produced by dizocilpine has been clearly demonstrated in *in vitro* preparations (Foster et al. 1987). The development of full antagonism requires the receptor-channel complex to be in its active state during exposure to the antagonist (Wong et al., 1986). However, by using an *in vivo* microelectrophoresis technique, Lodge's group noticed that the time course of NMDA antagonism by dizocilpine was not dependent on the frequency of agonist administration, indicating that the onset of such antagonism was not use-dependent *in vivo* (Church et al., 1986; Davies et al., 1988). They examined the possibility that endogenous agonists might provide enough activation of the NMDA receptor complex to allow the use-dependent access of dizocilpine to its site of action *in vivo*, even in the absence of exogenous agonists. Thus, by blocking any exogenous or endogenous activation of the NMDA receptor complex with a competitive NMDA antagonist (2-AP5), it was demonstrated that dizocilpine could gain access to its site of action *in vivo* even though the receptor complex was probably inactivated (Church et al., 1986; Davies et al., 1988).

These observations could partly justify the failure of *ex vivo* ^{3}H-dizocilpine binding to reflect NMDA receptor inactivation produced by NMDA antagonists in the present study. A possible explanation for the apparent discrepancy between *ex vivo* and *in vitro* results is the difference in temperature at which ^{3}H-dizocilpine binding occured in the two systems. *In vitro* autoradiography was performed at room temperature (23^{o}C) but it is possible that at body temperature ^{3}H-dizocilpine is able to gain access to its site even in the absence of activation of the NMDA receptor complex (Davies et al., 1988). At body temperature the ion channel associated with the NMDA receptor complex might adopt transient molecular configurations sufficient to allow brief channel openings and dizocilpine binding, without necessarily producing significant depolarization. Single channel currents mediated by NMDA receptors have multiple conductances (D'Angelo et al., 1990), and the association of ^{3}H-dizocilpine appears to have complex kinetics, indicating several intermediate states of the ion channel between fully closed and fully open (Cull-Candy et al., 1988; Jahr and Stevens, 1987; Javitt et al., 1990). The rate of interchange between different configurations could be accelerated by the increased temperature, and shifted towards the fully open state by the presence of NMDA receptor agonists. This hypothesis would explain, at least in part, why in the present study it was easier to demonstrate the positive modulation of *ex vivo* ^{3}H-dizocilpine binding by the glycine agonist D-serine, rather than negative modulations by NMDA antagonists. It would also justify the displacement of binding produced by non-competitive NMDA antagonists acting at level of the ion-channel (Price et al., 1988). All these considerations nevertheless suggest that *ex vivo* ^{3}H-dizocilpine binding does not represent a sensitive marker for modulation of the NMDA receptor complex under physiological conditions.

In conclusion this *in vivo* binding technique appears to be sensitive, only in some cases, to modulators of the NMDA receptor complex and the effect of NMDA antagonists seems to be more difficult to demonstrate. Our data also indicate that D-serine can represent a useful tool for studying the NMDA-coupled glycine receptor *in vivo*. This is related to its poor affinity for the glycine uptake carrier and for the strychnine-sensitive glycine receptor (Marvizon et al.,1986) and its stereospecificity for the glycine site associated to the NMDA receptor. An important observation in the present study was that the glycine site associated to the NMDA receptor complex may not be fully saturated *in vivo* and this may vary with the region. These observations might also suggest that glycine levels in the vicinity of receptors may be lower than those observed in CSF and perhaps that compartmentation of CSF glycine pools requires further study.

Thus, these findings from *in vivo* studies would suggest that the glycine site might not be maximally activated under certain conditions and it may still be open to modulation. The interest in this thesis concerns the role that this site may play in pathological conditions, with particular attention to the properties of novel systemically active antagonists.

CHAPTER 5: EFFECT OF NMDA RECEPTOR MODULATORS AND MCA-O ON SPATIAL LEARNING PERFORMANCE IN MICE

5.1. INTRODUCTION

5.1.1. Behavioural effects of NMDA antagonists

The potential beneficial effects of NMDA antagonists and their clinical use could be limited by the adverse neurobehavioural side effects exhibited by many of these drugs. Both competitive and non-competitive NMDA antagonists have been reported to induce "PCP-like" behavioural effects that include the induction of a complex motor syndrome that progresses to ataxia with increasing dose (Willets et al., 1991). Both competitive NMDA antagonists and channel blockers also possess amnesic properties and interfere with spatial and passive avoidance tasks (Wozniak et al., 1990, Danisz et al., 1988; Tonkiss et al., 1988). Initial suggestions on the involvement of NMDA receptors in learning and memory came from electrophysiological studies *in vitro* on LTP, a phenomenon hypothesized to be a model of memory formation (Bliss and Collingridge, 1993). Morris et al., (1986) first described the connection between NMDA receptors, LTP and spatial learning while showing that blockade of NMDA receptors prevented the induction of LTP in the hippocampus and impaired spatial learning. There has, therefore been considerable interest in the possibility that drugs acting upon this receptor complex may selectively alter learning and/or memory processes. One of the critical issue in this area is to determine whether drugs are selectively affecting learning and memory, or simply affecting the performance of a learned task. Thus, behavioural studies has clearly demonstrated that drugs acting at the NMDA receptor complex can disrupt learning and memory, but differences in the effects of NMDA antagonists may depend upon the site of antagonism and/or the type

of memory. Dizocilpine has been shown to impair learning of passive avoidance, brightness and olfactory discrimination and acquisition on a radial arm maze (Ward et al 1990; Wozniak et al., 1990; Kant et al., 1991). In addition several reports have used the Morris water maze task to assess effects of NMDA antagonists on learning and memory and have generally found that these compounds impair acquisition but not memory in this spatial task. The central issue for clinical trials is the concentration at which the behavioural changes are manifest relative to the therapeutic doses. With non-competitive antagonists such as dizocilpine, behavioural alterations are apparent at concentrations similar to those required for anti-ischaemic efficacy. For competitive antagonists (like CGS 19755, CGP 39551 or CGP 37849, Loscher et al., 1993) behavioural alterations occur at concentrations 3 to 10 times greater than those required for anti-ischaemic effects and there may be a wider separation for polyamine site antagonists (such as ifenprodil) or glycine site antagonists.

A wide variety of behavioural techniques have been used to characterize the effects of NMDA antagonists on learning and memory in rats. In particular, the radial arm maze has been widely used to investigate the effects of various NMDA antagonists. In general, NMDA antagonists have been found to disrupt performance in this task (Ward et al., 1990). The 8-arm radial maze is commonly used to investigate spatial memory in rodents (Olton and Samuelson, 1976). The radial arm maze is an advantageous tool for examining memory and its neural substrate in rodents. Capitalizing on the rat/mouse innate disposition to spontaneously alternate among spatial locations, this task requires the animal to systemically sample maze arms to obtain food rewards (Olton and

Samuelson, 1976). The radial maze technique can offer some advantages over other laboratory tests in that the task the animal is required to solve has a better ethological validity. Furthermore, it has been shown that the accuracy of performance in the maze is highly sensitive to the disruptive effect of the anticholinergic scopolamine (Eckerman et al., 1990) and depends largely on the integrity of the septo-hippocampal system (Olton and Papas, 1979). Nootropic pyrrolidonic drugs have been shown to facilitate learning and memory in this behavioural task, both in the normal animal and in various animal models of cognitive impairment (Poshel et al., 1983; Schindler et al., 1984). It has been suggested that the effects of this class of drugs could be mediated by central glutamatergic pathways (Pugliese et al., 1990). This hypothesis could explain the effect on cholinergic neurotransmission, since it is known that glutamate can positively modulate the activity of cholinergic neurons in various brain areas (Snell and Johnson, 1985; Nishimura and Boegman, 1990).

5.1.2. Behavioural effects induced by ischaemia

A description of the behavioural correlates of ischaemic damage is important because the principal goal of any stroke therapy in humans is the restoration of neuronal behavioural functions of the patients. Most of the behavioural work that has been conducted using the MCA-O model was focused upon simple reflex and motor function during the early phase of the infarction (i.e. first 24 hours). However, both histopathological damage and behavioural alterations produced by MCA-O presumably continue to evolve over time. MCA-O has been reported to produce long-term (1 month) spatial cognitive disturbances in rats and this cognitive deficit does not seem to

be due to hypermotility or to a disturbance in motor coordination (Markgraf et al., 1992). The general pattern of transient sensori motor and reflex deficits, but with more persistent cognitive impairments, is similar to that seen in humans following cerebral infarct. Thus, in the present study the effect of MCA-O on the 8-arm radial maze performance in mice has been examined over a period of 28 days.

The psychopharmacological effects produced by NMDA antagonists appears to depend, at least in part, on the site of action within the NMDA receptor complex. Numerous studies suggest that both competitive and non-competitive NMDA antagonists interfere with learning and memory (Woods et al., 1991; Bischoff and Tiedtke, 1992). By contrast, compounds acting at the glycine site of the NMDA receptor complex, such as 7-chlorokynurenic acid and D-cycloserine, failed to produce any learning impairment when administered i.c.v.(Chiamulera et al., 1990) or i.p. (Faiman et al., 1994). However, since most glycine site antagonists only poorly penetrate into the CNS, this would influence their effects on cognitive function after systemic administration. The advent of centrally acting glycine site antagonists makes it possible to test this hypothesis more effectively. In the present study we have compared the effect of systemic administration of the novel glycine antagonist, compound Z, with the effect produced by HA-966 and by the non-competitive NMDA antagonist dizocilpine on acquisition of a spatial orientation task in the 8-arm radial maze

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5.2. METHODS

5.2. Radial maze performance in mice after i.p. administration of NMDA antagonists

An 8-arm radial maze was used. Each arm (7x39 cm) radiated from an octagonal platform elevated 60 cm above the floor, placed in a uniformely illuminated room and maintained in a constant orientation during the whole experiment in order to minimize any extra-maze cues. Three days before the beginning of pre-training, animals were slightly food-deprived to bring the body weight to 85% of the initial level. On day 4, each mouse was placed on the central starting platform of the maze, and allowed to explore until it had consumed some of the food scattered on the whole maze. On the following day, the actual testing procedure started. All the arms of the radial maze were baited by a standard piece of food pellet placed at the distal end of each arm. Male CD1 mice (25-30g), 8 per group, went through one learning trial per day during 12 consecutive days, 30 min after injection (i.p.) of saline (control), dizocilpine (0.15 mg/kg), compound Z (1 mg/kg) and HA-966 (5mg/kg). On each trial the rank of occurrence of the first error (number of correct arm entries before an erroneous entry occurs) was recorded. Results were analyzed by one way analysis of variance (ANOVA).

5.2.2. Radial maze performance in mice subjected to MCA-O

The experimental procedure was the same as described above. After 6 days (one learning trial per day) one group of animals (n=8) was subjected to MCA-O and

another group of animals (n=8) was subjected to sham operation. Learning trials continued up to 28 days and the occurrence of the first error was recorded on each trial. When testing was complete, mice were sacrificed and the brains removed to assess histological damage

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5.3. RESULTS

5.3.1. Radial maze performace in normal mice after administration of NMDA antagonists

Radial maze performance is shown in Fig. 5.1. Daily injections of compound Z (1 mg/kg) and HA-966 (5mg/kg) produced no significant effect on the radial maze performance compared to control (saline treated) animals. By contrast dizocilpine (0.15 mg/kg) produced a significant impairment in the occurrence of first error, $p<0.05$ ANOVA) of performance at days 9-10 and 11-12. At this dose dizocilpine produced no significant muscle relaxation which might have contributed to its apparent amnesic action. This was determined using a rota-rod test (accelerating from 8 to 16 rpm over 360 s period) 15 and 30 min after injection of drugs.

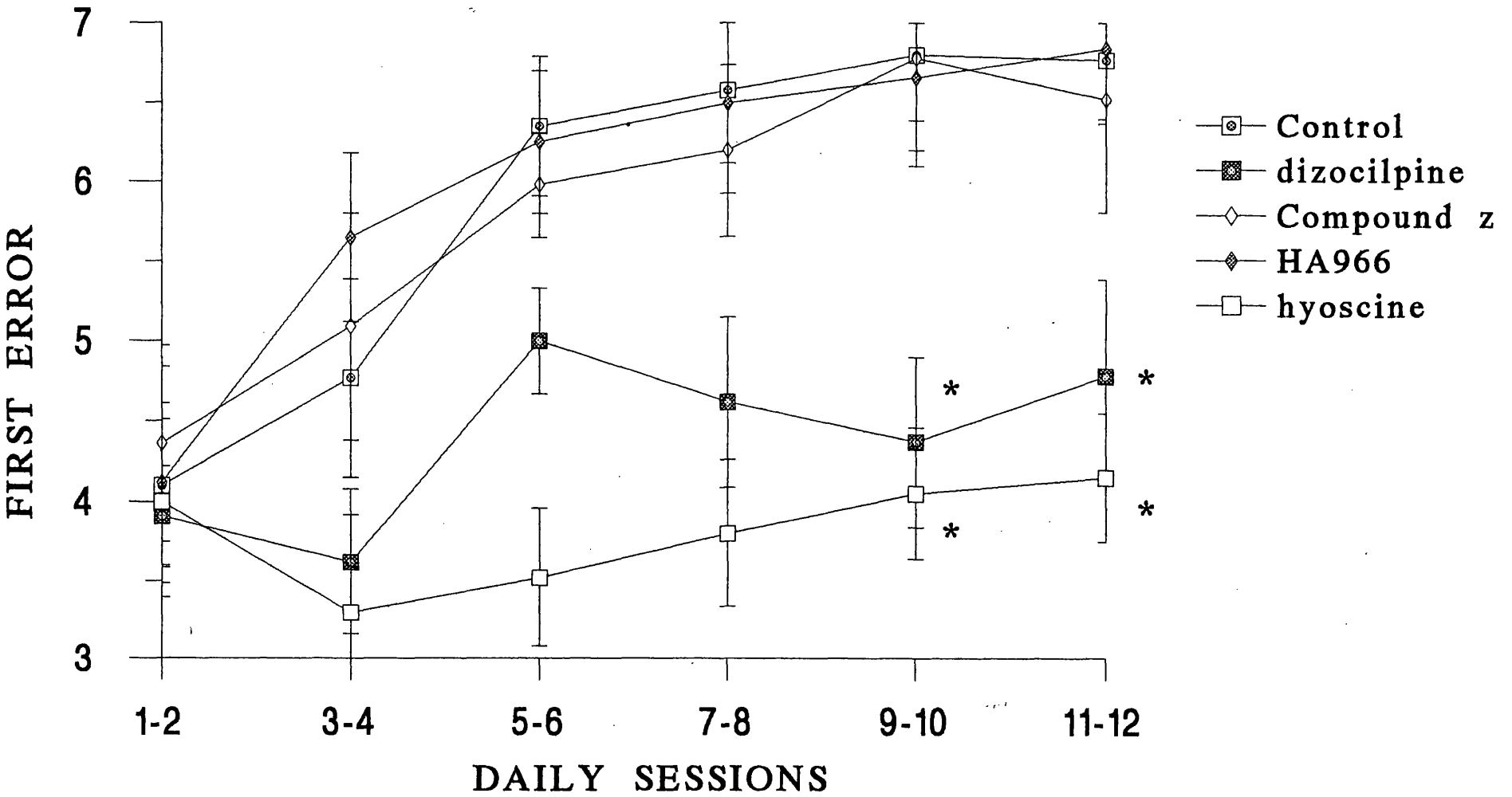
5.3.2. Radial maze performance in mice subjected to MCA-O

Results are summarized in Fig. 5.2. No significant difference in radial maze performance was observed between control and animals subjected to sham operation or MCA-O up to 28 days. An apparent impairment of performance was observed in MCA-O animals for few days after the surgical procedure. However, this was not statistically significant.

Fig.5.1.Radial maze performance in mice after i.p. administration of NMDA receptors modulators or hyoscine.

Animals (n=8/group) were subjected to 1 learning trial per day during 12 consecutive days, 30 min after injection (i.p.) of saline (control) or test compounds. Ordinate represents the number of correct arm entries before an erroneous entry occurs.

$*p < 0.05$ ANOVA


FIRST ERROR
7
6
5
4
3
1-2
3-4
5-6
7-8
9-10
11-12
DAILY SESSIONS
Control
dizocilpine
Compound z
HA966
hyoscine
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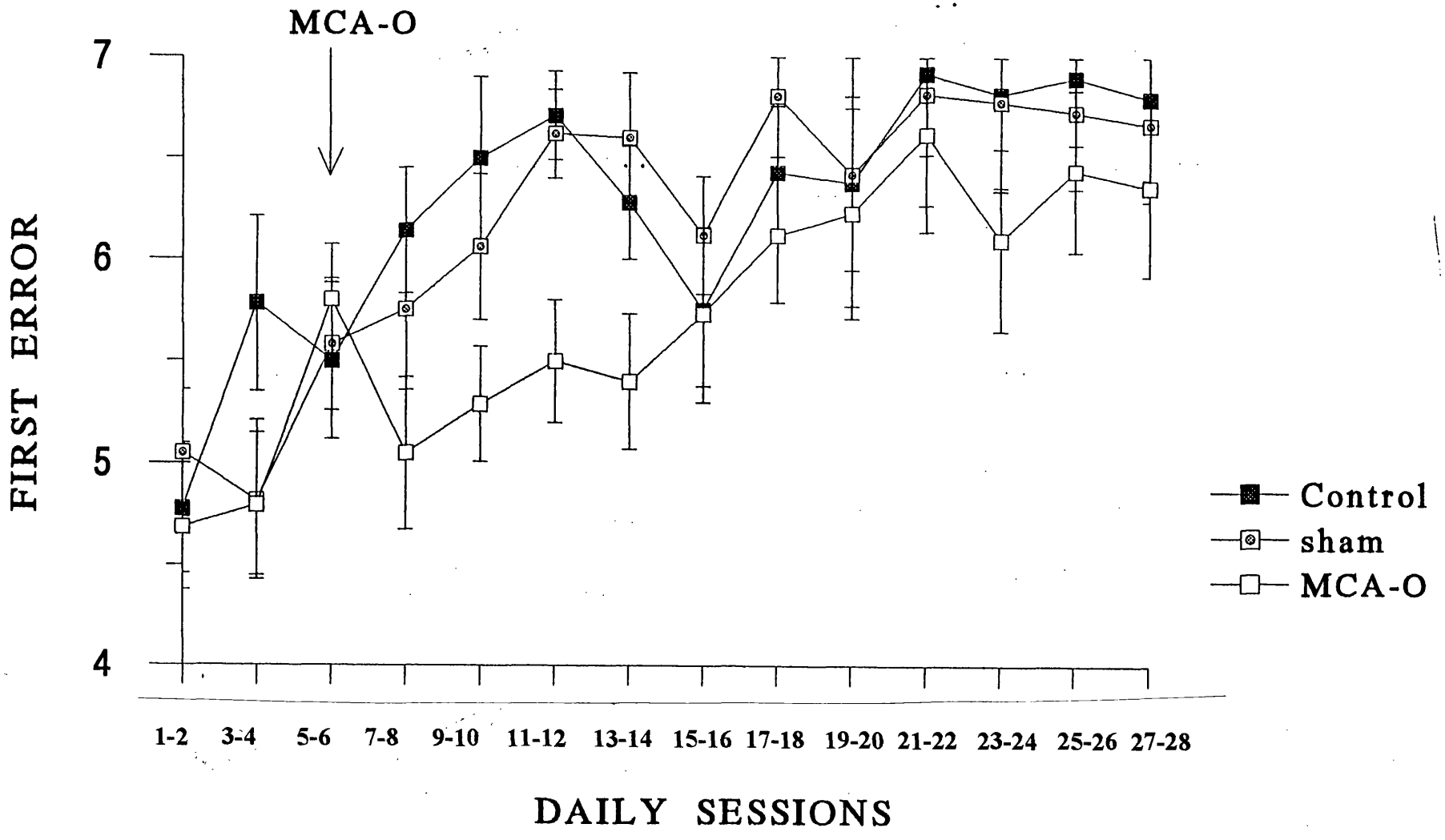
*

Fig.5.2. Radial maze performance in mice subjected to MCA-O.

At 6 days from the start of the experiment (one learning trial per day) one group of animals (n=8) was subjected to MCA-O and another group (n=8) was sham operated. Learning trials continued up to 28 days. Values represent the mean ± s.e.m. occurrence of the first error (number of correct arm entries before an erroneous entry occurs).


MCA-O
7
6
5
4
FIRST ERROR
1-2
3-4
5-6
7-8
9-10
11-12
13-14
15-16
17-18
19-20
21-22
23-24
25-26
27-28
DAILY SESSIONS
Control
sham
MCA-O

DISCUSSION

By quantifying behavioural deficits in an animal model of stroke, it might be possible to relate specific pathological mechanisms of cerebral ischaemic damage with specific behavioural impairments. Behavioural assessment is critical because histopathological measures alone (e.g. neuronal mortality) may not be sufficient to reveal subtle functional alterations. In order to model the types of behavioural deficits exhibited by humans, closer attention needs to be given to complex tasks (e.g. cognitive) which may be sensitive to subtle neuronal damage. This, however, is not indicative that the processes disrupted in a particular model underly the human neurodegenerative deficit, or that applying the treatment which is corrective in the animal model will have a beneficial effect in the human condition.

The standard method of proximal MCA-O in the rat or mouse leads to unilateral infarction in the somatosensory and portion of the frontal cortical areas, extending to neostriatum in the rat MCA-O model. This pattern is similar to that observed in humans and it has been reported that such damage in human patients leads to a variety of behavioural deficits, including language and cognitive problems (Nakayama et al., 1988). Results from the present study indicate no significant impairment on the radial maze performance in mice subjected to MCA-O up to 22 days after the induction of ischaemia. This might be related to the distinctive cerebral damage produced in the mouse model of MCA-O (mainly cortical) which is not associated with hippocampal injury. Studies conducted on global ischaemia models indicate that damage restricted to the CA1 hippocampal field is sufficient to produce significant memory impairment

(Okada et al., 1995). The ability of other brain structures to replace the compromised cortical functions might also account for the normal profile of radial maze performance observed in this study following MCA-O. Nevertheless, the radial maze task does not appear to reveal behavioural deficits which accompany cerebral ischaemic damage and, consequently, it is not suitable as a model within which to evaluate potential means of therapeutic intervention. A major problem is that complex behaviour rely on the appropriate interaction of many neural processes, and disruption of any one of severals different processes may lead to similarly appearing deficits. Although it may be relatively easy to produce behavioural deficits in experimental animals which superficially mimic deficits seen in human neurodegenerative conditions, the relevance of the models to the human condition is not always certain.

At doses close to those that protect against convulsant activity, both dizocilpine and PCP also induce a complex motor syndrome consisting of lateral head weaving, hyperlocomotion, body rolling, stereotypies and ataxia. The rank order of potencies for the induction of motor effects, while similar to, is not identical with the rank order of potencies to block NMDA-induced seizures: in particular, the doses of TCP required to induce head weaving are about 6- to 7- fold less than those required to block NMDA-induced seizures (Tricklebank et al. 1989). For competitive NMDA receptor antagonists, consistent with the findings of Compton et al. (1987) in rats and of Koek et al. (1987) in pigeons, the motor syndrome is again qualitatively similar to that seen with dizocilpine and PCP. In this case, however, the relationship between the doses inducing motor and anticonvulsant effects is, with the exception of 2-APV, the reverse

of that seen with TCP: anticonvulsant efficacy is achieved at doses lower than those necessary to induce head weaving. It is clear that dizocilpine shares many of the behavioural properties of phencyclidine and it seems likely, therefore, that these behavioural effects are mediated by a reduction of NMDA-mediated neurotransmission. Consistent with this hypothesis, structurally different antagonists acting competitively at the NMDA recognition site are also PCP-like in their ability to induce motor stimulation (e.g. CGP 37849, Loscher et al., 1993). It is notable, however, that with the competitive NMDA receptor antagonist CPP or CGP 37849, there is a significant window between the anticonvulsant properties of the compound and its motoric effects or its ability to generalize to the discriminative stimulus induced by dizocilpine or PCP (Jackson and Sanger, 1988; Willets and Balster, 1988; Loscher et al. 1993). Such differencies encourage the possibility of obtaining NMDA receptor antagonists devoid of the neurobehavioural effects of dizocilpine and PCP.

In vitro studies showed that NMDA antagonists inhibit the induction of long-term potentiation (LTP), which is regarded as the cellular mechanism underlying some forms of learning and memory (Collingridge and Bliss, 1987). *In vivo,* both competitive NMDA antagonists and non-competitive channel blockers have been shown to possess amnesic properties and to interfere with spatial and passive avoidance tasks. Compounds blocking the NMDA receptor activation *in vivo* via the glycine recognition site are currently few in number, due to the inability of these compounds to readily cross the blood-brain barrier,(Balster et al., 1993 (ACEA 1021) and Tricklebank et al., 1994 (L687,414)). However, it has been suggested that they might have distinct

advantages over other classes of NMDA antagonists (ACEA 1021 does not appear to substitute for phencyclidine in drug discrimination studies , Balster et al.,1993). Thus, the aim of the study in the present Chapter was to compare compounds acting at the glycine site of the NMDA receptor complex with the non-competitive NMDA antagonist dizocilpine on learning and memory.

Based on the present result, it is suggested that blockade by dizocilpine of the ion-channel coupled to the NMDA receptor complex impairs learning and memory functions at a dose which does not affect motor functions. By contrast, compound Z and HA-966, acting at the glycine site of the NMDA receptor complex, appear to be devoid of such undesired effects on cognitive processes. This observation is particularly relevant in view of the fact that compound Z has been demonstrated to be neuroprotectant in a mouse model of focal ischaemia after systemic administration (Chapter 2).

FINAL CONCLUSIONS

Over the past decade much research has focused on the role of NMDA receptors as it seems likely that their activation contributes to the excitotoxic mechanism associated to the permanent CNS damage which occurs following stroke, traumatic head or spinal injury, perinatal ischaemia or in hypoglycemia. The discovery of potent NMDA receptor antagonists has shown that such drugs have the potential to protect the CNS in these conditions and the development of safe and therapeutically useful compounds of this type is a major aim of many pharmaceutical companies. Several compounds acting as antagonists or modulators at the NMDA receptor complex are currently undergoing clinical trials to assess their efficacy in the acute treatment of stroke and head injury. Unfortunately, most well-characterized compounds of this type also produce serious side effects.

The proposed modulatory role for the glycine site in NMDA-mediated synaptic transmission makes it an attractive target for potential therapeutic neuroprotective agents. In the last few years rapid advances have been made in the development of highly potent and selective ligands for the glycine site and, like other NMDA receptor antagonists, these glycine antagonists have been demonstrated to protect against neuronal damage following an ischaemic process. However, the major problem currently facing medicinal chemists is obtaining in vivo activity, since the majority of glycine site ligands synthesized to date do not adequately penetrate the

blood-brain barrier. Nevertheless, recent years have seen significant improvements in CNS penetrability and bioavailability of novel compounds: in a MCA-O model of focal ischaemia in rodents, systemically administered glycine antagonists 7-chlorothiokynurenic acid, L-687,414, ACEA 1021 and compound Z yelded levels of protection similar to those observed in similar experiments using dizocilpine or other NMDA receptor antagonists.

It has been suggested that this class of NMDA receptor modulators may offer a better therapeutic window than agents acting at other recognition sites of the NMDA receptor complex. The glycine antagonists studied so far have failed to produce the behavioural and neuropathological changes observed after phencyclidine or dizocilpine administration. Overall, the glycine antagonists appear to be equally effective as other NMDA antagonists as neuroprotective agents, and they may offer advantages in terms of their side effect profile in the acute treatment of stroke.The clinical assessment of these compounds, however, is only just beginning and it is still not clear whether the glycine antagonists would be suitable for chronic treatment because of their possible long term effects on cognitive and motor functions.

The identification of multiple subtypes of EAA receptors represents a highly significant advance which is expected to have a major impact on drug discovery since it offers new possibilities for the development of subtype-selective agonist and antagonist drugs with improved side effect profiles. Preliminary data indicate

that EAA receptor subtypes show different central distributions, distinct functions and pharmacological specificity, and changes in expression during development. As a result, novel agents affecting specific subtypes of EAA receptors might be available in the near future.

Increased interest in alternative strategies for the treatment of cerebral ischaemia, including free radical scavengers, blockade of programmed cell death, antisense strategies or interactions with Na^{+} or Ca^{2+} channel conduction, can also be expected in the next few years.

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