THE RELATIONSHIP BETWEEN HIPPOCAMPAL LONG-TERM POTENTIATION AND SPATIAL LEARNING.

by

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D M Bannerman

To Mum and Dad.

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NMDA receptor-dependent 'Long-Term Potentiation' (LTP) is an experimental model of the type of synaptic plasticity which is considered by many to provide a neural substrate for learning. LTP has been particularly well studied in the hippocampus, a brain area which is strongly implicated in certain forms of learning and memory, and in particular in spatial learning. It has been proposed that LTP-like processes in the hippocampus may contribute to the neural mechanisms of spatial learning. Consistent with this "hippocampal LTP/spatial learning hypothesis" is the observation that the NMDA receptor antagonist, AP5, impairs performance on a spatial learning task in the watermaze across a comparable dose range to the inhibition of hippocampal LTP *in vivo*.

The aim of this thesis was to investigate further the relationship between hippocampal LTP and spatial learning in the watermaze by assessing (i) the effects on spatial learning of manipulating the inducibility of LTP through a different site on the NMDA receptor complex (the glycine site), (ii) the effects on spatial learning of preventing the expression of LTP at a site downstream from the NMDA receptor (the synthesis of the putative retrograde messenger, NO), and (iii) a further investigation of the effects of AP5 on spatial learning using a different watermaze protocol from those used previously.

Chronic intraventricular infusion of the glycine site antagonist, 7-chlorokynurenate (7CK), impaired performance in the watermaze, but did not prevent the induction of hippocampal LTP *in vivo*. Qualitative observations suggested that this impairment may well be due to non-mnemonic side effects of the drug treatment, and is unlikely to be a result of a direct action on the learning mechanism. The development of a novel analytical technique, using high performance liquid chromotography, enabled the determination of 7CK levels in tissue samples taken from animals participating in this study. This revealed that 7CK was rapidly excreted from the brain and was unlikely to reach sufficiently high levels in the hippocampus to prevent the induction of LTP. D-cycloserine, a partial agonist at the glycine site, did, however, produce a subtle enhancement of transfer test performance after spatial training in the watermaze.

The nitric oxide synthase (NOS) inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME), also failed to prevent the induction of hippocampal LTP *in vivo*, despite producing a 95% inhibition of the NOS activity in hippocampus, a result which suggests that NOS activity may not be required during LTP induction under normal physiological conditions. A behavioural investigation, conducted simultaneously, revealed that L-NAME impaired performance, early in training, on both spatial and non-spatial tasks in the watermaze when animals were given massed training trials. However, L-NAME did not affect spatial learning when rats were given just a single training trial per day. It seems unlikely, therefore, that L-NAME is acting directly on the mechanisms responsible for spatial learning.

In a final series of experiments, watermaze-experienced animals, which had been extensively trained, in the absence of any drug treatment, on a standard spatial reference memory task, were infused with either AP5 or artificial CSF and then trained on a spatial reference memory task in a second watermaze, located in a novel spatial environment. Although AP5 treated animals took consistently longer to escape from the pool during training, they were not significantly impaired when assessed using conventional 'transfer test' measures of performance. In contrast, rats with hippocampal lesions were impaired. Subsequent electrophysiological investigations revealed that the AP5 infusion had resulted in a near complete blocked of hippocampal LTP in these animals.

Neither 7CK, nor L-NAME, had any effect on the induction of hippocampal LTP in these experiments. Consequently, neither approach enabled us to test the hippocampal LTP/spatial learning hypothesis. In contrast, AP5 infusion did block the induction of LTP but produced only a mild behavioural impairment in the watermaze. The AP5 infused rats were capable of

considerable spatial learning, suggesting that the profile of impairment following AP5 treatment is different from that obtained after a hippocampal lesion. This result poses a serious challenge to the hippocampal LTP/spatial learning hypothesis.

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LIST OF ABBREVIATIONS

trans ACPD	1S, 3R aminocyclopentane dicarboxylate
aCSF	artificial cerebrospinal fluid
ANOVA	analysis of variance
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AP4	2-amino-4-phosphonobutyrate
AP5	2-amino-5-phosphopentanoate
AP7	2-amino-7-phosphoheptanoate
APV	2-amino-5-phosphonovalerate
CaMK II	Ca^{2+}-calmodulin -dependent multifunctional protein kinase II
CCP	3-((-)-2-carboxypiperazin-4-yl) propyl-1-phosphonate
cDNA	complimentary deoxyribonucleic acid
7-CK	7-chlorokynurenic acid
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CNS	central nervous system
5,7-DCK	5,7-dichlorokynurenic acid
DCS	D-cycloserine
DMTS	delayed matching to sample
DNMTS	delayed non-matching to sample
DNQX	6,7-dinitroquinoxaline-2,3-dione
EAA	excitatory amino acid
ecf	extracellular fluid
EGTA	ethylene-bis (oxyethylenenitrate) tetra-acetic acid
EPSP	excitatory post-synaptic potential
FAD	flavin adenine dinucleotide
FMN	flavin mononucleotide
GABA	γ-aminobutyric acid
g-DGG	g-D-glutamylglycine
HPLC	high performance liquid chromotography
icv	intracerebroventricular
ip	intraperitoneal
ITI	inter-trial interval
KYN	kynurenic acid
LTE	long-term enhancement (see also LTP)

LTP	long-term potentiation
mRNA	messenger ribonucleic acid
MK-801	(+)-5-methyl-10,11-dihydro-5h-dibenzo [a,d] cyclohepten-5,10-imine (also dizocilpine)
L-NAME	N^G-nitro-L-arginine methyl ester
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
NBQX	2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(F)quinoxaline
7-NI	7-nitro indazole
NMDA	N-methyl-D-aspartate
L-NMMA	N^G-monomethyl-L-arginine
NO	nitric oxide
L-NOArg	L-N^G-nitroarginine
NOS	nitric oxide synthase
NTB	nitrotetrazolium blue
OPA	o-phthalaldehyde
PCP	phencyclidine
PKC	Ca^{2+}/phospholipid-dependent protein kinase C
PPF	paired pulse facilitation
PPD	paired pulse depression
PTP	post-tetanic potentiation
SNP	sodium nitroprusside
STP	short-term potentiation
TCP	1-[-(2-thienyl)-cyclohexyl]piperidine
THF	tetrahydrofuran

Chapter 1 - Introduction

The way in which memories are laid down and stored is a central issue confronting neurobiologists today. One theory which is commonly ascribed to is that memories are contained within the synaptic connections between neurones (Hebb, 1949). More specifically, it has been suggested that information is stored as a distributed pattern of synaptic weights across a network of inter-connected neuronal elements (McNaughton and Morris, 1987). Implicit in these models of information storage is the ability of synapses to alter their efficacy. NMDA-receptor-dependent long term potentiation (LTP) is an experimental model of the type of plasticity envisaged by many to provide a neural substrate for learning (Bliss and Lømo, 1973; Bliss and Collingridge, 1993). The properties of LTP are particularly appealing with regard to a putative mnemonic mechanism. LTP is long-lasting and is induced by a brief period of activity (Bliss and Gardner-Medwin, 1973). Potentiation can be successfully induced using stimulation parameters which seem physiologically reasonable and which are within the normal firing range of neurones in the CNS (Rose and Dunwiddie, 1986; Larson and Lynch, 1986). Furthermore, NMDA-receptor-dependent LTP displays the properties of associativity, cooperativity and input-specificity which are characteristic of the type of 'Hebbian' synaptic mechanism thought to underlie learning (Andersen et al., 1977; Lynch et al., 1977; McNaughton et al., 1978; Levy and Steward, 1979; Larson and Lynch, 1986; Gustaffson et al., 1987). Taken together, the properties of LTP bear an intuitive similarity to those of associative learning and appear to provide a plausible mechanism through which co-incidence is detected, and for the subsequent storage of this information.

Consistent with the idea that LTP may resemble the sort of cellular changes that occur during learning is the fact that this experimental phenomenon is normally observed in areas of the brain which are thought to participate in the processing and storage of information. For example, LTP can be reliably induced in the hippocampus, a brain structure located in the temporal lobe of the forebrain, which is required for certain forms of learning and memory. The hippocampus appears to be specifically required for the processing of spatial and contextual information (Hirsh, 1974; O'Keefe and Nadel, 1978; Jarrard, 1993), and it is therefore not unreasonable to suggest that LTP-like events in the hippocampus might contribute to the mechanisms of spatial learning.

Despite the theoretical attractiveness of LTP as a putative substrate for information storage, obtaining experimental support for the involvement of LTP-like processes during learning has proved difficult. There are two independent but related hypotheses with which this

thesis is concerned, and which need to be tested experimentally. Firstly, there is the broad issue of whether or not LTP-like events provide the neural substrate for certain forms of learning. It is important at this stage to stress the fact that LTP, as induced using electrical stimulation in the laboratory, is an experimental phenomenon which may provide a model for certain types of synaptic plasticity. The relationship between LTP and any change in synaptic efficacy that might occur naturally during learning is far from clear. The hypothesis is not that LTP *is* the physiological basis of learning, but rather that the mechanisms involved in LTP may share common features with the mechanisms underlying certain forms of learning. From a general perspective, therefore, the hypothesis is that LTP-like events might contribute to the mechanisms responsible for certain forms of information storage. Secondly, and more specifically, there is the hypothesis that LTP-like processes in the hippocampus might subserve spatial learning. The aim of this thesis is to test this second hypothesis. Obviously, evidence supporting a role for hippocampal LTP-like events in spatial learning will also be consistent with the more general form of the hypothesis.

A common approach for testing the hippocampal LTP/spatial learning hypothesis has been to use pharmacological manipulations to prevent the induction of LTP and then assess the effects of these drugs on spatial learning. For example, the competitive NMDA receptor antagonist, D-AP5, impairs spatial learning at similar doses to those required to block the induction of LTP in the dentate gyrus *in vivo* (Morris et al., 1986a; Morris, 1989; Davis et al., 1992). The effects of D-AP5 infusion on behavioural performance in learning tasks are similar (but not identical) to those of lesioning the hippocampus (Morris et al., 1982; Morris et al., 1986b). Both experimental manipulations impair spatial learning in the watermaze but leave other forms of learning, such as the acquisition of a visual discrimination task in the watermaze, relatively intact. The ability of D-AP5-infused rats to acquire a visual discrimination task in the watermaze as quickly as control animals suggests that these rats are capable not only of swimming, climbing onto the platform and discriminating between the visual appearance of the two platforms, but also of certain other forms of learning. This behavioural dissociation goes some way to countering the argument that the D-AP5-induced deficit is due to non-associative side effects of the drug treatment on sensorimotor or motivational processes which may also affect levels of performance (Keith and Rudy, 1990). These studies have provided possibly the most reliable evidence that LTP-like events in the hippocampus might be involved in spatial learning.

Nevertheless, questions still remain as to the specificity of the D-AP5-induced behavioural

impairment and with regard to the selectivity of the D-AP5 effect on LTP relative to potential drug effects on other aspects of hippocampal physiology (Keith and Rudy, 1990; Morris and Davis, 1994). Although experiments of this type can never conclusively prove a causal link between a blockade of hippocampal LTP and a spatial learning impairment, further appropriate experimentation could either substantiate the claim that LTP-like events are involved in spatial learning or, conversely, disprove the hypothesis.

This need for further investigation has led me to adopt three different but related approaches to assess the relationship between hippocampal LTP and spatial learning. The experiments described in this thesis are all developments of previous studies which have demonstrated that intraventricular infusion of D-AP5 blocks the induction of hippocampal LTP and impairs the acquisition of a standard spatial reference memory task in the watermaze (Morris et al., 1986a; Morris, 1989; Davis et al., 1992).

The first set of experiments to be described (Chapter 4) involve using an alternative means of manipulating the NMDA receptor and, thus, of affecting the inducability of LTP. The NMDA receptor-associated glycine site influences the activity of the NMDA receptor ionophore (Johnson and Ascher, 1987) and antagonists of this receptor, such as 7-chlorokynurenate (7CK), prevent the induction of hippocampal LTP (Bashir et al., 1990; Oliver et al., 1990; Thiels et al., 1991). Along similar lines to previous studies conducted in this laboratory using D-AP5 (Davis et al., 1992), the effects of chronic intraventricular infusion of 7CK on spatial learning in the watermaze, and on the induction of LTP in the dentate gyrus *in vivo*, were assessed. In addition, the glycine site may also provide a means of up-regulating NMDA receptor-dependent processes, including LTP, within safe natural limits (Abe et al., 1990). Therefore, the possibility that the glycine agonist, D-cycloserine (DCS), might facilitate spatial learning in the watermaze was also investigated.

Whereas the experiments outlined in Chapter 4 involve affecting the induction of LTP by altering the activity of the NMDA receptor complex, the experiments described in Chapters 5 and 6 are concerned with a manipulation downstream from the NMDA receptor which may prevent the development and expression of LTP. The induction of LTP occurs post-synaptically (Lynch et al., 1983). There is now considerable evidence, however, that the expression of LTP is, at least in part, mediated by the pre-synaptic terminal (Bliss et al., 1986; Bekkers and Stevens, 1990a; Malinow and Tsien, 1990). This has led to the suggestion that an intercellular messenger is formed in the post-synaptic terminal, following the induction of LTP, and then diffuses back across the synaptic cleft and enhances the

release of neurotransmitter from the pre-synaptic terminal. There is evidence that nitric oxide (NO) might act as an intercellular messenger during the development of LTP. Indeed, a number of research groups have now shown that inhibitors of the enzyme NO synthase prevent the development of LTP in hippocampal slices (Böhme et al., 1991; O'Dell et al., 1991a; Schuman and Madison, 1991; Haley et al., 1992). The purpose of the experiments outlined in Chapter 5 is to determine whether or not inhibition of NO synthase (NOS) blocks the generation of hippocampal LTP *in vivo*. In a parallel series of investigations, the effects of NOS inhibitors on both spatial and non-spatial forms of learning in the watermaze are assessed (see Chapter 6).

In a final set of experiments (Chapter 7), the effects of the NMDA antagonist, D-AP5, on spatial learning in the watermaze are further investigated, but using a different behavioural protocol from those used previously in this laboratory (Morris et al., 1986a; Morris, 1989; Morris et al., 1989; Morris et al., 1990a; Davis et al., 1992). The experimental design involves training rats on a spatial learning task in two different watermazes in two distinct spatial environments. Extensive training in the first watermaze is carried out in the absence of any drug treatment with the intention that this will result in the animals being well practised in all the procedural aspects of the task (eg. learning to swim away from the side walls, learning that there is a means of escape via a platform, learning to successfully climb onto, and remain upon, the escape platform). In turn, it is hoped that this extensive spatial pre-training will minimise the behavioural consequences of any potential non-associative side effects of D-AP5 treatment. The effect of D-AP5 treatment on spatial learning is then assessed in a novel environment.

In summary, following on from previous studies in which the competitive NMDA antagonist, D-AP5, has been used as a means of investigating the relationship between hippocampal LTP and spatial learning (Morris et al., 1986a; Morris, 1989; Davis et al., 1992), the experiments described in this thesis adopt a number of alternative pharmacological and behavioural approaches for testing the hypothesis that LTP-like processes in the hippocampus are required for spatial learning. The aim of these experiments is to investigate (i) the effects on spatial learning of manipulating the inducibility of LTP through a different site on the NMDA receptor complex (the glycine site), (ii) the effects on spatial learning of preventing the development and expression of LTP at a site downstream from the NMDA receptor (the synthesis of the putative retrograde messenger, NO), and (iii) the effects of D-AP5 on spatial learning using a different behavioural protocol from those

used previously.

Before describing these experiments, it is first important to outline (i) the importance of the hippocampal formation for certain forms of learning and memory, namely spatial learning, (ii) the properties of LTP, particularly with regard to a putative role as the neural substrate of learning, and (iii) the underlying molecular mechanisms responsible for the induction and expression of LTP.

Chapter 2 - The hippocampus, long-term potentiation and spatial learning.

2.1 - Introduction

Learning is often defined as the acquisition of skills or of knowledge about the world. Memory is the storage of an internal representation of that knowledge. For many years the study of learning and memory (the two are not inextricably linked) has preoccupied biologists and psychologists alike. A considerable amount of time and resources have been, and continue to be, consumed in an attempt to understand the way in which sensory information is processed and then encoded as an internal representation of the world. A full investigation of the way in which animals learn and remember is a multidisciplinary study, requiring analysis at all levels of biological organisation, from the behaving organism, through the level of brain systems and neuronal circuits, to the cellular and, ultimately, molecular level. Such a study must cross the traditional boundaries of scientific disciplines and encompass psychology, anatomy, physiology, pharmacology, biochemistry and molecular biology. This thesis will endeavour to provide such a multi-level analysis, and hopefully illustrate the importance of combining data from these different scientific disciplines in an attempt to reach a common goal - the identification of the mechanisms underlying learning and memory. Ultimately, a knowledge of the biological mechanisms underlying memory formation, and of the brain structures involved, will be of great clinical importance and may provide a means of alleviating certain forms of amnesia.

Memory is unlikely to be a 'monolithic entity' (Squire and Zola-Morgan, 1983). The notion that all mnemonic functions are carried out by a single global system has been superseded by the idea that multiple memory systems exist and that it might be possible to dissociate the component memory systems from one another. These multiple memory systems are unlikely to be confined to a single brain structure and, along similar lines, may well be subserved by more than one underlying physiological and molecular mechanism. One brain region which is known to be required for certain forms of learning and memory, and which has attracted particular interest, is the temporal lobe of the cerebral cortex.

The temporal lobe, so called because it lies beneath the temporal bone of the skull, was first implicated in learning and memory through the work of the Canadian neurosurgeon, Wilder Penfield (1958). Penfield was attempting to determine the precise location of epileptic foci in the cerebral cortex, in order to allow the selective excission of diseased tissue, when he stumbled upon a fascinating discovery. During his investigations, the cerebral hemispheres were exposed in fully conscious patients, under local anaesthetic, and mild electrical

stimulation applied to the surface of the cortex. Penfield noticed that stimulation of certain areas of cortex (motor cortex) resulted in brief, uncontrollable twitching of a particular part of the patients body. Moreover, stimulation at a different point in this area of cortex resulted in twitching of a different part of the body. Further investigation revealed that stimulation of other regions of the cortex caused the patient to experience specific auditory, visual or tactile sensations. Stimulation of the temporal lobe, however, often resulted in the patient experiencing vivid recollections of past events. This lead Penfield to suggest that the temporal lobe may be a store of long-term memory.

Further, more dramatic evidence in support of this hypothesis, came from the study of an amnesic patient, 'H.M.' H.M. had long suffered severe grand mal seizures as the result of intractable temporal lobe epilepsy. In an attempt to relieve him of these convulsions, surgeons bilaterally removed his temporal lobes. Although the operation cured his epilepsy, H.M. was left with severe amnesia (Scoville and Milner, 1957). The operation had induced a severe anterograde amnesia, extending across a wide range of stilmulus presentations. Although H.M. is able to retain events in short term memory for seconds, even minutes, these memories are soon lost. In addition, he displays a degree of retrograde amnesia, although he is able to remember the more remote events of his early life, up until a few years before the surgery. Despite the severity of the amnesia, H.M. still retains all his language skills, his sensory faculties remain intact and his level of intelligence (as measured by standard tests) is preserved. Indeed, H.M. demonstrates some learning capabilities on tasks that have subsequently been described as 'procedural' or 'non-declarative' tasks (Milner, 1962; Corkin, 1968; Squire, 1987). At the same time, however, he is unable to recall the actual learning event itself.

There have been numerous reports of other amnesic patients with similar memory impairments as a result of damage to the medial temporal lobe. One particular case of note is that of 'R.B.', whose amnesia was the result of brain damage caused by an ischemic episode. A *post mortem* examination of the brain of R.B. revealed that the damage was almost exclusively restricted to the CA1 subfields of the hippocampus, a structure located just beneath the cortical surface of the temporal lobe (Zola-Morgan, Squire and Amaral, 1986). This individual case study raises the possibility that it is the hippocampus, in particular, which is responsible for certain forms of learning and memory.

2.2 - The Hippocampus: Anatomy and Function

2.2.1 - The Anatomy of the Hippocampus

The hippocampus is probably the most widely studied of all brain areas. The term 'hippocampus' comes from the Greek word for a seahorse and was first used in the sixteenth century by Arantius (1587) in a rather dubious attempt to describe the shape of this structure. In the mammalian brain, the hippocampal formation forms part of the temporal lobe of the cerebral cortex, lying deep in the telencephalon (forebrain), below the corpus callosum and behind the septum. Removal of the overlying posterior and temporal neocortex reveals the two hippocampi which are joined together at their most anterior point, near the midline, by the hippocampal commissure. The hippocampus extends first in the caudal plane from behind the septum before bending latero-ventrally around the thalamus, forming a curved mass that sits on the floor of the lateral ventricles (Figure 2.1).

The hippocampal formation (the term 'hippocampus' is often loosely used when referring to the hippocampal formation as a whole) consists of the hippocampus proper (cornu ammonis, or Ammon's horn), the dentate gyrus (fascia dentata) and the adjoining subicular complex and entorhinal cortex (Figure 2.2). The highly ordered arrangement of the principal cell types in the hippocampal formation results in a beautifully formed structure with the hippocampus proper and the dentate gyrus appearing as two interlocking U-shaped sectors. The mammalian hippocampus is a relatively simple form of cortex (archicortex) consisting of one major cell type, the pyramidal cell, which are packed together in one layer of what is essentially a three-layered structure. This is in contrast to the more complex organisation seen in the multi-layered neocortex. The mammalian hippocampus is separated from neocortex by the subicular complex and the entorhinal cortex which are often referred to as 'transitional' cortex. The transition can be clearly seen by taking a horizontal section through the posterior arch of the hippocampus.

Regions and Subfields of the Hippocampus

The hippocampus proper is composed of two major regions, which are differentiated on the basis of cell morphology (Cajal, 1911; Blackstad, 1956). The *regio superior* borders onto the subiculum and is characterised by a double row of medium sized pyramidal cells. The apical dendrites of these pyramidal cells do not bifurcate for several hundred microns and, even then, give off only small side branches. The giant pyramidal cells of the *regio inferior* are arranged in a semi-circular fashion adjascent to the dentate gyrus and the apical dendrites of these cells divide shortly after leaving the soma.

In addition, the hippocampus can also be divided into four subfields, based on the arrangement of the pyramidal cells (Lorente de No, 1934). Lorente de No's CA1 subfield corresponds to the *regio superior* whereas the combined area represented by the CA2 and CA3 subfields comprises the *regio inferior*. The CA4 subfield describes a group of cells, scattered in the hilus of the dentate gyrus. Although these cells are not strictly aligned as in other subfields, they are, nevertheless, generally considered as part of the hippocampus proper, in that they do possess many of the characteristics of pyramidal cells. The existence of a CA2 subfield has been a matter of some debate (Lorente de No, 1934; Blackstad, 1956; 1963), although it is now generally agreed that this region represents a transition zone between CA1 and CA3 and is only really visible in higher species.

The Dentate Gyrus

The dentate gyrus (*fascia dentata*) is a 'tooth-shaped' structure (a literal translation) which adjoins the hippocampus proper (Figure 2.2). The 'suprapyramidal' or 'buried' blade of the dentate gyrus lies adjacent to the hippocampal fissure and above the pyramidal cells of the CA3 and CA4 subfields. The opposite portion of the dentate gyrus, the 'infrapyramidal' or 'exposed' blade, lies below the pyramidal cells. The major cell type in the dentate gyrus are the granule cells, which like the pyramidal cells in the hippocampus proper, are arranged in a highly ordered and densely packed formation, resulting in a similar three-layered structure. The cell body layer (or 'granular' layer) curves from the suprapyramidal blade around into the infrapyramidal blade, creating a U-shaped structure that interlocks with the hippocampus proper. The 'molecular' layer is composed of the apical dendrites of the granule cells and their afferent connections, including the terminals of perforant path fibres which provide the main input pathway into the hippocampus. Finally, there is the 'polymorph layer' which

contains a population non-granular cells, including the basket cell interneurones. The granule cell axons begin to gather together in the polymorph layer, forming the 'mossy fibre' tract which provides the major efferent projection from the dentate gyrus.

The Intrinsic Connectivity of the Hippocampal Formation

The cells of the hippocampal formation receive inputs from a variety of sources :- (i) intrinsic afferent inputs from cells within the same hippocampal subfield, (ii) intrinsic afferent inputs from different subfields but within the same hippocampus, (iii) commisural afferents from the contralateral hippocampus, and (iv) extrinsic afferents from other brain structures, outwith the hippocampus. The principal circuit for relaying information through the hippocampal formation has been characterised as a 'tri-synaptic loop'. This internal circuit is said to begin with the perforant path fibres which originate in the entorhinal cortex and synapse onto the granule cells in the dentate gyrus. The axons of the granule cells themselves form the mossy fibre tract which makes contact with the CA3 pyramidal cells. The axons of the CA3 pyramidal cells, in turn, form the Schaffer collaterals which synapse onto the CA1 pyramidal cells. These neurones then project to the subiculum. In addition to providing a major source of output from the hippocampus, efferent fibres from the subiculum also project back to the entorhinal cortex and thus complete the loop. The participating fibres of the tri-synaptic circuit are all orientated along the transverse axis and thought to be sequentially activated, providing a fast, excitatory, unidirectional flow of information.

The major input to the hippocampus is through the entorhinal cortex. The entorhinal cortex receives information gathered from all the sensory modalities. With the exception of olfactory information, which is relayed directly from olfactory primary cortex, this sensory information is relayed through primary and secondary areas of sensory cortex, then via association cortex before arriving in the entorhinal cortex. Therefore, the entorhinal cortex receives information which is already highly processed and combines inputs from all the sensory modalities. This information is then relayed to the hippocampus proper via the perforant path. The fibres of the perforant path leave the entorhinal cortex and converge to form a compact 'angular bundle' on entering the white matter of the hippocampus. The major projection of the perforant path fibres is onto the dendrites of the granule cells in the dentate gyrus. In addition, there are also a limited number of projections onto the dendrites of pyramidal cells in both the CA3 and CA1 subfields.

The dentate granule cells also receive inputs from the contralateral dentate gyrus through the commisural fibres, from the basket cell interneurones, and indirectly from neighbouring granule cells via mossy cells in the polymorphic layer. The thin unmyelinated axons of the granule cells unite in the polymorph layer of the dentate hilus to form the mossy fibre tract which provides the major output from the dentate gyrus. The mossy fibres make contact, through *en passant* synapses, with the dendrites of the CA3 pyramidal cells. The 'mossy' appearance is a result of the large synaptic connections along the length of these axon fibres.

The axons of the CA3 pyramidal cells divide and give rise to a number of projections. These include associational projections to neighbouring CA3 cells within the same hippocampus and commissural projections to both CA3 and CA1 pyramidal cells in the contralateral hippocampus. A proportion of the CA3 axon fibres leave the hippocampus through the fimbria and project to the lateral septum. There is also a major fibre output, the Schaffer collateral pathway, which projects to CA1 pyramidal cells within the same hippocampus. The axons of the CA1 pyramidal cells, in turn, project to the subicular complex, leaving the hippocampus through the alveus.

The Lamellar Hypothesis

For many years the fibre projections contributing to the tri-synaptic loop were thought to constitute the predominant feature of the internal circuitry of the hippocampus. In 1971, Andersen (Andersen et al., 1971) proposed that the hippocampus could be theoretically divided up into extremely thin transverse slices, or lamellae, each containing a single tri-synaptic circuit. Each slice was thought to function virtually independently from the next, with only occasional excitatory or inhibitory connections between slices. Many of these transverse slices were then stacked together along the longitudinal axis of the hippocampus, with the major activity being within slices rather than between. The evidence in favour of this 'lamellar hypothesis' was mainly derived from neuroanatomical degeneration studies (Blackstad, 1956, 1958; Raisman et al., 1965; Blackstad et al., 1970; Hjorth-Simonsen and Jeune, 1972) and from electrophysiological experiments (Andersen et al., 1971). With respect to the former, this involved making a discrete lesion in part of the hippocampus and comparing the extent of the degeneration along the transverse (orthogonal) and longitudinal (septotemporal) axes. The pattern of degeneration appeared to support a lamellar organisation, with greater damage along the transverse axis. Further evidence came from Andersen's own *in vivo* electrophysiological experiments in which he used either a single

stimulating electrode and recorded from multiple sites along both the longitudinal and transverse axes, or, conversely, recording from a single site but stimulating at several different positions. The greater spread of activity in the direction of the transverse axis seemed to be consistent with the lamellar hypothesis.

As neuroanatomical techniques have improved, however, considerable opposition has arisen to the idea of a strictly lamellar organisation within the hippocampus. The development of tracer molecules, such as *Phaseolus vulgaris leucoagglutin* (PHA-L; Amaral and Witter, 1989), has provided a more discrete means of labelling specific fibre tracts and has greatly facilitated the study of the intrinsic connections within the hippocampus. The work of Amaral and Witter has revealed that, with the exception of the mossy fibre tract which does in fact show a distinctly lamellar organisation, "the major hippocampal projections are as extensive and highly organised in the long or septotemporal axis of the hippocampus as in the transverse axis" (Amaral and Witter, 1989). On the basis of this key finding, they have called into question the generality of the lamella hypothesis.

Nevertheless, Andersen's hypothesis has had a great influence on hippocampal research, particularly with regard to the widespread use of the *in vitro* hippocampal slice technique. Much of the work reviewed later in this chapter has been conducted using the *in vitro* hippocampal slice technique. The fact that a single lamella should contain a complete, functional tri-synaptic circuit (Andersen et al., 1971) means that thin (typically 350-400μm thickness), transverse hippocampal slices maintained under appropriate conditions can reveal many aspects of hippocampal circuitry *in vitro* (Skrede and Westgaard, 1971; Spencer et al., 1976). Many of the properties of the hippocampus, including the induction and expression of LTP (Schwartzkroin and Wester, 1975), are maintained in this preparation which provides a convenient and accessible means of investigating the physiology of this structure.

Extrinsic Afferent Projections to the Hippocampus

The major source of input to the hippocampus is from the entorhinal cortex. In addition, however, the hippocampus also receives projections from several sub-cortical structures. These projections appear to have a general modulatory influence on hippocampal activity. For example, fibres from the raphe nucleus originate in the midbrain and project to the hippocampus via the fornix. These fibres provide a serotonergic input and account for most of the 5-HT (serotonin) present in the hippocampus. Similarly, the locus coeruleus provides

a noradrenergic input to the hippocampus. These fibres course through the median forebrain bundle and the septum before entering the hippocampus via the retrosplenial cortex. Cells from the nucleus of the diagonal band of Broca and from the medial septal nucleus also project to the hippocampus via the fornix and provide a cholinergic / GABA-ergic input. The influence of the medial septal input is of particular significance in that it is responsible for the rhythmical slow activity (the theta-rhythm) which can be seen during hippocampal electroencephalographic (EEG) recordings as a regular waveform with a frequency of 3-12 Hz, and is believed to exert an important influence on the activity of cells in the hippocampus (Green and Arduini, 1954; Winson, 1974).

Extrinsic Projections from the Hippocampus

The axon fibres of CA3 and CA1 pyramidal cells are responsible for all the extrinsic projections from the hippocampus. A large proportion of fibres leaving the CA1 region do so via the alveus which terminates in the subicular complex. The cells of the subicular complex then project back to both the entorhinal cortex and the association cortices, the brain areas that provided the major source of input to the hippocampus. These reciprocal projections with the cortical areas that are involved in the initial processing of sensory information may allow the hippocampus to exert some degree of feedback control on the input it receives.

Having accumulated sensory information from a variety of sources, and processed this information accordingly, the hippocampus must then be able to influence the behaviour of the animal. This requires an output system to those areas of the brain that drive behaviour. Efferent fibres, leaving the hippocampus through the fornix, project to several sub-cortical structures which are thought to subserve this function. These structures include the mammillary bodies of the hypothalamus, both the septal region and the anterior nucleus of the thalamus, and the nucleus accumbens.

Summary

In summary, the hippocampus receives already highly processed information, derived from all the sensory modalities, and through projections to the structures of the limbic system, hypothalamus, nucleus accumbens and striatum, can exert a major influence on the behaviour of the animal. More detailed reviews of the anatomy of the hippocampus can be

found elsewhere (O'Keefe and Nadel, 1978; Gray, 1982; Seifert, 1983; Amaral and Witter, 1989).

2.2.2 - The Function of the Hippocampus

Although there remains some controversy regarding the precise role of the hippocampus, there is general agreement that this brain area is involved, in some way or another, with the processes of learning and memory. A detailed examination of the anatomy of this structure, in relation to other brain areas, reveals that the hippocampus can receive and then integrate information from all the sensory modalities and, subsequently, influence the behaviour of the animal. Therefore, the extrinsic connections, both to and from the hippocampus, are ideally suited to a mnemonic function.

The study of human amnesics has demonstrated, however, that not all forms of learning require an intact hippocampus. This implies that the hippocampus is involved in only a limited subset of learning processes. A survey of what is a vast literature regarding the role of this brain area in learning and memory suggests that the hippocampus is involved in certain, more complex forms of learning. A distinction is often made between these complex forms of information processing, sometimes referred to as 'cognitive' processes, and apparently simpler forms of learning such as classical Pavlovian conditioning which are subserved by simple associative mechanisms and which do not require the hippocampus.

Theories of Hippocampal Function

There have been numerous attempts made to define more precisely the role of the hippocampus in learning and memory. In the main, theories of hippocampal function have been formulated on the basis of experimental data, derived from studying the performance of hippocampal lesioned animals in a variety of learning and memory tasks. By comparing what these animals can and cannot do, it is hoped that the function of the hippocampus may be inferred. One major avenue of approach has been to assign a specific function to the hippocampus on the basis of the specific type of information utilised, namely spatial or contextual information (O'Keefe and Nadel, 1978; Hirsh, 1974). An alternative view, pursued by many, has been to think of the hippocampus as providing, or providing part of, a memory system that processes information in a specific way, but independently of the type of information (eg. working memory - Olton et al., 1979; temporary/intermediate-term

memory - Rawlins, 1985; declarative memory - Squire, 1987; configural learning - Sutherland and Rudy, 1989). A comprehensive review of the numerous theories of hippocampal function would be an exhaustive task and is beyond the scope of this thesis. As the behavioural studies described in this thesis are primarily concerned with spatial learning, 'the spatial mapping theory', will be pursued in more detail later in this chapter.

The Hippocampal Lesion

The lesion technique constitutes the major experimental approach by which theories of hippocampal function may be tested. The study of the behavioural consequences of hippocampal lesions arose, at least in part, through attempts to provide animal models of the human amnesic syndrome (Gaffan 1974; Mishkin, 1978). The effects of hippocampal lesions have been assessed in a wide variety of behavioural paradigms, and by a large number of researchers, often yielding conflicting results. This is reflected in the variety of theoretical accounts that have been postulated. There are a number of possible factors which might have contributed to this diversity of opinion.

Although the lesion technique involves removal of a discrete region of brain tissue, the hippocampus is not an isolated structure and its removal may, therefore, have indirect effects on other brain structures. Consideration of the extensive network of projections both to and from the hippocampus can only serve to highlight this possibility. These indirect effects on other brain structures may themselves have behavioural manifestations which will complicate the interpretation of experimental results.

A further difficulty which complicates the interpretation of lesion studies is that animals may have more than one means of solving a particular task. The concept of multiple memory systems suggests that there may in fact be parallel routes for processing information. This redundancy in the memory system may allow lesioned animals to use an alternative mechanism to solve a particular problem.

The complex nature of the anatomy of the hippocampus also raises the possibility that removing this structure may disrupt more than one memory system. It is possible, for example, that the hippocampus does not have a single function, but rather contributes to several cognitive processes. Consequently, attempts to formulate a unitary hypothesis of hippocampal function may be wholly inappropriate. It is maybe more appropriate, therefore, to consider the effects of a hippocampal lesion as constituting a behavioural syndrome rather

than trying to attribute all the experimental findings to a single mnemonic operation performed by the hippocampus.

Perhaps the greatest source of confound, however, concerns the type of lesion performed. A survey of the literature reveals considerable variety, both in terms of the lesion techniques used (eg. aspiration, electrolytic, radiofrequency, neurotoxic) and the precise area targeted (eg. the entire hippocampal formation, the hippocampus proper, the dentate gyrus, specific hippocampal subfields - CA1, CA3, the subiculum, the entorhinal cotex, the fimbria-fornix). Even within the subset of neurotoxic lesion techniques, different neurotoxins have been used (eg. ibotenate, NMDA, kainic acid, colchicine). Differences in terms of the type of lesion approach adopted are almost certain to have contributed towards the complicated state of the literature and may go some way towards explaining why, on occasions, different labs have obtained apparently opposite results on essentially the same tasks. Consequently, this has resulted in a number of different theories of hippocampal function.

A particular problem is that conventional lesion techniques (eg. aspiration, electrolytic, radiofrequency) cause additional damage to fibres of passage, adjacent areas of cortex and the cerebral vasculature. This damage, outwith the hippocampus, may contribute towards the behavioural effects of the lesion. For example, conventional lesions of the hippocampus (which, in addition, also cause damage to the underlying cortex) result in an impairment in performance on the delayed non-matching to sample task in non-human primates (DNMTS; Zola-Morgan and Squire, 1990). More recent studies, however, have shown that an equivalent impairment can be produced by lesioning just the area of underlying cortex, adjacent to the hippocampal formation, namely the perirhinal cortex and parahippocampal gyrus (Zola-Morgan et al., 1989). Furthermore, it has recently been reported that selective lesions of the hippocampus, which minimise the extent of extra-hippocampal damage, do not impair performance on the DNMTS task (O'Boyle et al., 1993).

The use of more selective lesioning techniques, that limit the extent of the extra-hippocampal damage, has been pioneered by Jarrard. He has developed a lesion technique in which the neurotoxin, ibotenic acid (a broad spectrum excitatory amino acid agonist), is used to destroy selectively the cells of the hippocampus proper and, at the same time, cause minimal damage to other cortical structures and fibres of passage (Jarrard, 1989). Ibotenic acid is injected in small quantities (0.05 or 0.10 μl) at 26 separate sites within the hippocampus and destroys only those cells whose cell bodies are within the immediate vicinity of the injection sites. Careful histological analysis has confirmed that this procedure results in a reproducable,

selective lesion of the hippocampus proper (the CA subfields and the dentate gyrus only), with minimal damage to the overlying cortex through which access is obtained.

Where applied, the use of this more restrictive lesion technique has had a profound impact on the outcome of a number of experiments (Jarrard, 1993). The result has been a reduction in the number of tasks that are impaired following this more selective lesion, compared with the conventional approaches. For example, the phenomenon of latent inhibition which is disrupted by electrolytic lesions (Kaye and Pearce, 1987) is left intact after an ibotenate lesion (Honey and Good, 1993). Similarly, ibotenate hippocampal lesions in monkeys do not affect the DNMTS task (O'Boyle et al., 1993), in direct contrast to the effects of conventional lesion techniques (Zola-Morgan and Squire, 1990).

Although several theories of hippocampal function appear to have 'failed the ibotenic acid lesion test', the idea that the hippocampus is involved in processing spatial or contextual information has remained relatively intact (Morris et al., 1990b; Selden et al., 1991; Honey and Good, 1993). There is little doubt, if any, that hippocampal lesioned animals exhibit impaired performance in tasks that, at least at an intuitive level, contain a spatial component. There is disagreement, however, as to whether the hippocampus is preferentially involved in processing spatial information, or whether this is just a subset of the operations performed by this structure.

Spatial Learning

In 1978 O'Keefe and Nadel published 'The Hippocampus as a Cognitive Map', in which they outlined a theory that ascribed an exclusively spatial mapping function to the hippocampus (O'Keefe and Nadel, 1978; Nadel, 1991). The theory is based, in part, on the notion of "the special nature of spatial information (and) is, first and foremost, a theory about memory for spatial layouts and the ways in which animals use such a memory system for adaptive behaviour in the world." (Nadel, 1991).

O'Keefe and Nadel proposed that animals have at their disposal at least two learning systems which can guide behavioural responses. The first of these, the 'taxon' system, enables animals to respond on the basis of egocentric representations (eg. approach, avoid, turn left) and does not require the hippocampus. The behavioural response is associated with a specific cue or stimulus, is relatively inflexible and is subserved by simple associative mechanisms. The stimulus-response association is strengthened or weakened in a

progressive manner with repeated learning experiences, dependent on reward or punishment. In contrast, the 'locale' system provides a much more flexible form of learning and requires the formation of a 'cognitive map' to represent the environment. A non-egocentric, allocentric representation is formed on the basis of the unique arrangement of external cues which allows the animal to reach its goal from any starting location. 'Locale' system learning is thought to occur independently of reward and is extremely rapid. Indeed, it may be that the spatial component of tasks, such as the watermaze, is acquired within a single trial, although the overall rate of acquisition may be limited by the acquisition of other 'procedural' components of the task (eg. learning that there is a means of escape from the water via the platform in the watermaze).

Furthermore, O'Keefe and Nadel proposed that the functions of the 'locale' system are mediated by the hippocampus and that spatial representations of the environment are contained within groups of pyramidal cells. The main line of evidence in support of this theory came from hippocampal single unit recordings made in freely moving rats. O'Keefe and Dostrovsky (1971) observed that certain cells in the hippocampus fire selectively when the animal is at a particular spatial location in the environment. These 'place cells' were identified as pyramidal cells in both the CA1 and CA3 subfields, and have also since been found in the dentate gyrus and entorhinal cortex (O'Keefe, 1979). The correlation between place cell activity and the specific spatial location associated with it (the 'place field'), appears to remain stable for as long as recording from a single unit can be maintained (Thompson and Best, 1990).

In terms of the anatomy and physiology of the hippocampus, O'Keefe and Nadel (1978) proposed that the dentate gyrus receives a multimodal sensory input from the entorhinal cortex and acts as a coincidence detector to recognise associations between stimuli. This information is then used to form a 'map' which is laid down in the CA3 region. The role of CA1 pyramidal cells is to act as a 'mismatch mechanism' and compare the incoming sensory information with existing information contained in the map and update if necessary. The hippocampal interneurones, which O'Keefe called 'displace' cells, fire independently of spatial location (O'Keefe, 1979). These cells do, however, fire in phase with the theta activity as it spreads across the hippocampus and, consequently, are also known as 'theta cells' (Fox and Ranck, 1975; Kubie et al., 1990). Because (i) inhibitory interneurones modulate pyramidal cell excitability, and (ii) theta activity is, in some instances at least, related to movement (Morris and Hagan, 1983), it has been suggested that the theta input

provides information to the pyramidal cells in CA1 and CA3 about the movement of the animal through its environment, and informs the mapping system that the spatial location is changing (ie. to prevent the mismatch mechanism from updating the 'wrong map').

Of all the theoretical attempts to define, precisely, the function of the hippocampus, the 'spatial mapping theory' has arguably stood the test of time better than any. Nevertheless, a number of questions remain. For example, it is still not fully understood how a combination of hippocampal pyramidal cells interact to set up a spatial representation and, furthermore, how one spatial representation is linked to the next in terms of the anatomy of the hippocampus. It is also uncertain whether pyramidal cell activity is related solely to spatial inputs, or whether non-spatial stimulus-response relationships can also elicit changes in pyramidal cell firing. For example, increases in pyramidal cell activity have been observed during 'nictitating membrane response' (NMR) conditioning in the rabbit (Berger et al., 1976). Indeed, Thompson and Best (1990) have shown that cells, previously identified as place cells, also exhibited correlated changes in firing rates during simple conditioning involving tone-footshock pairings. Furthermore, the place cell-place field relationship remained intact when the animal was returned to the original environment. This result suggests that hippocampal pyramidal cells may serve different functions under different conditions and that these functions may not be exclusively spatial. Finally, the relationship between place cells and spatial learning is not clearly defined. Colchicine lesions of the dentate gyrus impair spatial learning in a variety of tasks but leave place cell activity in the CA1 and CA3 subfields unaltered (McNaughton et al., 1989). This implies that the place cell-place field relationships alone are not sufficient for spatial learning.

Despite certain questions remaining unanswered, there is considerable support for the idea that the hippocampus is involved in processing spatial information. Although there is some support for this hypothesis from other avenues of research (Krebs et al., 1989), the major source of evidence favouring a role for the hippocampus in 'place' learning has come from the study of hippocampal lesioned animals in behavioural tasks with a presumed 'spatial' component. As with several other theories of hippocampal function, however, the spatial mapping theory suffers from the somewhat circular nature of the argument. For example, the hypothesis that the hippocampus is specifically involved in 'declarative' memory (Squire, 1987) is based on experiments in which hippocampal lesioned animals are impaired, relative to controls, on a set of learning tasks that are thought to require declarative memory. The basis of grouping these tasks together, however, and for claiming that they require a

declarative memory system, is that they are all disrupted by hippocampal lesions! Similarly, a role for the hippocampus in spatial learning is implied as a result of experiments in which hippocampal lesioned animals are impaired on spatial tasks. Yet, apart from our own intuition, the only reason for presuming these tasks to be spatial, is that they are affected by hippocampal damage.

In the rat, the most common way to assess spatial function is to look at maze learning. Both appetitive (eg. the radial maze - Olton and Samuelson, 1976; Olton et al., 1979; Jarrard, 1983, 1986; Jarrard et al., 1987) and aversive (eg. the watermaze - Morris, 1981, 1984) paradigms have been used to assess spatial learning with the same result, namely that hippocampal lesioned animals generally show deficits on these tasks. The behavioural task that will be used throughout this thesis is the open-field watermaze.

The Watermaze

The open-field watermaze was designed specifically to study spatial learning in the rodent (Morris, 1981, 1984; Stewart and Morris, 1993). A detailed description of the methodology is provided in Chapter 3 (see also Fig 3.1). Briefly, in the standard spatial reference memory task, the rat is placed in a large tank of lukewarm water (2m diameter; 25 ± 1°C) which is located in the middle of the laboratory. The rat finds this mildly aversive and, in order to escape from the water, has to locate an escape platform which is hidden 1-2 cm below the water surface. To ensure that the rat cannot see the escape platform the water is made opaque by the addition of milk powder. The rat is able to find the platform, which remains in a fixed location, using distal extra-maze cues, located around the laboratory.

During acquisition, performance is assessed by measuring the escape latency onto the platform. An additional measure of performance can be obtained with a 'probe trial' or 'transfer test'. During this 'transfer test', the platform is removed from the pool and the rat allowed to swim freely for 60 seconds. The amount of time a rat spends searching in the quadrant of the pool in which the platform had previously been located (the 'training' quadrant) provides a good measure of the rats' memory of the platform position. However, there are problems with both measures of performance. With regard to escape latency, for example, on any one trial there is always the possibility that a rat, with little knowledge of the goal location, might bump into the platform by chance after only several seconds. On many occasions, both drug treated and lesioned animals (including hippocampal lesions)

show a tendency to swim in circles around the pool at a set distance from the side wall (Morris et al., 1990b). By circling at the correct distance from the side wall they will, inevitably, find the platform. This proves to be quite an effective strategy and after several days of training, hippocampal lesioned rats can be escaping from the pool almost, if not quite, as quickly as controls. When then given a transfer test, however, the hippocampal lesioned rats show little, if any, spatial bias towards the former 'training' quadrant. This suggests that they no have no spatial memory for the platform position, having only learned that it is located at a certain distance from the side wall. The results of the transfer test can also be misleading. The transfer test can be considered as an extinction trial in that the rat is not rewarded for going to the previously correct location. After a certain amount of time searching in the vicinity of the former platform location, the rat may well begin to search elsewhere for an alternative means of escape. This will result in an underestimate of the rat's knowledge of the platform location. The significance of this increases the more often the animal is given a transfer test and can result in rats spending progressively less time in the training quadrant with each transfer test. There is a possibility that animals in the experimental group (drug-treated or lesioned) may react differently from controls to the absence of the platform. This will complicate the interpretation of results. Consequently, both escape latency and transfer test performance will be used routinely in all experiments to assess the extent of learning and firm conclusions drawn only when the pattern in both points to a treatment-induced deficit.

The Effects of Hippocampal Lesions on Performance in the Watermaze

Lesioning the hippocampus results in a reliable and profound deficit on spatial learning tasks in the watermaze. The striking nature of the impairment, irrespective of the type of lesion used, supports a preferential role for the hippocampus in spatial learning.

The effect of hippocampal damage on acquisition of the standard spatial reference memory task in the watermaze was originally demonstrated using aspiration lesions (Morris et al., 1982). Subsequently, a variety of other lesion techniques have been used with the same result. These include electrolytic lesions (Sutherland et al., 1983) and several types of neurotoxic lesion, using either kainic acid, colchicine (Sutherland et al., 1983) or ibotenic acid (Morris et al., 1990b). The striking deficit following ibotenic acid lesions of the hippocampus proper, is in contrast to the effects of this lesion on performance in several other tasks, previously shown to be disrupted by conventional lesion techniques (Jarrard,

1993). Aside from the hippocampus proper, lesions of the fornix (Morris, 1983), entorhinal cortex (Schenk and Morris, 1985), subiculum (Morris et al., 1990b) and dentate gyrus (McNaughton et al., 1989) also result in profound spatial learning impaiments.

The evidence which has accumulated over the past ten years, suggests that the hippocampal lesion-induced deficit is due to a selective effect on spatial learning and cannot be accounted for by some trivial explanation. These animals show no signs of any sensorimotor impairment, nor does their motivation to escape from the water appear to differ from that of controls. This is illustrated by the fact that hippocampal lesioned rats learned to swim towards, and climb onto, a visible escape platform as quickly as control rats (Morris et al., 1982; Schenk and Morris, 1985; Sutherland and Rudy, 1988). If the non-visible platform was then re-introduced at the same spatial location, however, the performance of the hippocampal lesioned animals deteriorated immediately, in direct contrast to the performance of controls. The fact that hippocampal lesioned rats showed some improvement in escape latency across trials during the standard spatial reference memory task suggests that they did, in fact, learn something about the task (eg. that there is a means of escape, to avoid the side walls). Indeed, their performance was comparable to that of control animals for which the platform position was randomly moved from one trial to the next (Morris, 1981, 1984). Furthermore, hippocampal lesioned animals were, in fact, able to learn a non-spatial task in the watermaze (Morris et al., 1986b). The rats were trained to discriminate between two visible platforms, one of which was rigid and provided a means of escape; the other was floating and offered insufficient bouyancy to support a rat (Figure 3.2; see also Chapter 3). Morris and co-workers showed that rats with aspiration lesions of the hippocampus were able to solve a discrimination based on the distinguishable, visual appearance of the two platforms. In contrast, these rats were impaired when the two platforms were visibly identical and the correct platform determined solely by its spatial location. Hippocampal lesioned rats exhibit a deficit on a working memory version of the spatial learning task (the 'delayed matching to place' DMTS; Morris et al., 1990b) and are also impaired on the retention and re-learning of a pre-operatively acquired spatial location (Schenk and Morris, 1985; Morris et al., 1990b). The extent of the retrograde amnesia following damage to the hippocampus and other temporal lobe structures is a matter of some debate (Squire, 1992). The study of human amnesics with damage to the temporal lobe (including the hippocampus), often reveals a temporally graded retrograde amnesia in which recent memories are often more impaired than remote memories (Scoville and Milner, 1957; Corkin, 1984: Squire et al., 1989). On the basis of these findings, it has been suggested that

the hippocampus has only a temporary role in information processing and that the site of long term information storage is elsewhere. As yet, there is little evidence of any time-dependent sparing of spatial memory following hippocampal damage when assessed in the watermaze (Bolhuis et al., 1994). A temporally graded retrograde amnesia (with sparing of remote memory) following electrolytic hippocampal lesions has, however, been demonstrated in the rat using a 'contextual fear' paradigm (Kim and Fanselow, 1992).

The Watermaze as a Behavioural Assay of Hippocampal Function

It is widely recognised that the watermaze procedure has certain limitations, notably, with regard to studying the psychological processes that underlie spatial learning (Biegler and Morris, 1993; Morris, 1993). The inability to control extramaze cues results in a situation where we have little indication of how these cues are used, or indeed, what specific cues are important. This should not detract, however, from the usefulness of the watermaze as a 'behavioural assay' of hippocampal function (or dysfunction). The central aim of this thesis is to investigate the possible involvement of 'LTP-like' processes in hippocampal-dependent forms of learning. To that end, all that is required is a task that is sensitive to hippocampal dysfunction. In this respect the watermaze is ideal. The standard spatial reference memory task is rapidly acquired, easy to run and highly sensitive to hippocampal damage. A precise description of the type(s) of learning mediated by the hippocampus becomes less important when the aim of the study is to determine the importance, if any, of synaptic plasticity to these forms of learning. Spatial learning in the watermaze is a task that requires the integrity of the hippocampus. LTP is a form of synaptic plasticity which can be reliably demonstrated in the hippocampus and which may bear a resemblance to endogenous mechanisms of learning and memory formation. The LTP/learning hypothesis can therefore be tested by assessing whether or not drugs which limit the induction of LTP also affect spatial learning in the watermaze.

There is, however, one major proviso. The experiments described in this thesis involve the use of drugs to manipulate the inducibility of LTP. The effects of these drugs on spatial learning are assessed in the watermaze. The effects of any drug on learning are only meaningful if, at the same time, one can demonstrate some degree of selectivity. A generalised impairment in performance across a wide range of tasks is likely to be of little interest and may well be the result of an action of the drug on some process other than the learning mechanism *per se*. For example, a drug-induced impairment of performance in the

watermaze might be due to an effect on any one of a number of behavioural processes (eg. altered sensorimotor capabilities, altered motivational state or a direct effect on the learning mechanism). It is important to remember that although lesioning the hippocampus results in, what appears to be, a specific impairment of spatial learning in the watermaze, spatial learning in the watermaze is not a 'hippocampus-specific' task. That is to say, disruption in any one of a number of other brain structures can also result in a deficit in performance. As Morris points out, "this lack of specificity should come as no surprise, bearing in mind that navigating to a learned but hidden location involves sensory processing of extramaze distal cues, learning to represent its location relative to these distal cues, the formation and consolidation of long term memory, and motor guidance through space to the remembered location. The procedural simplicity of the task belies its underlying functional complexity" (Morris, 1993). The fact that brain structures, other than the hippocampus, also contribute during spatial learning, either directly at the level of information processing and memory formation, or indirectly through sensorimotor or motivational processes, is of great importance when assessing the selectivity of drug-induced effects on learning.

In order to prove, or at least attempt to prove, that an impairment is due to a selective action of the drug on the learning mechanism, it is essential to demonstrate that the drug is without effect on a 'control' task in which the 'procedural' demands are similar to those of the main study (eg. equate the sensorimotor and motivational demands). For example, hippocampal lesioned animals are impaired on a spatial discrimination task in the watermaze but are unimpaired on a visual discrimination task using the same apparatus (Morris et al., 1986b). Such a 'dissociation' gives strong grounds for the claim that the effects of hippocampal damage are specific to spatial learning. Choosing the correct control task, or set of control tasks, requires some intuition as to the nature of the psychological processes and memory systems that may be called upon during the various behavioural tasks. The existence of multiple memory systems within the brain provides the basis for such 'dissociations' to be made between different forms of learning. The choice and use of control tasks in studying the effects of drugs on learning is of extreme importance.

Summary

It would appear that the hippocampus is required for certain forms of learning but not others. A precise description of hippocampal function, however, has proved elusive and is still a matter of considerable debate. The striking nature of the deficit in spatial learning tasks,

even when the most selective lesion techniques are used (Morris et al.,1990b), has led to the suggestion that the hippocampus is preferentially involved in spatial and contextual forms of learning. The spatial mapping theory (O'Keefe and Nadel, 1978), the contextual retrieval theory (Hirsh, 1974), the complex scene, or 'snapshot' theory (Gaffan and Harrison, 1989) and the configural theory (Sutherland and Rudy, 1989) all appear to have certain, intuitive, similarities. They share the common idea that the hippocampus is required for the formation of a complex representation in which the relationship between a number of cues in the environment must be established.

The lack of a concise psychological account of hippocampal function does not, however, preclude us from studying the molecular and cellular mechanisms that might underlie these memory processes. Having established that the hippocampus is required for spatial learning, and that the watermaze provides us with sensitive and convenient 'behavioural assay' of hippocampal function, we are now in a position to explore the possibility that synaptic plasticity, and in particular LTP, might bear some resemblance to the cellular mechanisms of learning in this brain structure.

2.3 - Long-Term Potentiation: A Model of Synaptic Plasticity

2.3.1 - Synaptic Plasticity as a Mechanism of Information Storage

'Plasticity' describes the process whereby the synaptic connections between neurones are either strengthened or weakened. Although the idea that memories may be stored at the connections between neurones in the brain was first alluded to by Cajal in the nineteenth century, it was not until the publication of Donald Hebb's 'The Organisation of Behaviour' (Hebb, 1949) that the putative role of synaptic plasticity, as a mechanism for information storage, was formally introduced. Hebb proposed that changes in the strength of synaptic connections between cells might occur as a consequence of coincident activity. "When an axon of cell A is near enough to excite a cell B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells such that A's efficiency as one of the cells firing B, is increased." (Hebb, 1949). The suggestion is that memories are stored as increases in synaptic efficacy following the simultaneous activation of two connected neurones. Hebb argued that representations of stimuli were contained within 'cell assemblies' in the brain. These 'cell assemblies' consist of groups of interconnected neurones, each exciting the next and thus forming a loop around which nerve impulses reverberate. If, for example, cell A is part of the cell assembly representing a tone and cell B is one of a group of neurones whose combined activity represents food, then the strengthening of the synaptic connection between A and B increases the probability that presentation of the tone will elicit the representation of food. Repeated tone-food pairings will incrementally strengthen the synaptic connection until presentation of the tone is sufficient, in itself, to activate the food representation and elicit the appropriate 'conditioned response', even in the absence of the food. This, of course, describes a potential series of events underlying Pavlovian conditioning.

Neural network models of cognitive processing suggest that the hippocampus possesses the required internal circuitry to store large quantities of information. Hebb's 'cell assemblies' have been expanded to encompass an entire distributive network, allowing numerous representations to be stored as a distributed pattern of synaptic weights across a set of neuronal elements, connected in parallel (McNaughton and Morris, 1987). Implicit in such models of information storage, is the ability of hippocampal synapses to alter their synaptic weights.

Changes in the strengths of synaptic connections as a result of learning episodes have been successfully demonstrated in simple invertebrate systems such as *Aplysia*. Learning processes such as habituation, sensitisation and classical conditioning are accompanied by changes in the size of synaptic responses, the amount of chemical neurotransmitter released, and the morphology of the participating synapses (Kandel et al., 1983; Kandel, 1991). In the mammalian CNS, however, things are, of course, a great deal more complicated. Although, as we shall see, demonstrations of synaptic plasticity in the hippocampus are commonplace, it is much more difficult to show that (i) the plasticity induced resembles a natural, on-going process, and (ii) this process may subserve learning. By far the most widely studied example of synaptic plasticity in the mammalian CNS is long-term potentiation (LTP).

Long-Term Potentiation

Long term potentiation, or long term enhancement (LTP, LTE), is an experimental phenomenon which provides a model of activity-dependent synaptic plasticity in the mamalian CNS. Although the initial observations were made as early as 1966 (Lømo, 1966), the phenomenon of LTP was first described in detail in 1973 by Tim Bliss and Terje Lømo (Bliss and Lømo, 1973). Using anaesthetised rabbits, Bliss and Lømo electrically stimulated the fibres of the perforant path and recorded evoked, extracellular, field potentials in the granule cell layer of the dentate gyrus (Figure 2.3). Following a baseline period, during which single pulses were given at low frequency (once every 2-3 seconds), a high frequency conditioning train or 'tetanus' was delivered to the perforant path (eg. 15 Hz stimulation for 10 seconds). Subsequently, when low frequency stimulation was resumed, the evoked response was now substantially larger for any given stimulus size (Figure 2.3). Bliss and Lømo observed increases in the amplitude of the field excitatory post-synaptic potential (epsp) and, in both the amplitude and latency of the population spike, suggesting an increase in both the amount of granule cell depolaristion resulting from a given stimulus and in granule cell excitability. This increase was immediate and lasted for up to ten hours in this preparation. In a parallel study, recording from chronically prepared unanaesthetised rabbits, the potentiation elicited remained above baseline levels for up to 16 weeks after the delivery of the conditioning trains (Bliss and Gardner-Medwin, 1973). More recent studies have confirmed the longevity of this effect (Barnes, 1979; Racine et al., 1983; Staubli and Lynch, 1987).

Since these initial reports in 1973, LTP has been intensely studied in an attempt to determine

not only the mechanisms underlying both its induction and maintenance, but also to ascertain whether or not this phenomenon is of any physiological significance. Following the initial discovery at perforant path-granule cell synapses, LTP has been demonstrated at numerous other synapses both within the hippocampal formation and in a number of other brain areas (see Table 2.1). While *in vivo* experiments such as those described by Bliss and Lømo are still of considerable importance, much of what is now known about LTP has been discovered using *in vitro* methods, in particular the hippocampal slice preparation. The hippocampal slice provides a convenient and accessable means of studying LTP and it would appear that the properties of LTP are conserved in this preparation. The properties of LTP and their relevance to any putative learning mechanism will be discussed. Before continuing, however, it is worthwhile determining precisely what is 'long-term potentiation' and defining exactly *what* is to be studied in the ensuing experiments.

First and foremost, LTP is an experimental phenomenon. The relationship between LTP, which is induced experimentally in the laboratory using electrical stimulation, and any potential change in synaptic efficacy that might occur naturally during learning (or any other endogenous on-going process for that matter), is far from clear. It is important to stress that the hypothesis is not that LTP *is* the physiological basis of learning, but rather that the mechanisms involved in LTP may share common features with the mechanisms responsible for learning. The aim of this thesis is to test the latter hypothesis.

Second, when a high frequency tetanus is delivered, the resulting potentiation consists of several distinct temporal components. These are post-tetanic potentiation (PTP) which lasts for seconds or, at most, a couple of minutes, short-term potentiation (STP) which lasts for minutes, and LTP. By convention, potentiation that remains stable and above baseline levels for at least one hour after induction can, legitimately, be considered as LTP (Bliss and Collingridge, 1993).

Third, LTP cannot be considered as a homogenous entity. This thesis is concerned, specifically, with N-Methyl-D-Aspartate (NMDA) receptor-dependent LTP. The induction of this particular form of potentiation is blocked by NMDA receptor antagonists (Collingridge et al., 1983; Harris et al., 1984; Morris et al., 1986a). Other forms of long term potentiation that do not require the NMDA receptor have also been reported (Harris and Cotman, 1986; Grover and Teyler, 1990; Aniksztejn and Ben-Ari, 1991; Bortolotto and Collingridge, 1993). The relevance of these forms of potentiation to learning, although undoubtedly of some importance, is beyond the scope of this investigation.

Fourth, this thesis is concerned particularly with potentiation of the synaptic component of the evoked response. Increases in synaptic efficacy in the dentate gyrus occur independently of the size of the population spike, a measure of granule cell excitability at the cell body (McNaughton et al., 1978). Accordingly, the initial slope of the field epsp will be reported in all experiments as this provides the purest measure of the synaptic component of the evoked response.

In summary, for the purposes of this thesis, LTP is defined as a NMDA receptor-dependent increase in the synaptic component of the evoked response, which remains stable and potentiated for at least an hour.

Properties of NMDA Receptor-Dependent LTP

NMDA receptor-dependent LTP has attracted such considerable attention because it appears to resemble the type of plasticity envisaged by Hebb to provide the physiological basis for information storage. Intuitively, there are certain similarities between NMDA receptor-dependent LTP and learning. For example, potentiation results from brief activity and, is both rapid in onset and long-lasting (Bliss and Gardner-Medwin, 1973; Barnes, 1979; Racine et al., 1983; Staubli and Lynch, 1987). The fact that LTP has been demonstrated in a number of brain areas, including the hippocampus, which are known to be involved in certain types of learning and memory is also consistent with the hypothesis that these mechanisms may contribute to learning. Furthermore, although LTP is normally elicited experimentally using a high frequency tetanus (which is often well beyond the normal physiological firing range of hippocampal neurones), it can also be successfully induced using stimulation parameters that are 'physiologically reasonable' and which might be expected to occur naturally within the CNS. For example, Rose and Dunwiddie (1986) have shown that LTP can be induced at Schaffer-collateral-CA1 synapses using 'primed burst' stimulation which mimics the complex spiking of CA3 pyramidal cells. Along similar lines, Lynch and colleagues have shown that patterns of stimulation delivered at a frequency within the range of the theta activity are particularly effective in inducing LTP (Larson et al., 1986; see also Pavlides et al., 1988). The potential importance of the theta rhythm with regard to hippocampal-dependent learning has already been discussed (Winson, 1978).

It is the properties of associativity, cooperativity and input-specificity, however, that have proved particularly attractive with regards to a putative learning mechanism. The properties

of associativity and cooperativity are a consequence of the intensity threshold for LTP induction. 'Associativity' describes how a weak, sub-threshold input will potentiate if it is paired with, and is therefore active at the same time as, a strong suprathreshold tetanus given to a separate but convergent input (McNaughton et al., 1978; Levy and Steward, 1979). Closely related to this is the idea of 'cooperativity' (McNaughton et al., 1978; Larson and Lynch, 1986). Consider a neuronal pathway consisting of a large number of afferent fibres projecting onto a post-synapatic target cell. Weak stimulation of this fibre tract activates relatively few input fibres and fails to reach the threshold for LTP induction. Stronger stimulation, however, which simultaneously activates numerous input fibres, will provide sufficient activation to exceed the threshold and result in LTP at all the activated synapses. This effect is known as 'cooperativity' and describes a mutual facilitation between different neurones within the same input pathway, whereas 'associativity' describes a mutual facilitation between independent but convergent inputs. Potentiation occurs only at those synapses that were active during stimulation and is therefore also described as 'input specific' (Andersen et al., 1977; Lynch et al., 1977; Larson and Lynch, 1986; Gustaffson et al., 1987). It is the properties of associativity, cooperativity and input-specificity which are indicative of a Hebbian synapse and provide the basis for an analogy between LTP and classical conditioning.

2.3.2 - The Induction of LTP

Many of the properties of LTP described so far are a consequence of the unique properties of a particular class of excitatory amino acid (EAA) receptor, the NMDA receptor. NMDA receptors appear to make only a limited contribution to synaptic transmission in the hippocampus under normal conditions. Consequently, NMDA receptor antagonists, such as D-AP5, have little effect on responses evoked by low frequency stimulation. In contrast, NMDA receptor antagonists prevent the induction of LTP, suggesting a crucial role for these receptors in altering the level of synaptic transmission (Collingridge et al., 1983; Harris et al., 1984).

Excitatory Amino Acid Neurotransmission in the CNS

Amino acids such as L-glutamate and L-aspartate are thought to provide the major source of excitatory neurotransmission in the mamalian CNS (Hayashi, 1956; Watkins and Evans,

1981). The actions of EAAs, such as L-glutamate, are mediated by at least five EAA receptor subtypes, exhibiting distinct electrophysiological, biochemical and pharmacological properties, and each with a unique anatomical distribution within the CNS (see Watkins et al., 1990). Normal fast excitatory synaptic transmission in the mammalian brain is primarily mediated by the AMPA receptor subtype (formerly the 'ionotropic' quisqualate receptor). Activation of AMPA receptors opens ion channels which are permeable to both sodium and potassium ions. Under normal conditions this results in depolarisation of the cell. AMPA receptors can be blocked pharmacologically with 'broad spectrum EAA receptor antagonists' such as kynurenate (Perkins and Stone, 1982: Ganong et al., 1983), γ-D-glutamylglycine (γ-DGG; Collingridge et al., 1984) and the quineloxidiones, 6-cyano-7-nitro-quinoxaline-2,3-dion (CNQX) and 6,7-dinitro-quinoxaline-2,3-dion (DNQX; Drejer and Honoré, 1988). A more specific block of AMPA receptors can be achieved with another quineloxidione, NBQX (Sheardown, et al., 1990). Co-localised with the AMPA receptors, on the same synaptic terminals throughout the CNS, is a second population of EAA receptors, the NMDA receptors (Figure 2.4).

The NMDA Receptor

The NMDA receptor is the most intensely studied of all the EAA receptor subtypes. The unique physiological and pharmacological properties of this receptor have generated great interest in both scientists and clinicians (Watkins and Collingridge, 1989,1994). The NMDA receptor is a complex molecular entity comprising several ligand binding sites, surrounding a high conductance, voltage-gated ion channel (Figure 2.5). Under appropriate conditions, activation of the ion channel results in a composite inward sodium/calcium current and an outward potassium current. The influx of sodium and calcium ions results in a depolarisation and also provides a molecular signal to initiate changes in cell activity. The NMDA receptor ionophore is both ligand- and voltage-gated. It is this unique, dual requirement which accounts for many of the fascinating properties of this receptor.

A Dual Requirement for Activation of the NMDA Receptor Ionophore

At normal resting membrane potentials (-65 mV) binding of glutamate to the NMDA receptor is not sufficient, on its own, to allow a current to flow through the ion channel. Conductance also requires that the cell is depolarised. This voltage dependence is due to the

blockade of the ion channel by magnesium ions (Mg^{2+}; Nowak et al., 1984; Mayer et al., 1984; Figure 2.5). The channel is blocked by concentrations of Mg^{2+} ions well below the physiological levels found in the extracellular fluid of the CNS (approx. 1mM; Ault et al., 1980; Scatton and Lehmann, 1982). At the resting membrane potential (or at hyperpolarising potentials), a Mg^{2+} ion sits in the channel and prevents other cations (Ca^{2+}, Na^{+} and K^{+}) from entering or leaving the cell. As the cell is depolarised, the relative build up of positive charge within the cell repels the Mg^{2+} ion out of the channel, thereby allowing current to flow. A membrane depolarisation of 20-30 mV is sufficient to allow Ca^{2+} and Na^{+} ions to enter the cell provided, of course, that glutamate is also present. Construction of a current-voltage (I-V) relationship for the NMDA receptor ionophore shows a region of 'negative slope conductance' at more negative membrane potentials (Figure 2.6; Nowak et al., 1984). As the cell is hyperpolarised from -40 to -80 mV, the conductance of the ion channel decreases as the Mg^{2+} blockade increases. This is in contrast to non-voltage gated channels which show a near-linear increase in conductance as the cell is hyperpolarised and the driving force acting on the ions increases. Experiments performed in a Mg^{2+} -free medium have revealed an I-V plot which is almost linear between -60 and +60 mV, confirming that in the absence of Mg^{2+} there is no voltage gating of the channel (Nowak et al., 1984).

The dual requirement for both glutamate to bind to the NMDA receptor and sufficient depolarisation to overcome the Mg^{2+} block explains the need for both pre-synaptic activity and a threshold level of post-synaptic depolarisation in order to induce LTP. These properties of the NMDA receptor account for the associativity, cooperativity and input-specificity of LTP. The NMDA receptor acts as a molecular coincidence detector which is sensitive to simultaneous pre- and post-synaptic activity.

The NMDA Receptor Current

The NMDA receptor ionophore is permeable to Na^{+}, K^{+} and Ca^{2+} ions. Activation of the NMDA receptor ion channel results in an influx of Ca^{2+} and Na^{+} ions into the cell. This influx of positive charge results in the depolarisation of the cell which, in turn, causes a further reduction in the voltage-dependent Mg^{2+} block. Consequently, more Ca^{2+} and Na^{+} ions can enter the cell, resulting in a regenerative depolarisation. The influx of calcium, which can be demonstrated experimentally using calcium-sensitive dyes (MacDermott et al., 1986), provides a molecular trigger which can initiate the changes in cell activity required to

increase synaptic efficacy. Intracellular calcium regulates many, if not all, cellular processes and is a key factor in determining the level of cell activity. The role of calcium in the induction of LTP is discussed below.

Multiple Ligand Binding Sites Associated with the NMDA Receptor

The NMDA receptor complex is a large protein with several distinct receptor binding sites (Figure 2.5). The binding of a ligand to a specific receptor site on this large protein complex can, through allosteric interactions, produce changes in the functional domain of the molecule, in this case the ion channel, and also alter the properties of other binding sites on the protein. A variety of ligands are capable of binding to the NMDA receptor complex and altering the activity of the ion channel. An analogy is often made with the inhibitory amino acid $GABA_A$ receptor complex which comprises an ion channel, permeable to chloride ions and blocked by picrotoxin or penicillin, surrounded by binding sites for GABA, barbiturates and benzodiazepines (Olsen, 1981). As a consequence of the multiple binding sites on the NMDA receptor complex, the activity of the ion channel may be modulated by a number of different ligands and the receptor may, therefore, provide a potential site of interaction for a number of inputs.

Agonists such as L-glutamate and NMDA bind to a receptor site on the extracellular surface of the cell membrane to activate the ion channel (Figure 2.5). The competitive antagonists D-2-amino-5-phosphonopentanoic acid (D-AP5), D-2-amino-7-phosphonopentanoic acid (D-AP7) and 3-3 (2-carboxypiperazine-4-yl-propyl-1-phosphonic acid) (CPP) also bind to this site and vie with the agonist for occupancy of the receptor.

Activation of the ion channel can also be prevented, non-competitively, by a group of compounds known collectively as the 'dissociative anaesthetics' (the term 'dissociative' refers to the lack of a correlation between the effects of these drugs on cortical and limbic EEG patterns). These include phencyclidine (PCP) and ketamine, both of which have been used clinically as general anaesthetics at one time or another. The effect of these drugs on NMDA receptor-mediated activity was first demonstrated by Lodge and Anis (1982). Subsequently, it has been shown that the dissociative anaesthetics bind to a site located deep in the ion channel (the 'PCP' site) which is distinct from the Mg^{2+} binding site (Kemp et al., 1987a; Lodge et al., 1988; Figure 2.5). Furthermore, blockade is 'activity-' or 'use- dependent' in that the agonist must be present and the membrane sufficiently depolarised in order to open

the ion channel and allow the channel blockers access to the PCP binding site (Wong et al., 1986; Loo et al., 1986; Kemp et al., 1986; Church et al., 1987; MacDonald et al., 1987; Fagg and Baud, 1988; Kloog et al., 1988). The most potent and selective of the dissociative anaesthetics, and consequently the most widely used as an experimental tool, is dizoclipine (MK-801; Wong et al., 1986). PCP, itself (also known as 'Angel Dust'), is a common drug of abuse with rather disturbing psychomimetic side effects and, consequently, is no longer in clinical use. The dissociative anaesthetics are not particularly selective for the NMDA receptor and display a wide range of other actions, with effects on a number of other neurotransmitter systems in the CNS (Lodge and Johnson, 1990). It is unclear to what extent the NMDA and non-NMDA mediated components contribute to the psychomimetic effects of these compounds.

NMDA receptor activity is enhanced by glycine, or a glycine-like compound through a distinct binding site on the NMDA receptor complex (Johnson and Ascher, 1987; Figure 2.5). This NMDA receptor-associated glycine site is distinct from the strychnine-sensitive glycine receptor which mediates inhibitory neurotransmission in the brain stem and spinal chord (Curtis et al., 1968; De Feudis et al., 1978; Snyder et al., 1976). Because experimental manipulations of this receptor constitute a major part of this thesis, the properties of the glycine site and its relationship to NMDA receptor activity will be discussed in greater detail in Chapter 4.

There is now a growing list of compounds which have been shown to bind to the NMDA receptor complex and influence the activity of the ion channel. For example, Zn^{2+} ions produce a voltage-independent block of NMDA receptor currents through an extracellular receptor site which is distinct from both the PCP and Mg^{2+} binding sites (Westbrook and Mayer, 1987; Figure 2.5). This effect is mimicked by high concentrations of tricyclic antidepressant drugs (Reynolds and Miller, 1988). Recently, considerable evidence has accumulated for a 'redox' site on the NMDA receptor complex. The NMDA response is substantially potentiated following exposure to reducing agents and, conversely, is reduced in the presence of oxidising agents (Lazarewicz et al., 1989; Aizenman et al., 1989; Aizenman et al., 1990). There is also support for a polyamine modulatory site (Ransom and Deschenes, 1990; Williams et al., 1991; Lerma, 1992) through which the cationic polyamines, spermine and spermidine, can enhance the binding of both glutamate and MK-801 (Steele et al., 1990; Pullan and Powel, 1991). As yet, it is not known whether there is an absolute requirement for polyamines (Reynolds and Miller, 1990). Similarly, there is

uncertainty as to the precise location of the polyamine binding site (Sprosen et Woodruff, 1990; Williams et al., 1991).

In summary, the NMDA receptor is a complex, highly regulated molecule. Further work is required to fully characterise the numerous recognition sites and to understand the way in which these sites interact to influence the activity of the ion channel.

Anatomical Distribution of NMDA receptors in the CNS

Quantitative autoradiography has been successfully used to determine the distribution of NMDA receptors in the CNS (see Young and Fagg, 1990). By looking at NMDA-displacable [^{3}H]-L-glutamate binding, Monaghan and Cotman (1985) were able to provide a detailed discription of the NMDA receptor distribution within the brain. In brief, NMDA receptors are found in high density in areas of the forebrain, in particular layers I to III of the frontal cortex, and in the hippocampus. In addition, the parietal, pyriform and cingulate cortices also show high NMDA receptor densities. Within the hippocampus, the CA1 subfield has the highest density of NMDA receptors found anywhere in the brain. There are also considerable numbers of NMDA receptors in both CA3 and the dentate gyrus, although NMDA binding sites are conspicuous by their absence in the terminal fields of the mossy fibre pathway. Outside the hippocampus, there are relatively high numbers of NMDA receptors in the amygdala, the nucleus accumbens, the striatum and the lateral septum. Moderate binding levels are found in the thalamus and hypothalamus. Generally speaking, the lowest densities are found in the midbrain and brainstem. Similar, but not identical, binding distributions have been observed using radiolabelled competitive NMDA antagonists, [^{3}H]-D-AP5 and [^{3}H]-CPP (Monaghan et al., 1984a, 1988). This small discrepancy has led to the suggestion that there may exist agonist- and antagonist- preferring forms of the NMDA receptor with different anatomical distributions in the brain. The fact that NMDA receptors are found in large numbers in several forebrain structures, is consistent with the hypothesis that these receptors may be important for certain forms of learning. These brain areas are also particularly prone to neurodegeneration and cell loss which supports the idea that NMDA receptors are involved in mechanisms of neuronal injury and cell death (Simon et al., 1984; Meldrum, 1985; Wieloch, 1985; Schwarz and Meldrum, 1985). Finally, it is worth pointing out that there is an extensive similarity between the anatomical distributions of NMDA and AMPA receptors (Monaghan et al.,1984b, 1989; Nielsen et al., 1988). The importance of this co-localisation will become apparent when

considering the way in which EAA receptor subtypes interact to bring about the induction of LTP.

NMDA Receptors are Required for LTP Induction

The first evidence that NMDA receptors are required for the induction of LTP was provided by Collingridge and co-workers using the *in vitro* hippocampal slice preparation (Collingridge et al., 1983). They showed that iontophoresis of AP5 onto CA1 pyramidal cells had no effect on the size of the field epsp evoked by normal low frequency stimulation of the Schaffer collateral pathway but did prevent the induction of LTP following a high frequency tetanus. Subsequent studies showed that this effect was stereoselective, dose-dependent (Harris et al., 1984) and reversible on washout of the drug (Collingridge et al., 1983). In addition, AP5 applied after the tetanus, had no effect on the size of the potentiated response (Davies and Collingridge, 1989). Similar results have also been obtained *in vivo*. The infusion of AP5 either directly into the dentate gyrus or into the lateral ventricle blocks the induction of LTP at perforant path - granule cell synapses in the anaesthetised rat (Morris et al., 1986a; Errington et al., 1987; Morris, 1989). A number of studies using other competitive and non-competitive NMDA antagonists (eg. CPP, MK-801, PCP and ketamine) have confirmed that NMDA receptors are required for the induction of LTP but make little, if any, contribution to responses evoked by normal low frequency stimulation, either before or after the tetanus (Stringer and Guyenet, 1983; Stringer et al., 1984; Abraham and Mason, 1988; Swartzwelder et al., 1989). The lack of an effect on the potentiated response implies that NMDA receptors are not required for the maintenance of LTP.

The putative role for NMDA receptors in the induction of LTP has also been tested by iontophoresing large, depolarising doses of NMDA or glutamate onto hippocampal slices (Collingridge et al., 1983; Kauer et al., 1988a). As expected, this caused a potentiation of the evoked response but the increase was not maintained and the result is only short-term potentiation (STP). This implies that activation of the NMDA receptor alone, may be insufficient to induce LTP and that other additional mechanisms may be required to convert STP into LTP (see Bortolotto and Collingridge, 1993).

The Mechanism of LTP Induction

The induction of LTP requires activation of the NMDA receptor ionophore, a voltage-gated,

ligand-gated ion channel. Consequently, the induction of LTP requires sufficient post-synaptic depolarisation to remove the Mg^{2+} block and allow Na^+ and Ca^{2+} ions to enter the cell. Evidence that post-synaptic depolarisation is required for the induction of LTP has been obtained from a number of experiments. For example, Wigström and Gustafsson (1983) showed that bathing hippocampal slices in a medium containing the $GABA_A$ antagonist, picrotoxin, facilitated the induction of LTP. Picrotoxin blocks inhibitory currents, allowing greater depolarisation of the post-synaptic cell during the tetanus, which facilitates the induction of LTP. Conversely, hyperpolarisation of the post-synaptic cell during tetanic stimulation has been shown to prevent the induction of LTP in hippocampal slices (Malinow and Miller, 1986). Finally, low frequency sub-threshold stimulation can produce LTP if paired with depolarisation of the post-synaptic cell (Wigström et al., 1986; Gustafsson et al., 1987). The depolarisation removes the Mg^{2+} block and allows Na^+ and Ca^{2+} ions to enter the cell. Altering the ratio of Mg^{2+} and Ca^{2+} ions also affects the inducibility of LTP, with low Mg^{2+} and high Ca^{2+} concentrations favouring induction (Dunwiddie and Lynch, 1979; Huang et al., 1988). The dual voltage and ligand gating of NMDA receptor activation accounts for the 'associativity' and 'cooperativity' of LTP. Mutual facilitation, either between different neurones within the same input pathway, or between independent but convergent inputs, provides sufficient depolarisation to overcome the Mg^{2+} block. The ligand-gated nature of NMDA receptor activation accounts for the 'input specificty' in that pre-synaptic activity is required to release glutamate which can then bind to the agonist recognition site.

The fact that AP5 has little, if any, effect on field epsps evoked by normal low frequency stimulation suggests that under these conditions the NMDA component of the synaptic response is minimal (Collingridge et al., 1983). As a result of normal low frequency stimulation, glutamate is released from the pre-synaptic terminal and can bind to both AMPA and NMDA receptors which are co-localised on the post-synaptic membrane (Figure 2.4). Activation of the AMPA receptor results in an inward sodium current which depolarises the post-synaptic neuron and accounts for a large part of the evoked field potential (Collingridge et al., 1983; Muller et al., 1988). This would appear to provide the necessary conditions for activation of the NMDA receptor (pre-synaptic glutamate release and post-synaptic depolarisation) yet low frequency stimulation does not result in LTP. This is because during normal low frequency stimulation, activation of GABA interneurones rapidly repolarises the cell and, therefore, there is insufficient depolarisation to remove the Mg^{2+} block. The slow time course of NMDA receptor activation, relative to repolarisation,

means that by the time the receptor can activate the ion channel the neuron has been hyperpolarised and the Mg^{2+} block restored. High frequency stimulation results in sufficient depolarisation to overcome this channel block. Although there are several potential contributing factors which might account for this, it is primarily due to a frequency-dependent depression of the inhibitory systems which is thought to involve a pre-synaptic $GABA_B$ autoreceptor-mediated reduction in GABA release (Davies et al., 1991). Removal of the channel block allows Na^+ and Ca^{2+} ions to enter the cell, producing further depolarisation and regeneration of the NMDA current (Figure 2.4). The slow time course of the NMDA current now results in efficient summation of the NMDA responses.

Although the high frequency tetanus is a very artificial means of stimulation which results in a level of activation that is unlikely to occur naturally, the same underlying mechanism may result in changes in synaptic efficacy during learning. If two convergent inputs are simultaneously active, and the resulting post-synaptic depolarisation is sufficient to activate the NMDA receptor channel, then these synaptic connections will be strengthened. Thus, the NMDA receptor provides a mechanism whereby coincidental inputs can be detected and stored as long term changes in synaptic efficacy.

2.3.3 - The Mechanisms of LTP Expression

NMDA receptors are required for the induction, but not the expression or maintenance of LTP. During induction, NMDA receptor activation results in an influx of Ca^{2+} ions (MacDermott et al., 1986) which provides the molecular signal to initiate changes in synaptic efficacy.

The Role of Calcium

The first indication that calcium might have an important role in the induction of LTP came from experiments conducted by Dunwiddie and Lynch (1979) using the *in vitro* hippocampal slice preparation. They found that lowering the Ca^{2+} concentration of the bathing medium preferentially blocked the development of LTP while, at the same time, permitting synaptic transmission, paired-pulse facilitation and post-tetanic potentiation. In a later study, Lynch and co-workers showed that injection of the calcium chelator, EGTA, into the post-synaptic neurone also prevented the induction of LTP (Lynch et al., 1983). These results provided the first evidence that Ca^{2+} ions entering the post-synaptic cell may constitute an important

physiological signal for the induction of LTP (Figure 2.4).

Another major body of evidence, supporting a Ca^{2+}-dependent mechanism of LTP induction, is that elevating Ca^{2+} levels facilitates the induction of LTP. In one such experiment, Malenka and co-workers loaded CA1 pyramidal cells with the photolabile Ca^{2+} chelator, nitr-5, which had previously been saturated with Ca^{2+} ions. In response to ultraviolet light, Ca^{2+} ions were released from the 'caged' compound, resulting in a significant potentiation of synaptic transmission (Malenka et al., 1988). In addition, it has been shown that raising the extracellular Ca^{2+} concentration can facilitate the induction of LTP (Huang et al., 1988) and, in some cases, may be sufficient on its own to induce potentiation (Turner et al., 1982; Reymann et al., 1986).

More recently, the development of Ca^{2+}-sensitive fluorescent dyes has greatly facilitated studies investigating the putative role of Ca^{2+} ions in LTP. Using the indicator dye, arsenazo III, MacDermott and co-workers were able to demonstrate that NMDA receptor activation resulted in an increase in intracellular Ca^{2+} activity (MacDermott et al., 1986). Subsequently, as techniques have improved, it has proved possible to directly visualise Ca^{2+} fluxes using dyes such as fura-2. Using these Ca^{2+}-imaging techniques, researchers have shown that tetanic stimulation causes a spatially localised, transient elevation in intracellular Ca^{2+} levels in the spines and dendrites of neurones (Regeher and Tank, 1990; Müller and Connor, 1991; Perkel et al., 1993).

The events that follow this initial Ca^{2+} influx are not yet fully understood. There is evidence to suggest that the induction of LTP may require a greater elevation of Ca^{2+} levels than that provided by the initial Ca^{2+} entry through the NMDA ionophore. For example, LTP induction may require the Ca^{2+}-dependent Ca^{2+} release from intracellular stores (Obenaus et al., 1989; Harvey and Collingridge, 1992). In addition, there is evidence that NMDA receptor activation may bring about an increase in Ca^{2+} entry through voltage-gated Ca^{2+} channels via a cAMP-dependent mechanism (Chetkovich et al., 1991). The situation is further complicated by reports that, under certain conditions, Ca^{2+} influx through voltage-gated Ca^{2+} channels can elicit potentiation, independently of the NMDA receptor (Grover and Teyler, 1990; Aniksztejn and Ben-Ari, 1991; Huang and Malenka, 1993). There is now strong evidence for a distinct compartmentalisation of intracellular Ca^{2+} ions within neurones (Regeher et al., 1989; Regeher and Tank, 1990; Müller and Connor, 1991; Guthrie et al., 1991), but the relationship between the various subcellular Ca^{2+} compartments and the requirements for LTP induction are not clear. Nevertheless, it still seems likely that NMDA

receptor activation causes a large Ca^{2+} transient, probably localised to the dendritic spines, which is ultimately responsible for the induction of LTP. The elevation of intracellular Ca^{2+} levels will activate a number of Ca^{2+}-dependent enzymes which are then capable of initiating long-term changes in cell activity and synaptic efficacy.

Intracellular Signal Transduction Mechanisms

A number of enzymes have been implicated in the signal transduction process whereby the Ca^{2+} influx ultimately leads to permanent changes in synaptic efficacy. Increasing the intracellular Ca^{2+} concentration is likely to activate a number of enzyme cascades. An early proposal was that the Ca^{2+} influx activates a Ca^{2+}-dependent protease, calpain, which, through the specific cleavage of membrane-associated proteins, could uncover additional glutamate receptor molecules (Lynch and Baudry, 1984). Although there is some support for this theory (Seubert et al., 1988; Oliver et al., 1989; Fazeli et al., 1990), it is unlikely that protease activity alone is sufficient for the development of LTP.

There is also evidence suggesting that a number of different protein kinase enzymes are required for the generation of LTP, including Ca^{2+}/phospholipid-dependent protein kinase C (Hu et al., 1987; Linden and Routtenberg, 1989; Malinow et al., 1988; 1989), cAMP-dependent protein kinase A (Chetkovich et al., 1991), Ca^{2+}/calmodulin-dependent protein kinase CaMKII (Lisman and Goldring, 1988; Malinow et al., 1988; 1989; Malenka et al., 1989; Silva et al., 1992a), tyrosine kinase (O'Dell et al., 1991b; Bading and Greenberg, 1991; Grant et al., 1992) and microtubule-associated protein 2 kinase (Bading and Greenberg, 1991). Similarly, the dephosphorylation of protein substrates could also be important (Halpain and Greengard, 1990). In addition, phospholipid metabolism is commonly involved in intracellular signalling processes and may contribute at some stage to the mechanisms of LTP expression (Linden et al., 1987; Williams and Bliss, 1988; 1989; Okada et al., 1989). The way in which these different enzyme systems interact and the precise requirements for the expression of LTP are not fully understood.

Downstream from these enzyme systems, there is a requirement for new protein synthesis in the maintenance phase of LTP (Krug et al., 1984; Frey et al., 1989). It is not clear what contribution the transcription of new mRNA species and/or an increase in the translation of existing mRNA make towards this new protein synthesis (Cole et al., 1989; Otani et al., 1989). More extensive reviews of the mechanisms involved in the development and

expression of LTP are provided elsewhere (Schwartz and Greenberg, 1987; Madison et al., 1991; Bliss and Collingridge, 1993).

The Locus of LTP Expression (Pre- versus Post- Synaptic Mechanisms)

Although there is little doubt that the induction of LTP occurs in the post-synaptic neurone, the locus of long-term expression is a matter of considerable debate. Theoretically, there are a number of possible ways in which an increase in synaptic efficacy might be maintained over a long period of time. For example, LTP could be mediated by a sustained increase in transmitter release from the pre-synaptic terminal. Alternatively, the expression of LTP may involve a purely post-synaptic mechanism. Possibilities include an increase in receptor number, receptor affinity or in the efficiency of the signal transduction mechanisms. Not surprisingly, in view of the great interest surrounding LTP, a sizeable research effort has been directed towards determining whether the mechanisms involved in maintaining long-term increases in synaptic efficacy are pre-synaptic, post-synaptic, or, indeed, a combination of the two. Before reviewing the evidence for either a pre- or post-synaptic locus of expression, it is worth briefly considering a couple of notable alternatives.

One possibility is that a reduction in the uptake of glutamate, resulting in an increase in the concentration of neurotransmitter in the synaptic cleft, could contribute towards an increase in synaptic efficacy (Yu et al., 1986). The action of the putative neurotransmitter, glutamate, is terminated, at least in part, by a Na^+/K^+-dependent, high-affinity uptake mechanism in glial cells, which avidly removes glutamate from the synaptic cleft (Brew and Atwell, 1987; Barbour et al., 1988). It has been suggested that the activation of glutamate receptors in the hippocampus may require the binding of two glutamate molecules (Mayer, 1989). Increasing the background level of glutamate in the synaptic cleft may, therefore, facilitate synaptic transmission by reducing the amount of glutamate that must be released from the pre-synaptic terminal in order to reach the level of receptor occupancy required to activate the ion-channels.

Alternatively, there is evidence to suggest that morphological changes, brought about by the induction of LTP, could contribute to a lasting enhancement of synaptic transmission (Fifkova and van Harreveld, 1977; Lee et al., 1980; Chang and Greenough, 1984; Desmond and Levy, 1986). It is not clear precisely what structural modifications are required for the expression of LTP but possibilities include an increase in spine number, a change in the

shape or type of spine, a growth response such that the pre- and post-synaptic elements become closer together, or a reduction in the width of the spine neck leading to a subsequent increase in conductance. It is also not clear whether these changes would be restricted to the post-synaptic element or whether changes in the pre-synaptic terminal could also occur.

A Post-Synaptic Locus of Expression

By the early 1980's, the experimental evidence available implicated the post-synaptic neurone in the induction of LTP (Lynch et al., 1983). It seemed reasonable, therefore, that the mechanisms responsible for the expression of LTP might also be post-synaptic. Early attempts to investigate this possibility experimentally were, however, inconclusive. For example, Lynch and Baudry (1984) proposed that the increase in synaptic efficacy was maintained by increasing the number of post-synaptic glutamate receptors. An earlier study appeared to provide some support for this claim. Lynch and colleagues had seen an increase in [^{3}H] glutamate binding to membrane fractions prepared from hippocampal minislices following the induction of LTP, relative to low-frequency or non-stimulated controls (Lynch et al., 1982). It soon became apparent, however, that this increase in [^{3}H]glutamate binding was not due to an increase in the number or affinity of glutamate receptors but was, in fact, due to an increase in glutamate uptake by reforming membrane vesicles (Pin et al., 1984; Kessler et al., 1987; Monaghan et al., 1989).

An alternative strategy was to examine the responsiveness of CA1 dendrites in hippocampal slices to the iontophoretic application of agonists, such as glutamate, before and after the induction of LTP. A post-synaptic mechanism for LTP expression would predict an increase in the size of responses to applied glutamate after the induction of LTP. The experimental evidence showed, however, that the application of glutamate resulted in, if anything, smaller responses after tetanic stimulation (Lynch et al., 1976; Murali Mohan and Sastry, 1985; Taube and Schwartzkroin, 1988).

A crumb of comfort for proponents of a post-synaptic locus for LTP expression was that the related phenomena of paired-pulse facilitation (PPF) and paired-pulse depression (PPD), which are believed to involve the pre-synaptic terminal, were unaffected following the induction of LTP (McNaughton, 1982). It was argued that if LTP and PPF/PPD shared a common pre-synaptic mechanism then one might expect some sort of interaction. As this was not the case, it implied (although not conclusively) that post-synaptic rather than pre-

synaptic mechanisms were involved. The lack of an interaction between NMDA receptor-dependent LTP and PPF has since been confirmed, both in the CA1 subfield and at synapses between associational-commissural fibres and CA3 pyramidal cells (Muller and Lynch, 1989; Zalutsky and Nicoll, 1990). This is in direct contrast to LTP induced at synapses between mossy fibres and CA3 pyramidal cells. Mossy fibre LTP occludes the amount of PPF that can be elicited, occurs independently of NMDA receptors and is thought to involve a pre-synaptic mechanism (Harris and Cotman, 1986; Zalutsky and Nicoll, 1990).

In 1988 two research groups reported data which provided the first strong evidence that the expression of LTP might be post-synaptic (Muller and Lynch, 1988; Muller et al., 1988; Kauer et al., 1988b). Both groups worked under the assumption that if LTP involved a pre-synaptic increase in transmitter release then both the NMDA and non-NMDA components of the evoked response would be similarly potentiated. Conversely, if following LTP induction only the non-NMDA component of the evoked response was potentiated, this would suggest a selective post-synaptic modification of the AMPA receptor. Using newly developed selective antagonists, and under conditions which allowed activation of the NMDA receptor/ion channel (eg. low Mg^{2+} or depolarising potentials), both groups were able to dissociate the NMDA and non-NMDA components of the evoked response. Following the induction of LTP, they both observed that only the non-NMDA component was potentiated, implying a post-synaptic change in the AMPA receptor population. This was in contrast to post-tetanic potentiation (PTP) and paired-pulse facilitation (PPF), which involve a pre-synaptic increase in transmitter release and resulted in a similar potentiation of both NMDA and non-NMDA components. It should be noted, however, that, although suggestive, this result is not definitive proof of a post-synaptic locus for LTP expression. It could be argued, for example, that increased transmitter release combined with downregulation of NMDA receptor/ion channel activity could account for the observed disparity between the potentiation of the NMDA and non-NMDA components. Indeed, there is some evidence to suggest that prior activation of the NMDA receptor does result in downregulation of subsequent NMDA-receptor mediated activity (Huang et al., 1992; Izumi et al., 1992a). Furthermore, recent observations that NMDA receptor mediated epsps do exhibit LTP has cast further doubt over these results and their interpretation (Tsien and Malinow, 1990; Bashir et al., 1991; Berretta et al., 1991; Asztely et al., 1992; X. Xie et al., 1992).

Perhaps the best evidence supporting a role for post-synaptic mechanisms in the expression of LTP has come from an experiment based on Lynch's earlier attempts to assess the effects

of LTP induction on the responsiveness of dendrites to the application of an exogenous agonist (Lynch et al., 1976). But whereas previous studies had failed to see an increase in the size of responses evoked by applied glutamate following LTP induction (Lynch et al., 1976; Murali Mohan and Sastry, 1985; Taube and Schwartzkroin, 1988), Collingridge and co-workers found that the responsiveness of CA1 neurones to iontophoretically applied AMPA did increase after tetanisation (Davies et al., 1989). The effect was not immediate with little change over the first 15-30 minutes. Then gradually the AMPA response increased reaching a peak approximately two hours after LTP induction. This gradual onset may explain why this effect had eluded other researchers who, in general, confined their investigations to the half hour period immediately after tetanic stimulation. Alternatively, the use of AMPA rather than glutamate as the applied agonist may have avoided complications with glutamate uptake systems and potential interactions with other glutamate receptor subtypes.

The mechanism by which an increase in the AMPA response is mediated remains to be determined. There is some evidence that a phosphorylation step, possibly of the AMPA receptor itself, may bring about a change in the functional characteristics of this receptor/ionophore (Reymann et al., 1990; Wang et al., 1991; Greengard et al., 1991). An exciting alternative possibility is that LTP induction initiates changes in the level of expression of different AMPA receptor subunits which confer different functional properties on the AMPA receptor complex as a whole, possibly leading to an increase in the conductivity of the ion channel (Sommer et al., 1990).

Although the results of Davies et al., (1989) suggest that post-synaptic modifications may be important in the later stages of expression, the immediate onset of LTP implies that pre-synaptic mechanisms may also be involved.

A Pre-Synaptic Locus of Expression

Although mechanistically, a pre-synaptic locus of LTP expression is harder to reconcile with a post-synaptic locus of induction, there is now substantial evidence to suggest that an increase in transmitter release may contribute to long-term increases in synaptic efficacy. The first experimental support for this hypothesis was provided by Skrede and Malthe-Sørenssen (1981). Having pre-loaded hippocampal slices with [^{3}H] D-aspartate, they noted that high frequency stimulation caused a significant, long-lasting increase in the efflux of the

radiolabelled amino acid. Both the background resting levels of release, and those evoked by low-frequency electrical stimulation, were increased post-tetanus. Although the actual identity of the natural neurotransmitter was not known, it was assumed that [^{3}H] D-aspartate would behave similarly and act as a marker for the endogenous ligand.

Much of the subsequent work investigating whether or not increases in transmitter release accompany the induction of LTP has been conducted by Bliss and colleagues. Using a miniature push-pull cannula to which a conventional Teflon-coated stainless-steel recording electrode was attatched, Bliss and co-workers were able to apply and collect small volumes of solutions, while simultaneously recording evoked field potentials *in vivo* (Errington et al., 1983). Using this technique, [^{3}H] glutamine, a precursor of the putative neurotransmitter L-glutamate, was infused into the dentate gyrus of anaesthetised rats (Dolphin et al., 1982). One hour later, perfusion of the dentate gyrus with an oxygenated medium was begun and samples of the resulting perfusate collected. [^{3}H] Glutamate was separated from radiolabelled glutamine and GABA on an anion-exchange column and then quantified by liquid scintillation. The induction of LTP was associated with a sustained increase in the stimulus-evoked release of [^{3}H] glutamate, relative to control animals which had received low-frequency stimulation throughout. Importantly, animals which received a high frequency tetanus but failed to exhibit LTP showed no corresponding increase in [^{3}H] glutamate release.

Using a similar experimental design, Bliss and co-workers also demonstrated an increase in the release of certain endogenous amino acids following LTP induction (Bliss et al., 1986). Samples of perfusate were collected, derivatised with o-phthalaldehyde, separated by reverse-phase high performance liquid chromatography (HPLC) and quantified by means of fluorescence detection (see Chapter 7). Their results revealed an increase in the release of both glutamate and aspartate after LTP induction but not of glutamine or glycine. Subsequent experiments have shown that the increase in glutamate release is prevented by manipulations which also block the induction of LTP, including simultaneous activation of the commissural fibres (Bliss et al., 1986), infusion of D-AP5 (Errington et al., 1987) or infusion of the lipoxygenase/phospholipase A_2 inhibitor, nordihydroguaiaretic acid (NDGA; Lynch et al., 1989). Similar results have been obtained both *in vitro* using the hippocampal slice preparation or the dentate gyrus 'minislice' (Bliss et al., 1990a), and in *ex vivo* studies (Lynch et al., 1985; Feasey et al., 1986).

Ex vivo experiments have enabled a comparison to be made between the decay time course

of LTP and that of the enhanced glutamate release (Bliss et al., 1987). Rats with chronically implanted electrodes were given unilateral, high frequency stimulation of the perforant path to induce LTP in the dentate gyrus. The magnitude of the resulting potentiation was then monitored for a number of days. After a pre-determined time interval, the rats were sacrificed and the dentate gyrus removed bilaterally. Slices were prepared from both the tetanised and non-tetanised hemispheres, and preloaded with [^{3}H] glutamate. The magnitude of the evoked glutamate release following LTP induction was then assessed relative to that from the non-tetanised control hemisphere. Animals sacrificed 3 days post-tetanus, still exhibited robust LTP and an increase in evoked glutamate release from the tetanised hemisphere. In contrast, 23 days after LTP induction, the size of the epsp had returned to baseline levels and there was no significant difference between the tetanised and non-tetanised slices in terms of evoked glutamate release. The strong correlation between potentiation and the increase in evoked glutamate release provides strong evidence that pre-synaptic mechanisms contribute to the expression of LTP. It should be pointed out, however, that another research group have reported a contradictory result, namely that the induction of LTP is not associated with a sustained, long-lasting increase in the release of either glutamate or aspartate (Aniksztejn et al., 1989). The reasons for the discrepancy between these studies is not clear.

A role for the pre-synaptic terminal in the expression of LTP may also be inferred from a number of other studies. For example, injection of the protein kinase inhibitor, H7, into the post-synaptic cell prevents the induction of LTP but is without effect on pre-established potentiation (Malenka et al., 1989; Malinow et al., 1989). In contrast, bath application of protein kinase inhibitors, including H7, affect both the induction and expression of LTP (Malinow et al., 1989). Taken together, these results suggest that protein kinase activity in the post-synaptic neurone is required for the induction of LTP but that protein kinase activity outwith the post-synaptic terminal is also required for subsequent LTP expression. Similarly, bath application of pertussis toxin, a G protein inhibitor, prevents the development of LTP, whereas deregulation of post-synaptic G-proteins by injecting guanosine-5'-O-(3-thiotriphosphate) (GTP-γ-S), a non-hydrolysable GTP analogue, does not affect potentiation (Goh and Pennefather, 1989). These results support a pre-synaptic involvement in the mechanisms of LTP expression and suggest that both G-proteins and protein kinases, outwith the post-synaptic terminal, are required. Furthermore, Nelson and colleagues (Nelson et al., 1989) have discovered a strong correlation between the persistence of LTP and the level of two phosphoproteins in the pre-synaptic terminal. These proteins have been identified as

synaptic vesicle proteins, suggesting that LTP induction may modify the machinery responsible for neurotransmitter release (see also Gianotti et al., 1992).

Quantal Analysis Studies

Quantal analysis is a well established method for determining whether changes in the level of synaptic transmission involve the pre- or post-synaptic terminal (Redman, 1990). It is possible that by applying the principles of quantal analysis to synaptic transmission, before and after the induction of LTP, one might be able to determine once and for all whether the expression of LTP occurs pre- or post-synaptically. The method derives from the 'quantal hypothesis of neurotransmitter release' which was first proposed by Bernard Katz in relation to synaptic transmission at the neuromuscular junction (Fatt and Katz, 1952; del Castillo and Katz, 1954). The hypothesis states that the neurotransmitter is released from the pre-synaptic terminal in small, discrete packets, of similar size called quanta. Each quantum corresponds to a single vesicle releasing its full load of transmitter into the synaptic cleft in an 'all or none' fashion. If one considers a single vesicle, transmitter will not be released every time the pre-synaptic terminal is activated. Rather, there is a finite probability (p), between 0 and 1, that following pre-synaptic activation any given vesicle will release its contents. The product of the total number of release sites available at a synapse (N) and the probability of release (p) defines the quantal content (m) which is the number of quanta released for a given pre-synaptic event. The unit post-synaptic response to a single quantum is known as the quantal size (a) and the product m.a constitutes the observed post-synaptic response.

Quantal analysis involves the statistical analysis of a large number of epsp magnitudes, under conditions which limit the amount of neurotransmitter released from the pre-synaptic terminal, in an attempt to estimate the quantal parameters m and a. Typically, these experiments involve stimulating the pre-synaptic terminal on a number of occasions and recording the resulting post-synaptic response. Over a large number of trials, a percentage of the pre-synaptic stimuli will result in 'failures', with no detectable post-synaptic response. The minimum measurable response corresponds to the release of a single quantum and provides a measure of the quantal size (a). A histogram can be plotted of the response amplitude against the frequency with which that response amplitude occurs. The result is an uneven distribution of response sizes, but if the release process obeys the quantal hypothesis then it should be possible to identify distinct peaks which correspond to integral multiples of

the unit post-synaptic event (Figure 2.7). Complex statistical analysis, which involves modelling the amplitude distribution to either a binomial or poisson distribution, allows estimates to be made of the quantal parameters m and a. If the enhancement of synaptic transmission is mediated by the pre-synaptic terminal then the quantal content (m) should increase. This may be the result of a change in either the probability of release (p) or the potential number of release sites (N). This will result in the biggest peak in the amplitude distribution shifting to the right with no change in the positions of the individual peaks or in the size of the maximum obtainable response (Figure 2.7). In contrast, if changes occur in the post-synaptic terminal then the quantal size (a) will increase and the amplitude distribution will be uniformly stretched out to the right (Figure 2.7). The biggest peak will still occupy the same position relative to the other peaks but the maximum obtainable response will be greater. By applying this method of analysis to LTP it should be possible to determine whether LTP expression is pre-synaptic, post-synaptic or both.

Early attempts to apply quantal analysis to synaptic transmission in the CNS hit upon a number of problems. The complex neuronal architecture in the CNS is unsuited to quantal analysis. For example, it is extremely difficult to isolate a pair of cells that are monosynaptically connected. In addition, in the CNS, the quanta that can be measured are much smaller and a great deal more variable than at the neuromuscular junction. Consequently, it has proved much harder to estimate accurately the size of a single quantal event. Furthermore, the poor signal to noise ratio obtained with conventional recording techniques rendered quantal analysis virtually impossible using CNS preparations. As a result, early attempts to apply these techniques to LTP were regarded with considerable scepticism (Voronin, 1983).

The development of whole cell patch clamping techniques provided a means by which some of these problems could be overcome (Neher and Sakmann, 1976). Whole cell patch clamp recording greatly reduces background noise, improving the signal to noise ratio to a point where quantal analysis becomes a feasible proposition in the CNS and can be performed reliably. In 1990, two independent research groups published reports describing how they had used the combination of whole cell patch clamping and quantal analysis to investigate the locus of LTP expression (Bekkers and Stevens, 1990a; Malinow and Tsien, 1990). Using conventional hippocampal slices, both groups attempted to achieve minimal pre-synaptic activation by employing very weak stimulation parameters, just slightly greater than the stimulus strength that resulted in 100% failures. Both groups acknowledged that, even then,

the likelihood was that multiple axonal inputs to the post-synaptic cell were being activated. Consequently, additional alternative approaches were sought. Bekkers and Stevens chose to study synaptic transmission in cultured hippocampal neurones, allowing pairs of cells connected by a single synapse to be easilly identified. Malinow and Tsien chose a more arduous route to achieve the same goal. Still using hippocampal slices, they impaled both a post-synaptic CA1 cell and also a pre-synaptic CA3 cell. Although it often took many attempts to find two monosynaptically connected cells, it did prove possible (see also Malinow, 1991). Before investigating the effects of inducing LTP, the quantal analysis techniques were validated by examining the changes in the quantal parameters in response to manipulations which were known to affect either the pre- or post-synaptic neurone exclusively (Malinow and Tsien, 1990). For example, raising the extracellular Ca^{2+} concentration increases transmitter release and consequently increases the quantal content (m). Conversely, application of a post-synaptic receptor antagonist (eg. CNQX) reduces the quantal size (a).

The results of these two studies were conclusive. Both groups were in agreement that LTP expression involved an increase in the quantal content (m), implying a pre-synaptic enhancement of transmitter release. Similar results were obtained in both slices and cultured neurones. Malinow and Tsien provided additional support for a pre-synaptic mechanism by showing that the number of 'failures' reduced from 63% to 17% following LTP induction. It is generally considered that a reduction in the number of failures is more likely to result from a change associated with the pre-synaptic terminal. Using cultured neurones, Bekkers and Stevens went one step further and suggested that the increase in quantal content was attributable to an increase in the release probability (p) and not in the number of available release sites (N).

Not surprisingly, however, it was not long before the controversy between a pre- and post-synaptic locus of expression was reopened. Within a year, a report was published in which quantal analysis was again used to assess the locus of LTP expression but with the opposite result, namely an increase in the quantal size (a) which is indicative of an increase in post-synaptic sensitivity (Foster and McNaughton, 1991). Subsequently, a number of research groups have attempted to determine the locus of LTP expression using quantal analysis, some supporting a pre-synaptic mechansim, some a post-synaptic change and some a combination of the two (Table 2.2). An explanation for the apparent contradictory nature of these results has not been forthcoming. It is possible that the initial release probability of the

synapses may have an influence on the outcome of the results. This in turn may be affected by experimental conditions such as the extracellular Ca^{2+} concentration. It has also been suggested that the age of the preparation, the bath temperature, the parameters used to induce LTP and the time point after LTP induction at which observations are made, may be contributing factors. Finally, questions still remain as to the validity of some of the assumptions that have been made about how synapses in the CNS behave, in order to vindicate the use of the various statistical methods employed in these quantal analysis studies (Korn and Faber, 1991).

In order to avoid some of these assumptions, two research groups adopted a different, but related strategy, looking at spontaneous 'miniature' post-synaptic currents in the absence of evoked pre-synaptic activity (Manabe et al., 1992; Malgaroli and Tsien, 1992). Unfortunately, however, these two studies also yielded conflicting results. While one group observed an increase in the size of the 'miniatures' supporting a post-synaptic change (Manabe et al., 1992), the other group reported an increase in the frequency with which the 'miniatures' occurred, favouring a pre-synaptic mechanism (Malgaroli and Tsien, 1992).

A Requirement for an Intercellular Messenger

A number of experimental approaches, including quantal analysis, have yielded evidence for the involvement of both pre- and post-synaptic mechanisms in the expression of LTP. There is, of course, no a priori reason why both terminals cannot contribute to the sustained increase in synaptic efficacy. Indeed, some of the quantal analysis studies do suggest that both pre- and post-synaptic mechanisms are important (Kullmann and Nicoll, 1992; Larkman et al., 1992). An interesting question that arises from a possible pre-synaptic contribution to the maintenance of LTP is, 'how does the induction of LTP in the post-synaptic neurone lead to an increase in transmitter release from the pre-synaptic terminal?'

This apparent anomaly has lead to the suggestion that the induction of LTP initiates the release of a 'retrograde messenger' from the post-synaptic cell which can then diffuse across the synaptic cleft and modify activity in recently active pre-synaptic terminals. It is not inconceivable that communication between post- and pre-synaptic neurones could be mediated by some means other than the generation of a retrograde messenger. For example, it has been suggested that the mass influx of Ca^{2+} ions into the post-synaptic neurone will result in a dramatic reduction in Ca^{2+} concentration in the synaptic cleft, to which the pre-

synaptic terminal may be sensitive (Bliss et al., 1990b). Alternatively, structural modifications of the cytoskeletal matrix, induced post-synaptically, may extend to the pre-synaptic terminal. However, it is the putative release of an intercellular retrograde messenger that has attracted most attention.

Investigations into the degree of synapse specificity of LTP induction also imply a requirement for an intercellular messenger (Bonhoeffer et al., 1989; Bonhoeffer, 1990). 'Input specificity' is an important property of LTP. Briefly, if LTP is induced at a synapse, neighbouring synapses on the same post-synaptic cell are only potentiated if their pre-synaptic terminals are active simultaneously (Gustaffson et al., 1987). Synapses on the post-synaptic cell for which the pre-synaptic afferents are inactive, remain unpotentiated. The specificity of LTP induction exhibited by the post-synaptic cell may not, however, be maintained along the pre-synaptic fibre. Bonhoeffer and co-workers have reported that potentiation is not restricted to the activated post-synaptic cell, suggesting that the existing Hebbian rule may need to be modified. The experiments were conducted using a hippocampal slice culture (Gähwiler, 1981) and required that intracellular recordings be made simultaneously from two post-synaptic neurones activated by the same pre-synaptic afferent input (Figure 2.8). Accordingly, two adjacent CA1 pyramidal neurones (25-60 μm apart) were impaled and an extracellular stimulating electrode positioned in the Schaffer collateral fibres. Before potentiating, the experimenters took great care to ensure that the two CA1 cells were not synaptically connected, and that depolarising one cell had no effect on the membrane potential of the other. LTP was induced by pairing post-synaptic depolarisation in the 'paired' cell (cell 1) with the delivery of a concurrent single afferent volley (a 'conditioning' stimulus) to fibre 'X' (Figure 2.8). This resulted in potentiation of synapse A but not of synapse B, as predicted by the conventional Hebbian rule. However, there was also potentiation of synapse C, between the 'unpaired' post-synaptic cell (cell 2) and the afferent input 'X' which had received the conditioning stimulus. This distributed pattern of synaptic potentiation suggests that LTP induction is not specific to the synapse at which the conjunctive event occurs and implies that an intercellular messenger may be released from the active post-synaptic neurone (cell 1) which can enhance the activity of all recently active pre-synaptic terminals along the input fibre X. The result was confirmed with optical recording techniques, using voltage-sensitive dyes, and has been repeated in slices of visual cortex.

Schuman and Madison (1994a) have conducted a similar experiment and included biocytin

in the recording electrodes. This allowed them to visualise the pyramidal cells from which recordings were made and to estimate the distance between cells. They found that if the distance between cells was in excess of 500 μm, then there was no potentiation in the unpaired cell. Furthermore, there was a significant negative correlation between the intersomatic distance and the amount of potentiation observed in the unpaired cell. This result is consistent with the idea that an unstable messenger molecule, with a relatively short half life, is being released from the activated post-synaptic neurone and is diffusing out to neighbouring pre-synaptic terminals and enhancing their activity.

The Identity of the Putative Intercellular Messenger

The identity of the putative intercellular messenger has attracted considerable attention. An early suggestion was that it may be proteinaceous in nature. A number of groups have detected an increase in the efflux of proteins following the induction of LTP (Duffy et al., 1981; Nyström et al., 1986; Charriaut-Marlangue et al., 1988; Fazeli et al., 1988; 1990; Otani et al., 1992). Fazeli and colleagues showed that the onset of LTP, induced either by tetanic stimulation or by raising the extracellular calcium concentration, was accompanied by an increase in the protein content of perfusates collected using the modified push-pull cannula (Fazeli et al., 1988). The increased protein efflux was blocked by infusion of D-AP5. Subsequent studies revealed that among the proteins released were protease enzymes, raising the attractive possibility that these enzymes were being released in order to bring about structural alterations to the synaptic elements. More recently, however, interest in proteins as intercellular messengers has waned somewhat, partly as a result of the slow time course of their release after LTP induction which is not consistent with the immediate onset of potentiation. A number of alternative candidates have been put forward, ranging from the ether phospholipid 'platelet-activating factor' (PAF; Arai and Lynch, 1992; Clark et al., 1992; Wieraszko et al., 1993) to the simple diatomic gas, carbon monoxide (CO; Verma et al., 1993; Zhuo et al., 1993; Stevens and Wang, 1993). It has also been suggested that K^+ ions, which can leave the post-synaptic cell through the NMDA ionophore during activation, might subserve this function by enhancing transmitter release from the pre-synaptic terminal, possibly through an interaction with metabotropic glutamate receptors (Collingridge, 1992). The two major candidates, however, for which there is the most evidence, are arachidonic acid and nitric oxide. An extensive review of the case for nitric oxide is provided in Chapter 5, but it also worthwhile briefly considering the possibility that arachidonic acid might

contribute as an intercellular messenger.

Arachidonic Acid as a Putative Intercellular Messenger

Arachidonic acid (AA) is a simple unsaturated fatty acid which can be liberated from membrane phospholipids by the enzymes phospholipase A_2 and phospholipase C. This small, lipid soluble molecule is freely diffusible with a relatively short half-life, making it an ideal candidate intercellular messenger. A role for AA as an *intracellular* messenger in *Aplysia* has already been demonstrated and the metabolic cascades involving AA in this system are well defined (Piomelli et al., 1987). It is still not clear, however, in either *Aplysia* or in the mammalian CNS, whether AA, or one of the lipoxygenase metabolites of AA, is the active agent (Piomelli et al., 1987; Axelrod et al., 1988; Piomelli and Greengard, 1990).

There are several lines of evidence supporting a role for AA in LTP and, at the same time, satisfying many of the criteria for a candidate retrograde messenger. For example, there is evidence that AA may be released through a NMDA receptor-dependent mechanism following LTP induction. AA is released from cultured neurones in response to NMDA. This has been observed in striatal (Dumuis et al., 1988), cerebellar (Lazarewicz et al., 1988) and hippocampal neurones (Sanfeliu et al., 1990). Similarly, there is an increase in the levels of AA in both the post-synaptic terminal (Clements et al., 1991) and in the ECF following LTP induction. Using the push-pull cannula method, Bliss and colleagues have observed a small but sustained increase in the concentration of AA, and two metabolites of AA, in perfusates taken after the induction of LTP in the dentate gyrus *in vivo* (Lynch et al., 1989; Bliss et al., 1990a,b).

Furthermore, inhibitors of phospholipase A_2, one of the enzymes responsible for liberating AA from the membrane, block the development of LTP (Linden et al., 1987). Nordihydroguaiaretic acid (NDGA), a phospholipase A_2/lipoxygenase inhibitor, blocks the induction of LTP both in the CA1 subfield of hippocampal slices *in vitro* (Williams and Bliss, 1988; 1989; Okada et al., 1989) and in the dentate gyrus *in vivo* (Lynch et al., 1989). NDGA also blocks the increase in glutamate release which normally accompanies the onset of LTP *in vivo* (Lynch et al., 1989).

Application of AA, on its own, does not result in potentiation in the dentate gyrus *in vivo* (Williams et al., 1989). In combination with weak activation of perforant path fibres, however, AA causes a long-lasting potentiation which develops slowly and is accompanied

by an increase in glutamate release. The AA-induced, activity-dependent potentiation is resistant to D-AP5, suggesting that AA bypasses the need for NMDA receptor activation. Moreover, the AA-induced potentiation occludes subsequent tetanus-induced LTP. Similar results have been obtained in both the dentate gyrus (Williams et al., 1989) and the CA1 region of hippocampal slices *in vitro* (Drapeau et al., 1990).

The mechanism by which AA might enhance synaptic efficacy is not entirely clear. There is reason to believe, in fact, that AA may have a number of actions, all of which contribute to the maintenance of LTP. As previously suggested, AA might act as a retrograde messenger during the development of LTP to bring about an increase in transmitter release from the pre-synaptic terminal. Application of AA increases the release of the putative transmitter, L-glutamate, both *in vivo* and *in vitro* (Williams et al., 1989). Similarly, AA enhances [^{3}H] glutamate release from synaptosomes prepared from hippocampal tissue (Lynch and Voss, 1990). Furthermore, AA mimicks the effects of LTP induction in elevating synaptosomal Ca^{2+} concentration and phosphoinositide turnover, indicative of a pre-synaptic mechanism (Lynch and Voss, 1991). One suggestion is that activation of pre-synaptic metabotropic glutamate receptors can enhance transmitter release through a protein kinase C-dependent mechanism, for which there is an absolute requirement for AA (Herrero et al., 1992).

In addition, AA has a number of other actions which may also contribute to the potentiation of synaptic transmission. AA potentiates the NMDA current in isolated cerebellar granule cells by increasing the probability of channel openings (Miller et al., 1992). This post-synaptic action of AA is not evidence for AA acting as an intercellular messenger but illustrates how a single molecular signal may facilitate an increase in synaptic efficacy through a number of divergent pathways. Similarly, AA also inhibits glutamate uptake by both glial cells and neurones, providing yet another potential mechanism through which synaptic transmission might be enhanced. This effect has been observed in retinal cells (Barbour et al., 1989) and in cultures of cortical cells (Yu et al., 1986) but the available evidence suggests, however, that AA has no effect on high affinity glutamate uptake in the dentate gyrus (Lynch and Voss, 1990).

Taken together, these results provide some support in favour of a role for AA in LTP. There are, however, certain other results which do not fit with the theory that AA acts as the retrograde messenger. For example, the fact that AA reduces neuronal Ca^{2+} currents and enhances K^+ currents would appear to work against a mechanism for increasing transmitter release, although it would appear that this may be overcome (Piomelli and Greengard, 1990;

Keyser and Alger, 1990). Potentially harder to reconcile with a role for AA as the retrograde messenger, is the slow onset of AA-induced potentiation. This is in contrast to the immediate increase in both the size of the epsp and in glutamate release and implies that AA is not involved in the early stages of LTP expression. This suggests that there may be a requirement for an additional intercellular signal to account for the immediate increase in synaptic efficacy. There is now considerable evidence that nitric oxide (NO) may perform this role (see Chapters 5 and 6).

2.4 - Long-Term Potentiation in the Hippocampus: A Role in Spatial Learning

NMDA receptor-dependent LTP is an experimental model of synaptic plasticity and may, therefore, bear some resemblance to the neural mechanisms that underlie learning. The attractiveness of LTP as a candidate mechanism of information storage has already been discussed. Briefly, LTP is long-lasting and can be induced by a brief period of activity using stimulation parameters which might be expected to resemble the firing characteristics of neurones under natural conditions. LTP also exhibits associativity, cooperativity and input specificity, properties which appear to satisfy many of the criteria required of a putative learning mechanism.

Furthermore, LTP can be reliably demonstrated in areas of the brain which have been implicated in learning and memory. For example, LTP is particularly robust in the hippocampus. As outlined earlier in this chapter, the hippocampus has been strongly implicated in certain forms of learning and memory, and particularly with regard to the processing of spatial and contextual information. It is not unreasonable, therefore, to suggest that LTP-like mechanisms in the hippocampus may constitute the neural substrate of spatial learning.

Evidence Supporting a Role for LTP-like Events in Spatial Learning

All in all, the properties of LTP provide a strong theoretical basis for the hypothesis that LTP-like events in hippocampus might contribute to the mechanisms of spatial learning. Despite this strong theoretical support, however, reliable experimental evidence for a link between hippocampal LTP and spatial learning has been less forthcoming. In the mammalian CNS, attempts to actually observe changes in synaptic efficacy during learning have proved notoriously difficult, both in terms of reliably measuring behavioural-induced changes in the strength of synaptic transmission and with regard to their subsequent interpretation (see Hargreaves et al., 1990; Moser et al., 1993a,b).

In the absence of any direct demonstration that changes in synaptic efficacy occur during learning, the evidence supporting the hippocampal LTP/spatial learning hypothesis has been derived from correlations between parameters associated with LTP and spatial learning. For example, comparisons made across groups of animals, in the absence of any experimental manipulation, have provided some indication that hippocampal LTP and spatial learning

might be correlated (Barnes, 1979; Barnes and McNaughton, 1980; 1985; Jeffery and Morris, 1993).

A more common approach, pursued by many researchers, has been to take manipulations which block LTP and assess their effects on spatial learning. Along these lines, three main experimental strategies have been adopted in an attempt to investigate the relationship between hippocampal LTP and spatial learning: (i) prior electrical saturation of hippocampal synapses to prevent further LTP induction (McNaughton et al., 1986; Castro et al., 1989; but see also McNamara et al., 1992; Robinson, 1992; Cain et al., 1993; Jeffery and Morris, 1993; Korol et al., 1993; Sutherland et al., 1993), (ii) the generation of mutant mice, deficient in specific enzymes which are required for the development of LTP (Silva et al., 1992a; Silva et al., 1992b; Grant et al., 1992), and (iii) the use of specific drugs to block the induction or expression of LTP. Correlations between the effects of these manipulations on LTP and spatial learning have provided the major source of experimental support for the hippocampal LTP/spatial learning hypothesis.

Pharmacological Manipulations of LTP and Spatial Learning

An obvious prediction of the LTP/learning hypothesis is that pharmacological manipulations which prevent the induction of LTP will also impair learning. More specifically, blockade of LTP-like events in hippocampus should disrupt hippocampal-dependent forms of learning, including spatial learning. Numerous attempts have been made to investigate, pharmacologically, the effects of blocking LTP on spatial learning, using a variety of drugs and a number of different behavioural paradigms. For example, intraventricular infusion of the calpain inhibitor, leupeptin, has been shown to prevent the development of LTP (Oliver et al., 1989) and also to impair performance on a spatial learning task in the radial maze (Staubli et al., 1984). More commonly, researchers have targeted the NMDA receptor as a means of manipulating the inducibility of LTP.

NMDA Receptor Antagonists Impair Spatial Learning in the Watermaze

Previous studies conducted in this laboratory have examined the effects of the selective, competitive NMDA antagonist, AP5, on learning in the open field watermaze. Chronic intraventricular infusion of D,L-AP5 (40 mM), by means of an osmotic minipump, has been shown to impair the acquisition of a standard spatial reference memory task in the

watermaze (Morris et al., 1986a; see also Morris, 1989). The AP5-infused rats took consistently longer to escape from the pool during training and showed little, if any, bias towards the former training quadrant during a transfer test, conducted after 15 training trials. In contrast, a separate group of AP5-treated rats were not impaired in learning a visual discrimination task in the watermaze. This behavioural dissociation is similar to that obtained following hippocampal lesions (Morris et al., 1982; Morris et al., 1986b) and suggests that the AP5-infused animals are capable of swimming relatively normally, of climbing onto the platform, of discriminating between the visual appearance of the two platforms, and of certain forms of hippocampal-independent learning. The AP5-induced impairment was stereoselective (the D isomer is active) and provided the first evidence that NMDA receptors might be required during spatial learning. Furthermore, Morris et al., demonstrated, in a separate group of animals, that the infusion of 40 mM D,L-AP5 also blocked the induction of LTP at perforant path-granule cell synapses in the dentate gyrus *in vivo*. This result confirmed previous reports from hippocampal slice studies that NMDA receptors are required for the induction of LTP and suggested a possible mechanism which might account for the AP5-induced spatial learning impairment.

AP5 Impairs Acquisition but not Expression of Spatial Learning

In a subsequent study, Morris showed that the infusion of AP5 prevented the acquisition but not the expression of spatial learning in the watermaze (Morris, 1989; see also Morris et al., 1990a). Rats were trained, in the absence of any drug treatment, to find a fixed location hidden escape platform, until they had reached a high level of performance. The animals were then divided into two groups and implanted with minipumps, delivering either artificial CSF or D,L-AP5 (40 mM). D,L-AP5 treatment did not affect the retrieval of a previously acquired platform location, a result which suggests that NMDA receptors may be required for the processing and/or strorage of spatial information, but are not required for the subsequent recall and expression of that learning. This result parallels the electrophysiological finding that NMDA receptors are required for the induction of LTP but are not involved in the expression or maintenance of potentiation. Taken together, these results are consistent with the idea that changes in synaptic efficacy are involved during the formation of memories but are not required for the subsequent retrieval of stored information.

The Dose-Response Study

One shortcoming of the original study (Morris et al., 1986a) was that only a single dose of D,L-AP5 (40 mM) was used. This left open the possibility that a lower dose of AP5 may still prevent the induction of LTP but, at the same time, leave spatial learning intact. Conversely, behavioural performance may be impaired at concentrations well below those required to block LTP in the hippocampus. Furthermore, the claim that the AP5-induced spatial learning deficit is due to a block of LTP-like events in the hippocampus would be substantiated if a learning impairment was found in the same animals which showed a block of LTP. In an attempt to address both these points, spatial learning and LTP induction were assessed within the same animals, across a range of D-AP5 concentrations (0-50 mM; Davis et al., 1992). After 30 spatial training trials (5 days/ 6 trials per day), the rats' knowledge of the platform location was assessed with a transfer test, conducted immediately after the last spatial training trial. The rats were then anaesthetised and an attempt made to induce LTP in the dentate gyrus. Whole tissue levels of D-AP5 were quantified using HPLC with fluorescence detection and the rats assigned to groups on the basis of the D-AP5 tissue concentration present in the hippocampus. The results revealed that the spatial learning impairment occurred across a comparable dose range to the blockade of dentate gyrus LTP *in vivo*. Behavioural performance correlated significantly with the magnitude of LTP measured 30 mins post-tetanus (escape latency - $r(48) = -0.62$; $p < 0.001$; transfer test performance - $r(48) = 0.45$; $p < 0.001$) and with the extracellular D-AP5 concentration (escape latency - $r(48) = 0.85$; $p < 0.001$; transfer test performance - $r(48) = -0.53$; $p < 0.001$). Importantly, this study found no evidence of a dose of D-AP5 that blocked the induction of LTP without affecting spatial learning. Furthermore, estimates of the D-AP5 concentration present in the extracellular fluid, obtained using microdialysis, suggested that the effects of D-AP5 on spatial learning and LTP *in vivo* occurred at similar extracellular concentrations to the blockade of LTP in the CA1 region of the hippocampal slice (Harris et al., 1984).

Summary

Intraventricular infusion of the competitive NMDA antagonist, AP5, impairs spatial learning in the watermaze across a comparable dose range to the blockade of LTP in the dentate gyrus *in vivo* (Davis et al., 1992). AP5 impaired neither visual discrimination learning in the watermaze (Morris et al., 1986a), nor the retrieval of previously acquired spatial information

(Morris, 1989; Morris et al., 1990a). These results suggest that AP5 may exert a selective effect on certain types of learning, including hippocampal-dependent spatial learning, while leaving other memory systems apparently in tact, and are consistent with the possibility that LTP-like events in the hippocampus contribute to the neural mechanisms of spatial learning.

These AP5 studies form a crucial component of the experimental evidence supporting the hippocampal LTP/spatial learning hypothesis. However, when considering these results, three key questions need to be addressed. Firstly, is the behavioural impairment arising from AP5 infusion due to a direct action on the neural mechanisms of learning or can it be attributed to an effect on some other sensorimotor or motivational process which will also influence performance? The lack of an effect on either visual discrimination learning or on the retrieval of previously acquired spatial information goes some way to arguing against a gross sensorimotor or motivational account of the AP5-induced behavioural deficit. The sensorimotor and motivational demands of both the visual discrimination task and the retrieval task are similar (but not identical) to those encountered during acquisition of the spatial reference memory task. The fact that performance on these tasks is spared provides some degree of control for potential non-associative 'side effects' of AP5 treatment which are known to occur at higher doses. Nevertheless, the possibility remains that a more subtle sensorimotor or motivational disturbance may still be present at these concentrations and could account for any behavioural impairment. Secondly, is the behavioural deficit due to an effect of AP5 in the hippocampus or to an action of the drug in some other area of the brain? For example, a number of other brain areas have been implicated in spatial learning and, therefore, even if the behavioural impairment is the result of a direct and selective effect on the learning mechanism, the possibility remains that AP5 is disrupting learning through an effect on some brain area other than the hippocampus. Thirdly, if the AP5-induced behavioural impairment is mediated through an effect on some aspect of hippocampal physiology, is the disruption of hippocampal function due to an effect on LTP-like processes or the result of some other effect of AP5 on the system properties of the hippocampus?

The results described so far provide some support for the hippocampal LTP/spatial learning hypothesis. Nevertheless, these results are far from conclusive and further investigation is required. For example, the use of other manipulations which prevent the development of LTP may provide a means of separating drug effects on LTP from drug effects on other system properties of the hippocampus. The aim of this thesis is to investigate further the relationship between hippocampal LTP and spatial learning in the watermaze by examining

(i) the effects on spatial learning of manipulating the inducability of LTP through a different site on the NMDA receptor complex (the glycine site), (ii) the effects on spatial learning of preventing the development and expression of LTP at a site downstream from the NMDA receptor (the synthesis of the putative retrograde messenger, NO), and (iii) a further investigation of the effects of D-AP5 on spatial learning but using a different behavioural protocol from those used previously. Further studies will either substantiate the hippocampal LTP/spatial learning hypothesis or, in the event of intact learning in the presence of a complete block of LTP, allow the hypothesis to be refuted.

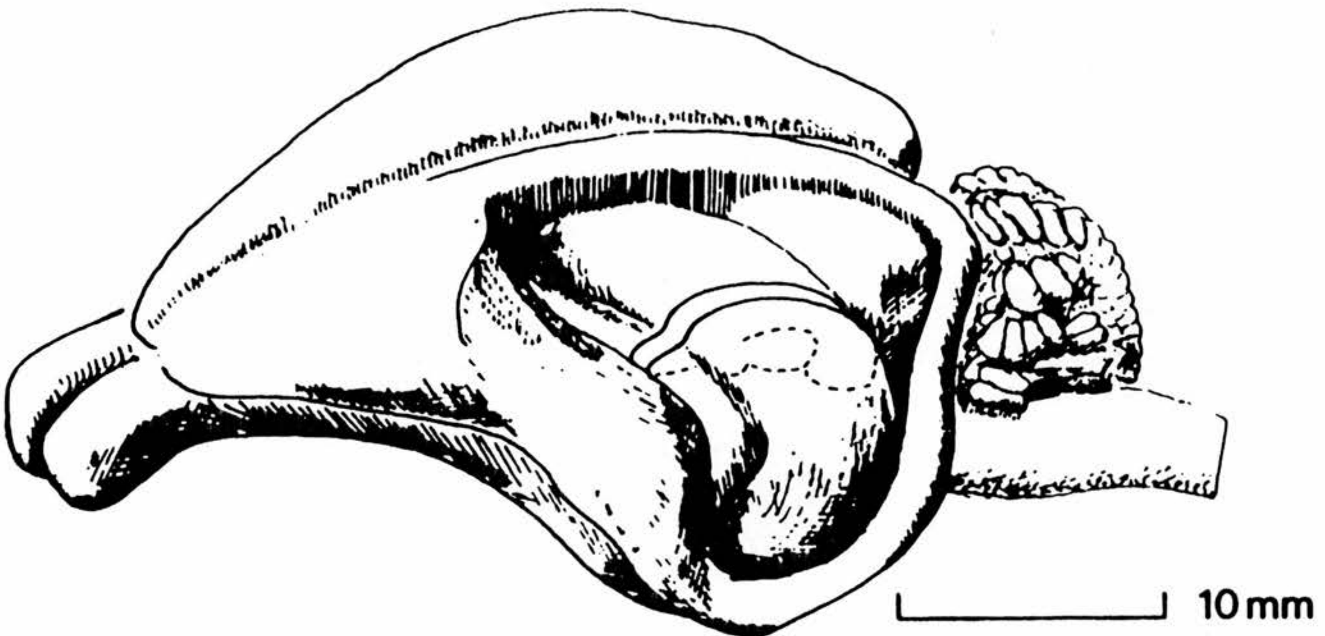


Fig. 2.1
The hippocampus. A lateral view of the rodent brain with the parietal and temporal neocortex removed to expose the hippocampal formation. The lamellar slice indicated has been presented separately in Fig. 2.2. (*From* Andersen et al., 1971).

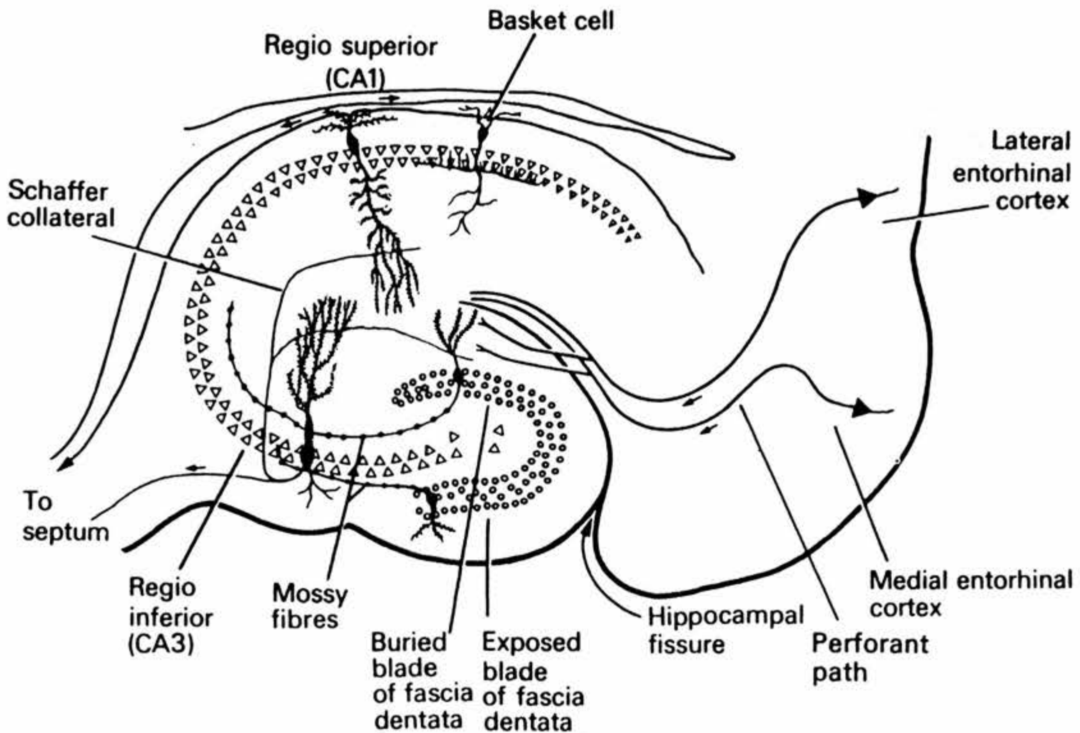


Fig. 2.2
A schematic diagram of the intrahippocampal connections. Horizontal section through the hippocampus (see Fig. 2.1). (*From* O'Keefe and Nadel, 1978).

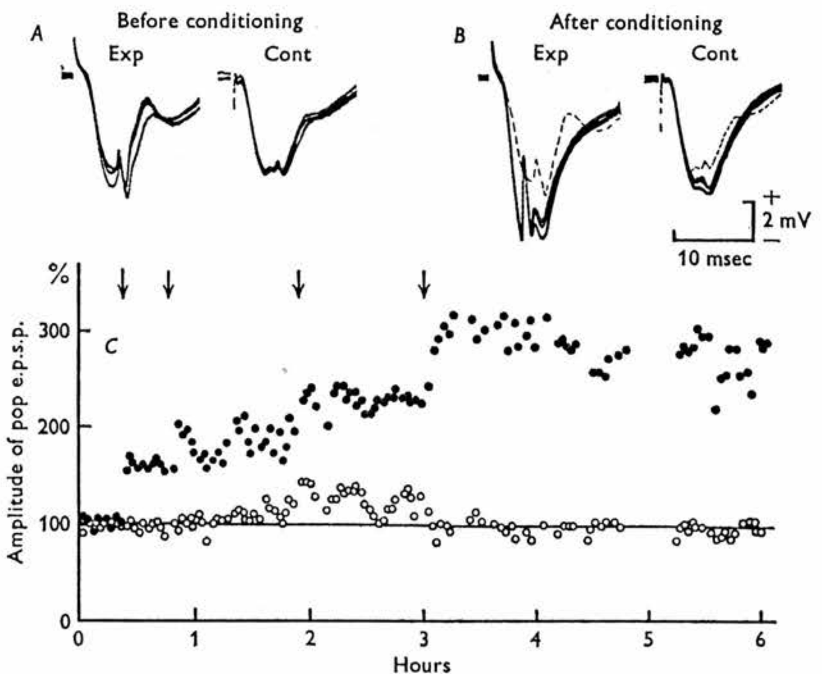


Fig. 2.3
Long-Term Potentiation. An experiment in which the standard parameters of the evoked response were potentiated. Three superimposed responses obtained in the synaptic layer for both the experimental and control pathways are shown in A (before conditioning) and in B (2.5 hr after the fourth conditioning train). C -A graph showing the amplitude of the pop. epsp for the experimental pathway (filled circles) and ipsilateral control pathway (open circles) as a function of time. (*From* Bliss and Lømo, 1973).

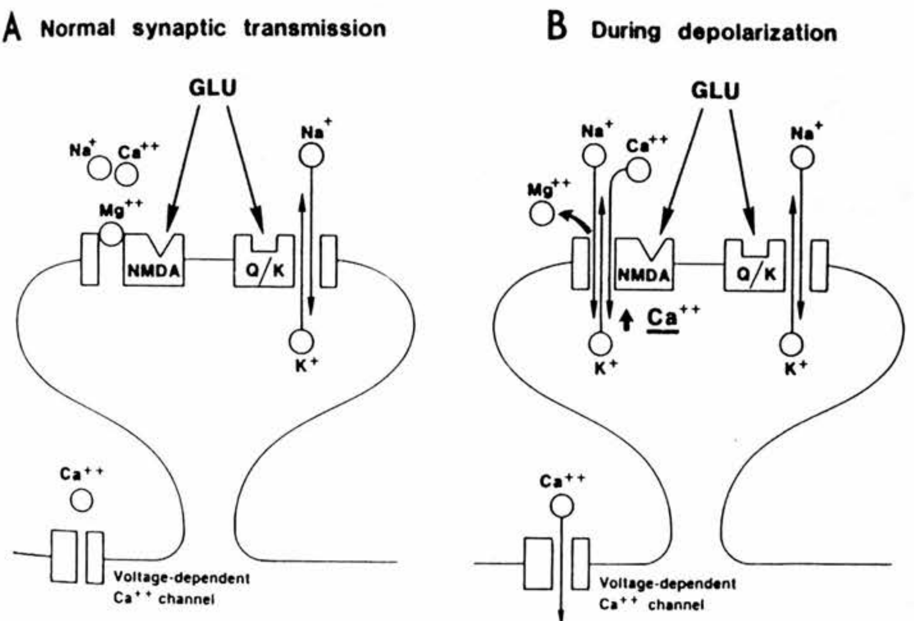


Fig. 2.4
Contrasting properties of NMDA and AMPA (Q/K) receptors which are co-localised on the post-synaptic terminal. A - The events occurring during low frequency synaptic transmission. B - The events occurring when when the post-synaptic membrane is depolarised, as would occur during a high-frequency tetanus. (*From* Nicoll et al., 1988).

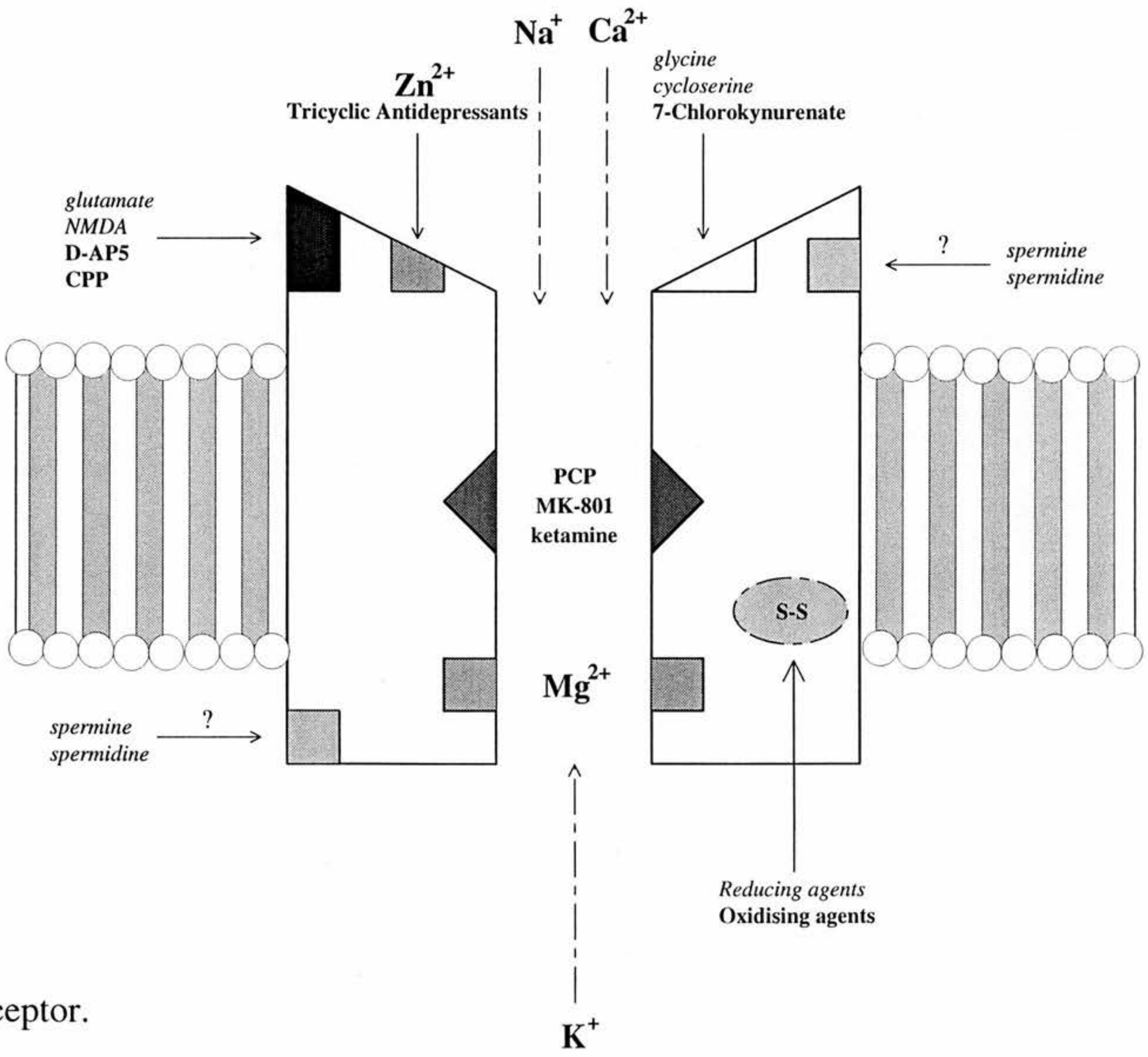


Fig. 2.5

The NMDA Receptor.

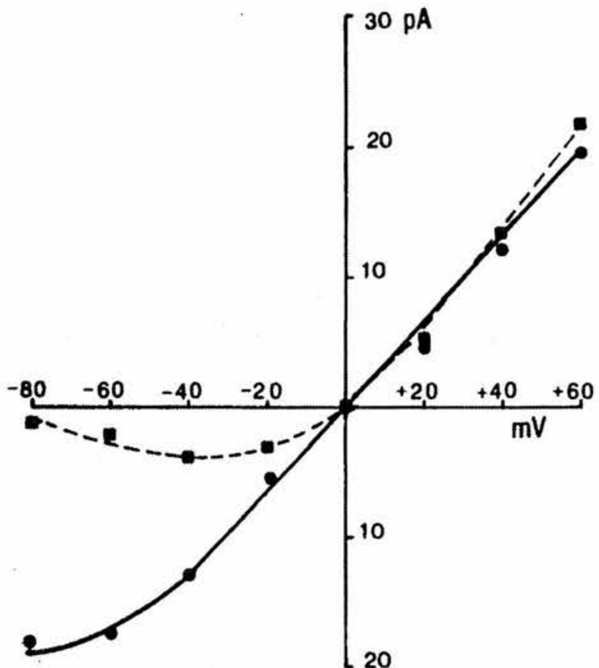


Fig. 2.6
The current-voltage relationship for the NMDA receptor. Glutamate-induced currents in the whole-cell recording mode. The glutamate-activated current was calculated at each potential as the difference between the current measured in control (Mg^{2+}-free or 500 μM Mg^{2+}) solutions and after addition of glutamate (10 μM). In a Mg^{2+} free solution (circles), the I-V relationship is approximately linear whereas in a Mg^{2+}-containing solution the I-V relationship shows a 'negetive resistance' region. (*From* Nowak et al., 1984).

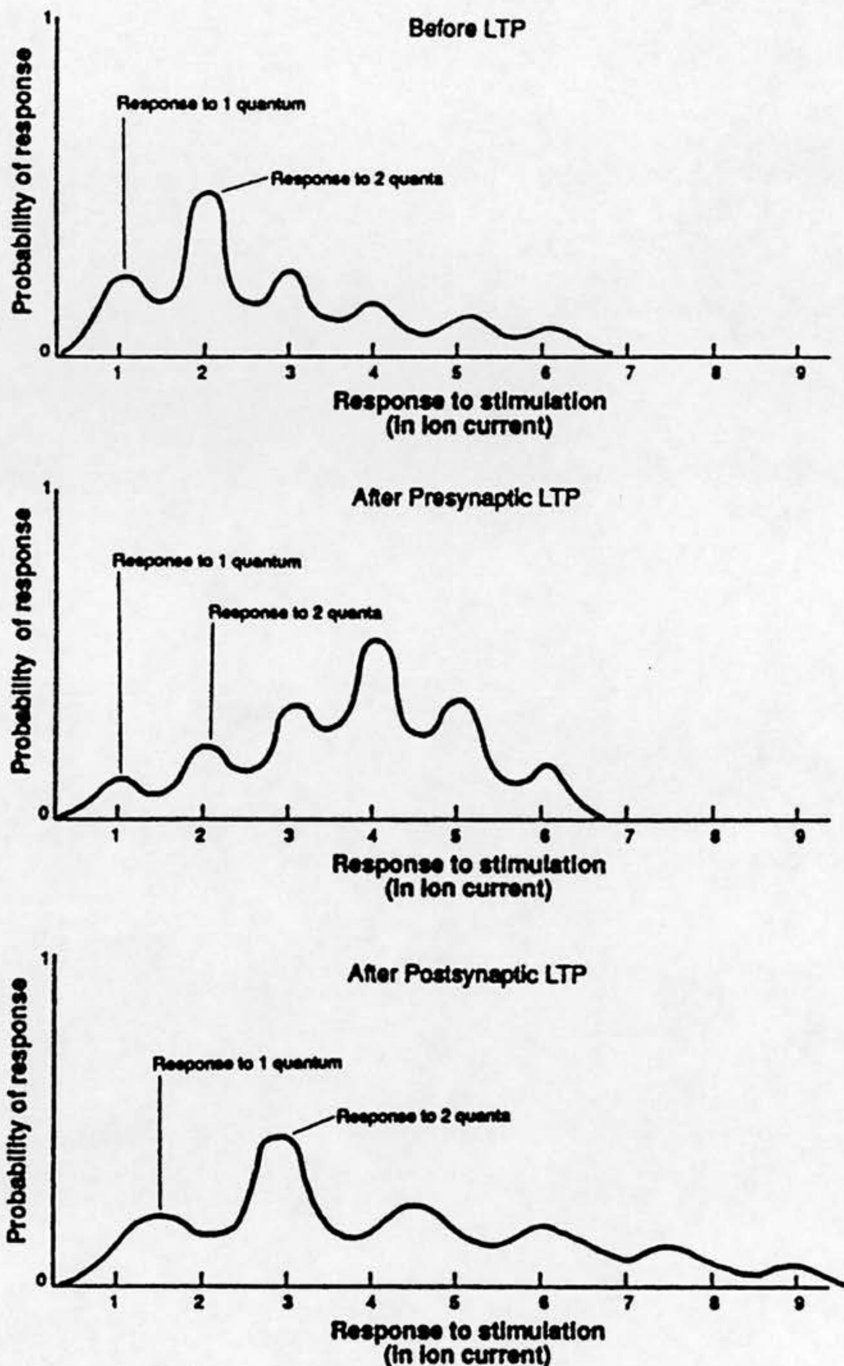


Fig. 2.7

These three idealised curves show how quantal analysis can be used to determine whether LTP is due to pre-synaptic or post-synaptic modification. The y-axis of each curve shows the frequency with which a certain number of quanta are released from the pre-synaptic neuron. The x-axis shows the response to that number of quanta. (*From* Barinaga, 1990).

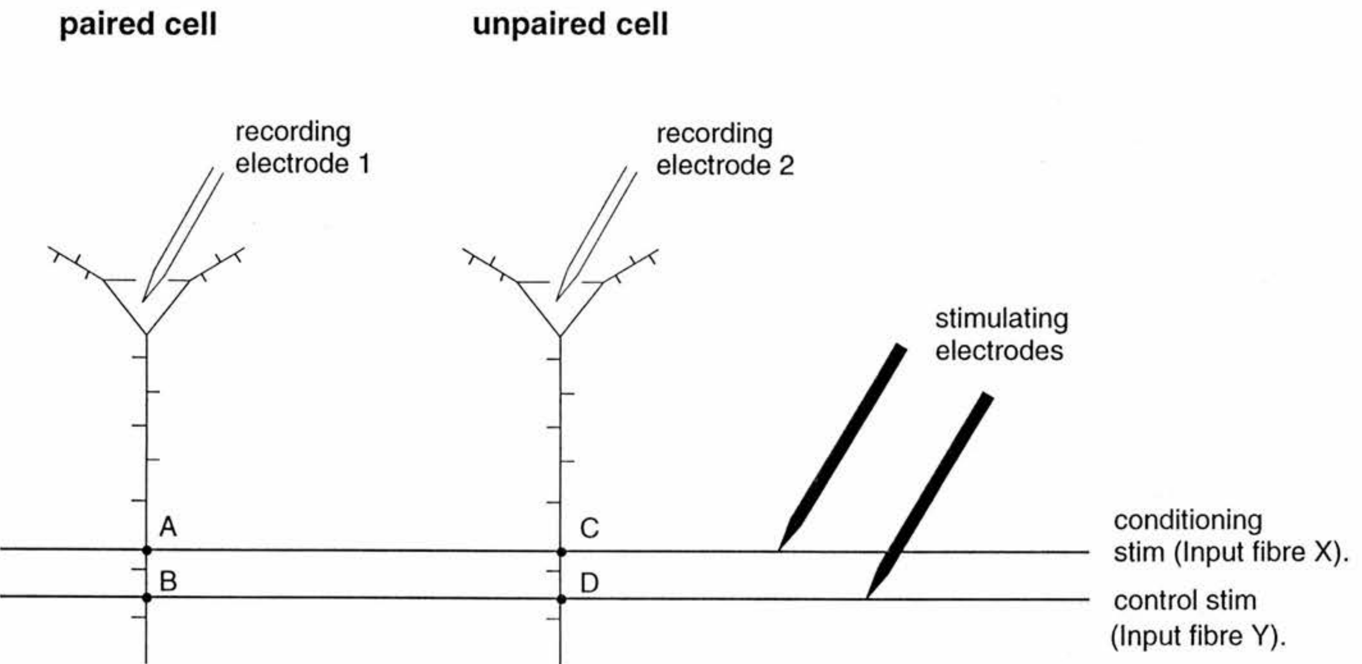


Fig 2.8

The synapse specificity of LTP induction is not maintained along the pre-synaptic input fibre. Potentiation is observed at both synapses A and C, but not at synapse B (reconstructed from Bonhoeffer et al., (1990) Cold Spring Harbour Symposia on Quantitative Biology, Volume LV).

Table 2.1 Long-Term Potentiation in the Mammalian CNS

Region	Reference
Hippocampus	
Perforant path - granule cells	Bliss and Lømo, 1973
Mossy fibre - CA3	Harris and Cotman, 1986 Zalutsky and Nicoll, 1990
Schaffer collateral - CA1	Schwartzkroin and Wester, 1975 Bliss et al., 1983
Commissural - CA1	Buzsaki 1980 Bliss et al., 1983
Perforant path - CA1	Doller and Weight, 1985
Visual Cortex	Artola and Singer, 1987
Pyriform Cortex	Stripling and Patneau, 1985
Neocortex	Komatsu et al., 1981 Lee 1982 Wilson and Racine, 1983 Sakimoto et al., 1986 Bindman et al., 1987
Amygdala	Racine et al., 1983 Chapman et al., 1990 Clugnet and Le Doux, 1990
Septal area	Racine et al., 1983
Subiculum	Racine et al., 1983
Entorhinal cortex	Racine et al., 1983
Cerebellum	Racine et al., 1986
Medial geniculate nucleus	Gerren and Weinberger, 1983
Superior cervical ganglion	Briggs et al., 1985

Table 2.2 - Quantal Analysis of Hippocampal Long-Term Potentiation

Voronin (1983)	*Neurosci 10;1051*	Pre-synaptic
Malinow and Tsien (1990)	*Nature 346;177*	Pre-synaptic
Bekkers and Stevens (1990)	*Nature 346;724*	Pre-synaptic
Foster and McNaughton (1991)	*Hippocampus 1;79*	Post-synaptic
Malinow (1991)	*Science 252;722*	Pre-synaptic
Baskys et al., (1991)	*Neurosci Letts 127;169*	Pre-synaptic
Voronin et al., (1992)	*Expl Brain Res 89;288*	Pre-synaptic
Kullmann and Nicoll (1992)	*Nature 357;240*	Pre and Post
Larkman et al., (1992)	*Nature 360;70*	Pre and Post
Liao et al., (1992)	*Neuron 9;1089*	Pre and Post

Chapter 3 - General Methods

Subjects

Male Lister-hooded rats (200-600 g) obtained from the breeding colony in the Department of Pharmacology, University of Edinburgh, were used in all experiments. They were individually housed with *ad libitum* access to food and water. A 12 hour light/dark cycle was maintained (0700-1900) with all testing carried out during the "light" phase. All rats were weighed daily throughout all experiments.

Data Analysis

All data are analysed using the statistical packages *CLR ANOVA* and *Statview* (Mackintosh), and are expressed throughout this thesis as mean ± S.E.M., both in the text and graphically. The Tukey's Honestly Significant Difference (HSD) test is used as a conservative post hoc comparison throughout the data analysis.

Behavioural Methodology

Watermaze Apparatus

All behavioural testing was carried out in an open field watermaze (Morris, 1981,1984; Figure 3.1) consisting of a large circular tank (diameter 2.0 m, depth 0.6 m) containing water at 25 ± 1°C to a depth of 0.3m. The water was made opaque by the addition of powdered milk which not only prevents the animal from seeing the submerged escape platform but also allows efficient tracking of swim paths. The pool was located in the centre of a room containing various prominent cues (wall posters, wall cupboards, a rig of electrophysiological equipment, a set of white curtains and a large metal frame). The room was diffusely illuminated by 4 floodlights located in the 4 corners of the room. The swim paths taken by the animals in the pool were monitored by a video camera mounted in the ceiling. The resulting video signal was relayed to a video recorder allowing both on- and off-line analysis and from there to an image analyser (HVS VP112). The x and y co-ordinates of the rats' position were sampled at 10 Hz by an Archimedes computer (using specialised software called "Watermaze" written by R.Spooner) and stored on disc. This program can provide measures of latency, pathlength, swim speed, annulus crossings (the number of times the rat crosses the former platform location during a "transfer test") and the distribution of time spent in a defined region of the pool (eg. quadrant, annulus from the side

wall).

In certain experiments, the rats were trained in 2 separate watermazes located in 2 different rooms. Although the layouts of the 2 rooms were similar, they contained distinguishable extramaze cues creating 2 distinct spatial environments.

Non-Spatial Pretraining

All rats (excepting those in the visual discrimination study - Expt. 6.4) received 1-3 days of non-spatial pre-training. This consisted of a certain number of trials during which the extramaze cues were obscured from view by pulling a set of white curtains around the pool. A hidden escape platform (diameter 10 cm), submerged 1-1.5 cm below the water surface, was moved randomly to a different position in the pool between trials. The rat was placed in the water, facing the side wall, at a random start position and allowed to swim freely around the pool. Rats which failed to find the platform after a pre-determined time period (60 or 120 sec) were guided to its location; they were then allowed to stay on the platform for 30 sec between trials. This pre-training was intended to allow the animals to become accustomed to the apparatus under conditions which prevented any spatial learning but allowed them to learn that there was a means of escape from the water via the platform. Pre-training was conducted in the absence of any drug, prior to any surgery and before the start of spatial training.

Spatial Learning Tasks

In spatial learning tasks, the white curtains were pulled back, exposing the extramaze cues. The rats were trained to find the hidden escape platform (see above) whose location was now fixed. The animals were placed in the pool, from a random start position, facing the side wall (nominally N,S,E or W) and allowed to swim freely until they located the platform or until 120 sec after the start of the trial at which point (when necessary) they were guided onto the platform. They were then allowed to remain on the platform for 30 sec. A variety of training paradigms were used throughout this thesis varying the number of trials per day, the inter-trial interval (ITI) and the number of days of training.

Performance was generally assessed in terms of escape latency during acquisition and by means of a 'transfer test' (probe trial, extinction trial) during which the platform was removed from the pool and the rat allowed to swim freely for 60 sec. The amount of time a rat spent

searching in the quadrant of the pool in which the platform had been located (the 'training' quadrant) provided a good measure of its memory of the platform position. During the transfer test, the number of times the rat crossed the former platform location (annulus crossings) was also recorded, providing an additional index of performance for some experiments.

Cue Task

In certain experiments, the rats were also trained on a 'cue' task during which they were required to escape from the water by climbing onto a visible grey platform (diameter 10 cm, extending 1-1.5 cm above the water surface). Both the platform location and the rats' starting position were randomly changed from one trial to the next. The white curtains were pulled around the pool to obscure the extramaze cues. The 'cue' task is a relatively quick and simple control task to determine if there are any gross sensorimotor or motivational effects resulting from a drug treatment or brain lesion. It is important to appreciate that it is not a measure of visual discrimination learning capacity.

Visual Discrimination Task

An additional control task involved training the animals to discriminate between two distinguishable platforms (diameter 10 cm), whose visible surfaces were 1-1.5 cm above the water surface, in order to escape from the pool. One of these platforms (grey or black-and-white stripes) was rigid and provided an escape from the water; the other was floating and offered insufficient bouyancy to support a rat. The floating platform was tied to a weight resting on the bottom of the pool in order to maintain its position (Figure 3.2). The white curtains were pulled around the pool to occlude the extramaze cues. The animals received 10 trials per day, with an ITI of 5-10 min, until a pre-determined criterion level of performance (90% correct choices over 2 consecutive days; 18 out of 20) had been attained. On each trial, the rat was placed into the water facing the sidewall at a point across the pool from the two visible platforms; these were moved randomly around the pool between trials in order to vary the spatial location of the rewarded platform. In addition, a pseudorandom sequence was used to alternate the left/right orientation of the rewarded/non-rewarded platforms. In a final test, conducted the day after reaching criterion, the rats were presented with 2 visibly identical platforms, only one of which was rigid and a means of escaping from

the water. If the rats were attending to the visual appearance of the platfoms to solve the task, then performance should fall to chance.

Surgery

Implantation of Alza osmotic minipumps

As D-AP5 and 7CK do not readily cross the blood brain barrier, both drugs must be infused directly into the brain. The technique used throughout this thesis involved chronic infusion via a surgically implanted osmotic minipump in order to attain sufficiently high levels of the drug within the brain. The rats were anaesthetised with tribromoethanol (Avertin - 0.29 g/kg) and placed in a stereotaxic frame (Kopf) with the skull horizontal. An inscision of the scalp was made along the midline to reveal the skull surface. An L-shaped stainless steel cannula (23 gauge) was lowered into the right lateral cerebral ventricle (AP from Bregma = 0.9 mm; ML = 1.3 mm; DV = 4.5 mm from skull surface) and secured by means of acrylic dental cement and 3 small watchmakers screws. A small length (3 cm) of Silicone rubber tubing connected the cannula to an Alza osmotic minipump (Model 2002) which was implanted, subcutaneously, between the scapulae. The minipump enables the drug or vehicle (eg. aCSF) to be infused continuously at a rate of 0.5 µl/hr for a period of up to 14 days. In certain experiments, electrophysiological recordings were made in animals with osmotic minipumps in place. During implantation of the minipump, the positions for subsequent acute electrode placements were marked on the skull surface (see Electrophysiological Methodology below). The rats were then left for 2 days to recover from the acute effects of surgery and to allow the intracerebral drug levels to reach a steady state before the start of behavioural testing.

7CK is rapidly excreted from the brain and consequently there is an assymetrical distribution of drug levels between the two hemispheres of the brain (see Chapter 4). It was, therefore, necessary to make electrophysiological recordings from the same side of the brain as the infusion cannula. In order to do this a 'double-L' shaped cannula was lowered into the left lateral ventricle and cemented to the right side of the skull. Electrophysiological recordings could then be made from the left hemisphere.

Excitotoxic lesions of the hippocampus

The procedure used to kill cells in the hippocampus was similar to the technique reported by

Jarrard (1989). The rats were anaesthetised with tribromoethanol (Avertin - 0.29 g/kg) and placed in a stereotaxic frame (Kopf). An incision of the scalp was made along the midline and the bone overlying the neocortex was removed. Injections were made with a 1 μl Hamilton syringe mounted on the stereotaxic frame. Ibotenic acid (Tocris Neuroamin) was dissolved in phosphate-buffered saline (pH 7.4) at a concentration of 10 mg/ml. Injections of ibotenic acid (0.05-0.10 μl) were made over approximately 30 seconds at each of 26 sites (for the stereotaxic co-ordinates of the injection sites, see Jarrard, 1989). Rats in the sham operated control group underwent a similar surgical procedure with the exception that no ibotenic acid was injected and the needle tract limited to the overlying cortex.

Electrophysiology

All electrophysiological experiments were performed under urethane anaesthesia (1.5 g/kg) with the rat mounted in a stereotaxic frame (Kopf) with the skull horizontal. The animal's temperature was monitored by a rectal probe and maintained at 36.2 ± 0.2°C using an isothermic heating blanket (Harvard Apparatus). Teflon coated stainless steel electrodes (Goodfellow 75 μm) were lowered into the hippocampal formation in order to record positive-going field-potentials. A bipolar stimulating electrode was positioned in the angular bundle of the perforant path (AP = -7.5 mm, LAT = 4.1 mm, DV = approx. 2.0 mm) and a monopolar recording electrode (AP = -3.8 mm, LAT = 2.2 mm, DV = approx. 2.8 mm) in the hilus of the dentate gyrus. After optimising the electrode positions in order to maximise the size of the field epsp, the animal was left for at least 30 mins (and more commonly for an hour) without any stimulation to ensure a stable baseline by allowing the tissue to recover from the electrode implantation. Perforant path test pulses were delivered at low frequency (0.1 or 0.05 Hz) and consisted of either monophasic (100 μs pulse duration) or biphasic (100 μs half-pulse duration) pulses at a stimulus intensity of 300 - 700 μA. Stimulation was controlled by a Neurolog system capable of delivering either single pulses or trains of pulses via a stimulus isolator. The field epsps were amplified using a polygraph (Grass Instruments) and monitored on-line by computer (either a MINC 11/23 or an Acorn Archimedes 5000) and stored on disc. In all experiments, the initial slope of the field epsp (measured using linear regression between 2 fixed time-points) was used as the main measure of the evoked response. The amplitude of the population spike (measured between the trough of the negative-going spike and a tangent subtending the two local maxima of the field-potential) was also recorded and will be reported for some experiments.

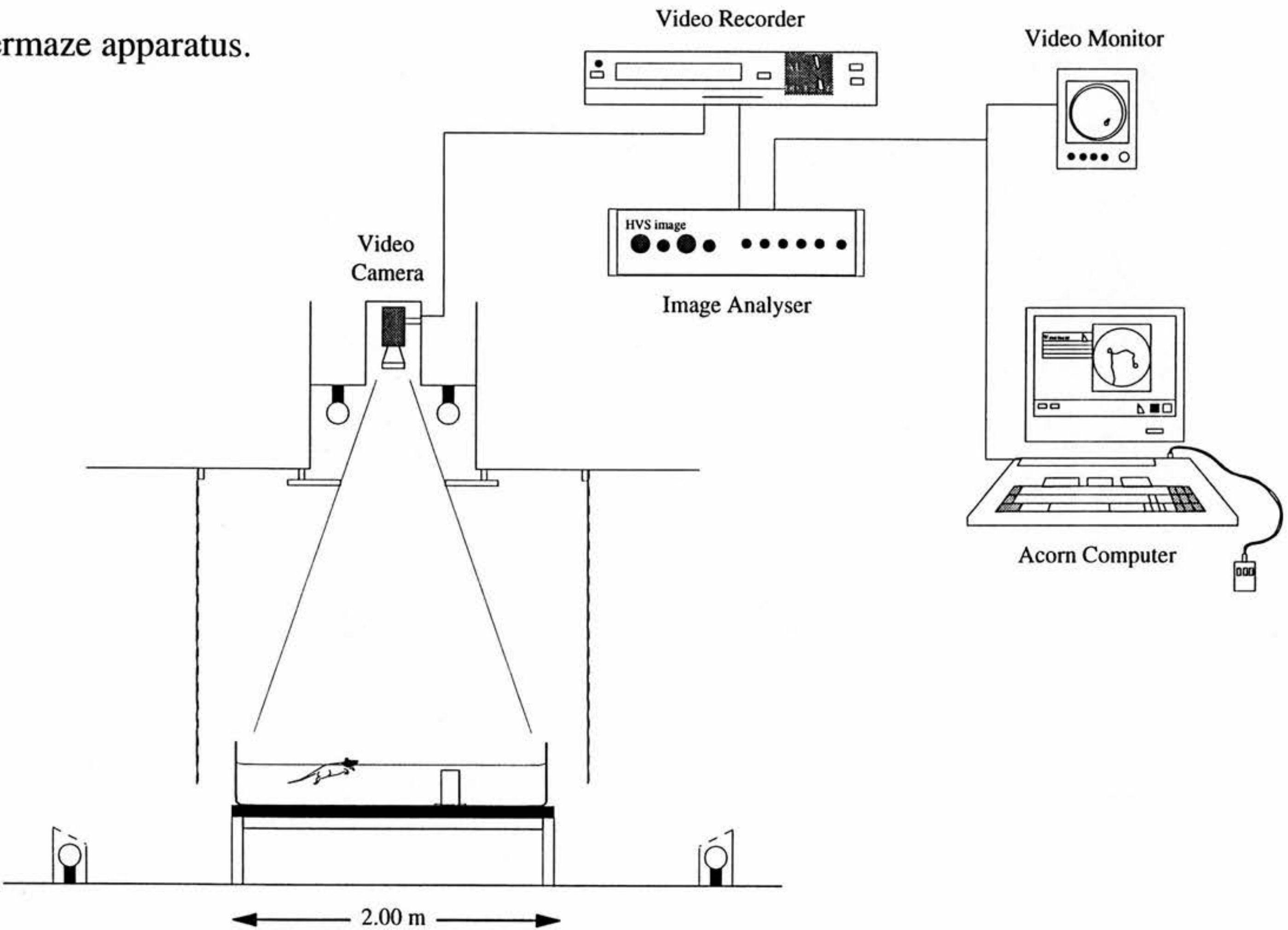
In most electrophysiological experiments, the aim was to compare the amount of LTP that could be induced in different groups of animals. Following a baseline period of low frequency stimulation (10-20 min), an attempt was made to induce LTP by delivering a brief period of high frequency stimulation (a tetanus). In most experiments, this consisted of 4 trains of 33 pulses at 250-400 Hz, at a stimulus intensity of 700 μA with an inter-train interval of 10-20 seconds. These tetanus parameters reliably produce considerable and robust LTP. In some cases, however, it is more desirable to use weaker stimulation parameters, closer to the threshold for LTP induction (ie. parameters that produce a minimal but measureable amount of LTP). Accordingly, a 'weak' tetanus consisting of less trains (3), fewer pulses (25), a lower tetanic frequency (100 Hz) and smaller stimulus intensity (300-600 μA) was used in some experiments. After the tetanus, low frequency stimulation resumed and responses were typically monitored for at least an hour. LTP is expressed as the percentage increase in the size of the initial slope of the field epsp at a set time point post-tetanus relative to the pre-tetanus baseline period. Immediately before and after this 'main series', an input/output (I/O) function was constructed during which a series of low-frequency test pulses were delivered (eg. 4 pulses at each stimulation intensity from 0-1000 μA, 0.1 or 0.05 Hz).

Histology

At the end of behavioural testing, rats with hippocampal lesions were injected with Euthetal (200 mg/kg sodium pentobarbital) and perfused transcardially with physiological saline and 10% formol saline. Their brains were removed and placed in formol saline solution. Subsequently, the brains were embedded in egg yolk, soaked in a succrose-formalin solution for 24 hr, frozen, and then sectioned in the horizontal plane (30 μm sections). In Expt 4.2 a cresyl violet stain was used to determine cell loss and selected sections were stained according to the Fink-Heimer procedure (Fink and Heimer, 1967). In Expt 7.2, the sections were stained with thionine.

Rats implanted with minipumps were killed at the end of electrophysiological testing and their brains removed on ice. Having removed tissue samples for the analysis of drug levels (D-AP5 or 7CK), a coronal wedge of tissue, taken from the region immediately adjacent to the infusion cannula, was removed, retained in formalin, frozen, cut in 30 μm coronal sections and stained with cresyl violet. This allowed histological determination of the cannula position and assessment of any damage caused by the cannula and/or infusion.

Fig. 3.1
The watermaze apparatus.



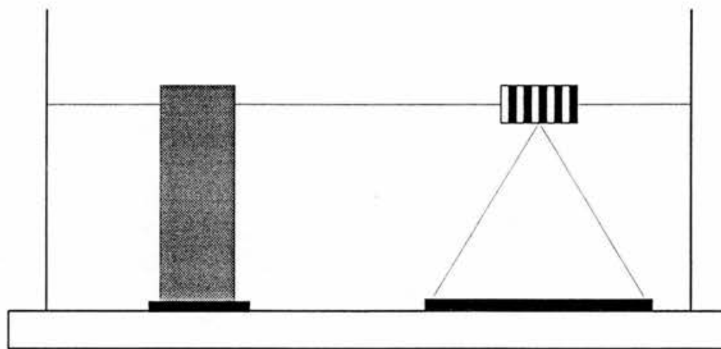


Fig 3.2

Visual discrimination in the watermaze. The rats are trained to discriminate between two visible platforms, one of which is rigid and provides a means of escape; the other is floating and offers insufficient bouyancy to support the rat.

Chapter 4 - The effects of agonists and antagonists of the NMDA receptor associated glycine site on LTP *in vivo* and spatial learning.

The importance of NMDA receptors for the induction of LTP has been discussed. A variety of competitive and non-competitive NMDA antagonists have been found to block the induction of LTP (Collingridge et al., 1983; Stringer and Guyenet, 1983; Stringer et al., 1984; Abraham and Mason, 1988; Swartzwelder et al., 1989). The glycine modulatory site on the NMDA receptor complex provides an alternative means of manipulating the inducibility of LTP. Before describing the experiments in which glycine site ligands have been used to investigate the role of NMDA receptor-dependent mechanisms in spatial learning, a brief review of glycine neurotransmission in the CNS is provided. This review is intended for the interested reader as a background to the experiments included in this chapter.

Glycine as a neurotransmitter in the CNS

Glycine, in terms of structure, is the simplest of all the amino acids. Glycine is a neutral molecule which is found in all body fluids and tissue proteins in large amounts, and is a common intermediate in many metabolic processes. A role for glycine as an inhibitory neurotransmitter in the brain stem and spinal cord has been well documented (Curtis et al., 1968; De Feudis et al., 1978; Snyder and Bennet, 1976). For example, glycine is the neurotransmitter released by 'Renshaw cells', a population of inhibitory interneurones found in the ventral root of the spinal cord (Davidoff et al., 1967; Werman et al., 1967). The inhibitory action of glycine is blocked by the convulsant drug, strychnine (Curtis et al., 1968; De Feudis et al., 1978; Graham et al., 1985), and is mediated by an increase in post-synaptic chloride conductance (Hamill et al., 1983; Bormann et al., 1987). The activation of inhibitory glycine receptors hyperpolarises the neuron and thus moves the membrane potential away from the firing threshold.

Autoradiographic studies revealed that the distribution of binding sites labelled by the antagonist, [^{3}H]-strychnine, did not strictly match the pattern of receptors labelled by [^{3}H]-glycine (De Feudis et al., 1978; Kishimoto et al., 1981; Bristow et al., 1986). These studies showed that while strychnine-sensitive binding sites predominate in the brain stem and spinal cord, there are a large number of strychnine-insensitive glycine binding sites especially in the forebrain. The reason for this anomaly became apparent following an accidental discovery by Johnson and Ascher (1987).

The NMDA receptor-associated glycine site

Johnson and Ascher (1987) were recording whole cell currents in cultured neurones in response to the application of NMDA. They noticed that the size of the NMDA response was dependent upon the rate at which the drug solution was delivered. More specifically, the slower the perfusion rate, the larger the NMDA response. They reasoned that the cultured cells, either the neurones themselves or glial cells, were releasing some factor which was capable of augmenting the NMDA response. At slower perfusion rates this factor was allowed to accumulate to a level sufficient to potentiate the NMDA response. In order to test this hypothesis, Johnson and Ascher harvested the 'conditioned medium' in which the cultured cells were kept and applied this, along with NMDA, to a whole cell patch preparation. The conditioned medium significantly enhanced the NMDA response but had little, if any, effect when applied alone. In an attempt to identify the causative agent, the conditioned medium was then extensively analysed. The potentiating factor was found to be resistant to heating at 90°C for 10 minutes and could pass through dialysis tubing of a pore size that indicated a relative molecular mass of less than 700. This suggested an amino acid and a systematic investigation of the effects of numerous amino acids on the NMDA response revealed that glycine was the most likely candidate. Glycine augmented the NMDA response at concentrations equivalent to those accumulating in the conditioned medium. Furthermore, when cells were perfused with a solution containing saturating levels of glycine, the size of the NMDA response was independent of the perfusion rate. The potentiation occured at glycine concentrations as low as 10nM and saturated at micromolar levels. This effect was not blocked by strychnine and was specific to the NMDA response, with no potentiation of responses to either kainate or quisqualate.

This discovery prompted Bowery (1987) to write a letter to *Nature* in which he put forward further, compelling evidence for a glycine binding site associated with the NMDA receptor. Comparing previously published autoradiographic studies, Bowery highlighted the strong correlation between the localisation of supraspinal, strychnine-insensitive [^{3}H]-glycine binding sites (Bristow et al., 1986) and NMDA-displacable [^{3}H]-glutamate binding sites (Monaghan and Cotman, 1985). In a subsequent study, Bowery showed that the distribution of strychnine-insensitive glycine binding sites was also similar to that of the non-competitive NMDA antagonist, MK-801, with the highest density in cortex, hippocampus and striatum (Bowery and Hudson, 1986; Bowery et al., 1988).

These results suggested that there is a glycine binding site associated with the NMDA

receptor complex through which glycine, or a glycine-like compound, can enhance NMDA receptor ion channel activity. It is thought that the NMDA receptor complex possesses several ligand binding sites which are linked together by a series of complex, reciprocal, allosteric interactions through which the binding of a ligand at one site can influence the affinity for ligands at other binding sites on the receptor complex (Foster and Fagg, 1987). This hypothesis is supported by the fact that concentrations of glycine, equivalent to those shown by Johnson and Ascher (1987) to enhance the electrophysiological response, can alter the binding properties of other sites on the receptor complex and vice-versa. Glycine and other glycine agonists, such as D-serine, enhance the binding of L-glutamate and NMDA while reducing the binding affinity of competitive NMDA antagonists (Fadda et al., 1988; Compton et al., 1990). A reciprocal relationship is maintained with L-glutamate and NMDA increasing glycine binding affinity (Kessler et al., 1989). This effect is reversed by NMDA antagonists such as AP5 and CPP. Furthermore, both Ca^{2+} and Mg^{2+} ions also enhance strychnine-insensitive glycine binding (Marvizón and Skolnick, 1988). The glycine mediated enhancement of L-glutamate binding appears to occur through a competitive mechanism, with an increase in the binding affinity of the NMDA receptor (decreased K_d) but no change in the number of receptor sites (B_{max}). Glycine also facilitates NMDA-induced stimulation of binding to PCP receptor sites, located deep in the ion channel (Fagg and Baud, 1988). In well washed cortical membrane preparations, glycine can cause up to a four-fold enhancement of NMDA-induced stimulation of [^{3}H] MK-801 or [^{3}H] TCP binding (Bonhaus et al., 1987; Reynolds et al., 1987; Snell et al., 1987; Wong et al., 1987; Bénavidès et al., 1988; Johnson et al., 1988; Ransom and Stec, 1988; Thomas et al., 1988; Monahan et al., 1989a; Sircar et al., 1989). The presence of L-glutamate or NMDA is an absolute requirement for glycine enhancement and competitive NMDA antagonists, such as AP5 and CPP, completely block any effect. The observation that glycine enhances [^{3}H] MK-801 binding in solubilised membrane preparations, implies that the glycine receptor is an integral part of the NMDA receptor complex (McKernan et al., 1989).

Pharmacology of the glycine site

Following the discovery that glycine potentiated the actions of NMDA through a strychnine-insensitive mechanism (Johnson and Ascher, 1987), the search began for selective antagonists of this novel receptor site. The first compound to be identified as a potential antagonist was the tryptophan metabolite, kynurenate. For several years kynurenate had

been considered as a 'broad spectrum' EAA antagonist, blocking both NMDA and non-NMDA receptor mediated responses (Perkins and Stone, 1982; Ganong et al., 1983). With the observation that kynurenate competitively reduced strychnine-insensitive [^{3}H] glycine binding in rat forebrain membranes (Kessler et al., 1989), it seemed reasonable to suggest that this compound was inhibiting NMDA responses through an action on the glycine site. This was supported by the demonstration that the kynurenate inhibition of NMDA responses was reversed by glycine (Birch et al., 1988a, 1988b; Watson et al., 1988; Bertolino et al., 1989; Fletcher et al., 1989). Kynurenate is found in the brain at sub-micromolar concentrations, leading to suggestions that it may have an endogenous role as an anti-convulsant or neuroprotective agent (Moroni et al., 1988; Swartz et al., 1990).

The lack of specificity exhibited by kynurenate means that this drug is of limited use as an experimental tool for studying the glycine receptor. For example, several groups have shown that in the presence of saturating concentrations of glycine, kynurenate still has a residual inhibitory effect on the NMDA response which suggests an additional direct action at the NMDA/glutamate recognition site (Evans et al., 1987; Kemp et al., 1987b; Burton et al., 1988; Fletcher et al., 1989). Ascher and co-workers have studied kynurenate antagonism using the whole-cell voltage clamp technique with cultured mouse cortical neurones (Ascher et al., 1988). Low concentrations of kynurenate (3-30 μM) inhibit the NMDA response in a manner which is competitive with respect to glycine and non-competitive with respect to NMDA, with no reduction of the maximal NMDA response. At concentrations above 100 μM, however, kynurenate depressed the maximal NMDA response. This confirms the fact that at higher concentrations there is a direct action on the NMDA/glutamate site and specificity is lost. Consequently, a great effort has been made to develop more potent and selective antagonists.

Using kynurenate as the parent molecule, a number of compounds were synthesised with a view towards finding more selective antagonists (Kemp et al., 1987a). The most successful of these has proved to be the chlorinated derivative, 7-chlorokynurenate (7CK; Kemp et al., 1988). In rat cortical slices, 7CK (10-100 μM) potently inhibits the depolarisation evoked by application of NMDA, with a 100 μM dose producing almost a complete block of the NMDA response. This antagonism is non-competitive with respect to NMDA but is competitive with respect to glycine. The 7CK inhibition of the NMDA response is almost completely reversed by co-administration of either glycine or D-serine (both 100 μM). This is in contrast to the parent compound, kynurenate (Evans et al., 1987; Kemp et al., 1987b;

Burton et al., 1988; Fletcher et al., 1989) and suggests that the side effects of 7CK on the NMDA/glutamate recognition site are minimal. Furthermore, in whole-cell patch clamp recordings made from cultured cortical neurones, 7CK completely abolishes the NMDA/glycine mediated response. This effect can be reversed by increasing the glycine concentration but not the NMDA concentration. Radioligand binding studies have confirmed the improved selectivity of this compound (Kemp et al., 1988). In rat cortical membrane preparations, the binding affinity of 7CK for the strychnine-insensitive [^{3}H]-glycine binding site (IC_{50} = 0.56 μM) is considerably greater than for NMDA (IC_{50} = 169 μM), AMPA (IC_{50} = 153 μM) or kainate sites (IC_{50} > 1000 μM). In contrast, the IC_{50} for the inhibition of [^{3}H] glycine binding by kynurenate is 41 μM. Estimates, made on the basis of these binding studies, suggest that the 7-chloro substitution of kynurenate results in a selective 70-fold increase in affinity for the glycine site (Kemp et al., 1988), providing a potent and selective experimental tool for blocking this receptor.

A number of amino acids, in particular serine and alanine, can mimic the actions of glycine at the NMDA receptor (Bonhaus et al., 1987; Fadda et al., 1988; Ransom and Stec, 1988; Snell et al., 1988). Interestingly, the order of relative potency displayed by these amino acids at the NMDA receptor-associated glycine site is different from the order of relative potency at the strychnine-sensitive site. In addition, the action of serine and alanine at the NMDA receptor-associated glycine site shows a marked stereoselectivity, in direct contrast to the action of these compounds at the strychnine-sensitive site. Although less potent, D-serine is often chosen as the 'applied agonist' in preference to glycine because it has less affinity for both the strychnine-sensitive receptor and glycine uptake mechanisms, and is, therefore, more selective.

The categorisation of glycine site ligands as either agonists or partial agonists has proved difficult. Because all these compounds are less efficacious than glycine itself, the effect they produce when applied to a preparation will depend upon the existing concentration of glycine present. For example, consider an amino acid which can activate the receptor but is less potent than glycine. In the presence of low levels of glycine, this amino acid will act as an agonist and enhance the NMDA response (eg. enhance glycine-induced [^{3}H] TCP binding). At higher glycine concentrations, this compound now competes with glycine for the binding sites and, being less efficacious, reduces the effect of the full agonist (eg. reduce glycine-induced [^{3}H] TCP binding). In this situation the amino acid is now acting as a weak antagonist. These compounds are commonly referred to as 'partial agonists' and are

exemplified by the cyclic analogue of glycine, D-cycloserine (DCS; Hood et al., 1989; Watson et al., 1990). In the absence of glycine, DCS produces a dose-dependent enhancement of [^{3}H] TCP binding to rat forebrain membrane preparations (Hood et al., 1989). Similarly, DCS enhances NMDA responses during electrophysiological recording from the *Xenopus* oocyte preparation (Watson et al., 1990). Both studies show that the maximal enhancement elicited by DCS is only 40-50% of the maximal enhancement produced by glycine. At glycine concentrations below 0.1μM, DCS appears to act as an agonist. At higher glycine concentrations, however, DCS causes a dose-dependent reduction of the glycine-mediated enhancement.

Is glycine a co-agonist or a modulator of the NMDA receptor?

The demonstration that the selective glycine antagonist, 7CK, completely blocks the NMDA response in both cortical slices and whole cell patch clamp recordings from cultured cortical neurones (Kemp et al., 1988) calls into question the idea that glycine is simply a modulator of the NMDA response. In fact these results suggest that there is an absolute requirement for glycine which acts as a co-agonist at the NMDA receptor complex. The major problem in distinguishing between these two possibilities is that in nearly all preparations there will be at least a small but significant basal concentration of glycine present which can provide tonic activation of the receptor. This may be as little as 10 nM in a rapidly superfused single cell preparation (Ascher and Johnson, 1989) but may reach micromolar levels *in vivo* (Ferraro and Hare, 1985; Skilling et al., 1988). In order to circumvent this problem, Kleckner and Dingledine (1988) used the *Xenopus* oocyte preparation to study the effects of glycine on NMDA responses. Injection of rat brain mRNA into the frog egg results in expression of NMDA receptors which possess all the normal characteristics of the neuronal receptor (Verdoorn et al., 1987). This provides a glycine free environment for studying the NMDA response. Voltage clamp recordings made from the oocyte preparation revealed that in the absence of added glycine there was no response to NMDA, implying that glycine is, in fact, a co-agonist at the NMDA receptor and that there is an absolute requirement for this amino acid transmitter.

Mode of action

The mechanism by which glycine promotes NMDA receptor/channel activity is not fully

understood. Glycine enhances the binding of L-glutamate or NMDA to the receptor and simultaneously reduces the binding of NMDA antagonists (Fadda et al., 1988; Compton et al., 1990). This is brought about through a competitive mechanism, involving an increase in the binding affinity (K_d) with no change in the number of binding sites (B_{max}). It is thought that the glycine site and the NMDA/glutamate site are allosterically linked and that binding of glycine induces a conformational change that increases the affinity for the agonist at the glutamate recognition site.

Radioligand binding studies comparing L-[^{3}H] glutamate and [^{3}H] CPP binding have provided evidence for two anatomically distinct forms of the NMDA receptor, an 'agonist-preferring' form and an 'antagonist-preferring' form (Monaghan et al., 1988). The 'agonist-preferring' sites predominate in the striatum and septum whereas the 'antagonist-preferring' receptors are more numerous in the thalamus and cerebral cortex. Apart from the relative affinities for agonists and antagonists, the pharmacological profiles of the two sites are very similar. Monaghan and co-workers have shown that glycine causes a greater percentage increase in L-[^{3}H] glutamate binding in areas where the antagonist-preferring form is more common, namely in the thalamus and cerebral cortex. They hypothesised that glycine was enhancing the NMDA response by converting 'antagonist-preferring' receptors to 'agonist-preferring' receptors. This is consistent with the binding studies that showed an increase in glutamate or NMDA binding and a concomitant reduction in NMDA antagonist binding in the presence of glycine (Fadda et al., 1988; Compton et al., 1990). In order to test this hypothesis L-[^{3}H]-glutamate and [^{3}H]-CPP binding were compared in the presence of saturating concentrations of glycine (100 μM; Monaghan, 1990). If the hypothesis is correct then glycine should convert all the receptors into the 'agonist-preferring' form, resulting in a homogenous receptor population. In the presence of saturating concentrations of glycine, however, the distinct 'agonist-preferring' and 'antagonist-preferring' binding profiles were maintained. It would seem, therefore, that rather than converting 'antagonist-preferring' receptors to 'agonist-preferring' receptors, glycine enhances the binding affinity for glutamate of both receptor populations. A uniform increase in glutamate binding affinity will appear as a greater percentage increase in areas where the initial glutamate binding affinity was lower, namely in regions where the 'antagonist-preferring' receptors predominate.

An entirely different proposal is that glycine potentiates NMDA responses by accelerating the recovery of the NMDA receptor from desensitization (Mayer et al., 1989). Desensitization is defined as a decrease in response size following prolonged exposure to the

agonist and is thought to occur as a consequence of saturating intracellular calcium levels (Mayer and Westbrook, 1985). Using a fast perfusion system, Mayer and colleagues co-applied NMDA and glycine to cultured hippocampal neurones. Application of NMDA resulted in a desensitization, the onset of which was relatively independent of the glycine concentration. Increasing the glycine concentration (30-300nM) did, however, markedly enhance the rate of recovery from desensitization and resulted in a corresponding increase in the peak current size of the NMDA response. This raises the possibility that glycine might potentiate the NMDA response by facilitating the recovery of receptors from desensitization. There is disagreement, however, as to the extent to which such a mechanism might contribute to the glycine-mediated potentiation of NMDA responses. Other researchers have failed to see any effect of glycine on desensitization of the NMDA receptor (Ascher and Johnson, 1989) and it seems unlikely that a desensitization-based mechanism alone could account for the observed potentiation. For example, glycine potentiates NMDA responses at concentrations of NMDA or glutamate which are below the threshold for desensitization. Furthermore, the fact that there appears to be an absolute requirement for glycine (Kleckner and Dingledine, 1988) is difficult to reconcile with a mode of action which solely involves an increase in the rate of recovery from desensitization. It remains possible, however, that glycine contributes to NMDA receptor/ion channel activity in more than one way.

Physiological actions of glycine-site ligands

Glycine modulation of NMDA receptor function has now been observed in cortex (Thompson et al., 1989; Drejer et al., 1989), thalamus (Salt, 1989), striatum (Ransom and Deschenes, 1989), cerebellum (Danysz et al., 1989; Nicoletti and Canonico, 1989; Wroblewski et al., 1989), spinal cord (Birch et al., 1988a, 1988b), retina (Drejer et al., 1989) and in the myenteric plexus (Regianni et al., 1989). In hippocampus, glycine enhances the NMDA response both in cultured neurones (Forsythe et al., 1988) and in slices (Minota et al., 1989). Glycine receptor ligands also modulate NMDA-induced [^{3}H] noradrenaline release from hippocampal slices (Ransom and Deschenes, 1988).

In the whole animal, administration of glycine potentiates strychnine-induced convulsions in mice (Larson and Beitz, 1988). This effect is blocked by AP5, suggesting a NMDA receptor-dependent mechanism. Similarly, D-serine potentiates NMDA induced seizures in mice and reverses the anti-convulsant effects of 7CK in an audiogenic seizure model (Singh et al., 1990). Glycine antagonists, including 7CK, show neuroprotective effects in both

striatum and hippocampus in response to injections of quinolinate or NMDA (Foster et al., 1989). These results suggest that it should be possible to modulate NMDA receptor/channel activity through the glycine receptor using antagonists such as 7CK, agonists such as glycine itself, or partial agonists such as D-cycloserine (DCS).

The glycine site and long term potentiation

Glycine receptor antagonists block the induction of hippocampal LTP both *in vitro* (Bashir et al., 1990; Oliver et al., 1990) and *in vivo* (Thiels et al., 1991). In the CA1 region of the rat hippocampal slice, 7CK (20-30 μM) blocks the induction of LTP and STP (Bashir et al., 1990; Oliver et al., 1990). This is reversed by co-administration of D-serine (0.2-2.0 mM) and, like the D-AP5 block of LTP, appears to involve an effect on the induction process and not the mechanisms of expression (Oliver et al., 1990). In the anaesthetised rat, an intrahippocampal infusion of 7CK (400 μM) has been reported to block the induction of LTP at CA1 pyramidal cell-commissural synapses (Thiels et al., 1991). This was also reversed by co-administration of D-serine (1 mM). D-serine alone slightly enhanced the amount of potentiation seen over the first 15 minutes post-tetanus, although 20 minutes after the tetanus the magnitude of LTP was indistinguishable from controls. In contrast, glycine (0.05-0.5 mM) has been shown to facilitate the induction of LTP in hippocampal slices (Abe et al., 1990).

The glycine receptor as a novel route for investigating the relationship between LTP and spatial learning

The use of drugs that act at the glycine site may represent a novel means by which to investigate physiological and behavioural processes that may involve NMDA receptors. The glycine receptor antagonist 7CK prevents the induction of LTP in a comparable manner to D-AP5 (Bashir et al., 1990; Oliver et al., 1990; Thiels et al., 1991) and may, therefore, provide an alternative experimental tool for investigating the relationship between LTP and spatial learning. Furthermore, glycine agonists may facilitate NMDA dependent processes including the induction of LTP. This raises the exciting possibility that these compounds might also enhance cognitive processes such as spatial learning.

The first three experiments reported in this chapter investigated the effects of intraventricular infusion of 7CK upon both spatial learning in the watermaze and LTP induction *in vivo*. The

experimental design was based upon the within-subject protocol used by Davis et al., (1992) to investigate the effects of D-AP5 on LTP induction and spatial learning. The strength of this protocol is that, for any individual subject, the level of behavioural performance can be directly compared with (i) the ability to induce LTP in hippocampus and (ii) the tissue levels of drug present in various brain regions. A dose of 75mM was chosen on the basis of a pilot study which indicated that lower doses of 7CK were without effect on performance in the watermaze.

The final experiment described in this chapter (Expt 4.4) investigated the effects of D-cycloserine (DCS), a partial agonist of the glycine receptor, on spatial learning in the watermaze. Using glycine receptor agonists or partial agonists, it might be possible to enhance ongoing NMDA-receptor mediated processes within safe natural limits along similar lines to the way in which benzodiazepines modulate $GABA_A$ receptor-mediated activity (Olsen, 1981). In fact there are already several reports in the literature claiming that glycine site agonists or partial agonists can facilitate certain forms of learning (Monahan et al., 1989b; Thompson et al., 1992). These drugs may be of great clinical importance for treating the cognitive impairments associated with certain forms of neurological disorder.

Development of a novel analytical technique to quantify 7CK tissue levels in the brain

One of the major strengths of previous work using D-AP5 to investigate the relationship between hippocampal LTP and spatial learning (Davis et al., 1992) was that, for any individual animal, the behavioural and electrophysiological results could be related to the exact concentration of the drug present in the brain. This was possible due to the measurement of D-AP5 levels in brain tissue samples by high performance liquid chromotography (HPLC) with fluorescence detection. The exact determination of D-AP5 levels in brain tissue was important, not only for assigning animals to groups, but also to verify that the minipump has successfully delivered the drug into the brain. This enables the experimenter to exclude any animal from the study for which there is no drug present, as a consequence of the minipump failing. Furthermore, comparing the amount of the drug present in different brain regions with the animal's learning ability might give some indication as to the possible site(s) of action of the drug. Therefore, bearing this in mind, an attempt was made to develop a similar analytical technique in order to quantitatively monitor the levels of 7CK present in the brain following chronic intraventricular infusion. A brief outline of some of the steps involved in the development of a method for both the separation

of 7CK from other tissue constituents and its subsequent detection is provided in Appendix A.

Experiment 4.1 - The effects of 7-Chlorokynurenate on spatial learning and LTP *in vivo* with electrophysiological testing carried out in the hippocampus contralateral to the drug infusion cannula.

Drugs

All drug solutions were made up in pyrogen free water. A 100 mM 7-chlorokynurenate (7CK; Tocris Neuramin) solution was made up in equimolar NaOH. "Spiking" with small aliquots (1-2 µl) of 5M NaOH was often required to ensure that the drug fully dissolved. This was then diluted in artificial cerebrospinal fluid (aCSF) to give a final concentration of 75 mM. The aCSF (Alza methodology) was also made up in pyrogen free water with the following final ionic concentrations (mM): Na^+, 150.0; K^+, 3.0; Ca^{2+}, 1.4; Mg^{2+}, 0.8; PO_4^{3-}, 1.0; Cl^-, 155.0 (pH: 7.3 ± 0.1).

Procedure

7CK (75 mM) was chronically infused via an Alzet osmotic minipump into the right lateral cerebral ventricle of rats (n = 9) which had previously received non-spatial pre-training in the open field watermaze (3 days, 4 trials/day, 120 sec max. swim time). The minipump was implanted (see Chapter 3, Surgery) on the day after non-spatial pre-training. The day on which surgery was performed will be referred to as day 0. The assignment of animals to groups was counterbalanced with respect to both performance during pre-training and the location of the platform during spatial training. Having allowed 2 days for the animals to recover from the acute effects of surgery and for the intracranial drug levels to reach a steady state, the rats were trained to find a fixed location, hidden escape platform, positioned in the centre of either the NE or SW quadrant of the pool (3 days, 6 trials/day, 120 sec max. swim time, 30 sec ITI). On day 6, 24 hours after the last spatial training trial, the rats' memory of the platform location was assessed in a transfer test (see Chapter 3 - Spatial learning tasks). A control group (n = 8), comprising both unoperated (n = 4) and aCSF infused (n = 4) animals, was also included.

Immediately following the transfer test, the animal were anaesthetised with urethane (1.5 g/kg) and an attempt made to induce LTP in the left hippocampus (contralateral to the infusion cannula). The animals were prepared as described previously (see Chapter 3 - Electrophysiological methodology). Following a 20 min baseline period of low frequency stimulation (0.05 Hz, 700μA monophasic pulses of 100 μs duration), a high frequency tetanus (4 trains of 33 pulses at 400 Hz, 700 μA monophasic pulses of 100 μs duration, inter-train interval of 20 sec) was delivered in an attempt to induce LTP. Low frequency test pulses continued for a further 100 min.

Following the completion of electrophysiological testing, the rats were killed and their brains removed on ice. Coronal wedges of brain tissue in the region of the cannula were taken from animals implanted with minipumps. For 7CK infused animals, tissue samples (right and left hippocampus, frontal cortex, visual cortex and anterior striatum) were dissected out, frozen on dry ice and stored at -80°C.

The experimental procedure consisted of a series of phases (eg. non-spatial pre-training, surgery, spatial training, electrophysiology, tissue analysis and histology) which were conducted on each animal. As the electrophysiological phase was the rate limiting step of the protocol, with only 1-2 rats completed on each day, the experiment was run in a staggered manner.

Determination of 7CK levels in brain tissue by 'High Performance Liquid Chromotography'.

Tissue samples were thawed and then extracted in 0.1M perchloric acid. 7CK was separated from other tissue constituents by HPLC and detected by fluorescence (see this chapter - 'Development of a novel analytical technique to quantify 7CK tissue levels in the brain', and Appendix A). Separation was achieved on a C8 Spherisorb column. The mobile phase consisted of 3 parts 50mM sodium acetate (adjusted to pH 6.2 with glacial acetic acid) to 1 part methanol and was pumped at a flow rate of 1 ml/min by a Varian Vista pumping system. The endogenous fluorescence of 7CK was enhanced to detectable levels by post-column application of 0.5M zinc acetate at a flow rate of 1 ml/min. 7CK was detected using an Applied Biosystems fluorescence detector (excitation wavelength 331 nm, emission wavelength > 398 nm) and the peak size quantified using a microcomputer-based integration package (Midas, Comas Instruments). The identity of the 7CK peak in the tissue samples was determined by comparison of retention times, having previously been identified by

"spiking" with a standard 7CK sample and by the absence of such a peak in samples from aCSF infused rats.

Results - Behaviour

The control subjects were all capable of swimming around the pool until the platform was located and then climbing onto it. They rapidly learned to swim away from the side walls and directly to the platform, resulting in a progressive reduction in escape latency across trials (Figure 4.1.1). In contrast, the 7CK treated rats appeared to show some degree of sensorimotor impairment, reminiscent of that displayed by high doses of D-AP5 (Davis et al.,1992). The 7CK rats showed prolonged righting reflexes, spent more time at the side walls (a pronounced thigmotaxis) and had difficulties climbing onto, and then remaining on, the platform. They also showed little, if any, improvement across trials (Figure 4.1.1) and often failed to find the platform at all within the 120 sec swim time. In many cases performance actually deteriorated across the 6 trials of each training session, with the animals appearing to tire very quickly and experiencing great difficulty in swimming on the later trials. Perhaps surprisingly, however, the average swim speed of both groups of rats on the first day of spatial training (averaged over trials 1-6) was 0.30 ± 0.01 m/s ($t < 1$; $p > 0.20$).

Inspection of the data revealed that there were no systematic differences between the unoperated, and aCSF infused, control subjects. For example, a t-test comparing the amount of time spent in the training quadrant during the transfer test by both groups showed no significant difference (% time; unoperated controls = 42.6 ± 7.0; aCSF infused controls = 38.9 ± 5.5; $t < 1$; $p > 0.20$). Previous studies using minipumps have shown that aCSF infused animals perform indistinguishably from unoperated controls on spatial learning tasks in the watermaze (Morris, 1989; Davis et al., 1992). In addition, analysis of performance of unoperated, sham lesioned, and aCSF infused controls in a subsequent experiment (Expt 4.2), revealed no group differences, confirming that implantation of a minipump and the infusion of aCSF does not affect spatial learning in the watermaze. For the purposes of further analysis, therefore, the unoperated, and aCSF infused rats will be combined to form one control group.

An ANOVA of escape latencies for the 18 spatial training trials revealed an overall effect of group ($F(1,15) = 24.61$; $p < 0.0005$), an effect of trial ($F(17,255) = 2.02$; $p = 0.01$) but no

groups by trials interaction ($F (17,255) = 1.28$; $p > 0.20$). A separate ANOVA of the escape latencies of the 7CK group alone showed no significant effect of trial ($F (17,136) = 1.45$; $p > 0.10$), consistent with the observation that the 7CK rats showed little improvement across trials. In contrast, the control rats did show a significant improvement across trials ($F (17,119) = 2.75$; $p < 0.001$). There was no significant difference between the groups on trial 1 (controls (s) = 52.2 ± 12.8; 7CK = 80.1 ± 17.2; $t (15) = 1.27$; $p > 0.20$) but by trial 2 control animals were escaping from the pool significantly faster than the 7CK rats (controls (s) = 25.5 ± 11.0; 7CK = 73.4 ± 16.5; $t (15) = 2.34$; $p < 0.05$) and this was maintained throughout training.

During the transfer test, control rats searched persistently in the region of the former platform location whereas 7CK rats searched randomly about the pool and showed no spatial bias towards the former 'training' quadrant (Figures 4.1.2 and 4.1.3). An ANOVA revealed a significant effect of quadrant ($F (2,45) = 6.72$; $p < 0.005$) and a groups by quadrants interaction ($F (2,45) = 4.32$; $p < 0.025$). A subsequent analysis of the percentage time spent in the training quadrant only, confirmed that control rats were spending significantly longer in the training quadrant than the 7CK rats (% time; controls = 40.8 ± 4.2 ; 7CK = 26.8 ± 2.5; $t (15) = 2.93$; $p < 0.05$). In summary, 7CK rats were impaired relative to controls both in terms of escape latency during training and in the transfer test.

Electrophysiology

The chronic infusion of 7CK (75 mM) into the contralateral ventricle did not affect the induction of LTP in the left hippocampus. Baseline field epsps, evoked by low frequency stimulation of the perforant path (700 μA. 0.05 Hz), did not appear to be grossly affected by the drug (Figure 4.1.4). The mean absolute slope of the pre-tetanus baseline field epsps (averaged over the 10 min period immediately prior to the tetanus) did not differ between the 2 groups (control = 3.84 ± 0.64 mV/ms; n = 7; 7CK = 2.76 ± 0.65 mV/ms; n = 8; $t (13) = 1.18$; $p > 0.20$). For each animal, the mean absolute slope for the 10 min prior to the tetanus was then used as a within-subject baseline (= 100%) and the data normalised with respect to this value (Figure 4.1.5). 7CK infusion had no significant effect on the initial potentiation seen 0-4 min after the tetanus (control = 137.4 ± 9.3%; 7CK = 129.2 ± 8.6%; $t < 1$; $p > 0.20$). Neither was there an effect on the amount of potentiation seen 1 hour after the tetanus (mean % potentiation 50-60 min post-tetanus; control = 126.5 ± 5.9%; 7CK = 127.4 ± 9.0%; $t < 1$; $p > 0.20$).

7CK tissue levels

The mean levels of 7CK present in the various brain regions examined were as follows (pmol/mg wet weight): right hippocampus = 54.6 ± 22.9; left hippocampus = 19.8 ± 14.4; visual cortex = 5.5 ± 3.3; frontal cortex = 3.4 ± 1.5 and anterior striatum = 5.7 ± 4.0. There were detectable levels of 7CK present in the right hippocampus of all the animals implanted with minipumps infusing the drug. The range of concentrations resulting from the infusion of an identical dose of 7CK (75 mM) was, however, extremely wide (eg. right hippocampus; range = 2.1 - 218.8). In the other brain regions studied, 7CK was found in only a proportion of the animals implanted (left hippocampus = 67%; visual cortex = 44%; frontal cortex = 44% and anterior striatum = 33%).

Histology

Histological examination of the wedge of tissue taken from implanted animals showed that the cannula was correctly located in the right lateral ventricle (Figure 4.1.6). In a small number of animals there was some enlargement of the ventricles. In addition, in some animals there was a little damage to the fornix immediately below the ventricle. By necessity, there was also a small amount of damage to the overlying cortex, but as the performance of aCSF infused animals was indistinguishable from unoperated controls, this is unlikely to be important. There was no apparent difference in terms of the amount of damage or ventricular enlargement between aCSF and 7CK infused rats.

Experiment 4.2 - The effects of 7-Chlorokynurenate on spatial learning and LTP *in vivo* with electrophysiological testing carried out in the hippocampus ipsilateral to the drug infusion cannula.

In the light of the results of the previous study (Expt 4.1), a second experiment was conducted along similar lines but with several important changes in experimental procedure. Analysis of 7CK levels in brain tissue samples revealed that the drug concentration was considerably higher in the right (ipsilateral) hippocampus than in the left (contralateral) hippocampus. As electrophysiological testing was conducted in the left hippocampus, this could account for the failure to observe a block of LTP in animals which, nevertheless, show

a behavioural deficit. Bearing this in mind, the following experiment investigated whether or not LTP could be induced in the hippocampus ipsilateral to the infusion cannula. In addition, during Expt 4.1 the behavioural performance of drug treated animals often deteriorated across the 6 trials of each training session, with the rats appearing to tire very quickly and experiencing difficulty in swimming on later trials. Therefore, in an attempt to reduce the consequences of non-associative drug effects, such as increased fatigue or anxiety which may be contributing to the observed deficit in performance, the spatial training protocol was altered. The intertrial interval (ITI) was extended from 30 seconds to 4 hours in order to allow animals to recover fully between trials (see Morris et al., 1986). A group of rats with ibotenate lesions of the hippocampus were also included for comparison.

Procedure

All rats received one day of non-spatial pre-training (6 trials, 120 sec max. swim time). On the following day (day 0), a minipump was implanted infusing 7CK (75mM) into the left lateral cerebral ventricle through a double-L-shaped cannula. The cannula was cemented to the skull in such a way as to allow electrophysiological testing to be conducted in the left hippocampus. Beginning on day 3, the rats were trained to find a fixed location, hidden escape platform (5 days, 3 trials/day, 120 sec max. swim time, 4 hr ITI, platform pos. = NE or SW). The rats were allowed to remain on the platform for 30 sec before being returned to their home cage for 4 hours. On day 8, 24 hours after the last spatial training trial, the rats' memory of the platform location was assessed with a transfer test. Immediately after the transfer test, an attempt was made to induce LTP in the left hippocampus (ipsilateral to the infusion cannula). Otherwise, the experimental protocol was identical to that used in Expt 4.1.

Following the completion of electrophysiological testing, the rats were killed and their brains removed on ice. Coronal wedges of brain tissue in the region of the cannula were taken from animals infused with aCSF. Although obtaining such tissue from the 7CK infused rats would have been desirable, such samples were required for the quantification of drug tissue levels by HPLC analysis. Tissue samples (right and left hippocampus, frontal cortex, visual cortex and striatum) were dissected out, frozen on dry ice and stored at -80°C.

Separate groups of rats receiving either ibotenate lesions of the hippocampus (HPC; see Chapter 3- Excitotoxic lesions of the hippocampus) or sham hippocampal lesions were also

prepared. Both the hippocampal and sham operated animals had already participated in a separate study looking at the context specificity of latent inhibition and conditioning in an operant chamber (Honey and Good, 1993). Consequently, these animals received their non-spatial pre-training post-operatively. At the end of behavioural testing, animals with hippocampal lesions were perfused with formol saline, their brains removed, frozen, sectioned and stained (see Chapter 3 - Histology). The sham operated animals were not used for electrophysiological testing.

The final group sizes were as follows: 7CK (n = 14), HPC (n = 9), aCSF (n = 7), unoperated controls (n = 8) and sham lesioned (n = 8). It should be noted that not all animals contribute data to all phases of the experiment (eg. animals died before completion of electrophysiological testing).

Results - Behaviour

All animals swam well in the pool and learned to use the platform as a means of escape from the water. Although there was still some evidence of a sensorimotor impairment in the 7CK animals (eg. slower righting reflex, some difficulty staying on the platform following a prolonged swim time), this was considerably less than that seen in Expt 4.1. Examination of the average swim speeds over the first 6 trials of spatial training revealed a weak trend towards control rats swimming slower than both HPC and 7CK animals (swim speed (m/s): controls = 0.30 ± 0.01; HPC = 0.32 ± 0.01; 7CK = 0.32 ± 0.01; $F(2,43) = 3.14$; $0.05 < p < 0.10$). During training, all animals spent progressively less time at, or near, the side walls and rapidly learned to find the platform. By the end of spatial training animals from all 3 groups were escaping from the pool in, on average, less than 20 seconds (Figure 4.2.1).

Consistent with previous reports (see Expt 4.1 - Results; Morris, 1989; Davis et al.,1992), the performance of unoperated, sham lesioned and aCSF infused animals was indistinguishable. An ANOVA of escape latencies for these 3 groups of animals revealed no effect of group ($F(2,20) = 1.16$; $p > 0.20$), a significant effect of trial ($F(14,280) = 17.94$; $p < 0.0001$) and no groups by trials interaction ($F < 1$; $p > 0.20$). Furthermore, an ANOVA of the percentage time spent in the training quadrant by these animals also showed that performance of the three control groups was indistingushable (unoperated = 46.4 ± 4.3; sham lesioned = 48.4 ± 4.0; aCSF infused = 44.2 ± 3.9; $F < 1$; $p > 0.20$). For the purposes of further analysis, therefore, these 3 groups will be combined to form one control group (n = 23).

An ANOVA of escape latencies for the 15 spatial training trials revealed a highly significant overall effect of group ($F(2,43) = 22.60$; $p < 0.0001$), trial ($F(14,602) = 17.73$; $p < 0.0001$) and a groups by trials interaction ($F(28,602) = 2.70$; $p < 0.0001$; Figure 4.2.1). Subsequent analysis showed that there was no difference between the groups on either the first trial of spatial training (controls = 96.3 ± 7.0 secs; 7CK = 72.2 ± 13.9 secs; HPC = 72.6 ± 15.3 secs; $F(2,43) = 1.41$; $p > 0.20$) or on the last day of training (trials 13-15; $F < 1$) . A Tukey's HSD pairwise comparison, looking at the effect of group on escape latency, showed that both the 7CK and the HPC groups were significantly different from controls ($p < 0.01$). There was no significant difference between the 7CK and HPC groups.

Control rats spent a considerable time searching in the training quadrant during the transfer test (46.4 ± 2.3%; Figure 4.2.2 and 4.2.3). The HPC animals showed no spatial bias and searched randomly all over the pool (28.9 ± 2.3%). Some of the 7CK infused rats displayed a small bias towards the training quadrant but this was less pronounced than the bias shown by control animals (35.1 ± 3.7%). An ANOVA revealed a significant effect of quadrant ($F(2,129) = 28.45$; $p < 0.0001$) and a groups by quadrants interaction ($F(4,129) = 5.83$; $p < 0.0001$). A second ANOVA looking solely at the time spent in the training quadrant showed a significant difference between the groups ($F(2,43) = 8.31$; $p < 0.001$). Subsequent Tukey's HSD pairwise comparisons confirmed that both the HPC group ($p < 0.01$) and the 7CK group ($p < 0.05$) were spending less time in the training quadrant than the controls. There was no difference, however, between the HPC and 7CK groups ($p > 0.05$). In summary, 7CK and HPC rats were impaired, relative to control animals, in a spatial learning task in the watermaze, both in terms of escape latency during acquisition and in a transfer test.

Electrophysiology

The chronic infusion of 7CK (75 mM) into the ipsilateral ventricle did not affect the induction of LTP in the left hippocampus. Baseline field epsps were not affected by the drug. The mean absolute slope of the pre-tetanus baseline field epsps (10 min period prior to tetanus) did not differ between the 2 groups (control = 1.29 ± 0.17 mV/ms; n = 9; 7CK = 1.60 ± 0.29 mV/ms; n = 11; $t < 1$; $p > 0.20$). The absolute slope values in this experiment were, however, significantly lower than those obtained in Expt 4.1 ($F(1,31) = 17.33$; $p < 0.0005$). This may be a consequence of recording from the same hemisphere into which the cannula was implanted. Mechanical trauma or ventricular enlargement, resulting from either the implantation or infusion process, may make it harder to obtain sizable potentials in the

ipsilateral hippocampus, as a consequence of either tissue displacement or a direct physical effect. As in Expt 4.1, the data was normalised relative to the 10 min pre-tetanus slope value (Figure 4.2.4). The initial amount of potentiation (0-4 min post-tetanus) did not differ between the groups (control = 138.0 ± 7.9%; 7CK = 146.9 ± 6.7%; $t < 1$; $p > 0.20$). After 1 hour (50-60 min post-tetanus), there was also no difference in the amount of LTP observed (control = 124.6 ± 3.8%; 7CK = 126.2 ± 4.6%; $t < 1$; $p > 0.20$).

7CK tissue levels

The mean tissue concentration of 7CK present in the left hippocampus from all drug-infused animals participating in the behavioural study was 11.9 ± 2.9 pmol/mg wet weight (n = 14; Table 4.2.1). There were detectable levels of 7CK in the left hippocampus of all but one of these animals. Subject 8410 had no 7CK in the left hippocampus but was included in the study because there was drug present in the striatum (7.27 pmol/mg wet weight). There was 7CK present in all but one of the striatal samples (mean = 3.0 ± 0.7 pmol/mg wet weight; n = 14). In the other brain regions studied, however, 7CK was only rarely detected. In the right hippocampus, only one sample contained 7CK (rat 8418 = 3.2 pmol/mg wet weight). Three animals had 7CK in the visual cortex (mean = 4.2 ± 1.0 pmol/mg wet weight; n = 3) and there was no drug present in the frontal cortex of any of the subjects. These values are considerably lower than those reported in Expt 4.1.

Correlation analysis

7CK impairs performance in a spatial learning task in the watermaze. This same group of animals exhibit LTP which is indistinguishable from controls, suggesting that the impairment in performance is not due to a disruption of LTP-like processes in the hippocampus. It is possible, however, that within this group of animals (n = 14) there are some rats which display relatively less LTP and also show a bigger impairment in the behavioural task. Inspection of the data, however, suggests that this is not the case (Table 4.2.1; Figure 4.2.5). Analysis revealed no significant correlation between the proportion of time spent in the training quadrant during the transfer test and the amount of LTP induced 1 hour post-tetanus ($r = 0.16$; $p = 0.64$). Somewhat more surprisingly, there was also no correlation between the amount of LTP induced and the concentration of 7CK in the left hippocampus ($r = 0.00$; $p = 1.00$). This suggests that the concentration of 7CK in the

hippocampus resulting from the infusion of 75 mM 7CK is well below that required to block the induction of LTP. In addition, there was no significant correlation between the proportion of time spent in the training quadrant and the 7CK concentration in the left hippocampus ($r = -0.31$; $p = 0.28$). It is possible that the behavioural effects of the drug are due to an action in some other brain region. Of the other brain regions analysed, only the striatum had detectable levels of 7CK in the majority of subjects (13 out of 14). There was, however, no correlation between striatal levels of the drug and the proportion of time spent in the training quadrant during the transfer test ($r = 0.20$; $p = 0.50$). The possibility remains that performance in the behavioural task is correlated to the amount of 7CK present in some other brain region not included in the tissue analysis study.

Histology

Histological examination of the wedge of tissue taken from aCSF infused animals shows that the cannula is correctly located in the left lateral ventricle (Figure 4.2.6). As in Expt 4.1, in some animals there is some enlargement of the ventricles, and a small amount of damage to both the overlying cortex and the fornix immediately beneath the ventricle. No histology was performed on the brains of 7CK infused animals due to the need for this tissue for the determination of brain levels of 7CK. However the results of Expt 4.1 suggest that there is no difference between infusing aCSF or 7CK in terms of the extent of brain damage or ventricular enlargement. This, coupled with the fact that performance of aCSF infused animals is indistinguishable from unoperated controls or sham lesioned animals, suggests that the behavioural impairment resulting from the infusion of 7CK is unlikely to be due to the effects of the surgery, the presence of the minipump or the non-specific effect of the infusion process *per se.*

The extent of damage in hippocampal lesioned animals was assessed and used to determine whether or not an animal was accepted into the study. In order to be included, the hippocampal damage had to fulfill certain criteria which were assessed without any knowledge of the behavioural performance of that subject. A more complete histological report is provided elsewhere (Honey and Good, 1993) but briefly, for animals to be included it was necessary for almost all of the CA1 - CA4 pyramidal cells and most of the granule cells in the dentate gyrus to have been removed with no, or little, damage to adjacent areas (Figure 4.2.7).

Experiment 4.3 - A study of the time course of 7-Chlorokynurenate brain tissue levels following chronic intraventricular infusion.

In both Expts 4.1 and 4.2 the infusion of 7CK has resulted in a behavioural impairment with no subsequent effect on the induction of LTP. One possible explanation is that 7CK levels, having reached a peak early in behavioural testing, decrease over the course of the experiment so that by the time an attempt is made to induce LTP, the drug concentration has fallen below the threshold level required to inhibit induction. In order to assess this possibility a time course study was conducted to investigate whether 7CK tissue levels vary during the course of the experiment.

Procedure

In order to determine whether the intracranial levels of 7CK remain constant throughout the duration of behavioural and electrophysiological testing, a separate group of animals were implanted with minipumps (see Chapter 3- Surgery) infusing 7CK (75 mM) into the right lateral ventricle. The rats were then sacrificed after various time intervals;- 3 days ($n = 10$), 6 days ($n = 9$), 9 days ($n = 8$) or 12 days ($n = 9$). Tissue samples from both the right and left hippocampus were dissected out on dry ice and whole tissue levels of 7CK determined by HPLC with fluorescence detection (see this chapter - Determination of 7CK tissue levels in brain tissue by high performance liquid chromotography). Striatal tissue samples were also taken from a subset of the animals and from the remaining animals a wedge of tissue was removed for histological verification of the cannula placement.

Results

The results of the time course study establish that from day 3 to day 12 (encompassing the period of behavioural and electrophysiological testing) the levels of 7CK in the brain remained constant. In one animal (from the day 9 group) there was no 7CK detectable in any of the brain areas analysed and, consequently, this subject was excluded from the study. Because the cannula was implanted in the right ventricle, the levels of 7CK were substantially higher in the right hippocampus (mean 7CK tissue concentration for all 36 animals in the time course study (pmol/mg wet weight) - right hippocampus = 15.6 ± 2.7; left hippocampus = 2.8 ± 1.1). It is worth noting that 7CK was detected in only 28% of samples taken from the left hippocampus. In 90% of the striatal samples analysed there were

detectable levels of 7CK present (mean tissue concentration (pmol/mg wet weight) = 4.3 ± 0.8; n = 21).

The mean levels of 7CK present in the ipsilateral hippocampus in animals sacrificed at various time intervals after implantation were as follows (pmol/mg wet weight) - day 3 = 17.6 ± 5.4; day 6 = 14.7 ± 2.7; day 9 = 19.7 ± 9.7 and day 12 = 10.5 ± 2.7 (Figure 4.3.1). An ANOVA revealed no significant difference between days ($F < 1$; $p > 0.20$). A further comparison was made between the drug levels detected in the day 9 group of the time course study (19.7 ± 9.7; n = 8) and the mean 7CK tissue concentration found in the ipsilateral hippocampus of rats in Expt 4.2, in which the animals were sacrificed on day 8 (12.9 ± 2.9; n = 13; excluding subject 8410 in which there was no 7CK detectable in the left hippocampus). A t-test showed no significant diffence in 7CK levels between animals in the time course study and those in Expt 4.2 ($t < 1$; $p > 0.20$). These values are, however, considerably lower than those reported in Expt 4.1 (mean 7CK tissue level in the ipsilateral hippocampus (pmol/mg wet weight) = 54.6 ± 22.9).

7CK levels in the striatum also remained fairly constant between days 3 and 12 (Figure 4.3.2). The mean tissue concentration for each time interval were as follows (pmol/mg wet weight): day 3 = 6.8 ± 2.6 (n = 5); day 6 = 3.9 ± 1.8 (n = 4); day 9 = 3.1 ± 1.0 (n = 8) and day 12 = 4.2 ± 1.4 (n = 4). An ANOVA showed no significant effect of day ($F < 1$; $p > 0.20$).

In summary, the levels of 7CK in the brain appear to have remained constant throughout the period of behavioural and electrophysiological testing. The levels of 7CK detected in the time course study are comparable to those seen in Expt 4.2 but are considerably less than those found in Expt 4.1.

Experiment 4.4 - The effects of D-Cycloserine on spatial learning.

Despite the failure of these experiments to provide further support for the hippocampal LTP/spatial learning hypothesis, the possibility remains that glycine receptor agonists or partial agonists may facilitate spatial learning. The following study investigated the effects of DCS on the acquisition of a spatial learning task in the watermaze.

Drugs

D-Cycloserine (DCS; D-4-amino-3-isoxazolidone) is a ring compound which is stable in alkaline solution but is rapidly destroyed when exposed to neutral or acidic pH (Goodman and Gilman, 1990). Therefore, DCS was made up at a concentration of 0.6 mg/ml in phosphate buffered saline (PBS; 0.1M, pH 8) every 3-4 hours. The rats were injected i.p. with 10 ml/kg of this solution, resulting in a dose of 6 mg/kg.

Procedure

Following one day of drug-free, non-spatial pre-training (day 1; 6 trials, 60 sec max. swim time), the rats were trained to find a fixed location, hidden escape platform (3 days, 6 trials/day, 120 sec max. swim time, 30 sec ITI, platform pos. = NE or SW). The rats were injected daily with DCS (6 mg/kg) or an equivalent volume of the vehicle (0.1M PBS) 30 mins prior to the start of behavioural testing. The assignment of animals to groups was counterbalanced with respect to performance during pre-training and to the location of the platform during spatial training. On day 5, 24 hours after the last spatial training trial and 30 mins after injection, the rats were given a transfer test. The experiment was run in 2 replications, the first comprising of 24 animals (12 per group) and the second comprising of 20 animals (10 per group). For the purposes of analysis the results from the 2 replications were combined, resulting in a total of 22 subjects per group.

Results

Both groups of animals acquired the task rapidly, showing a dramatic reduction in escape latency across trials (Figure 4.4.1). An ANOVA of escape latencies over the 18 trials for both groups showed no effect of group ($F(1,42) = 2.10$; $p > 0.10$), but an effect of trial ($F(17,714) = 43.81$; $p < 0.0001$) and a groups by trials interaction ($F(17,714) = 1.71$; $p < 0.05$). Inspection of the data, however, shows that although there are small differences in mean escape latency between the 2 groups on certain trials, neither group performed consistently better than the other (Figure 4.4.1). This suggests that the interaction is most likely due to random fluctuations in performance rather than to some substantial underlying cause. There were no significant differences in escape latencies between the 2 groups on trial one (vehicle $= 97.7 \pm 7.1$; DCS $= 81.9 \pm 10.1$; $t(42) = 1.28$; $p > 0.20$). Comparison of mean swim speeds on the first day of spatial training (averaged over trials 1-6) showed that both vehicle and

DCS injected rats had an average swim speed of 0.27 ± 0.01 m/s ($t < 1$; $p > 0.20$).

During the transfer test, animals from both groups spent a large proportion of time searching in the training quadrant (Figure 4.4.2). Analysis of the time spent in the 4 quadrants of the pool during the entire 60 seconds of the transfer test indicated that the DCS injected rats were spending, on average, more time in the training quadrant, relative to the controls (vehicle = 46.1 ± 2.7%; DCS = 52.8 ± 3.0; Figure 4.4.3). An ANOVA revealed a hugely significant effect of quadrant ($F (2,126) = 115.35$; $p < 0.0001$) and a trend towards a groups by quadrants interaction ($F (2,126) = 2.78$; $0.05 < p < 0.10$). A t-test comparing the amount of time spent in the training quadrant did not, however, reveal a significant difference between the groups ($t (42) = 1.65$; $p > 0.10$). Analysis of the first 30 seconds of the transfer test was more suggestive of the possibility that DCS might, indeed, enhance performance (Figure 4.4.4). An ANOVA again showed a significant effect of quadrant ($F (2,126) = 113.77$; $p < 0.0001$) but also a significant groups by quadrants interaction ($F (2,126) = 3.43$; $p < 0.05$). Comparison of the time spent in the training quadrant showed a trend towards a bigger spatial bias in the DCS group (vehicle = 49.9 ± 3.4%; DCS = 59.0 ± 3.4%; $t (42) = 1.90$; $0.05 < p < 0.10$). Finally, analysis of the first 15 seconds of the transfer test revealed a significant effect of quadrant ($F (2,126) = 65.23$; $p < 0.0001$), a groups by quadrants interaction ($F (2,126) = 5.65$; $p < 0.005$), and, when compared directly, there was a significant difference between the groups in terms of the amount of time spent in the training quadrant (vehicle = 48.0 ± 5.2%; DCS = 66.3 ± 4.6%; $t (42) = 2.65$; $p = 0.01$; Figure 4.4.5). These results suggest that the drug-treated rats show a stronger bias towards the training quadrant during the early part of the transfer test which may be indictive of a DCS-mediated learning enhancement. As the transfer test proceeds, the DCS rats might be learning that the platform is no longer in its expected location and spend less time searching in the training quadrant (Figure 4.4.6), thus explaining the lack of a difference between the groups when analysing the full 60 seconds of the transfer test.

DISCUSSION

Intraventricular infusion of 7CK impairs spatial learning but does not block the induction of hippocampal LTP

The results of Expts 4.1 and 4.2 indicate that the intraventricular infusion of 7CK (75mM) disrupts the acquisition of a spatial reference memory task in the watermaze but does not block the induction of hippocampal LTP in the same animals. At first glance this result appears to be at odds with the hippocampal LTP/spatial learning hypothesis in that animals showing a distinct spatial learning impairment, nevertheless, exhibit normal LTP. This dissociation, however, does not compromise the hypothesis. Performance on complex learning tasks, such as the watermaze, can be disrupted in a number of different ways which are independent of the learning process *per se*. A major criticism of the D-AP5 studies (Morris et al., 1986a; Davis et al., 1992) is that this drug might disrupt performance on spatial learning tasks by some means other than directly inhibiting the learning mechansim (Keith and Rudy, 1990). It has been suggested that the drug might interfere with sensorimotor or motivational processes, resulting in impaired performance. Likewise, a similar argument may now account for the observed 7CK-induced learning deficit in the absence of a block of LTP. Clearly, therefore, this dissociation between the effects of 7CK on spatial learning and LTP in no way excludes a possible role for LTP-like events as part of the mechanisms of spatial learning. These results do, however, illustrate the problems associated with using drugs to investigate the biological mechanisms of memory. The most critical aspect of these studies is to determine whether a drug-induced impairment in performance is due to a direct action on the learning mechanism or to an effect on some other behavioural process which indirectly influences the rate of learning. These non-associative drug effects are sometimes called 'performance effects' and may well be responsible for the impaired performance in rats following chronic infusion of 7CK.

The failure to block LTP in the dentate gyrus following infusion of 7CK

The chronic intraventricular infusion of 7CK (75mM) did not prevent the induction of LTP in the dentate gyrus. The only previously published study investigating the effects of glycine receptor antagonists on hippocampal LTP *in vivo* reported that 7CK, infused directly into the hippocampus, blocked induction at CA1 pyramidal cell-commissural synapses (Thiels et al.,

1992). Similarly, the majority of other studies investigating this issue *in vitro* have studied LTP induction at CA1 pyramidal cell-Schaffer collateral synapses (Bashir et al., 1990; Oliver et al., 1990). It is possible that the failure to observe a block of LTP at perforant path-granule cell synapses *in vivo* is due to the fact that in the dentate gyrus, the inducibility of LTP cannot be modulated through the glycine receptor. This seems extremely unlikely, however, in that (i) there is no evidence to suggest that the population of NMDA receptors in the dentate gyrus is different from that in CA1, (ii) there are no *a priori* grounds to believe that LTP in the dentate gyrus is fundamentally different from that in CA1, and most importantly (iii) it has recently been shown that 7CK does in fact block the induction of LTP in the dentate gyrus in hippocampal slices *in vitro* (Watanabe et al., 1992). It therefore seems extremely unlikely that dentate gyrus LTP *in vivo* differs from both CA1 LTP *in vivo* and dentate gyrus LTP *in vitro*.

An alternative explanation for the failure to observe a block of LTP may be derived from recent studies which have indicated that the stimulation parameters used to induce LTP may crucially determine whether or not a drug-induced inhibition is seen (see Chapter 5 this thesis; Chetkovich et al., 1993; Haley et al., 1993). In contrast to the lack of an effect of 7CK on LTP induced by a strong tetanus, it is possible that LTP induced by a weak tetanus may indeed be sensitive to 7CK. The antagonist competes with glycine for vacant glycine binding sites on the NMDA receptor complex. It is possible, therefore, that a stronger tetanus may result in higher concentrations of glycine in the synaptic cleft which can overcome the 7CK-induced receptor blockade, thus allowing the induction of LTP.

Perhaps the most plausible explanation, however, for the failure to block LTP is that the infusion protocol used does not result in sufficiently high levels of 7CK in hippocampus to block the NMDA receptor associated glycine site and thus prevent LTP induction. The lack of a significant correlation between 7CK levels in the hippocampus and the magnitude of LTP induced implies that the 7CK concentration, resulting from the infusion of a 75mM solution, is well below that required to block LTP.

Whole tissue levels of 7CK are surprisingly low and may account for the failure to block LTP

The actual concentration of 7CK available to glycine receptors in the hippocampus is difficult to estimate, making it hard to compare these results with previous experiments

investigating the effects of 7CK on LTP induction in hippocampal slices. Davis et al., (1992) showed that the extracellular concentration of D-AP5 *in vivo* (8.3 μM), measured using microdialysis, is comparable to the bath concentration in *in vitro* hippocampal slice experiments at which LTP is blocked (Harris and Cotman, 1984). Unfortunately, the concentrations of 7CK resulting from this infusion protocol were far too low to even contemplate a microdialysis study. The whole tissue levels of 7CK in brain samples taken from these experiments were right on the limit of detection for our analytical system. Bearing in mind the fact that whole tissue levels can greatly exceed extracellular drug concentrations (30 fold difference for D-AP5; Davis et al., 1992), it is extremely unlikely that detectable levels of 7CK would be obtained with microdialysis.

The whole tissue levels of 7CK can, however, be compared with the whole tissue levels obtained following chronic infusion of D-AP5. A previous study conducted in this laboratory found that chronic infusion of 30mM D-AP5, using the same Alzet minipump technique, resulted in whole tissue drug levels of 180 ± 30 pmol/mg wet weight of tissue in the hippocampus contralateral to the infusion cannula and 200 ± 40 pmol/mg wet weight in the hippocampus adjacent to the infusion cannula (Bolhuis and Reid, 1992). In Expts 4.1 and 4.2, the infusion of 75 mM 7CK resulted in whole tissue drug levels, measured in the hippocampus ipsilateral to the infusion cannula (the brain area with the highest recorded levels in the study), of 54.6 ± 22.9 and 11.9 ± 2.9 pmol/mg wet weight of tissue respectively. In the case of Expt 4.2, the 7CK levels were nearly 20 times less than those obtained with D-AP5 despite the fact that the 7CK solution in the minipump was more than twice the concentration of D-AP5 infused using the same technique. These results suggest that 7CK is rapidly removed from the brain. As a result, 7CK is only found in consistently detectable levels in brain areas adjacent to the infusion cannula. It is not clear whether 7CK is broken down enzymatically in the brain or excreted directly. Analysis of brain tissue samples did not reveal any potential breakdown products although the possibility that 7CK is metabolised, forming non-fluorescent derivatives cannot be ruled out. The rapid excretion of 7CK is in agreement with that reported for the parent compound, kynurenate (KYN; Turski and Schwarcz, 1988). KYN, injected directly into the hippocampus, is rapidly excreted from the brain with only 0.2% of the drug remaining in the hippocampus after two hours. Consistent with these results, KYN was excreted unmetabolised.

The rapid excretion of 7CK from the hippocampus probably accounts for the absence of a block of LTP in these experiments. It is extremely difficult to make comparisons between

these experiments and studies conducted with hippocampal slices, in the absence of extracellular drug concentrations obtained using microdialysis. It is clear, however, from comparisons with D-AP5 that 7CK is rapidly excreted from the brain and unlikely to be present in sufficient amounts to block LTP. In the D-AP5 dose-response study (Davis et al., 1992), a whole tissue concentration of 270 $\pm$ 30 pmol/mg wet weight corresponded to a complete block of LTP ('high concentration' group), whereas a tissue concentration of 150 $\pm$ 30 pmol/mg wet weight resulted in a 63% blockade of LTP ('medium concentration' group), and 60 $\pm$ 30 pmol/mg wet weight caused no impairment of LTP induction whatsoever ('low concentration' group). In Expts 4.1 and 4.2 the inducability of LTP was assessed in the hippocampus with whole tissue drug levels of 19.8 $\pm$ 14.4 and 11.9 $\pm$ 2.9 pmol/mg wet weight respectively. The tissue levels of 7CK are approximately one order of magnitude lower than the levels of D-AP5 required to see any effect on LTP. Examination of the existing literature suggests that there are no *a priori* reasons for assuming that 7CK is substantially more potent than D-AP5 at blocking LTP. For example, Watanabe and colleagues found that bath application of 10 μM 7CK resulted in a complete block of LTP at perforant path-granule cell synapses in 4/4 slices tested (Watanabe et al., 1992). 1 μM 7CK blocked LTP in 2/3 slices. The dose range for 7CK inhibition of LTP in hippocampal slices appears to be comparable to that of D-AP5 (Harris and Cotman, 1984). It seems reasonable, therefore, to conclude that 7CK tissue levels are considerably lower than the levels that might be required to inhibit the induction of LTP *in vivo*. The only study investigating the effects of 7CK on hippocampal LTP *in vivo* showed that continuous infusion of 400 μM 7CK directly into the hippocampus, with a total injection volume of approximately 150 nl, blocked the induction of LTP (Thiels et al., 1992). This corresponds to a total of just 60 pmoles which is surprisingly low in the light of these results. It is not clear, however, whether the drug is distributed throughout the hippocampus or remains in the tissue immediately adjacent to the infusion cannula and the recording electrode. Interestingly, a similar injection of 100 μM D-AP5 was also sufficient to block LTP.

Comparing these results with the D-AP5 dose response study may also shed some light on the nature of the behavioural impairment. A significant spatial learning impairment was seen with D-AP5 in the 'high concentration' group but not in either the 'medium' or 'low concentration' groups (Davis et al., 1992). The fact that the whole tissue levels of D-AP5 found in the hippocampus of the 'high concentration' group greatly exceed those obtained with 7CK, suggests that the behavioural impairment seen in these experiments is unlikely to be due to an effect of 7CK on NMDA receptors in the hippocampus. It is interesting to note

that the 7CK-induced behavioural impairment occurs at much lower whole tissue drug levels than that seen with D-AP5.

Finally, there is no obvious explanation for the discrepancy between the tissue levels of 7CK attained in Expt 4.1 and those recorded in both Expts 4.2 and 4.3. There is a huge variation in the whole tissue levels resulting from the infusion of a single dose of 75mM 7CK, especially in Expt 4.1. The rapid excretion of the drug, coupled with small differences in the placement of the cannula, may result in large differences in the distribution of 7CK within the brain and thus contribute to this variation. The difference in 7CK levels between the two hippocampi in Expt 4.1 could account for the presence of a spatial learning impairment in the absence of an effect on LTP in the hippocampus contralateral to the infusion cannula. The purpose of Expt 4.2 was to determine whether or not LTP induction would have been blocked in the hippocampus adjacent to the infusion cannula in Expt 4.1. In this respect, Expt 4.2 is redundant in that the 7CK concentration found in the hippocampus in which LTP induction was assessed was lower than in Expt 4.1.

The nature of the 7CK-induced spatial learning impairment

The observation that chronic intraventricular infusion of 7CK causes a behavioural impairment which disrupts spatial learning in the watermaze is in agreement with a recently published study (Watanabe et al., 1992). Along similar lines to Expts 4.1 and 4.2, Watanabe and co-workers showed that acute injections of 7CK (10 nmol) into the lateral ventricles 10 min prior to behavioural testing caused a marked impairment in the acquisition of a spatial lerning task in the watermaze. In certain respects this result is also consistent with the observation that D-AP5 impairs spatial learning (Morris et al., 1986; Davis et al., 1992), but although both NMDA receptor antagonists disrupt performance in the watermaze, the underlying nature and the specificity of the impairment induced by 7CK may well differ from that obtained with D-AP5.

Like the impairment seen with D-AP5 (Davis et al., 1992), there is some evidence to suggest that 7CK infusion does not completely inhibit spatial learning. Although there were no significant differences between the HPC group and the 7CK group, either in terms of escape latency or in the transfer test (Tukey's HSD pairwise comparisons; $p > 0.05$), the group means (% time in training quadrant) did suggest that there may have been a trend for the 7CK infused rats ($35.1 \pm 3.7\%$) to have learned more than the HPC lesioned animals ($28.9 \pm$

2.3%) about the spatial location of the platform. Furthermore, inspection of Table 4.2 reveals that a number of animals within the 7CK group do exhibit a considerable bias towards the training quadrant.

A key issue is to assess whether or not the observed learning impairment is due to a specific disruption of spatial learning, or rather, is the result of an indirect effect on some other behavioural process which also influences the animal's level of performance. Making such a distinction between non-associative 'performance' effects and specific learning impairments is not always easy. There is, however, some suggestion from Expts 4.1 and 4.2 that behavioural processes, other than learning, are disrupted following 7CK infusion. For example, 7CK treated rats often displayed a sensorimotor impairment reminiscent of that resulting from high doses of D-AP5 (Davis et al., 1992). The 7CK rats often exhibited a prolonged righting reflex and had difficulties climbing onto, and then remaining on, the escape platform. Occasionally, the 7CK infused rats appeared to tire very quickly in the water and experience some difficulty in swimming on the latter trials of a training session. This was not, however, reflected in a gross reduction in swim speeds (see also Watanabe et al., 1992). One possible reason for this is that control rats also slow down as they learn the procedural elements of the task and begin to search systematically for the platform. The observed sensorimotor impairment in 7CK animals was reduced in Expt 4.2 compared with Expt 4.1, possibly as a consequence of changing the training protocol and extending the ITI which might allow drug-treated rats to recover between trials. It should be noted, however, that the improvement seen in Expt 4.2 may simply reflect the lower tissue levels of 7CK that were attained in this study compared with Expt 4.1. Nevertheless, whatever the underlying cause of this improvement, a subtle sensorimotor impairment was still evident in Expt 4.2. There were still a number of occasions when 7CK rats fell off the platform after a prolonged swim and most of the animals in this group still displayed a prolonged righting reflex. It seems plausible, therefore, that the observed impairment in spatial learning resulting from 7CK infusion may well have been due to effects of the drug on aspects of behaviour unrelated to learning *per se*. A prediction, therefore, is that the 7CK-induced deficit may extend to other non-spatial learning tasks such as the visual discrimination task and possibly even the single visible platform task. Although additional studies may help to determine the specificity (or lack of) of the 7CK-induced behavioural impairment, the expense of these experiments, both in terms of terms of time and financial resources, probably outweighs their importance.

The behavioural impairment resulting from the infusion of 7CK occurs at doses below those required to block the induction of hippocampal LTP with commonly used stimulation parameters (Davis et al., 1992). The locus and underlying mechanism responsible for the behavioural impairment, whether specific to spatial learning or not, remain unidentified. It may still be the case that the behavioural impairment is due to a disruption of synaptic plasticity in the hippocampus. The possibility that LTP induced by weaker forms of stimulation may, in fact, be sensitive to blockade by these doses of 7CK cannot be ruled out (see also Chapter 5 this thesis; Chetkovich et al., 1993; Haley et al., 1993). It is possible that the LTP-like events that underly spatial learning are more akin to potentiation induced by weaker stimulation parameters.

An alternative explanation which could account for the observed pattern of results, is that chronic exposure to 7CK results in the development of a tolerance to the drug. Initially 7CK can block changes in synaptic plasticity and thus prevent spatial learning, but by day 8, sufficient drug tolerance has developed to allow the induction of LTP. The results of Expt 4.3 indicate that the failure to block LTP, in animals which nevertheless showed a spatial learning impairment, is not due to a progressive decline in 7CK levels over the course of the experiment. This appears to rule out an explanation based on the development of a metabolic tolerance resulting in increased break down or excretion of the drug but does not, however, exclude some form of molecular tolerance involving an up-regulation of NMDA receptor complex activity in order to overcome the 7CK-induced blockade of the glycine receptor. It seems more likely, however, that 7CK does not block hippocampal synaptic plasticity at any stage of the experiment but disrupts spatial learning through some other mechanism.

Despite the apparent inability to block LTP, the possibility remains that these doses of 7CK are interfering with some other aspect of hippocampal physiology which could contribute to the spatial learning impairment. It has been suggested that the NMDA receptor dependence of spatial learning may be due to the involvement of these receptors in the expression of the theta rhythm and not in the induction of LTP (Leung and Desborough, 1988). It could be the case, therefore, that this dose of 7CK is disrupting hippocampal theta and that this is responsible for the behavioural impairment. Alternatively, there is some evidence from both *in vitro* and *in vivo* studies that 7CK may have a small but significant effect on normal low frequency neurotransmission in the hippocampus. For example, bath application of 7CK caused a small depression of normal low frequency synaptic transmission (a reduction in the

size of the epsp slope) in hippocampal slices which was not reversed by co-administering D-serine. This suggests that this effect is not mediated by the glycine receptor and is likely to involve an action at the AMPA receptor (Oliver et al., 1990). Bashir and colleagues (Bashir et al., 1990) showed that the amplitude of the population spike was also reduced following bath application of 7CK to slices. Similarly, intrahippocampal infusion of 7CK reduced the size of the population spike evoked by normal low frequency stimulation *in vivo* (Thiels et al., 1992). None of the effects of 7CK on low frequency neurotransmission were reversed by D-serine. Although an effect on some aspect of hippocampal physiology as the cause of the behavioural impairment cannot be ruled out, this seems unlikely bearing in mind the lack of a significant correlation between hippocampal tissue levels of 7CK and behavioural performance.

Of the other brain regions analysed, only the striatum had detectable levels of 7CK present in the majority of subjects. There was, however, no significant correlation between striatal levels of 7CK and the degree of learning as assessed by the transfer test. The possibility remains, of course, that 7CK is having an effect in some brain area not included in the tissue analysis. Indeed, 7CK may prevent the induction of LTP in a brain area, other than hippocampus, which is required for spatial learning. Nevertheless, the lack of a significant correlation between drug tissue levels and performance is somewhat surprising. The absence of such a correlation may suggest that the 7CK levels are either well below or well above the range of tissue levels which affect learning. The fact that there is a significant group difference between 7CK-infused and control animals, however, suggests that the drug concentrations are not well below the levels required to impair performance. The possibility that tissue levels of 7CK are well above those required to affect behaviour in the watermaze cannot be ruled out although the results of pilot studies suggested that lower doses of 7CK were ineffectual. Alternatively, it could be that a by-product of 7CK metabolism or some other consequence of the implantation procedure, or of the drug infusion, is responsible for the behavioural impairment although this seems unlikely bearing in mind that the performance of aCSF infused animals was indistinguishable from that of unoperated controls. Alternatively, the % time in the training quadrant may be an inappropriate measure to use in looking for a correlation. A number of other contributing factors will influence this score and may mask any drug-performance correlation.

Conclusions - Glycine antagonists, LTP and spatial learning

The results of Expt 4.1 and 4.2 demonstrate a behavioural deficit in the absence of a block of LTP. This pattern of results does not invalidate the hippocampal LTP/spatial learning hypothesis. This dissociation between hippocampal LTP and spatial learning may arise as a result of any one of a number of drug effects on behavioural processes which need not directly involve the learning mechanism. The necessity for LTP-like processes in spatial learning can only be tested using an experimental manipulation which successfully prevents (or enhances) the induction of LTP.

The possibility remains that higher concentrations of 7CK will successfully block the induction of LTP and that this would in fact disrupt the learning mechanism. Whether or not this would increase the magnitude of the behavioural deficit is unknown although, clearly, this approach would be of little use for investigating the relationship between LTP and learning. A higher dose of 7CK might prevent LTP induction and cause a greater learning impairment but it would, no doubt, be impossible to determine whether the increased behavioural deficit was due to a block of LTP-like events in the hippocampus or to an enhanced drug effect on the behavioural processes responsible for the learning impairments seen at lower doses in Expts 4.1 and 4.2. An alternative approach would be to infuse 7CK directly into the hippocampus, a protocol which is known to result in sufficient drug levels to block the induction of hippocampal LTP *in vivo* (Thiels et al., 1992). Furthermore, infusion of the drug directly into the hippocampus may result in less spread of the drug to other brain areas, compared with infusion into the ventricles. For example, injection of radioloabelled [^{3}H] AP4 directly into the hippocampus resulted in a pattern of distribution in which drug diffusion was mainly restricted within the hippocampus (Morris et al., 1989). It is possible, therefore, that the hippocampus is able to retain a drug effectively within a localised area, allowing higher drug levels to accumulate and limiting the spread to other brain areas. Even so, the possibility of drug effects on processes unrelated to the induction of LTP cannot, however, be ruled out. For example, 7CK may affect normal low frequency neurotransmission in the hippocampus and there is still the possibility that 7CK may diffuse outside the hippocampus. These concerns suggest that 7CK will be of little, if any, use as experimental tool for investigating the relationship between hippocampal LTP and spatial learning.

The glycine receptor may still, however, provide such an opportunity. Since these experiments were undertaken, further chlorination of the parent compound, kynurenate, has

resulted in an even more efficacious and selective antagonist, 5,7-dichlorokynurenic acid (5,7DCK; Baron et al., 1991). This compound is probably a better prospect than 7CK for inhibiting the NMDA receptor-associated glycine site, but there is still the concern that 5,7DCK will be subject to many of the same problems associated with 7CK, such as rapid excretion from the brain and effects on processes other than hippocampal LTP.

Despite the failure of these experiments to substantiate the hippocampal LTP/spatial learning hypothesis, they do, however, illustrate the importance of a within-subject protocol in which behaviour, LTP induction and drug tissue levels are assessed in the same animals. For example, Watanabe and co-workers showed that intraventricular infusion of 7CK caused a behavioural deficit in a spatial learning task in the watermaze and that bath application of 7CK onto hippocampal slices blocked the induction of LTP *in vitro* (Watanabe et al., 1992). From their results they conclude that the "impairment of spatial learning by 7-Cl-Kyn observed in the present study may be, at least partly, attributable to blockade of hippocampal LTP." The results of Expts 4.1 and 4.2 suggest that this is not necessarily the case and advise more caution when interpreting drug-induced learning deficits.

Does D-Cycloserine enhance spatial learning in the watermaze?

Expt 4.4 investigated the effects of DCS on spatial learning in the watermaze. Inspection of the data (Figure 4.4.1) revealed that there was no consistent difference in terms of escape latency during acquisition between rats given DCS and vehicle injected controls, and no indication whatsoever that this drug might facilitate learning. In the transfer test, however, there was some suggestion that the drug group may have learned more than the control animals about the location of the hidden escape platform during training (Figure 4.4.6). Analysis of performance in the transfer test revealed that during the initial stages (comparing the first 15 seconds), the DCS treated rats show a stronger spatial bias towards the training quadrant relative to vehicle injected animals. As the transfer test proceeds the difference between the two groups reduces and analysis of the full 60 sec of the probe trial failed to reveal a significant effect. One interpretation of these results is that during acquisition DCS does facilitate learning but as the escape latencies are so rapid this is not seen as a reliable difference between the groups (a "ceiling" effect). With the transfer test, however, the greater learning by the DCS group is reflected in a stronger initial bias towards the training quadrant. As the probe trial continues the rats learn that the platform is no longer present in its former location and begin to search elsewhere. The rate of extinction will be determined,

in part, by the strength of the original learning and so it is not inconceivable that the DCS-treated group extinguish faster. It is also possible that the DCS effect on spatial learning is part of a generalised learning facilitation which may also extend to the rate of extinction.

It should be noted, however, that although significant, the difference between DCS-injected rats and controls is minimal. Indeed, using the conventional analysis (escape latencies during training and 60 sec transfer test) there were no significant differences between the groups. The effect of DCS is only seen at one particular time point during testing, on one measure of performance, and relies heavily on one particular interpretation of the animals' behaviour during the transfer test. Therefore, the difference maybe should not be overemphasised.

D-Cycloserine does not produce a robust spatial learning enhancement

There are a number of possible reasons why DCS produced only a small spatial learning enhancement in the watermaze, if any. It could of course simply be that NMDA receptors are not required for spatial learning, in which case there is no *a priori* reason for assuming that glycine agonists should enhance learning. An alternative explanation is that the standard spatial reference memory task in the watermaze is just too easy and acquisition by control animals is so rapid that any potential learning enhancement remains undetected. It may be possible to overcome this ceiling effect by making the task harder. One approach might be to reduce the number, or salience, of the extramaze cues.

A completely different approach would be to reduce the level of performance in control animals by co-administering a second drug which retards the rate of learning. This would effectively lower performance away from the 'ceiling'. There is an apparently endless list of pharmacological agents which disrupt perfomance in the watermaze (McNamara and Skelton, 1993). An obvious candidate, for example, is D-AP5 (Morris et al., 1986a). A recent study by Fishkin and colleagues has adopted this approach using the muscarinic cholinergic antagonist, scopolamine. They found that DCS (3.0 or 10.0 mg/kg i.p; 30 mins prior to testing) reversed deficits induced by scopolamine, in both a standard spatial learning task in the watermaze and in a T-maze reinforced alternation procedure (Fishkin et al., 1993). Interestingly, the authors found no enhancement in the DCS alone group in the watermaze, in terms of escape latency which is in agreement with this study. No transfer test data was reported.

It remains to be seen whether higher doses of DCS cause a further enhancement of spatial learning using this paradigm. The choice of DCS, a partial agonist at the glycine receptor, may also contribute to the lack of a robust effect. The possibility remains that a more efficacious agonist, such as D-serine or glycine itself, may have produced a greater learning enhancement. The lack of a robust learning enhancement, however, may not be due to either the task difficulty (or lack of it) of the standard watermaze procedure or the choice of ligand but, rather, may be a result of the fact that the NMDA receptor-associated glycine site is naturally saturated by endogenous levels of glycine.

Is the Glycine Site Naturally Saturated?

Estimates of the glycine concentration present in the ECF indicate endogenous levels in excess of 1 μM (Skilling et al., 1988; Ferraro and Hare, 1985). The actual concentration of glycine available to the receptor, however, is unknown. If μM concentrations of glycine are available then this would, undoubtedly, saturate the receptor. This is somewhat surprising in that it would appear to rule out the possibility that the endogenous role of glycine is to modulate the NMDA receptor. This does, however, leave open the possibility that the receptor can be endogenously regulated by an antagonist such as kynurenate, but the levels of kynurenate present in the brain would be of little consequence in the presence of such high glycine concentrations (Moroni et al., 1988; Swartz et al., 1990). With *in vitro* experiments much will depend upon the nature of the preparation. A number of studies have shown that adding exogenous glycine has no effect on the NMDA response and that glycine-mediated potentiation can only be observed in the presence of an antagonist (Kemp et al., 1988; Fletcher and Lodge, 1988; Watson et al., 1988). In contrast, other researchers have demonstrated that the addition of glycine or D-serine can enhance neuronal responses to NMDA in the absence of antagonists (Salt, 1989; Thompson et al., 1989; Danysz et al., 1989; Forsythe et al., 1988; Lovinger and Weight, 1988). Factors such as the rate of perfusion (eg. Johnson and Ascher, 1987), regional variations in glycine concentrations and the ease with which the applied agonist can gain access to the receptors are likely determinants of whether or not a glycine-mediated enhancement of NMDA receptor-dependent processes is seen. It has also been pointed out that these experiments could be complicated by the fact that applied agonists, such as NMDA, may stimulate the release of endogenous glycine (Kemp et al., 1988; Shalaby et al., 1989). With regard to the situation *in vivo*, administration of glycine or D-serine enhances several physiological processes

mediated by NMDA receptors including convulsant effects (Larson and Beitz, 1988; Singh et al., 1990). This suggests that at least some glycine receptors are unsaturated *in vivo*. There are conflicting reports concerning the ability of glycine agonists to modulate the inducibility of LTP. Intrahippocampal injection of D-serine did not cause a significant increase in the magnitude of LTP that could be induced at CA1-commissural synapses *in vivo* (Thiels et al., 1992). In contrast, Abe and co-workers showed that glycine facilitated the induction of LTP in hippocampal slices (Abe et al., 1990). It is not clear, however, what effect continuous perfusion of slices *in vitro* will have on the levels of glycine available to the receptors.

Finally, if glycine receptors in the hippocampus are naturally saturated with endogenous glycine then an alternative prediction is that the injection of a partial agonist, such as DCS, might be expected to compete with glycine for these receptors and reduce NMDA receptor-mediated activity. Consequently, DCS treatment might be expected to cause a spatial learning impairment. The results of Expt 4.4 reveal that this is not the case. The observation that DCS produces a small enhancement in performance is consistent with the idea that the glycine receptor may not be fully saturated.

Conclusions - D-Cycloserine and learning enhancement

As with learning impairments, the underlying cause of a learning enhancement is not always easy to identify. The rate of learning may be enhanced through general increases in non-associative behavioural processes such as arousal, motivation and excitability. Non-specific effects of DCS on brain systems involved in arousal might be expected to facilitate performance in a variety of learning tasks. Examination of the existing literature suggests that DCS does, indeed, enhance a number of different forms of learning, including trace eyeblink conditioning in rabbits (Thompson et al., 1992), passive avoidance in rats and reversal of place learning in a T-maze in rats (Monahan et al., 1989b). The variety of learning tasks affected by DCS could be taken as evidence for a generalised increase in arousal or motivation. Alternatively, this may simply reflect a common mechanism (possibly involving NMDA receptors), shared by a number of different forms of learning, all of which will be affected following an i.p injection of DCS. As far as the potential clinical importance of these compounds is concerned, however, the precise mechanism by which cognition is enhanced is, in some respects, irrelevant.

As it stands, this experiment provides only limited circumstantial evidence that NMDA receptors might be involved in the processes underlying spatial learning. Further studies are required to determine whether or not hippocampal-independent forms of learning in the watermaze are also enhanced (eg. visual discrimination; see Expt 6.4). In addition, it also remains to be seen firstly, whether DCS will facilitate the induction of LTP *in vivo*, and secondly, whether any effect on LTP occurs at similar doses to those which enhance learning.

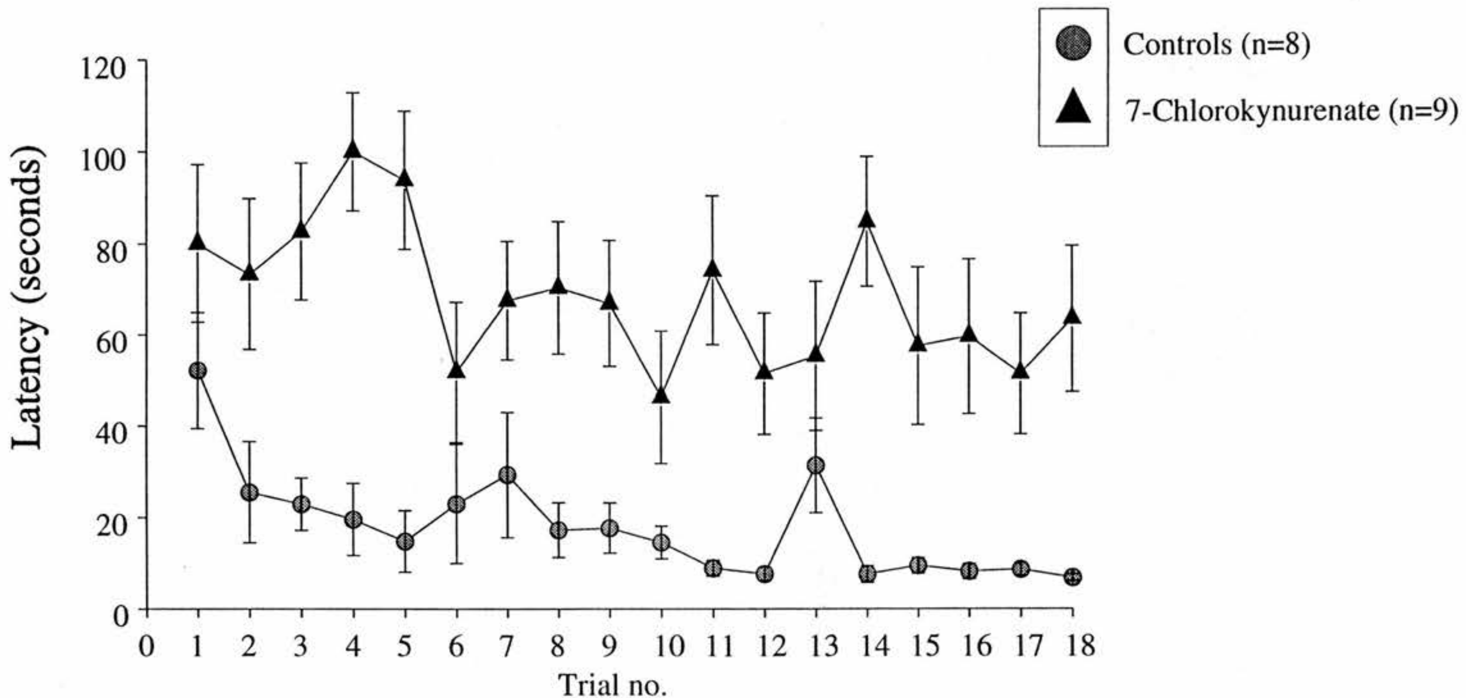
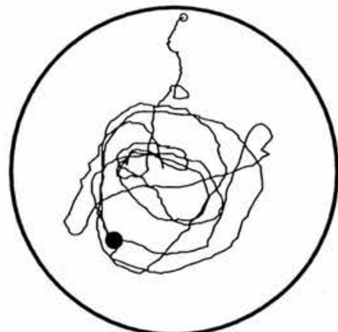


Fig. 4.1.1

Mean escape latency (+/- 1 SEM) during acquisition of a standard spatial reference memory task in the watermaze (Expt. 4.1).

Control



7-Chlorokynurenate

Fig. 4.1.2

During the transfer test the platform is removed from the pool and the rat allowed to swim freely for 60 seconds. The above are representative pathways of subjects taken from both experimental groups (Expt 4.1).

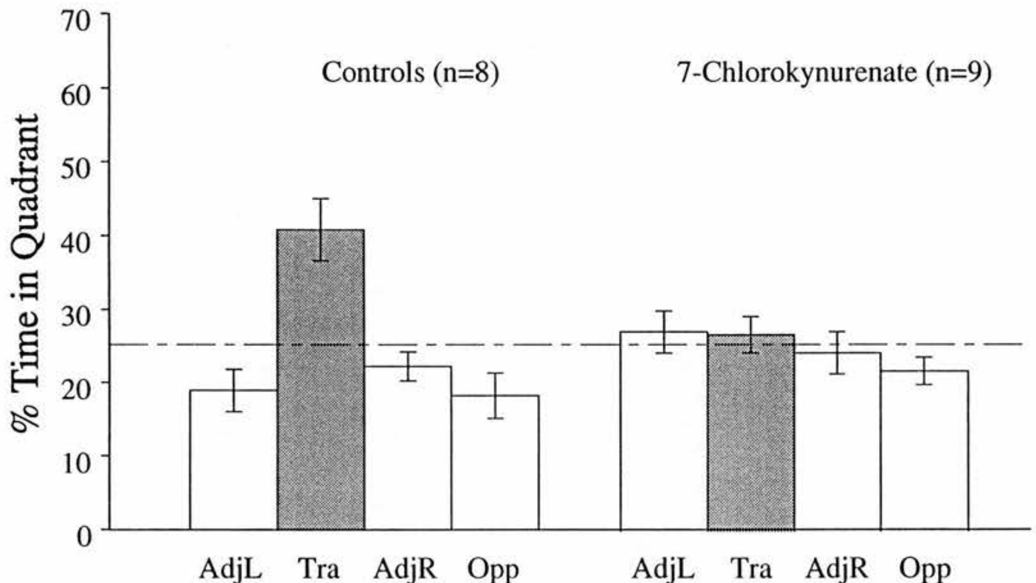


Fig. 4.1.3

The percentage time spent in each quadrant of the pool during the 60 sec transfer test (Expt 4.1). (AdjL = quadrant adjacent to, and to the left of the quadrant to which the animals were trained; Tra = the training quadrant; AdjR = quadrant adjacent to, and to the right of the training quadrant; Opp = the quadrant opposite the training quadrant).

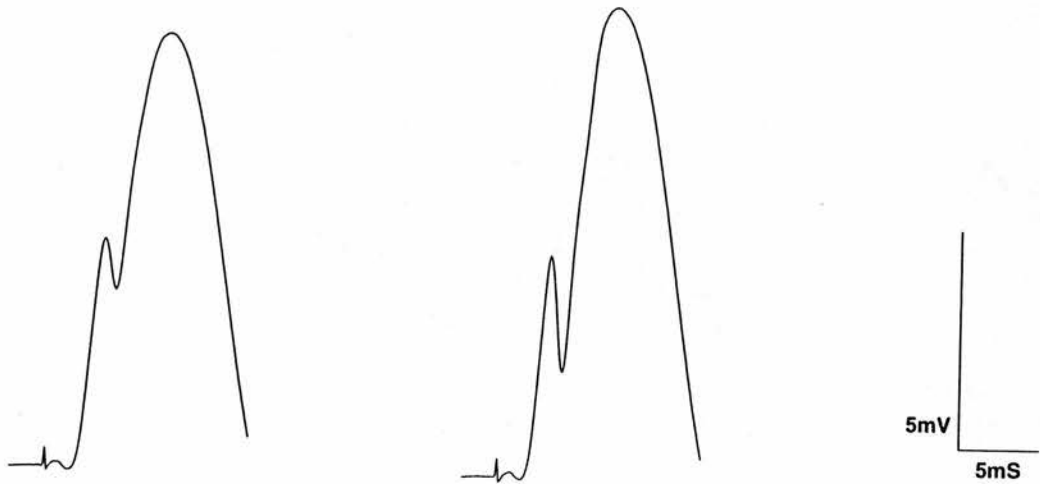


Fig. 4.1.4

Representative field epsps recorded in the presence of 75 mM 7CK from the granule cell layer of the dentate gyrus following perforant path stimulation before (left) and after (right) tetanic stimulation (Expt 4.1).

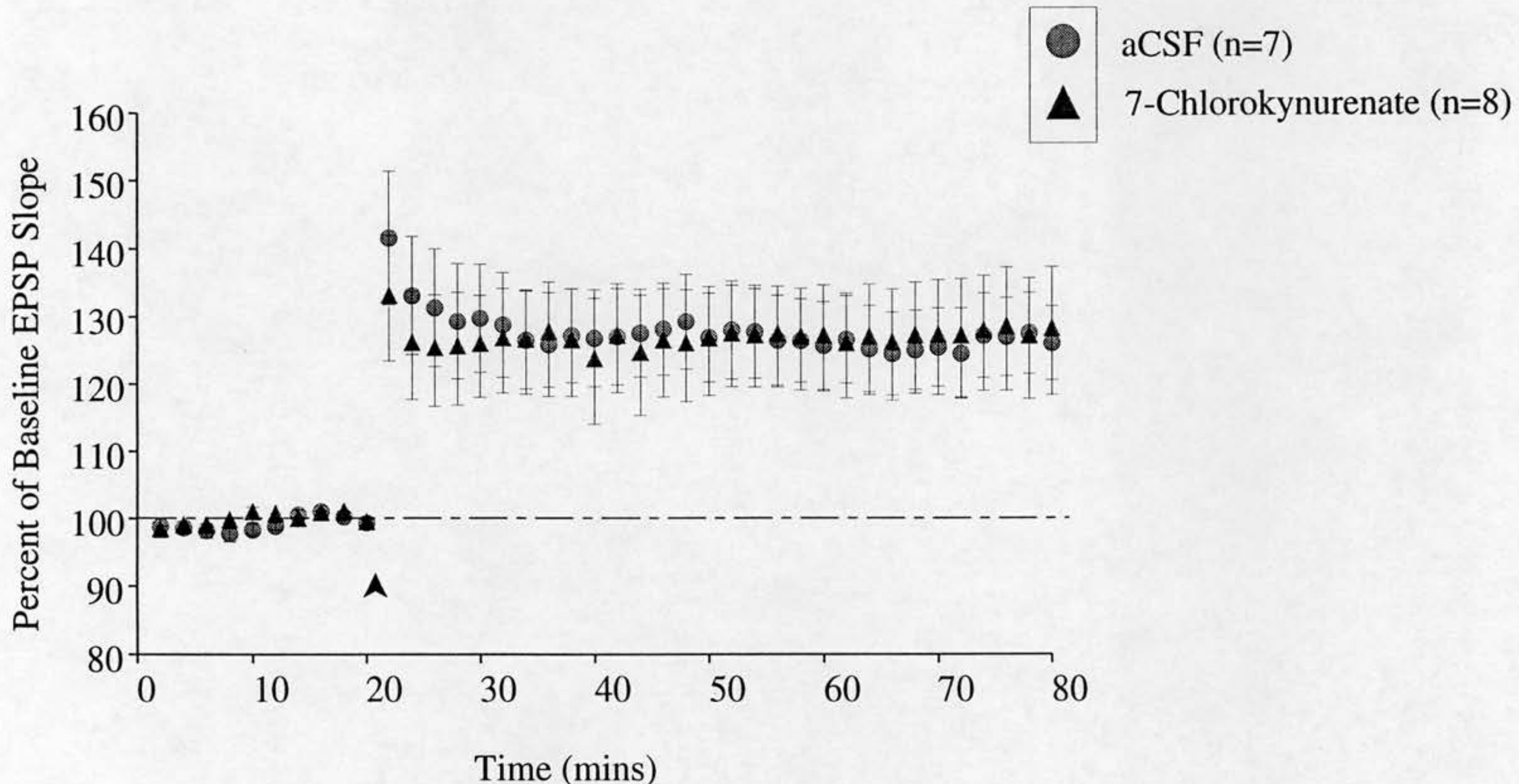


Fig. 4.1.5

The infusion of 75 mM 7-Chlorokynurenate into the lateral ventricle contralateral to the stimulating and recording electrodes failed to block the induction of LTP (Expt 4.1). (▲= tetanus).

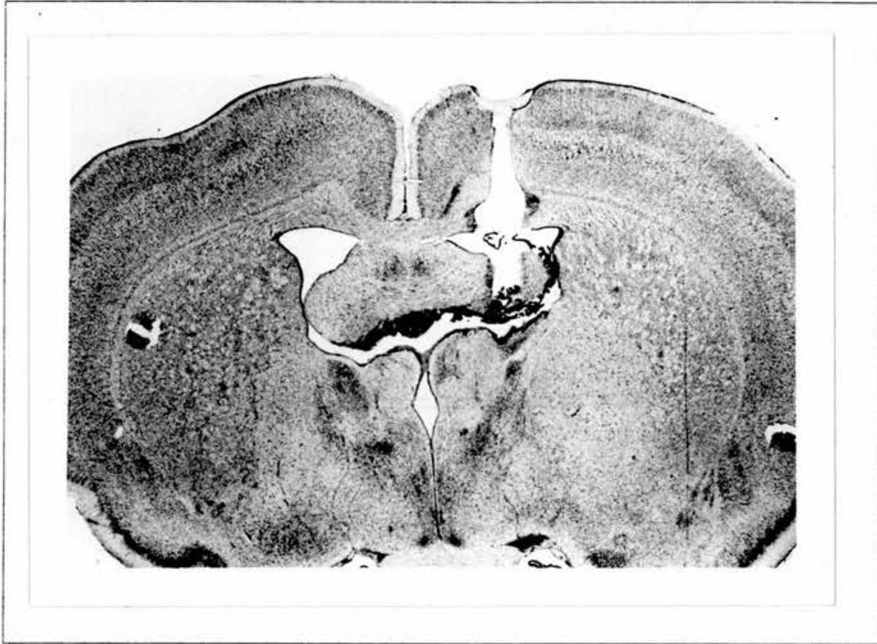


Fig. 4.1.6
Photomicrograph showing the location of the cannula in the right lateral ventricle (Expt 4.1).

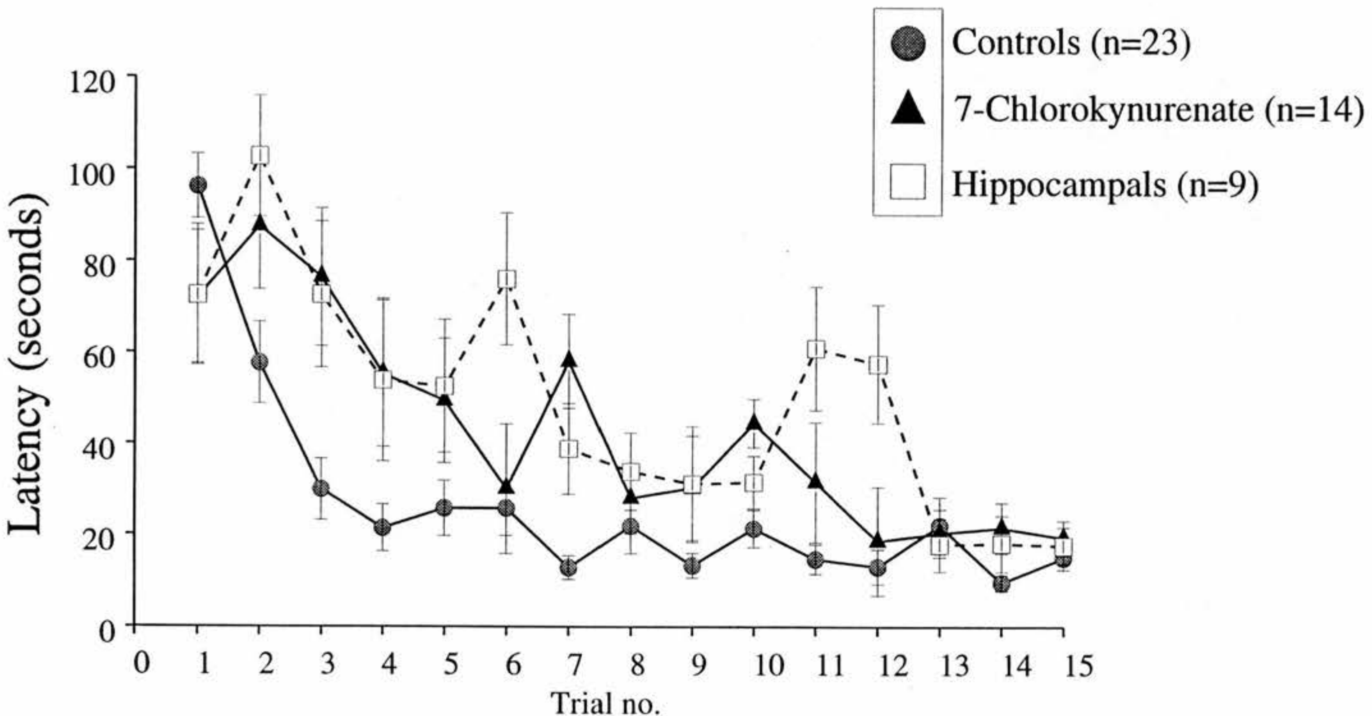
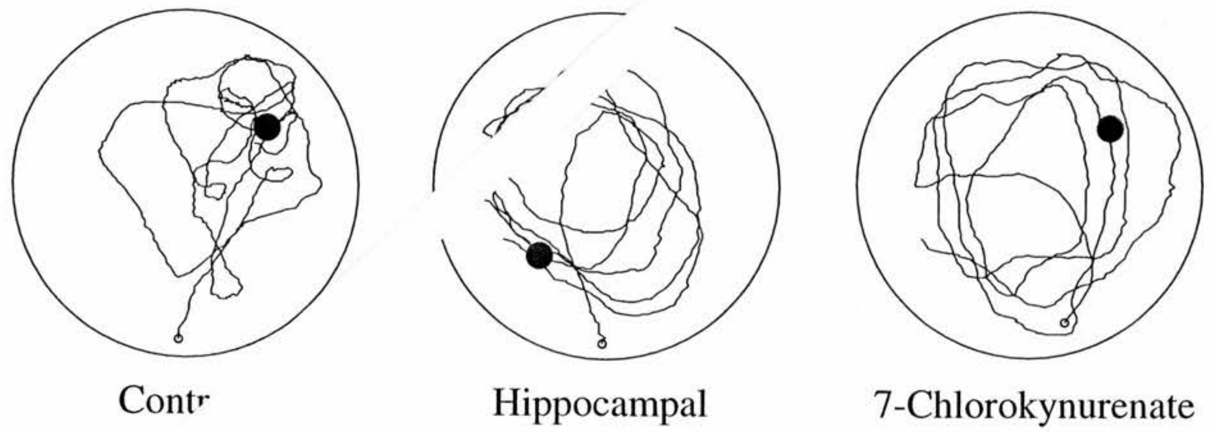


Fig. 4.2.1

Mean escape latency (+/- 1 SEM) during acquisition of a standard spatial reference memory task in the watermaze (Expt. 4.2).



.g. 4.2.2

During the transfer test the platform is removed from the pool and the rat allowed to swim freely for 60 seconds. The above are representative pathways of subjects taken from the 3 experimental groups (Expt 4.2).

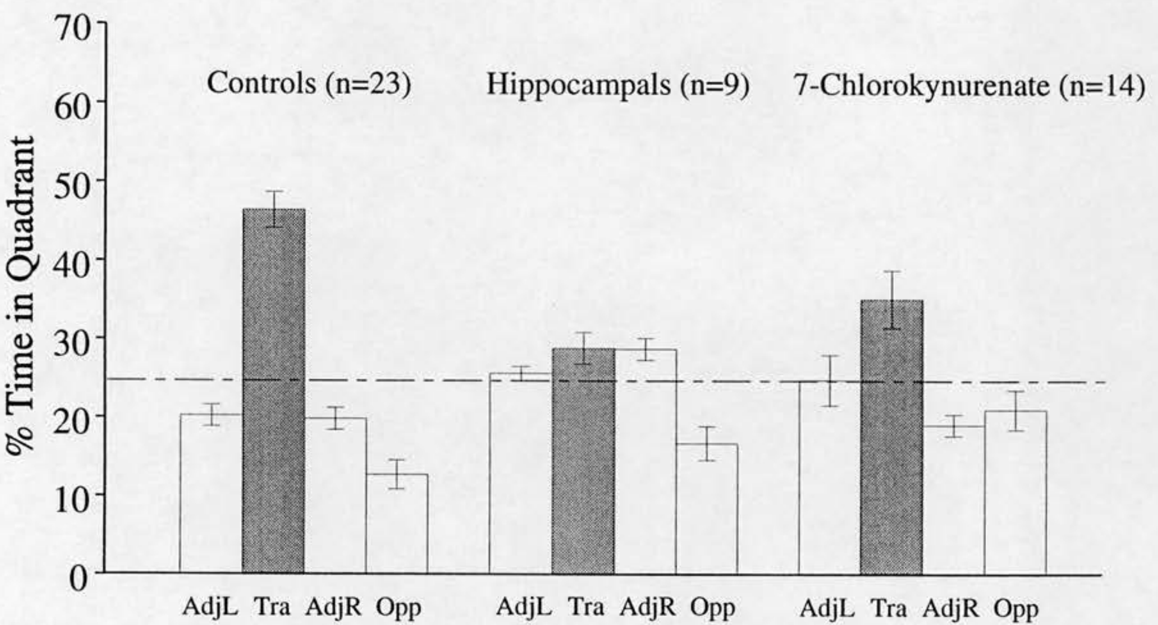


Fig. 4.2.3

The percentage time spent in each quadrant of the pool during the 60 sec transfer test (Expt 4.2). (AdjL = quadrant adjacent to, and to the left of the quadrant to which the animals were trained; Tra = the training quadrant; AdjR = quadrant adjacent to, and to the right of the training quadrant; Opp = the quadrant opposite the training quadrant).

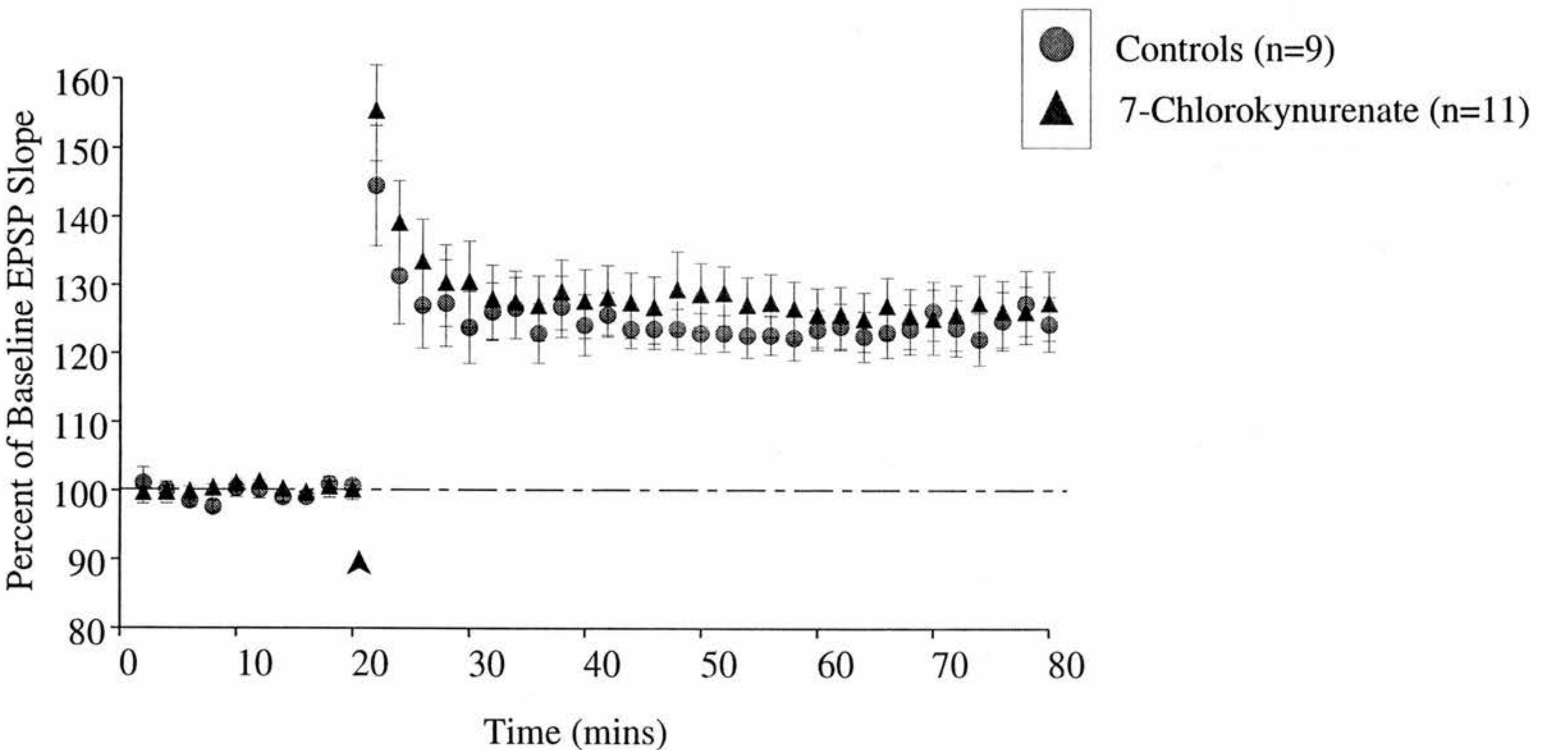


Fig. 4.2.4

The infusion of 75 mM 7-Chlorokynurenate into the lateral ventricle ipsilateral to the stimulating and recording electrodes failed to block the induction of LTP (Expt 4.2). (▲= tetanus).

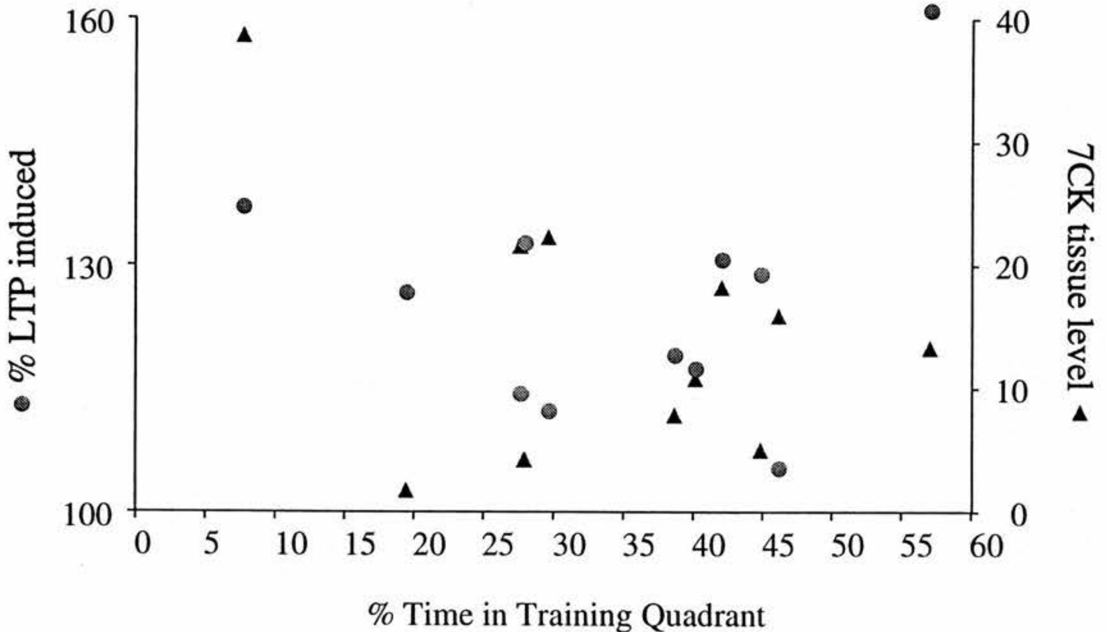


Fig. 4.2.5

Correlation of behavioural perfomance during the transfer test with inducability of LTP and 7CK tissue levels for rats (n = 11) infused with 75 mM 7CK (Expt 4.2).

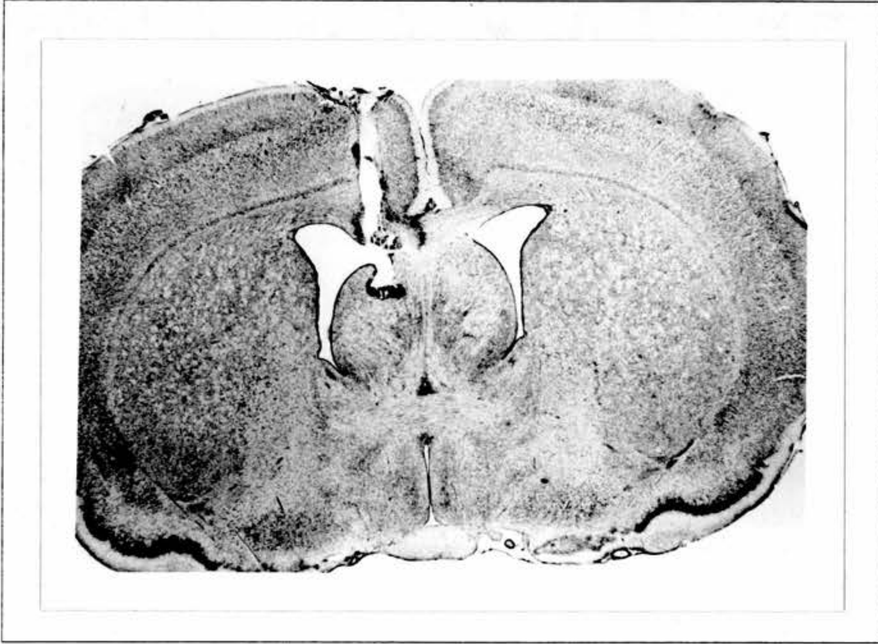
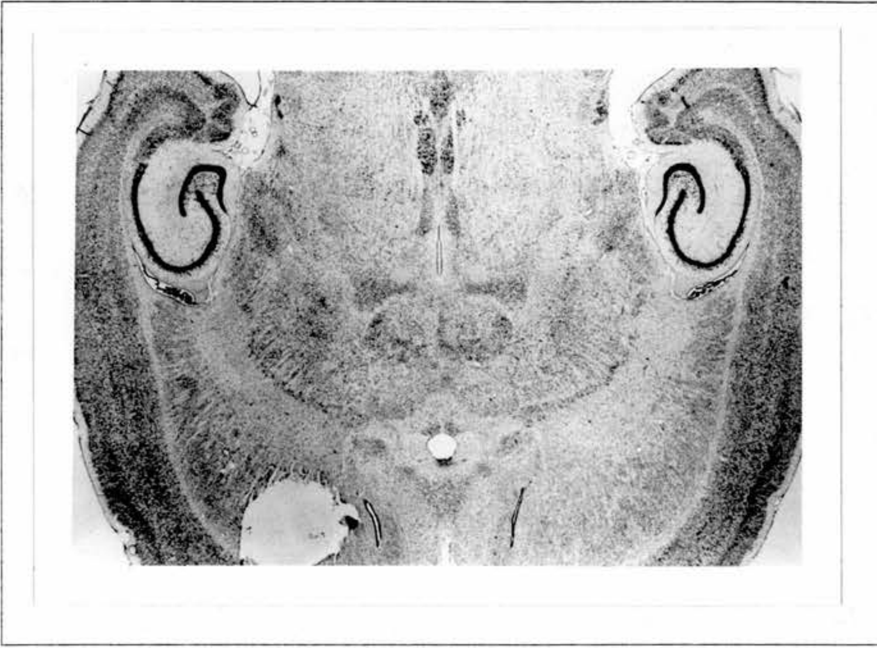


Fig. 4.2.6
Photomicrograph showing the location of the cannula in the left lateral ventricle (Expt 4.2).



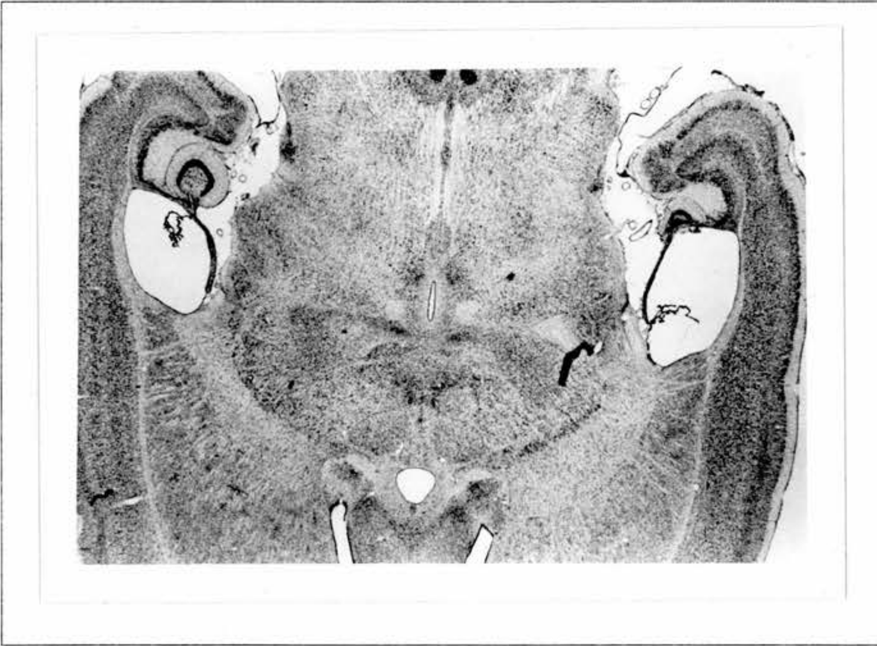


Fig. 4.2.7
Photomicrographs showing representative normal (above) and hippocampal lesioned (below) brains.

Table 4.2.1

Experiment 4.2- Summary Table for Individual Subjects

Subject	% Time in Training Quadrant	%LTP induced [1] after 60 mins	7CK in Left Hippocampus (pmol/mg wet weight)	7CK in Striatum (pmol/mg wet weight)
8406	28.0	132.99	4.26	2.36
8407	46.2	105.59	15.93	9.30
8408	55.3	-	5.00	1.97
8409	38.7	119.32	7.84	1.90
8410	27.4	-	*	7.27
8413	40.2	117.68	10.80	3.77
8418	44.9	129.21	5.04	3.17
8419	7.8	137.34	38.63	1.35
8420	27.4	-	2.50	3.80
8421	19.5	126.96	1.78	1.37
8422	42.1	130.99	18.24	1.63
8423	57.1	161.34	13.31	2.29
8428	29.7	112.50	22.30	*
8430	27.7	114.62	21.59	1.58

- no usable evoked response

* 7CK tissue levels below detectable limit

[1] LTP is expressed as a percentage of the pre-tetanic baseline response (100%)

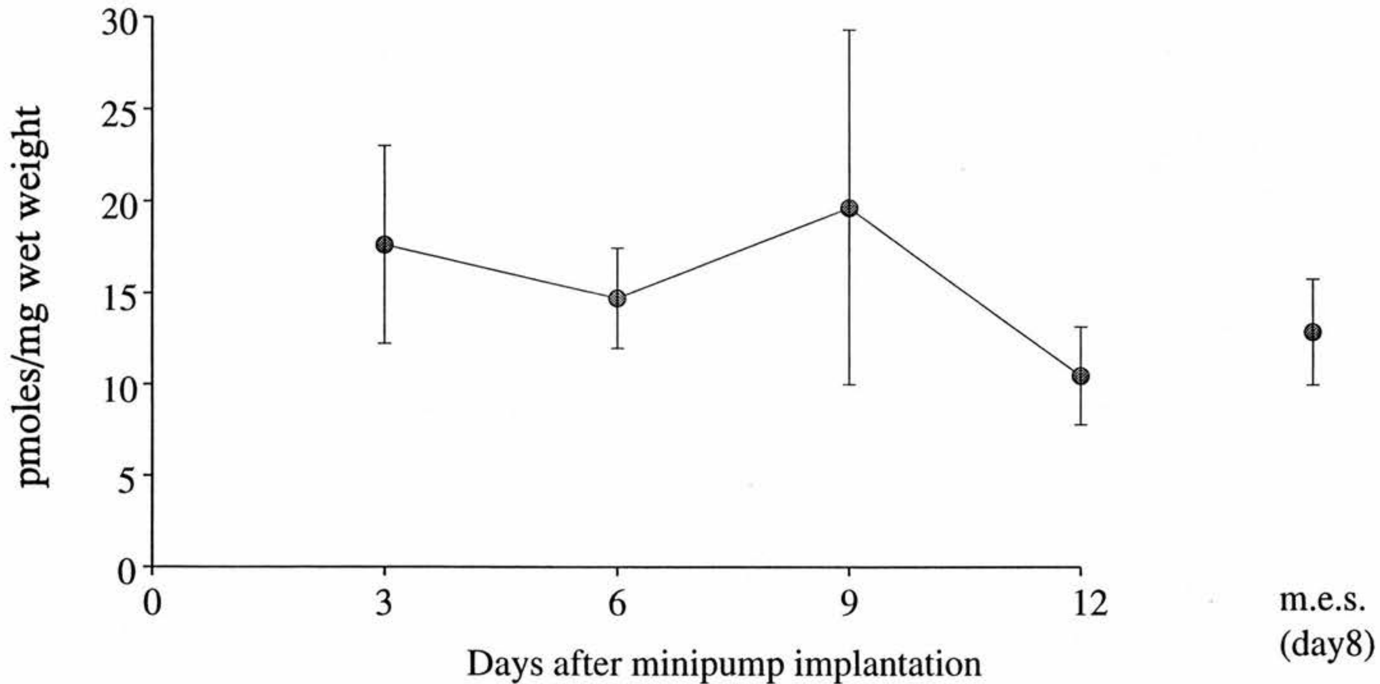


Fig 4.3.1

A time course study for 7CK whole tissue levels in the left hippocampus (ipsilateral to the infusion cannula; Expt 4.3). The value for day 8 is the mean 7CK tissue concentration in the ipsilateral hippocampus in Expt 4.2.

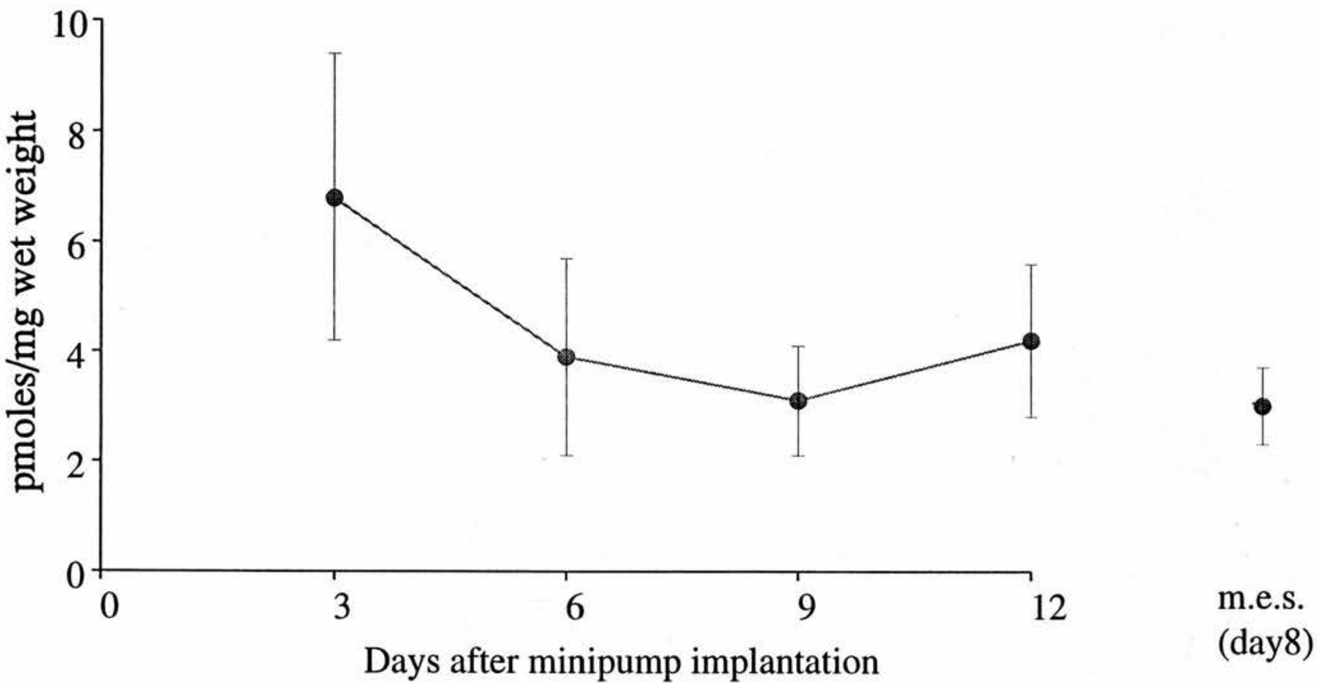


Fig 4.3.2

A time course study for 7CK whole tissue levels in the striatum (Expt 4.3). The value for day 8 is the mean 7CK tissue concentration in the striatum in Expt 4.2.

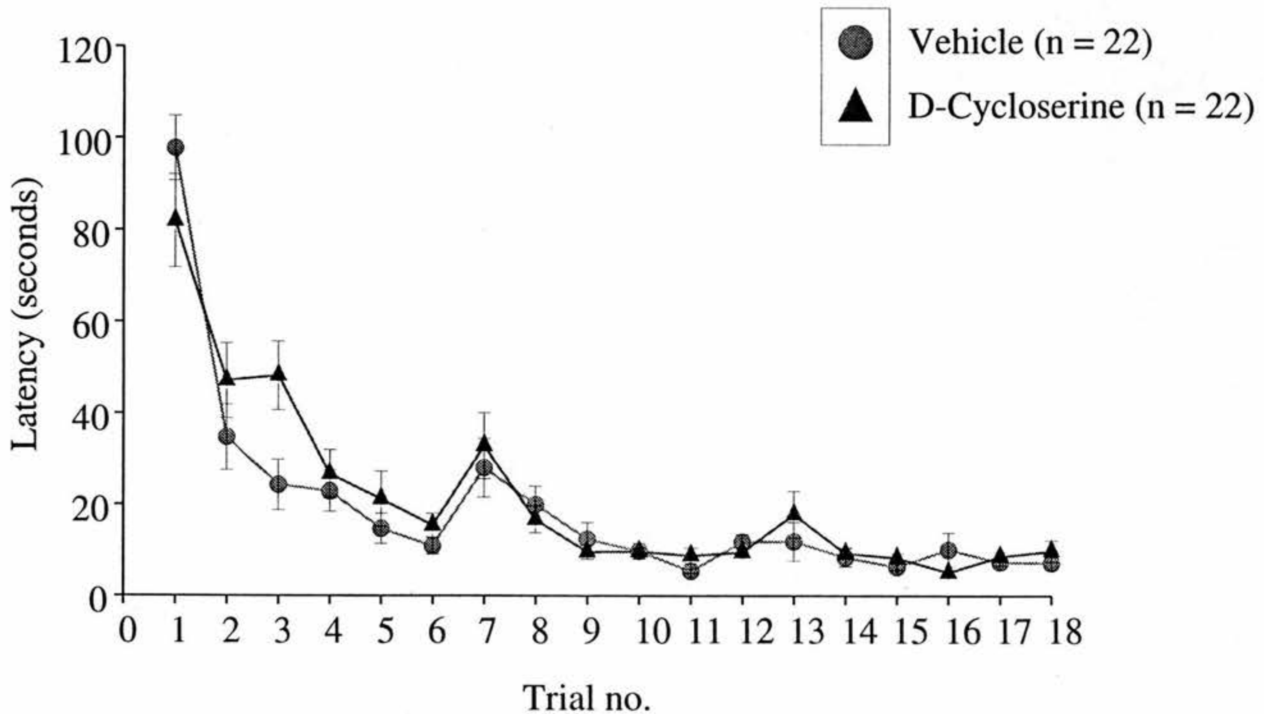
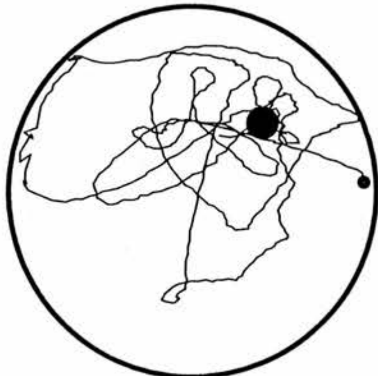


Fig. 4.4.1

Mean escape latency (+/- 1 SEM) during acquisition of a standard spatial reference memory task in the watermaze (Expt. 4.4).

Vehicle



D-Cycloserine

Fig. 4.4.2

During the transfer test the platform is removed from the pool and the rat allowed to swim freely for 60 seconds. The above are representative pathways of subjects taken from both experimental groups (Expt 4.4).

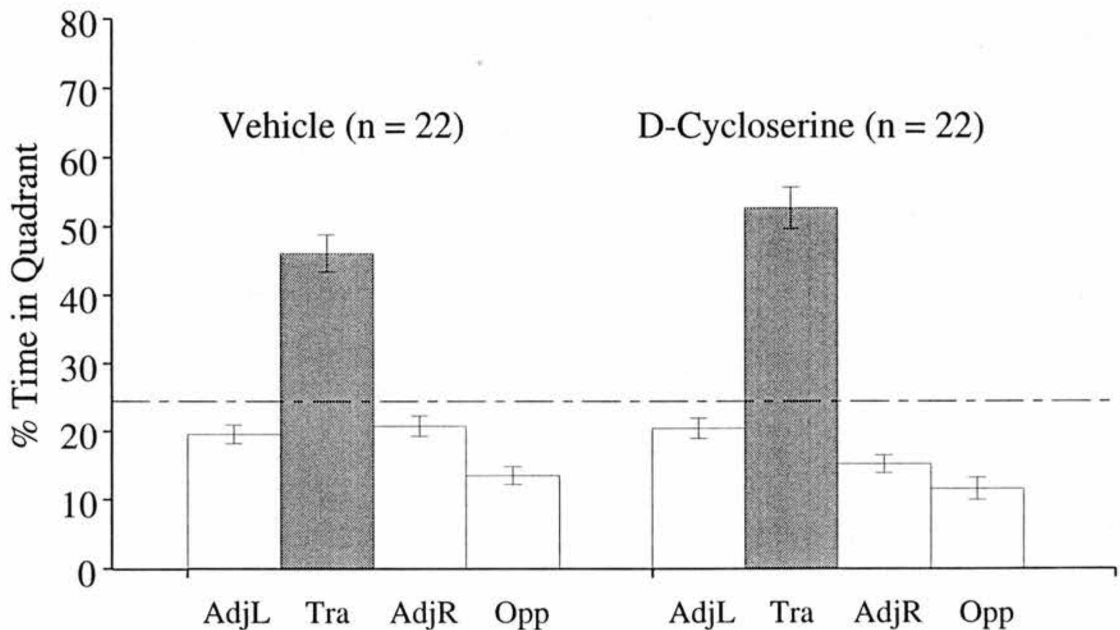


Fig. 4.4.3

The percentage time spent in each quadrant of the pool during the full 60 sec of the transfer test (Expt 4.4). (AdjL = quadrant adjacent to, and to the left of the quadrant to which the animals were trained; Tra = the training quadrant; AdjR = quadrant adjacent to, and to the right of the training quadrant; Opp = the quadrant opposite the training quadrant).

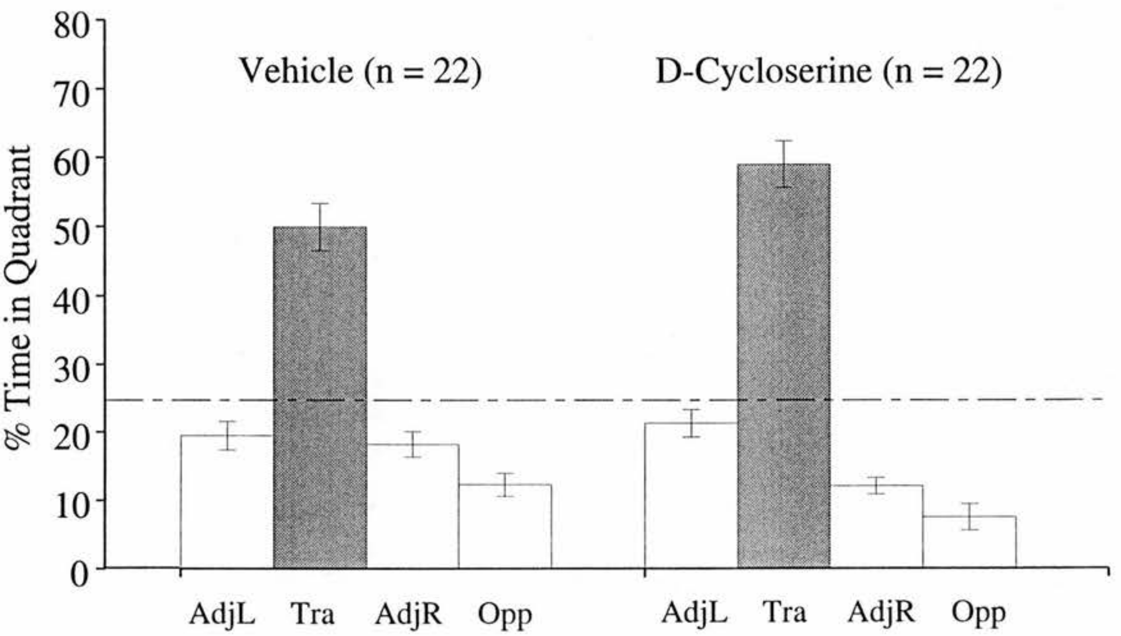


Fig. 4.4.4

The percentage time spent in each quadrant of the pool during the first 30 sec of the transfer test (Expt 4.4). (AdjL = quadrant adjacent to, and to the left of the quadrant to which the animals were trained; Tra = the training quadrant; AdjR = quadrant adjacent to, and to the right of the training quadrant; Opp = the quadrant opposite the training quadrant).

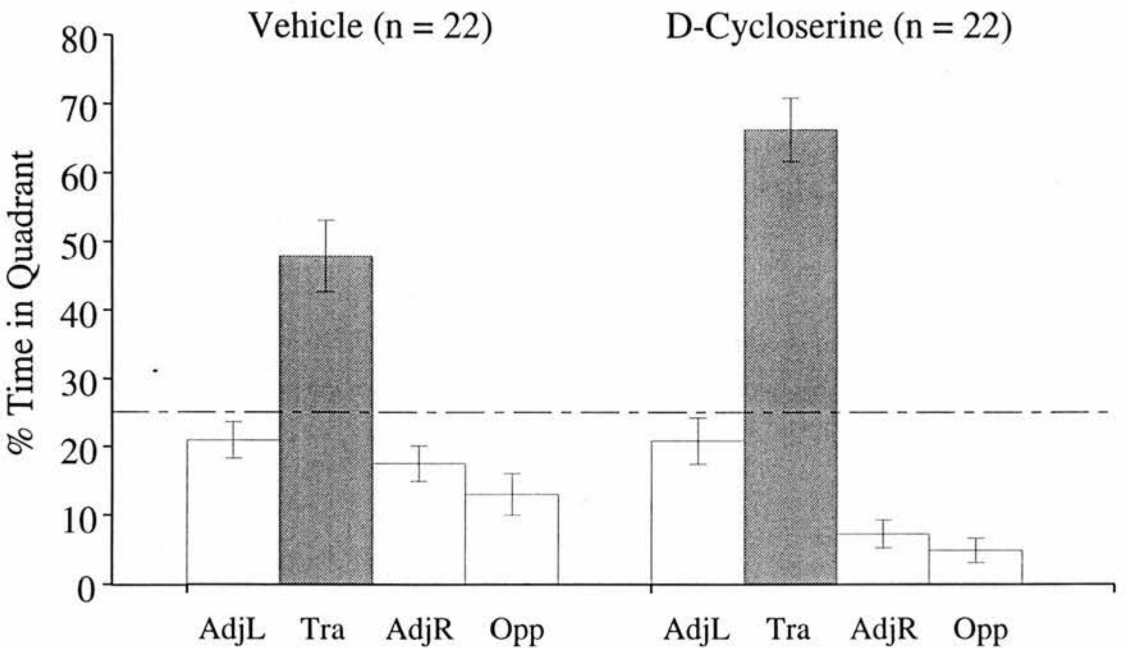


Fig. 4.4.5

The percentage time spent in each quadrant of the pool during the first 15 sec of the transfer test (Expt 4.4). (AdjL = quadrant adjacent to, and to the left of the quadrant to which the animals were trained; Tra = the training quadrant; AdjR = quadrant adjacent to, and to the right of the training quadrant; Opp = the quadrant opposite the training quadrant).

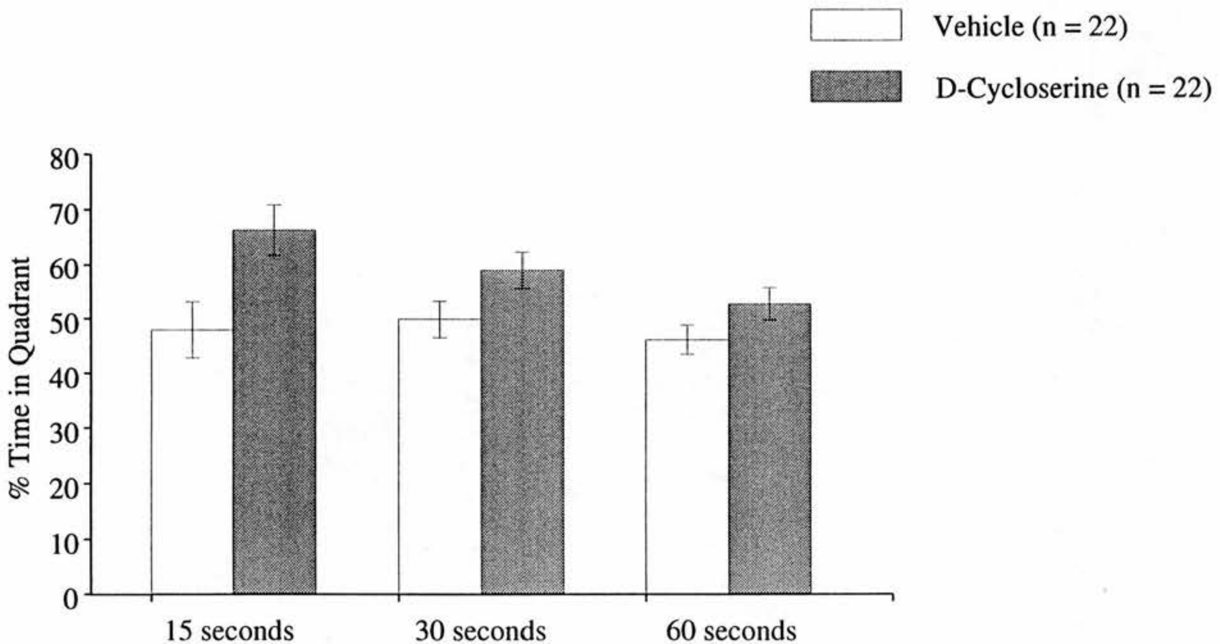


Fig 4.4.6

A comparison of the amount of time spent in the training quadrant during the first 15 sec, first 30 sec or the full 60 sec of the transfer test (Expt 4.4).

Chapter 5 - The effects of the nitric oxide synthase inhibitor, L-NAME, on the induction of hippocampal LTP *in vivo*.

Evidence that the locus of LTP expression is, at least in part, pre-synaptic (Dolphin et al., 1982; Bliss et al., 1986; Bekkers and Stevens, 1990a; Malinow and Tsien, 1990), coupled with the observation that the synapse specificity of LTP induction is not maintained in pre-synaptic terminals (Bonhoeffer et al., 1989), has led to suggestions that there may be a requirement for an intercellular messenger during the development of LTP (see Chapter 2). Although there is some experimental support favouring a role for arachidonic acid (AA) as an intercellular messenger during the later stages of LTP (Williams et al., 1989), the slow onset of the AA-induced potentiation implies that a second intercellular messenger molecule may be required to account for the immediate increase in synaptic efficacy. Recently, a considerable amount of evidence has accumulated suggesting that nitric oxide (NO) may also function as an intercellular messenger during the development of LTP.

Nitric Oxide

Nitric oxide (NO) is a simple, diatomic, gaseous compound which is thought to subserve a number of physiological functions, including that of a putative neurotransmitter in both the peripheral and central nervous systems. NO is a free radical species with an unpaired electron. Consequently, NO is a highly reactive and highly toxic molecule which under physiological conditions has a half-life of only 3-5 seconds. This lack of stability ensures that NO can travel only a minimal distance from the point of synthesis, restricting its biological influence to a limited number of neighbouring cells. This, coupled with the fact that it is freely diffusible and readily crosses cell membranes, makes NO an ideal candidate intercellular messenger. Before considering the role of NO as a putative neurotransmitter, and more specifically as a candidate retrograde messenger in LTP, it is worthwhile reviewing, briefly, some of the other aspects of the biology of NO.

Nitric Oxide Synthesis

In biological systems NO is formed as a result of the oxidation of one of the terminal guanidino nitrogens on the amino acid, L-arginine. The reaction also results in the formation of L-citrulline and is catalysed by the enzyme, nitric oxide synthase (NOS). The activity of the enzyme can easily be assayed by measuring the stoichiometric conversion of [^{3}H] L-arginine to [^{3}H] L-citrulline (Bredt and Snyder, 1990). Initial attempts to purify NOS from rat cerebellum by means of ion-exchange chromatography resulted in the loss of enzyme

activity. The activity was recovered by co-administering soluble brain extracts along with the isolated protein, suggesting that some essential co-factor was being lost during the purification process. The essential co-factor was subsequently identified as the calcium binding protein, calmodulin (Bredt and Snyder, 1990). This absolute requirement for Ca^{2+} -calmodulin provides a potential mechanism by which NMDA receptor activation might stimulate NOS. Furthermore, Knowles and co-workers have shown that at normal, resting intracellular free Ca^{2+} concentrations (approx. 50 nM), NOS is inactive (Knowles et al., 1989). Elevation of cytosolic free Ca^{2+}levels (> 80 nM) does, however, activate the enzyme with half-maximal reaction rates occuring at a Ca^{2+} concentration of approximately 160 nM. Therefore, the enzyme only becomes active when the cytosolic free Ca^{2+} concentration is elevated well above normal resting levels. This will, of course, be the case following a high frequency tetanus which results in a large Ca^{2+} influx through the activated NMDA receptor-ionophore. The reaction also requires molecular oxygen (Kwon et al., 1990) and a number of additional enzymic co-factors, including a series of electron donors, NADPH, FAD, FMN and in some cases, tetrahydrobiopterin (Bredt et al., 1991a).

Because NOS avidly binds NADPH, Bredt and Snyder (1990) were able to successfully purify the enzyme using affinity chromatography with a 2',5'-ADP-linked agarose column. Subsequently, the amino acid sequence of the protein has been determined by means of a polymerase chain reaction (PCR) cloning strategy (Bredt et al., 1991a). The brain NOS isoform is a 160 Kd protein which shows substantial homology to the rat cytochrome P-450 reductase enzyme. In addition to binding sites for calmodulin and various electron donors, brain NOS has several phosphorylation sites through which a number of protein kinases could potentially regulate the activity of the enzyme (Bredt et al., 1991a, 1991b). There is now evidence to suggest that protein kinase A, protein kinase C and CaM kinase II are all capable of phosphorylating the enzyme at distinct residues on the protein, although the regulatory effects of these kinases on NOS activity are not yet fully understood (Bredt et al., 1991b; Brune and Lapetina, 1991; Nakane et al., 1991).

The brain NOS is one of four distinct isoforms of the enzyme that have now been identified. A second NOS isoform, found in the endothelial lining of blood vessels, bears great similarity to the protein in brain (Lamas et al., 1992; Marsden et al., 1992; Sessa et al., 1992). Both isoforms are constitutively expressed and are Ca^{2+}-calmodulin-dependent. In contrast, the two isoforms of the enzyme found in macrophages (Q. Xie et al., 1992; Lowenstein et al., 1992) and in hepatocytes (Geller et al., 1993) show considerably less

homology to the brain NOS and possess a number of different properties. For example, these isoforms show a much weaker Ca^{2+}-dependence and their activity is inducible. The induction of the enzyme is elicited following exposure to stimuli such as microbial agents or cytokines and involves the synthesis of new protein. Following induction, there is little regulation of the enzyme activity and large quantities (nmol) of NO are produced over long periods of time. This is in contrast to the constitutively expressed enzyme, found in brain and endothelium, which produces much smaller amounts of NO (pmol) in short bursts of activity.

Both the inducible and the constitutively expressed forms of the enzyme are competitively inhibited by structural analogues of the enzyme substrate, L-arginine. The first compound to be widely used in this respect was the methylated form of the amino acid, N^G-monomethyl-L-arginine (L-NMMA; Moncada et al., 1989). More recently, other L-arginine derivatives, including L-N^G-nitroarginine (L-NOArg; Moore et al., 1990) and N^G-nitro-L-arginine methyl ester (L-NAME; Rees et al., 1990), have emerged as more potent inhibitors of NOS. The enzyme inhibition is stereoselective and can be reversed by co-administration of L-arginine. In general, these arginine derivatives do not discriminate between the various isoforms of NOS, although a recent report has claimed that 7-nitro indazole (7NI) may show some selectivity for the brain enzyme (Moore et al., 1993). The physiological actions of NO can also be prevented by haemoglobin. NO binds avidly to the Fe^{2+} ion in the haem moiety of this protein. Because haemoglobin is such a large molecule, it is unable to cross cell membranes and, therefore, only chelates NO which has been released from cells into the extracellular space. Finally, a number of compounds actually release NO and, therefore, provide useful experimental tools for investigating the putative biological actions of this gas. These include the nitrovasodilators, a group of compounds including sodium nitroprusside and nitroglycerin (glyceryl trinitrate), which have been used clinically for many years in the acute treatment of cardiac failure and angina pectoris.

The Distribution of Brain Nitric Oxide Synthase

Having isolated and purified the NOS enzyme from rat cerebellum, Bredt and co-workers were able to raise antibodies to this protein in rabbits (Bredt et al., 1990, 1991b). Using this antisera, they were then able to localise the NOS protein by means of immunohistochemistry and map the distribution of the enzyme in the rat brain. They found that NOS in the brain is localised exclusively in neurones, with the highest density being found in the cerebellum and

olfactory bulbs. Moderate enzyme staining was also seen in a number of other brain regions including the dentate gyrus. In the cerebral cortex there is only a light staining for the NOS enzyme, associated with isolated neurones. Antibodies raised to rat brain NOS also display immunoreactivity in a number of locations in the periphery including the myenteric plexus of the intestine, the retina, the adrenal medulla and the endothelial layers of large blood vessels. The distribution of NOS in brain has been confirmed using anti-sense oligonucleotide probes to map the mRNA for this protein, and this correlates highly with both the immunohistochemical mapping and the catalytic activity in various brain regions as determined by measuring the conversion of [^{3}H] L-arginine to [^{3}H] L-citrulline (Bredt et al., 1991b). The localisation of the NOS enzyme is also identical to the distribution of NADPH diaphorase staining in both rats and primates (Bredt et al., 1991b; Dawson et al., 1991; Vincent and Kimura, 1992). This histochemical staining technique uses nitrotetrazolium blue (NTB) which is reduced by NOS, in the presence of NADPH, to give a blue colour. Indeed, it now seems likely that NOS catalytic activity accounts for all NADPH diaphorase staining in the brain (Dawson et al., 1991).

Effectors of Nitric Oxide

It is generally believed that NO exerts most of its biological actions through the soluble enzyme, guanylate cyclase (Arnold et al., 1977; Miki et al., 1977). This enzyme takes guanosine triphosphate (GTP) as a substrate and synthesises cyclic guanosine monophosphate (cGMP), an intracellular second messenger molecule, structurally similar to cyclic adenosine monophosphate (cAMP). Because NO is freely permeable, it can diffuse across cell membranes and enter cells where it binds to the iron-containing haem group of the guanylate cyclase enzyme. NO binds directly to the Fe^{2+} ion at the centre of the porphyrin ring of the haem moiety. This pulls the Fe^{2+} ion out of the plane of the porphyrin ring, causing a conformational change in the protein which activates the enzyme (Wolin et al., 1982). In turn, cGMP may then contribute to a number of signalling cascades (Goy, 1991). For example, cGMP can activate its own cGMP-dependent protein kinase, phosphodiesterases, and may even act directly on ion channels to alter their conductivity. Examination of the respective distributions of NOS and guanylate cyclase, across a number of brain regions, reveals an interesting pattern. In cerebellum, striatum, hippocampus and cortex, NOS appears to be found almost exclusively in populations of interneurones, with little staining for the enzyme in the principal cell types such as purkinje cells in cerebellum

and pyramidal cells in hippocampus and cortex. Conversely, guanylate cyclase is found in the principal cell types and not in the interneurones. The localisation of the two enzymes in two different populations of neurones implies that NO is, indeed, acting as an intercellular messenger. Furthermore, it is unlikely that the generation of NO and the activation of guanylate cyclase can occur within the same cell (or cellular compartment). The concentration of Ca^{2+} ions required to activate NOS will simultaneously inhibit guanylate cyclase which is consistent with the idea that the site of action of NO is in a second cell. The possibility that NO may act through other effector systems within the same cell cannot, however, be ruled out.

Because the distribution of NOS does not match perfectly with that of guanylate cyclase (Nakane et al., 1983), there is the distinct possibility that NO may also act through alternative transducer mechanisms. For example, there is evidence to suggest that NO can activate ADP-ribosyltransferase enzymes which, in turn, can covalently modify existing protein substrates (Brune and Lapetine, 1989). Another interesting possibility is that NO may actually enhance gene transcription (Peunova and Enikolopov, 1993). Alternatively, NO can bind to the haem moiety of other enzymes that possess this prosthetic group, including mitochondrial enzymes involved in electron transport reactions and the tricarboxylic acid cycle, or the enzymes required for DNA synthesis. The possibility that NO might bind to the iron-sulphur centres of these essential enzymes and disrupt their function could account for the cytotoxic properties of this compound. Being a highly reactive free radicle species, however, there are numerous potential ways in which NO could irreparably disrupt cell function, leading to cell death.

Finally, in the CNS, it appears that NO can act on pre-synaptic terminals to modulate the release of conventional neurotransmitters. There is evidence to suggest that NO can evoke or enhance the release of acetylcholine (Prast and Philippu, 1992; Lonart et al., 1992), noradrenaline (Lonart et al., 1992) and dopamine (Hanbauer et al., 1992; Zhu and Luo, 1992), from a number of different CNS preparations. This raises the interesting possibility that NO might act on pre-synaptic terminals to enhance EAA neurotransmitter release during LTP. Indeed, it has recently been shown that NO, produced in response to the application of NMDA, causes an increase in the release of aspartate in neonatal cerebellar slices (Dickie et al., 1992). This result is entirely consistent with the idea that NO might act as a retrograde messenger in LTP to bring about an increase in transmitter release.

Biological Actions of Nitric Oxide

The evidence supporting a physiological role for NO arose, primarily, from two independent lines of research in macrophages and in blood vessels. A number of experimental findings pointed towards a role for nitrate metabolism in the macrophage-mediated immune response to infection. First, the levels of nitrates excreted in the urine were found to increase dramatically with infection (Wagner et al., 1983). Second, the urinary excretion of nitrates was, nevertheless, maintained in germ-free rats and was independent of any dietary intake (Green et al., 1981a; 1981b). Third, mutant mice which are selectively deficient in macrophages have very low urinary nitrate levels (Stuehr and Marletta, 1985). These findings prompted researchers to study the activation of macrophages in tissue culture (Iyengar et al., 1987; Hibbs et al., 1987a; 1987b). It became apparent that the activation of macrophages resulted in the production of nitrates and nitrites. Furthermore, both the production of nitrates and the bactericidal actions of macrophages were dependent on the presence of L-arginine in the culture medium. These results suggest that the production of NO by macrophages may be a crucial step in the immune response to infection. This has subsequently been confirmed with the observation that NOS inhibitors prevent the bactericidal and tumoricidal actions of macrophages (Nathan and Hibbs, 1991). It is generally believed that the free radical properties of NO provide an ideal mechanism for destroying foreign or cancerous cells.

With regard to blood vessels, the importance of NO also became apparent during the late 1980's. A number of agents, including acetylcholine and bradykinin, elicit vasodilation through relaxation of the smooth muscle cells present in the walls of blood vessels. The importance of endothelial cells in mediating this vasodilation had been recognised for several years. Furchgott and Zawadski (1980) had shown that removal of these cells from the inner lining of blood vessels prevented vasodilation. Furthermore, smooth muscle relaxation could be restored with the re-addition of the endothelial cells. In addition, vasodilation was Ca^{2+}-dependent (Griffith et al., 1986) and accompanied by an increase in cGMP levels in the smooth muscle cells but not in the endothelial cells themselves (Rapaport et al., 1983). In the light of these findings, it was proposed that endothelial cells responded to activation by producing a substance which was termed 'endothelium-derived relaxing factor' (EDRF). EDRF could then diffuse into neighbouring smooth muscle cells and elicit a relaxation through a guanylate cyclase-dependent mechanism. The identity of EDRF, however, remained a mystery. A clue to its identity came from the observation that

nitrovasodilators, which were thought to act by releasing NO, caused relaxation of the smooth muscle in blood vessels in a manner similar to EDRF, but in the absence of endothelial cells (see Furchgott, 1988). Subsequent studies have investigated the possibility that NO might act as the endogenous vasodilator, EDRF (Palmer et al., 1987; Ignarro et al., 1987). Comparisons revealed that both NO and EDRF were extremely unstable with a half-life of just a few seconds. The biological activity of both species was blocked by haemoglobin and enhanced by superoxide dismutase (an enzyme which mops up superoxide free radicles and thus prolongs the half-life of NO). Furthermore, direct application of NO to blood vessels mimicked precisely the effect of EDRF. Finally, the demonstration that endothelial cells actually released NO, in sufficient amounts to account for the observed vasodilation, confirmed that the two molecules were one and the same.

The importance of NO as a signal in blood vessels is now well established. It is generally accepted that NO produces a tonic vasodilation of blood vessels in addition to mediating the acute effects of endothelium-dependent vasodilators. Not surprisingly, therefore, administration of NOS inhibitors causes a profound increase in blood pressure (Moncada, 1991). An intriguing possibility is that NO from other sources may also be capable of modulating local blood flow. For example, NO, produced by macrophages in response to bacterial agents, could account for the profound vasodilation of blood vessels accompanying septic shock. Similarly, neuronally produced NO may provide a mechanism by which local changes in neuronal activity within the brain can be matched to changes in local cerebral blood flow (Edelman and Gally, 1992; Iadecola, 1993). The coupling of metabolic activity to blood flow is a fundamental aspect of the cerebral circulation. There is now considerable evidence to suggest that NO might play an integral part in matching the metabolic demands of active neurones with local blood supply (Goadsby et al., 1992; Kelly et al., 1994).

Nitric Oxide as a Neurotransmitter

The possibility that NO, a simple gaseous molecule, might act as an intercellular messenger between endothelial cells and smooth muscle cells in blood vessels was something of a novel physiological concept. Similarly, the idea that NO might function as a neurotransmitter between cells in the nervous system was also somewhat unconventional. NO appears to satisfy few, if any, of the criteria normally required of a conventional neurotransmitter (Orrego, 1979). For example, NO is not stored in cells ready for immediate release in response to an activating signal. There is no packaging of this gaseous molecule in nerve

terminals nor any special release mechanism. Furthermore, NO does not act through conventional receptors present on the extracellular surface of the post-synaptic membrane. In contrast to other neurotransmitters, NO is formed on demand, diffuses across cell membranes and directly influences the activity of intracellular proteins within the target cell (eg. guanylate cyclase).

The first suggestion that NO might act as a novel type of neurotransmitter came from the work of John Garthwaite and colleagues (Garthwaite et al., 1988). The elevation in cGMP levels following EAA receptor activation in the cerebellum is well documented (Drumond, 1983). Garthwaite and Garthwaite (1987) had previously provided evidence suggesting that although the response to EAA's was mediated primarily by the cerebellar granule cells, the actual increase in cGMP levels occured elsewhere, either in glial or purkinje cells. This implied that some form of intercellular communication may be required. In view of the observation in the vascular system that EDRF, now identified as NO, was capable of elevating cGMP levels in adjacent cells, Garthwaite reasoned that EDRF/NO could be released from granule cells following EAA receptor activation, diffuse into neighbouring cells and activate guanylate cyclase. Using a primary culture of neonatal cerebellar cells, Garthwaite and co-workers showed that the EAA-induced increase in cGMP levels was mediated by NMDA receptors and required the Ca^{2+}-dependent release of an unstable, diffusible messenger which appeared to be identical to EDRF/NO (Garthwaite et al., 1988). For example, the EAA-induced increase in cGMP levels was enhanced by superoxide dismutase, mimicked by the NO donor, sodium nitroprusside, and blocked by NO chelators such as haemoglobin. In addition, when opened rings of rat aortic tissue were incubated with cultured cerebellar cells, in the presence of NMDA, the strips of muscle exhibited a marked relaxation, identical to that elicited by EDRF/NO. Subsequent studies have revealed that the NMDA-induced elevation of cGMP levels is accompanied by an increase in the conversion of [^{3}H] arginine to [^{3}H] citrulline (Bredt and Snyder, 1989; Kiedrowski et al., 1992a) and is blocked by a number of arginine-derived NOS inhibitors (Bredt and Snyder, 1989; East and Garthwaite, 1990). These results confirmed that the response of cerebellar cells to NMDA involves the synthesis and release of NO which can then activate guanylate cyclase in neighbouring cells.

Since this discovery in the cerebellum, considerable evidence has accumulated to suggest that NO may act as an atypical neurotransmitter at a number of other sites, both in the central and peripheral nervous systems. In the periphery, NO is a plausible candidate for the non-

adrenergic, non-cholinergic (NANC) neurotransmitter of the autonomic nervous system. For example, there is a major population of NOS-containing neurones in the myenteric plexus of the small intestine (Bredt et al., 1990) and there are now reports that NO may mediate the NANC relaxation of the intestine (Bult et al., 1990; Desai et al., 1991; Kanada et al., 1992). Similarly, NO has been implicated in a number of other functions mediated by the autonomic nervous sytem including penile erection (Rajfer et al., 1992) and the parasympathetic innervation of the pigmented epithelium and choroid blood vesels in the retina (Sandell, 1985; Bredt et al., 1990). In the peripheral autonomic nervous system, NOS is located in pre-synaptic terminals and NO is released in response to the calcium influx following depolarisation. NO, released from these pre-synaptic terminals, then diffuses into target smooth muscle cells and activates guanylate cyclase, causing a relaxation. As yet, there is no evidence that NO is co-localised with any other conventional neurotransmitter in these fibres, raising the possibility that there exists a population of specific NO-producing neurones in the periphery.

In the CNS, the observation by Garthwaite and colleagues that the activation of NMDA receptors can result in the release of a molecule with the properties of NO, may be of particular relevance to the mechanisms of synaptic plasticity.

Nitric Oxide and Synaptic Plasticity

Taken together, the properties of NO and of the brain isoform of NOS, are consistent with the possibility that this molecule might act as an intercellular messenger in the CNS and could, therefore, play an important role in certain forms of synaptic plasticity, including hippocampal LTP. In summary, NO is a small molecule which can freely diffuse across cell membranes. The short half-life of NO ensures that its effects are limited both spatially and temporally. There is evidence that NO is produced by the enzyme NOS following the activation of NMDA receptors, not only in the cerebellum (Garthwaite et al., 1988) but also in the hippocampus (East and Garthwaite, 1991). This is further supported by the fact that activation of NOS requires that the intracellular Ca^{2+} concentration be elevated above resting levels in a manner which is consistent with the Ca^{2+} influx that occurs following activation of the NMDA receptor/ionophore (Knowles et al., 1989). NO can then diffuse into adjacent cells and act through any one of several effector systems to bring about changes in synaptic efficacy, possibly by increasing transmitter release from the pre-synaptic terminal (Dickie et al., 1992).

One proposed mechanism, therefore, is that NO, formed in the post-synaptic neurone following NMDA receptor activation, can diffuse across the synaptic cleft into the pre-synaptic terminal and initiate an increase in transmitter release (Figure 5.1). Thus, NO might function as a retrograde messenger and initiate changes in the pre-synaptic terminal required for the expression of LTP, through either guanylate cyclase- (Haley et al., 1992; Chetkovich et al., 1993) or ADP-ribosyltransferase-dependent mechanisms (Schuman et al., 1992). However, the immediate pre-synaptic neurone may be only one of several potential targets for NO. By acting on nearby blood vessels, NO could bring about an increase in blood flow to accomodate the additional demand resulting from enhanced neuronal activity. Furthermore, NO may also diffuse into neighbouring glial cells and post-synaptic neurones and modulate their activity. There is, of course, the possibility that NO may also interact with other neighbouring pre-synaptic terminals and initiate an increase in transmitter release from these neurones (see Bonhoeffer et al., 1989).

The possibility that NO, released via an activity-dependent mechanism, can influence more than just the immediate pre-synaptic neurone may have important consequences for the formation of cortical maps during development (Gally et al., 1990; Read-Montague et al., 1991; Edelman and Gally, 1992)). As the cortex develops, neighbouring neurones whose firing patterns are temporally correlated become partitioned together into clumps or strips of neuronal tissue, containing groups of cooperatively interactive cells. The maxim "fire together, wire together" has often been used to describe the way in which these cortical columns or fields are formed. Computer modelling of the development of the cortex has suggested that NO, or a molecule with the properties of NO, would be ideal for recruiting neighbouring neurones into columns and for sharpening and refining the boundaries of these cortical fields.

Nitric Oxide as an Intercellular Messenger for Hippocampal LTP

Theoretically, NO would appear to be an ideal candidate intercellular messenger, well suited to acting as the retrograde signal during the development of hippocampal LTP. Although by no means conclusive, there is now experimental evidence to support this possibility. If NO is required for the development of LTP then one would predict that NOS inhibitors should reduce or prevent the increase in synaptic efficacy following tetanic stimulation. Initial studies, carried out in four independent laboratories, appeared to confirm this prediction (Böhme et al., 1991; Schuman and Madison, 1991; O'Dell et al., 1991a; Haley et al., 1992).

Using the *in vitro* hippocampal slice preparation these researchers showed that bath application of several different NOS inhibitors blocked the development of LTP at CA1-Schaffer collateral synapses in response to a high frequency tetanus. Similarly, the injection of NOS inhibitors directly into the post-synaptic cell also prevented the induction of LTP using a combination of post-synaptic depolarisation and low frequency stimulation of pre-synaptic afferent fibres (Schuman and Madison, 1991; O'Dell et al., 1991a). This suggested that the locus of NO synthesis was in the post-synaptic neurone. The block of LTP by NOS inhibitors was stereoselective and reversed by co-administration of L-arginine. Furthermore, NOS inhibitors only prevented the development of LTP if applied during or immediately after the tetanus. For example, Böhme and co-workers showed that L-NOArg blocked LTP when administered either 15 minutes before, or 5 minutes after tetanic stimulation (Böhme et al., 1991). In contrast, Haley and colleagues showed that application of L-NAME 30 minutes after high frequency stimulation had no effect on pre-established LTP (Haley et al., 1992). These results imply that NO is required for the initial induction process but not for the long-term expression of potentiation. This has been confirmed by Schuman and Madison (1991). They showed that the inhibiton of LTP by L-methyl arginine was not reversed by the addition of the enzyme substrate, L-arginine, when applied one hour post-tetanus. In agreement with the other studies, however, LTP was induced successfully when L-arginine was co-administered at the time of the tetanus. If the expression of LTP requires the continued, persistent activation of NOS, then the addition of L-arginine should reverse the enzyme blockade and result in the immediate expression of LTP. As this was not the case, Schuman and Madison reasoned that NO is only required during the early development of LTP.

All four research groups also showed that bathing slices with the NO chelator, haemoglobin, prevented the induction of LTP (Schuman and Madison, 1991; O'Dell et al., 1991a; Haley et al., 1992; Bon et al., 1992). In contrast, the related compound methaemoglobin, which has a much lower affinity for NO, had no effect on LTP (O'Dell et al., 1991a). Haemoglobin is a large protein which cannot cross cell membranes. The fact that this molecule blocks the induction of LTP is consistent with the hypothesis that NO is being released from the post-synaptic terminal into the extracellular space.

With evidence for the production of NO in the post-synaptic neurone and for its subsequent release into the extracellular space, one remaining question is whether or not this molecule can influence the release of neurotransmitter from pre-synaptic terminals. There are a

number of reports suggesting that NO can effect the release of several different neurotransmitters including the EAA, aspartate, from cerebellar slices in response to NMDA (Dickie et al., 1992). This conclusion was based on the fact that both NOS inhibitors and haemoglobin prevented the NMDA-evoked release of [^{3}H] aspartate from this preparation. This study does not, however, directly test whether NO can elicit an increase in EAA transmitter release or bring about an increase in synaptic efficacy. Because NO is such an unstable species, it is likely to prove very difficult to attain sufficient levels of the gas in tissues in order to see a significant potentiation. O'Dell and co-workers (1991a) have overcome this problem, at least in part, using the whole-cell patch-clamp recording technique with hippocampal neurones in dissociated culture, to which NO was applied by bubbling the gas into helium distilled water until saturation. Although exposure to NO ($\geq$ 40 nM) usually resulted in cell death, brief application of a lower concentation (5-10 nM) mimicked the application of L-glutamate in producing an increase in the frequency of miniature excitatory post-synaptic currents (mEPSC's). This result is indicative of an increase in pre-synaptic transmitter release. The increase in mEPSC frequency was long lasting, in some cases still present one hour after NO application, and was independent of NMDA receptors, occurring in the presence of D-AP5. In a subsequent study, the application of NO to hippocampal slices resulted in a long lasting enhancement of synaptic transmission when paired with a weak tetanus (50 Hz for 0.5 sec; Zhuo et al., 1993). Neither the application of NO, nor the weak tetanus, alone, produced potentiation. Indeed, NO applied 5 minutes after the tetanus failed to elicit potentiation, suggesting a synergistic interaction rather than a simple additive effect. Potentiation was specific to pathways receiving the weak tetanus, occluded subsequent attempts to induce further LTP with a strong tetanus and was elicited independently of NMDA receptor activation. Potentiation was also observed following pairing of the weak tetanus with the NO precursor, L-arginine. The strength of the accompanying tetanus appears to be of considerable importance in determining whether or not LTP is obtained. In their manuscript the authors allude to unpublished experiments in which the pairing of NO with low frequency stimulation resulted in long-term depression (LTD; Zhuo et al., 1993). Clearly, the importance of the tetanus parameters is a matter for further consideration. Furthermore, it is also somewhat surprising in the light of the previous experiment of O'Dell and co-workers in which NO increased the frequency of mEPSC's recorded from cultured hippocampal neurones (O'Dell et al., 1991a), that the application of NO alone fails to result in some potentiation of synaptic transmission. An alternative strategy, pursued by Böhme and colleagues, has been to use NO donors as a

source of the gas and investigate the effects of these compounds on synaptic transmission in hippocampal slices (Böhme et al., 1991; Bon et al., 1992). Bath application of either sodium nitroprusside (SNP; Böhme et al., 1991) or hydroxylamine (Bon et al., 1992) resulted in a small but significant, transient depression of low frequency transmission followed by a pronounced potentiation. The increase in synaptic transmission lasted for at least an hour after washout of the drugs and occluded subsequent attempts to induce further LTP with a high frequency tetanus. It is worth noting that potentiation was observed in the presence of these compounds with just low frequency stimulation. In contrast, in the experiments of Zhuo and colleagues (Zhuo et al., 1993), in which NO gas was bubbled onto slices, a weak tetanus was also required before LTP was obtained. The reason for this apparent inconsistency is not clear although an obvious possibility is that the levels of NO to which neurones are exposed in the presence of NO donors differs from that obtained by bubbling the gas onto the slice. The concentration of NO might have a significant effect on whether or not additional tetanic stimulation is required.

In summary, there is now considerable experimental support, obtained from *in vitro* studies, in favour of the hypothesis that NO might act as an intercellular messenger during the development of LTP. Both NOS inhibitors and the NO chelator, haemoglobin, can block the induction of LTP in hippocampal slices. In addition, there is also evidence that NO might enhance synaptic transmission, either alone or when accompanied by a weak sub-threshold tetanus.

NOS inhibitors, hippocampal LTP and spatial learning

As previously outlined in Chapter 2, a major component of the evidence supporting a role for the mechanisms of LTP in learning is derived from the comparable effects of drugs on LTP and learning. Possibly the strongest support has come from studies using the competitive NMDA antagonist, D-AP5, which impairs spatial learning across a comparable dose range to the inhibition of hippocampal LTP *in vivo* (Davis et al., 1992). Along similar lines, there is now evidence to suggest that NOS inhibitors impair certain forms of learning, including spatial learning (Chapman et al., 1992). Taken together with the work described in the previous section, these findings are consistent with the hippocampal LTP/spatial learning hypothesis.

Two important issues, however, are still unresolved. First, it remains to be seen whether NO

synthase inhibitors prevent the induction of hippocampal LTP *in vivo* at doses similar to those that produce a spatial learning impairment. Second, the behavioural specificity of the observed learning impairment has yet to be fully investigated. This and the following chapter of the thesis describe a series of experiments attempting to address both of these issues. The experiments outlined in this chapter investigate the effects of the NOS inhibitor, L-NAME, on the induction of LTP at perforant path-granule cell synapses in the dentate gyrus *in vivo*. A concurrent series of behavioural experiments, investigating the effects of this compound on both spatial and non-spatial learning tasks in the watermaze, are described in Chapter 6. The electrophysiological studies described here form part of a more thorough investigation into the physiological effects of NOS inhibition which also included an examination of the effects of L-NAME upon cerebrovascular function (Bannerman et al., 1994a *in press*; see Appendix C). These experiments were conducted by Dr. Paul Kelly and are outlined briefly in Appendix B.

Experiment 5.1: The effect of L-NAME upon NOS activity.

Before proceeding with the behavioural and electrophysiological studies, it is essential to establish a dose of the enzyme inhibitor that is sufficient to inhibit NOS.

Procedure

NOS activity was assayed by measuring the conversion of [^{3}H] arginine to [^{3}H] citrulline (as previously described by Bredt and Snyder, 1990). Rats (250-450 g; n=32) were given a single i.p. injection (n=18), of either 0.9% physiological saline or L-NAME (10 or 75 mg/kg), or a series of eleven i.p. injections (n=14), of either saline or L-NAME (75 mg/kg). All solutions were delivered in an injection volume of 10 ml/kg. One hour after the (last) injection, the rats were sacrificed and their brains removed on ice. Tissue samples from the hippocampus, cerebellum and cortex were dissected out and homogenised in 10 volumes of ice cold 50 mM Tris HCl, 1 mM EDTA (pH 7.4). The homogenate was then centrifuged at 10,000 rpm for 1 minute and the supernatant retained on ice. 50µl of the crude supernatant was then incubated for 10 minutes in 50 mM Hepes buffer (pH 7.4), 1mM NADPH, 1mM EDTA, 1.25 mM $CaCl_2$, 1mM dithiothreitol, 10 µM (6R) BH_4·2HCl, 10µg/ml calmodulin, 1 µM L-arginine and 0.2 µCi [^{3}H] L-arginine in a total volume of 0.15 ml at 37°C. The reaction was stopped by the addition of 900µl of stop buffer (10 mM EDTA, 100 mM

Hepes; pH 5.5) and the samples stored on ice. The samples were then loaded onto a 1 ml column of Dowex resin (50X8-200, Na^+ form) which had previously been equilibrated with stop buffer. The [^{3}H] citrulline was eluted with 2 ml of water and quantified by liquid scintillation using Ultima Gold scintillant.

L-[2,3,4,5-^{3}H] arginine monohydrochloride (62 Ci/mmol) was obtained from Amersham. (6R)-5,6,7,8-Tetrahydro-L-biopterin dihydrochloride ((6R)BH_4·2HCl) was obtained from Semat. Ultima Gold Scintillant was obtained from Canberra Packard. All other materials were obtained from Sigma.

Results

L-NAME treatment resulted in a dose related inhibition of brain NOS activity. The results (Figure 5.1.1) are expressed as percentage inhibition relative to control levels of NOS activity, measured in tissue samples taken from saline injected rats (n=11). The measured enzyme activity in the saline injected controls was in hippocampus = 2.09 ± 0.21, cortex = 1.40 ± 0.11 and cerebellum = 3.11 ± 0.25 (dpm x 10^5). A single i.p. injection of the 10 mg/kg dose of L-NAME (n=5) resulted in only a partial blockade of enzyme activity (percent inhibition of synthase activity in hippocampus = 68.2 ± 1.7%, cortex = 67.3 ± 3.1% and cerebellum = 52.6 ± 1.9%). However, a single i.p. injection of 75 mg/kg L-NAME (n=6) resulted in a near complete inhibition of brain NOS (hippocampus = 92.9 ± 1.3%, cortex = 93.0 ± 1.6% and cerebellum = 89.0 ± 1.1%). Chronic i.p. injections of 75 mg/kg L-NAME (n=10) resulted in a slightly greater inhibition of NOS activity (hippocampus = 96.6 ± 0.1%, cortex = 96.6 ± 0.3% and cerebellum = 95.1 ± 0.6%).

Experiment 5.2: The effect of L-NAME upon LTP induced by a strongly suprathreshold tetanus.

The effect of the NOS inhibitor, L-NAME, upon the induction of LTP *in vivo* was examined at perforant path-granule cell synapses in the dentate gyrus. Initially, a dose of L-NAME (75 mg/kg) was chosen which produces > 90% inhibition of NOS in hippocampus (Expt 5.1). Potentiation was induced using tetanic stimulation parameters which produce reliable, robust and D-AP5-sensitive LTP (see Chapter 3).

Procedure

The animals were prepared as described previously (see Chapter 3 - Electrophysiological methodology). The induction of LTP was then examined in rats given a single i.p. injection of L-NAME (75 or 750 mg/kg) one hour prior to the LTP-inducing tetanus (n=8 per group). Control animals (n=8) were given an equivalent volume of physiological saline (10 ml/kg). Following a 10 min baseline period of low frequency stimulation (0.1 Hz, 700μA biphasic pulses of 100μs half pulse duration), an attempt was made to induce LTP using a strong tetanic stimulus (consisting of 4 trains of 33 pulses at 250 Hz with 10 sec between trains, 700μA biphasic pulses of 100μs half pulse duration). After the tetanus, low frequency test pulses were resumed for a further 50 min.

Results

The mean absolute slope of the pre-tetanus baseline field epsps (averaged over the 5 min period immediately prior to tetanus; 30 recordings) did not differ across groups (L-NAME, 75 mg/kg = 2.94 ± 0.29 mV/ms; L-NAME, 750 mg/kg = 3.05 ± 0.48 mV/ms; saline = 3.46 ± 0.62 mV/ms; $F < 1$; $p > 0.20$). For each animal, this absolute slope value was then used as a within-subject baseline (= 100%) and the data normalised with respect to this value. The potentiation elicited by this tetanus was unaffected by L-NAME. The mean slope of the field epsp measured over the 5 min period, 45-50 min after the tetanus was 121.4 ± 5.2% for L-NAME (75 mg/kg) animals, and 121.7 ± 3.6% for controls (Figure 5.2.1). Animals given 750 mg/kg of L-NAME (n=8), 10 times the initial dose, also demonstrated normal LTP (117.1 ± 5.0%; Figure 5.2.2). An ANOVA of the %LTP obtained in these three groups showed that they did not differ ($F < 1$; $p > 0.20$).

Experiment 5.3: The effect of L-NAME upon LTP induced by a just suprathreshold tetanus.

In the light of results obtained from *in vitro* studies (Böhme et al., 1991; Schuman and Madison, 1991; O'Dell et al., 1991a; Haley et al., 1992), the failure to block LTP following an injection of L-NAME was somewhat surprising. One possibility is that NO might act to modulate the threshold for LTP induction and, consequently, that NOS inhibitors may be capable of blocking LTP induced by a 'weak near-threshold' tetanus but are unable to prevent potentiation induced by 'strong suprathreshold' stimulation. In view of the inability

of L-NAME to affect LTP induced by a strongly suprathreshold tetanus, the high frequency stimulation parameters were altered to a protocol close to the threshold for LTP induction. The number of tetanic trains, pulses per train, train frequency and pulse intensity were all reduced until only a minimal but measurable amount of LTP was observed in control animals.

Procedure

The experimental protocol was changed such that test pulse frequency was reduced to 0.05 Hz and test pulse intensity to between 300 and 600 μA (the intensity required to elicit a response that was 50% of the maximum epsp slope). One hour prior to tetanisation, the rats were injected i.p. with either saline (n=7), 10 mg/kg L-NAME (n=6), 75 mg/kg L-NAME (n=10), 75 mg/kg D-NAME (Bachem; n=8), or 75 mg/kg L-NAME co-administered with 225 mg/kg L-Arginine (Sigma; n=4). All drug solutions were made up in 0.9% physiological saline and delivered in an injection volume of 10 ml/kg. Baseline measurements were taken over a 10 min period immediately prior to the tetanus and the response monitored for a further hour after high frequency stimulation. The 'weak' tetanus consisted of 3 trains of 25 pulses (300-600 μA biphasic pulses of 100 μs half pulse duration), delivered at a frequency of 100 Hz with 10 sec between trains. The amount of potentiation was measured over a 10 minute period, 50-60 min post-tetanus.

Results

The mean absolute slope of the baseline field epsps (averaged over the 10 min period immediately prior to tetanus) did not differ between the L-NAME (75 mg/kg; 2.86 $\pm$ 0.35 mV/ms) and saline groups (2.75 $\pm$ 0.34 mV/ms; $t < 1$; $p > 0.20$). For each animal, the data was then normalised with respect to this pre-tetanus value (= 100%). L-NAME had no statistically significant effect on the amount of initial potentiation seen 0-4 min after the tetanus ($t < 1$; $p > 0.20$) with, if anything, there being a trend towards greater potentiation in the L-NAME-treated rats (121.2 $\pm$ 3.4%) than in the saline group (116.3 $\pm$ 3.3%; Figures 5.3.1). The slope of the field epsp declined in both groups over the course of the next hour. This decline was substantially greater, however, in the L-NAME group: (1) the mean slope potentiation 50-60 min post-tetanus shown by the saline group (110.5 $\pm$ 4.7%) was significantly greater than that of the L-NAME group (98.0 $\pm$ 2.9%; $t(15) = 2.50$, $p < 0.025$);

(2) the saline group showed significant potentiation relative to its pretetanus baseline ($t(6) = 2.31$, $p < 0.05$) whereas the L-NAME group did not ($t < 1$; $p > 0.20$); (3) the L-NAME group showed a significantly greater decline in slope between 0-4 min post-tetanus and 50-60 min post-tetanus ($t(15) = 3.21$, $p < 0.01$); and (4) if we define LTP as a 5% increase over baseline 50-60 min after the tetanus, only 2 out of 10 L-NAME treated animals (20%) showed LTP while 5 of 7 saline treated animals (71%) did so ($\chi^2 = 4.50$, df=1, $p < 0.05$).

These results raise the possibility that L-NAME may limit the induction of LTP induced by a weak, near threshold, tetanic stimulus. This effect appears to be dose-related, stereospecific and reversed by co-administration of L-arginine. A lower dose of L-NAME (10 mg/kg) resulted in weak potentiation 50-60 min post-tetanus ($105.2 \pm 4.8\%$; Figure 5.3.2). LTP of a magnitude comparable to that induced in the saline group was seen in both the D-NAME group ($108.0 \pm 5.6\%$) and in rats co-administered with both L-NAME (75 mg/kg) and L-arginine (225 mg/kg; $109.8 \pm 5.9\%$).

Experiment 5.4: The effect of AP5 upon LTP induced by just suprathreshold tetanus

To investigate whether the potentiation induced by a weak tetanus (Expt 5.3) was NMDA-receptor dependent, an attempt was made to induce LTP, in the presence of D-AP5, using the same weak tetanus.

Procedure

A further group of animals (n=4) were prepared for electrophysiological recording (see Chapter 3) and a drug infusion cannula lowered into the lateral cerebral ventricle (AP = -0.9mm, LAT = 1.3mm, DV = -4.5mm from skull surface). Having ensured a stable baseline, the rats were given an acute intracerebroventricular infusion of D-AP5 (10µl of 30 mM, infused at 0.05 µl/min over 20 minutes). D-AP5 (Tocris) was made up at a concentration of 100 mM in equimolar NaOH and diluted with artificial cerebrospinal fluid (aCSF) to a final concentration of 30 mM. Following a baseline period of low frequency stimulation, a weak tetanus was delivered and the response monitored for a further hour as in Expt 5.3.

Results

Both LTP and STP were blocked by D-AP5 (Figure 5.4.1). The size of the field epsp, relative to the pre-tetanus baseline, was 105.9 ± 4.1% between 0-4 min post-tetanus and 100.2 ± 3.3% between 50-60 min post-tetanus.

Experiment 5.5: The effect of chronic treatment with L-NAME upon induction of LTP by a just supra-threshold tetanus.

In the parallel series of behavioural experiments, investigating the effects of NOS inhibitors on learning and memory (see Chapter 6), L-NAME was injected on a daily basis over a number of days. It was, therefore, of interest to know whether the apparent blockade of weak tetanus induced LTP in the presence of L-NAME would persist until the end of the behavioural study. Chronic injection of L-NAME has previously been shown to produce a greater inhibition of NOS (Expt 5.1).

Procedure

Accordingly, a group of animals, given daily injections of either L-NAME (75 mg/kg; n=7) or saline (n=9) for 10 to 20 days (the rats were taken from the behavioural Expts 6.3, 6.4 and 6.5), were tested for LTP induction by a weak tetanus using the same protocol as that of Expt 5.3. The rats received a final injection, under anaesthetic, one hour before the tetanus was delivered (and 24 hr after the penultimate injection).

Results

Surprisingly, in the light of the results of Expt 5.3, no impairment of LTP was observed. The mean absolute slope of the baseline field epsps was 2.91 ± 0.18 mV/ms (L-NAME) and 3.29 ± 0.39 mV/ms (saline; $t < 1$; $p > 0.20$), and the mean potentiation observed 50-60 min post-tetanus was 111.4 ± 5.4 % (L-NAME) and 113.5 ± 3.1 % (saline; $t < 1$; $p > 0.20$; Figure 5.5.1).

Experiment 5.6: The effect of L-NAME upon an extended 2 hr baseline in the absence of a tetanus.

Repeated difficulties in maintaining stable baselines in L-NAME treated rats, coupled with the observation that 4 of the 10 animals in the L-NAME group of Expt 5.3 showed field epsps which fell at least 5% below baseline 1 hour post-tetanus, alerted us to the necessity to examine the effects of L-NAME upon an extended baseline covering the full period during which potentiation was being monitored.

Procedure

Animals were prepared for recording as before. Following a 20 min baseline, L-NAME (75 mg/kg; n=11) or saline (n=9) was injected i.p.. In addition, a group of rats which had received chronic L-NAME treatment were also included in this study (n = 6). As in Expt 5.5, the rats were given a series of 10 daily injections of L-NAME (75 mg/kg). On the day of electrophysiological testing, the animals were prepared for recording and, following a 20 min baseline period, were then given a final injection of L-NAME. For all 3 groups, low-frequency test pulses (0.05 Hz, as in Expt 5.3) then continued for a further 120 min but no tetanus was given.

Results

The mean absolute slope of the baseline field epsps prior to drug injection was 3.23 $\pm$ 0.34 mV/ms (acute L-NAME), 4.61 + 0.71 mV/ms (chronic L-NAME) and 3.53 $\pm$ 0.28 mV/ms (saline; $F(2,23) = 2.91$; $p > 0.05$). Following injection, however, the acute L-NAME treated rats showed a short-term elevation of the baseline (8-12 min post-injection: acute L-NAME = 107.0 $\pm$ 2.4 %; saline = 100.2 $\pm$ 1.6 %; $t(18) = 2.27$, $p < 0.05$) followed by a long gradual decline, while the saline treated rats showed a slight but gradual increase (Figure 5.6.1). One hour after drug injection, the mean slope of the acute L-NAME treated rats was 91.3 $\pm$ 3.6% of baseline, falling to 89.3 $\pm$ 4.1 % after two hours. One hour after injection, the mean slope of the saline treated rats was 105.4 $\pm$ 1.8% of baseline, rising to 112.1 $\pm$ 3.3 after two hours. These differences were highly significant both 1 and 2 hours after injection (1 hour: $t(18) = 3.28$, $p < 0.005$; 2 hours: $t(18) = 4.23$, $p < 0.001$). Acute L-NAME treatment caused a change in field epsps which followed a well defined time-course, with most of the decline occurring during the first 60 min after drug injection. Interestingly, the chronically treated

L-NAME animals showed neither the short-term increase immediately after the last injection, nor the gradual decline thereafter (Figure 5.6.1). In fact, the time course of the slope changes in this group did not differ from those of the saline treated group (50-60 min: $t < 1$; 110-120 min: $t(13) = 1.09$; $p > 0.10$).

For purposes of comparison with previous LTP experiments in this study (eg. Expts 5.3 and 5.5), it is important to measure the change in "baseline" during the *second* hour starting 60 min post-injection. A further ANOVA was therefore conducted comparing the mean slopes 50-60 min post-injection with those 110-120 min post-injection, equivalent to the analysis used in Expt 5.3. This showed that the slope function for the acute L-NAME treated rats declined by 1.7 ± 3.5 % while the saline treated rats increased by 6.3 ± 2.2 % ($t(18) = 1.86$, $0.05 < p < 0.10$). While this change is not significant in itself, the absolute difference between the acute L-NAME and saline treated rats amounts to 8.0 %. This is sufficient to account for the apparent blockade of LTP observed in Expt 5.3. The lack of a difference in the baselines between the saline and chronically treated L-NAME groups would, therefore, explain the results of Expt 5.5, and is also consistent with this account.

Experiment 5.7: The effect of L-NAME upon induction of LTP by a weak tetanus using a non-tetanised hemisphere as a within-subject control pathway.

In the light of the results of Expt 5.6, it is possible that the apparent blockade of weak tetanus LTP (Expt 5.3) can be explained by underlying changes in the baselines. However, Expts 5.3 and 5.6 were conducted using separate groups of animals. Accordingly, a two-hemisphere experiment was undertaken in which the perforant path of one hemisphere received a weak tetanus while the other served as a within-subject control pathway (Bliss and Lømo, 1973).

During the course of this study, two separate groups reported that i.c.v. administration of L-NAME attenuated LTP of the population spike in the dentate gyrus *in vivo* (Mizutani et al., 1993; Iga et al., 1993). Changes in the amplitude of the population spike can occur for several reasons other than an alteration in synaptic efficacy (McNaughton et al., 1978). Nevertheless, LTP of the population spike was examined in a subset of the animals participating in this study.

Procedure

The animals were prepared in the usual way excepting that stimulating and recording electrodes were implanted bilaterally. Potentials were elicited by low-frequency test pulses to each hemisphere (0.05 Hz, as in Expt 5.3), interlaced such that the rats received 1 stimulus every 10 sec. After a 20 min baseline, the rats were injected with L-NAME (75 mg/kg; n=12) or saline (n=12) and, 60 min later, a weak tetanus (as in Expt 5.3) was delivered to one hemisphere only. Test pulses continued for a further 60 min.

Results

There were no significant differences in the mean absolute slope values of the pre-injection baselines of the L-NAME and saline treated animals, or between the tetanised and non-tetanised hemispheres (L-NAME: tetanised = 4.38 $\pm$ 0.51 mV/ms; non-tet = 3.47 $\pm$ 0.38 mV/ms; saline: tet = 3.80 $\pm$ 0.35 mV/ms; non-tet = 3.47 $\pm$ 0.54 mV/ms; Groups $F < 1$; $p > 0.20$; tetanised/nontetanised $F (1,22) = 3.20$, $0.05 < p < 0.10$). All potentials were initially normalised with respect to the pre-injection baseline (10 min period immediately prior to injection, i.e., 10 to 20 min in Figure 5.7.1). The first result of note is that, over 2 hours, L-NAME had a comparable effect upon field epsps to that seen in Expt 5.6. Figure 5.7.1 shows the drug induced change on what served throughout as the non-tetanised control pathway; L-NAME caused an immediate increase in slope followed by a gradual decline, while the saline pathway showed a slow but steady increase. A comparison of the two hemispheres during the first hour after injection, revealed a significant positive correation in terms of the % change in the size of field epsps in the L-NAME group ($r (11) = 0.62$; $p < 0.05$; Figure 5.7.2). In contrast, there was no significant correlation between the % change seen in the two hemispheres in saline injected rats ($r (11) = 0.36$; $p > 0.20$; Figure 5.7.2). There was a 16.1% difference between the L-NAME and saline groups at the 80 min time-point on Figure 5.7.1 (i.e., one hour after injection and the point at which a tetanus was delivered to the experimental pathway). This difference has to be taken into account when assessing the amount of LTP in the two groups, a point shown graphically in Figures 5.7.3 and 5.7.4 which plot the results for both hemispheres in the L-NAME and saline groups respectively. These graphs appear to show some potentiation following tetanisation in both groups of animals, but it is difficult to assess this quantitatively due to the gradually changing baselines. If one looks solely at the experimental pathway and normalises with respect to the 10 min period prior to the tetanus (70-80 min), the outcome is a result similar

to the finding reported in Expt 5.3, in that the weak tetanus produces apparently greater potentiation in the saline group (Figure 5.7.5). In contrast, however, if the non-tetanised pathway (also normalised with respect to the 70-80 min time period) is used as a within-subject control at each time-point (t) throughout the experiment, (ie. final $value_t$ = ($experimental_t$ / $control_t$) x 100%), a very different result is obtained. As shown in Figure 5.7.6, it now appears that, if anything, L-NAME is associated with slightly greater potentiation than that shown by the saline group. A comparison of the % potentiation analysed in this way, one hour after the tetanus (ie. 130-140 min), revealed that both groups showed LTP, but that there was a trend for there being slightly greater potentiation in the L-NAME group (L-NAME = 116.8 $\pm$ 5.0 %; saline 106.5 $\pm$ 1.9 %; $t(22) = 1.91$; $0.05 < p < 0.10$). It should be noted that our protocol was designed to produce a minimal but measurable LTP. The within-subjects (Figure 5.7.5) rather than the between-subjects variability (Figures 5.7.3 and 5.7.4) provides the appropriate error term with which to assess the presence or absence of potentiation. Comparisons between the tetanised and non-tetanised hemispheres, for both saline and L-NAME treated animals, showed that both groups displayed significant LTP (L-NAME: $t(11) = 3.12$; $p < 0.01$; saline: $t(11) = 2.83$; $p < 0.025$).

The best extracellular measure of changes in excitatory feedforward synaptic efficacy in the molecular layer of the dentate gyrus is the early rising slope of the field potential. This parameter has been reported throughout the thesis so far. Nonetheless, in the light of recent reports (Mizutani et al., 1993; Iga et al., 1993), LTP of the population spike was also examined in a subset of animals participating in this study. To be included, an animal had to have a population spike of > 1 mV pre-tetanus. The results (Figure 5.7.7) indicate that, 50-60 min post-tetanus, as much population spike LTP was shown by the L-NAME treated rats (166.9 $\pm$ 16.9 %; n=9) as by the saline group (150.2 $\pm$ 12.3 %; n=9). These values did not differ ($t < 1$; $p = 0.44$).

DISCUSSION

This series of experiments showed that i.p. injections of L-NAME caused a dose-related inhibition of NOS with both a single acute, and a series of chronic, 75 mg/kg injection(s) resulting in > 90% inhibition of enzyme activity in hippocampus. At this and a 10 fold

higher dose, L-NAME was without effect upon LTP induced by a strong tetanus and recorded in the dentate gyrus *in vivo*. In contrast, L-NAME caused an apparent inhibition of LTP *in vivo* when induced by a weak tetanus, but subsequent experiments using a within-subject control pathway suggest that this is primarily a consequence of an underlying decrease in the size of the baseline field epsp. Finally, the results of an associated study (by Dr. P. Kelly; see Appendix B) revealed that L-NAME also caused a reduction in cerebral blood flow but with no change in cerebral glucose utilisation.

Inhibition of hippocampal NOS

As the main finding of this chapter is that an inhibitor of NOS, L-NAME, does not appear to block the induction of LTP *in vivo* induced by either weak or strong tetanic stimuli, it is essential to establish that the drug administration regimes used were sufficient to inhibit the enzyme. The results of the NOS assay show that a single 75 mg/kg i.p. injection of L-NAME produced a 93% inhibition of the enzyme in hippocampus. The lower dose of 10 mg/kg L-NAME resulted in only 68% inhibition. Dwyer et al., (1991) have suggested that a more effective method of inhibiting NOS is to give repeated injections of a synthase inhibitor over several days. Their results show that a 50 mg/kg injection of L-N^G -nitroarginine (NOArg) caused a 50% inhibition in cerebellum after a single injection, but 95% inhibition after 4 days of twice daily injections. This raised the possibility that an even greater inhibition of NOS is obtained with repeated daily injections of L-NAME. The results of Expt 5.1 extend Dwyer et al's finding to a different NOS inhibitor, revealing that a slightly greater inhibition of NOS (96%) was achieved following chronic L-NAME (75 mg/kg) treatment. Nevertheless, the enzyme assay (Expt 5.1) showed that a single injection is sufficient to produce a near complete inhibition of the enzyme in hippocampus.

It should be recognised, however, that L-NAME would have been active both peripherally and centrally. Evidence that L-NAME was active in inhibiting NOS peripherally derives from the observation that there was a dramatic increase in blood pressure sustained throughout the period (> 60 min) for which electrophysiological observations were made (see Appendix B). L-NAME also caused measurable changes in baseline field-potentials in the same animals that failed to show a blockade of LTP (Expt 5.7), although it is not known whether this was primarily a central or peripheral effect. The use of an intracerebroventricular route of administration may allow such a dissociation to be made.

L-NAME induced changes in baseline field-potentials

An acute injection of L-NAME resulted in a prolonged, gradual decrease in the size of the field epsp, preceded by a small transient rise. These changes were somewhat surprising in that previous experiments conducted in hippocampal slices demonstrate no significant effect of NOS inhibitors on baseline synaptic responses (Haley et al., 1993). This suggests that the decline in evoked potentials seen reliably in the dentate gyrus following L-NAME injection *in vivo* is either a property of networks of neurons too large to be preserved in slices, for example, those providing cholinergic or catecholaminergic afferents to the hippocampus which may be regulated by the release of NO (Wood et al., 1992; Lonart et al., 1992; Hanbauer et al., 1992), or a direct or indirect consequence of L-NAME-induced changes in cerebral blood flow.

The uncoupling of cerebral blood flow from cerebral metabolism may contribute to the L-NAME induced decrease in the baseline, although it is not clear what the consequences of the relative oligaemia which develops following L-NAME treatment might be for the maintenance of normal hippocampal function. Whilst the level of blood-flow in hippocampus remains considerably higher than that which would be expected to produce hypoxic dysfunction or ischemic damage (Siesjo, 1978), and at normal levels of perfusion there is considerable excess in the delivery of energy substrates, the resulting reduction in blood flow may be sufficient to alter normal synaptic transmission (eg. the size of the baseline field epsps).

In contrast to the decrease in the size of field epsps that follows injection of L-NAME, animals which received injections of saline showed a small gradual increase. One possible explanation to account for this phenomenom, which has been observed previously (Bliss and Lømo, 1973), is that the level of anaesthetic, in this case urethane, may decrease slightly over time resulting in a gradual increase in the level of synaptic transmission. Alternatively, the rising baseline might be explained by the gradual recovery of brain tissue disturbed during electrode implantation. The gradual rise in the size of the field epsp is unlikely to be due to an increase in brain temperature as core temperature was maintained at 36.2 ± 0.2 °C throughout recording, and no corresponding decrease in the size of the population spike was observed (N.B. Moser et al., 1993a). The lack of a significant correlation between the two hemispheres in saline injected animals during the hour after injection (Expt 5.7; Figure 5.7.2) may give some indication as to the underlying cause of the baseline change in controls. Factors such as brain temperature and the level of anaesthesia might be expected to

affect the magnitude of field epsps equally on both sides of the brain. In contrast, the effects of electrode implantation will vary between hemispheres and between animals. The lack of a significant correlation may, therefore, indicate that the gradually rising baseline is due to the gradual recovery of brain tissue from the implantation of electrodes.

L-NAME has little effect on hippocampal field potentials in chronically treated animals

Treatment with L-NAME over the course of several days should afford ample opportunity for synaptic transmission to stabilise at a new lower baseline. Further injection of L-NAME would not be expected to produce additional decrements in the size of the field potentials. This was indeed the case (Expt 5.6). Thus, if the apparent blockade of weak tetanus LTP seen in Expt 5.3 were really an artefact of a gradually changing baseline, one would not expect such LTP to be impaired in chronically treated animals. This result is exactly what was obtained.

An alternative explanation for the failure to block LTP following chronic administration of L-NAME is that tolerance develops to the effects of the inhibitor. However, this seems extremely unlikely. The data reported in Expt 5.1, and that of Dwyer et al., (1991), show that the level of NOS inhibition increases with repeated injections, indicating that chronic treatment results in cumulative effects rather than the development of a tolerance to the inhibitor.

The significance of the tetanus strength used to induce LTP in detecting a blockade by NOS inhibitors

The results of Expt 5.2 showed that LTP induced by strong tetanic stimuli *in vivo* was unaffected by high doses of L-NAME (75 and 750 mg/kg). Following the reports that inhibition of NOS blocked the induction of LTP *in vitro* (Böhme et al., 1991; Schuman and Madison, 1991, 1994a; O'Dell et al., 1991a; Haley et al., 1992), this result was somewhat surprising. This, however, may have been due to the fact that the tetanus used was strong enough to recruit alternative cellular mechanisms which may be sufficient to support LTP independently of NO. It is possible, therefore, that LTP would be blocked by L-NAME if a near threshold tetanic stimulus was used for its induction, a stimulus that, arguably, might be more physiologically relevant. NO might, for example, have a role in setting the threshold for the establishment of LTP. A number of other research groups have pursued this line of

thought, namely that the presence or absence of a block of LTP is dependent upon the pattern and intensity of the tetanic stimulation. Unfortunately, these studies have yielded conflicting results. For example, two groups have reported that NOS inhibitors block LTP induced by a 'weak' tetanus but have no effect on potentiation evoked by 'stronger' stimulation parameters (Chetkovich et al., 1993; Haley et al., 1993). Surprisingly, although Haley and co-workers observed a dissociation between the weak and strong tetanus in terms of whether or not NOS inhibitors blocked LTP, the amount of potentiation evoked by the two stimulation paradigms did not differ. If the tetanus parameters are important, the question remains as to whether the magnitude of LTP induced is the important factor in determining whether or not NOS inhibitors block LTP, or rather some unknown consequence of the pattern of stimulation, unrelated to the size of any increase in synaptic efficacy, may be crucial. In contrast to these results, Gribkoff and Lum-Ragan (1992) found that NOS inhibitors reduced the magnitude of LTP evoked by a 'strong' tetanus but were ineffectual against 'weak' tetanus-induced potentiation.

In this investigation, the first experiment examining the effects of L-NAME on LTP induced by weak tetanic stimuli (Expt 5.3) gave suggestive results, but, following the finding that LTP induction was unaffected in rats chronically treated with L-NAME (Expt 5.5), and the observation that several L-NAME treated rats showed post-LTP field-potentials well below the pre-injection baseline (Expt 5.3), an investigation was conducted into the effects of L-NAME upon an extended non-tetanised baseline (Expt 5.6). This revealed a gradual decline in the size of baseline field epsps in L-NAME treated rats that was measurable over 1-2 hr but not detectable over shorter baseline periods of 10-20 min beginning about an hour after the drug injection. Taken together with the gradually increasing baseline exhibited by saline-injected controls, the change in the dentate field potentials of the two groups over 1-2 hr seemed to be sufficient to account for the apparent inhibition of LTP induced by a weak tetanus in Expt 5.3. Moreover, the critical further experiment, in which recordings were made from both a tetanised and non-tetanised hemisphere within the same animal, provided strong evidence in support of this possibility (Expt 5.7). These findings, therefore, offer no support for the intuition that inhibition of NOS may be more effective in blocking LTP induced by a weak tetanic stimulus in the dentate gyrus *in vivo*.

L-NAME fails to block dentate gyrus LTP in vivo

The results of these experiments suggest that the magnitude of LTP, induced by either a 'weak' or a 'strong' tetanus, is unaffected by the i.p. injection of the NOS inhibitor, L-NAME. These results are in agreement with those of Barnes et al., (1994a), using unanaesthetised rats, who also reported a failure to block LTP with i.p. injections of an NOS inhibitor. Repeated i.p. injections of 50 mg/kg L-NOArg, which resulted in > 90% inhibition of the NOS enzyme, did not affect the magnitude of LTP induced at perforant path-granule cell synapses *in vivo*.

There are at least three possible explanations for the absence of a blockade of LTP with systemic administration of L-NAME. First, the residual 4-7% of NOS activity, present following L-NAME treatment, may be sufficient to support normal LTP. Indeed, it may prove near impossible to ensure a complete blockade of NOS activity by pharmacological means. It was hoped that gene knockout experiments of brain NOS would provide such an opportunity but a recent report by Huang et al., (1993) has revealed that in mutant mice, deficient in NOS, there is still a low level residual catalytic activity. The possibility cannot be ruled out that NO is, indeed, required for the induction of LTP and that this small amount of NO production is sufficient to support potentiation. Second, NO may play a threshold modulatory role in LTP induction such that other messengers (e.g. CO - Stevens and Wang, 1993; arachadonic acid - Williams et al., 1989; calcium-calmodulin dependent kinase II - Malinow et al., 1989) could contribute to the induction of potentiation under these experimental conditions. Third, NO may have no role in the induction of LTP *in vivo*.

With respect to the last alternative, despite the fact that four independent laboratories have reported a blockade of LTP with NOS inhibitors in hippocampal slices, a number of other well established research groups have either failed completely to observe this effect or found that NOS inhibitors only block LTP under certain experimental conditions (pers. comm.). A number of factors may be important in determining whether or not a blockade of LTP is observed *in vitro*. Tim Bliss's research group have systematically investigated a number of these potential variables (Williams et al., 1993). Using slices prepared from young rats (70-100g) and maintained at room temperature (24°C), they observed that the induction of LTP at CA1-Schaffer-collateral synapses was reduced by both NOS inhibitors and haemoglobin. In contrast, however, at 29°C neither the enzyme inhibitors nor the NO chelator had any effect on the amount of LTP induced. The failure to block LTP at 29°C, which could not be overcome by increasing the concentration of the enzyme inhibitor, suggests that there may

not be an absolute requirement for NO at more physiological temperatures. Temperature, however, is not the sole determining factor. In slices prepared from adult rats (300-350g), there was no blockade of LTP at either temperature. Moreover, in a further study carried out using young slices in which the inhibitory currents had been blocked with bicuculline, NOS inhibitors failed to block LTP even at 24°C. A similar result, using picrotoxin, has been reported elsewhere (see Zhuo et al., 1993). Examining the data obtained from the various experiments included in their study and performed at 24°C, Williams and colleagues noted that there was an inverse relationship between the magnitude of LTP and the degree of blockade by NOS inhibitors. The greater the LTP induced, the less of a blockade observed with the enzyme inhibitor. There is an intuitive similarity between these findings and some of the results obtained with weak and strong tetani (Haley et al., 1993; Chetkovich et al., 1993). Indeed, there is a general consensus of opinion that under conditions which favour the induction of LTP, such as disinhibition, higher temperatures and stronger tetanic stimulation, the probability of seeing a block of LTP with NOS inhibitors *in vitro* is reduced.

Bearing in mind the importance of experimental conditions such as temperature, age of the animal and tetanus parameters in determining whether or not L-NAME blocks the induction of LTP (Gribkoff and Lum-Ragan, 1992; Haley et al., 1993; Chetkovich et al., 1993; Williams et al., 1993), it is not too surprising that the procedural differences of this study (eg. *in vivo* vs *in vitro,* systemic administration vs bath application) have resulted in no block of LTP. A separate consideration is the use of perforant path-dentate gyrus synapses rather than the Schaffer collateral-CA1 synapses which are used more frequently in hippocampal slices. The synapses in the dentate gyrus were chosen because they exhibit a relatively high density of NOS (Bredt et al., 1990; 1991), and because this is a frequently used and reliable method of studying hippocampal LTP *in vivo.* In addition, there are no *a priori* grounds to believe that LTP in perforant path-dentate gyrus synapses is fundamentally different from that of Schaffer collateral-CA1 synapses (eg. NMDA receptor dependency). Even so, the possibility that these results may not apply to other hippocampal subfields cannot be ruled out and a study of CA1 LTP *in vivo* would be worthwhile.

Two research groups have recently reported that infusion of L-NAME into the contralateral lateral ventricle blocks the induction of LTP of the population spike in the dentate gyrus *in vivo* (Mizutani et al., 1993; Iga et al., 1993). The data presented in Expt 5.7 does not show any blockade of LTP either in terms of the slope of the field epsp or of the population spike (Figure 5.7.7). There are a number of differences in procedure that may account for these

anomolous results. For example, the strain and age of the animals may be important (n.b. Williams et al., 1993). Alternatively, the i.c.v. route of drug administration may result in higher levels of enzyme inhibition in the hippocampus than the > 90% inhibition achieved following an i.p. injection. In addition, it is possible that the use of a stronger tetanus, resulting in greater spike potentiation in control animals (eg. as used in Expt 5.2), may reveal an attenuation of spike potentiation in L-NAME treated animals. Unfortunately, population spike data were not collected during Expt 5.2. One further point, worthy of note is that Mizutani et al., (1993) report significantly smaller initial potentiation in animals treated with NOS inhibitors, which is in contrast to most other studies both *in vitro* and *in vivo*. This raises the possibility that i.c.v. administration may have additional physiological effects.

Nevertheless, the possibility also remains that inhibition of NOS may affect spike potentiation in the hippocampus *in vivo*. However, although this may represent an important physiological action of NO, this thesis is particularly concerned with the synaptic component of LTP. The early rising slope of the extracellular field-potential provides the best index of excitatory drive that can be recorded *in vivo*, being relatively unaffected by alterations in excitability that affect the size of the population spike or by feed-forward inhibition which, though present, is small in magnitude. The results of this investigation certainly offer no support for an involvement of NO in the synaptic component of LTP *in vivo*.

The failure to observe a block of LTP in the presence of NOS inhibitors in these experiments may not come as a complete surprise. To many researchers, the putative role of NO in the generation of LTP is highly questionable. Perhaps the major concern for proponents of the NO/LTP hypothesis is the apparent lack of the NO synthase enzyme in CA1 pyramidal cells. The majority of studies have been carried out in hippocampal slices and have examined the role of NO during the induction of LTP at synapses between Schaffer-collateral fibres and CA1 pyramidal cells. If NO is acting as an intercellular messenger, then it should be synthesised in the post-synaptic neurone in response to activation of NMDA receptors. Indeed, the injection of NOS inhibitors directly into CA1 pyramidal cells blocks the induction of LTP (O'Dell et al., 1991a; Schuman and Madison, 1991). Despite this result, the majority of anatomical studies examining the distribution of NOS in the brain have failed to find the NOS enzyme in the very cells where it is apparently required. For example, immunohistochemical techniques for detecting the enzyme protein (Bredt et al., 1990), oligonucleotide probes specific for NOS mRNA (Bredt et al., 1991b) and NADPH diaphorase staining (Vincent and Kimura, 1992) have all shown little, if any, NOS present in

these cells. Anatomical studies have, however, identified the NOS enzyme in the dentate gyrus of the hippocampal formation, although the issue as to whether or not NO is required for the development of LTP in this region is clearly controversial.

One possible explanation for the apparent anomaly in CA1 is that the pyramidal cells contain an, as yet unidentified, isoform of NOS which remains undetected by the antibodies and oligonucleotide probes that have been used so far. For example, the antibodies used were prepared against cerebellar NOS and may, therefore, fail to detect the enzyme in hippocampus. Furthermore, it has been found in immunoprecipitation studies that only 90% of brain enzyme activity is removed (Bredt et al., 1991b; Fazeli, 1992). It is possible that this residual 10% corresponds to a distinct isoform of NOS. Some support for this possibility has been derived from two recent studies which claim that there is, in fact, some NOS localised in CA1 pyramidal cells, identified using both immunohistochemical (Schweizer et al., 1993) and NADPH diaphorase staining techniques (Wallace and Fredens, 1992). These results cannot, however, explain the apparent absence of the co-product, citrulline (Pasqualotto et al., 1991), or of any enzyme that might use or remove citrulline, in CA1 neurones (Vincent et al., 1991).

The possible absence of NOS in CA1 pyramidal cells does not, however, completely discount a role for NO in the development of LTP. NOS has been identified in hippocampal interneurones (Bredt et al., 1990; Bredt et al., 1991b; Vincent and Kimura, 1992) and in septal fibres projecting to the hippocampus (Pasqualotto and Vincent, 1991). Stimulation of the septum increases cGMP levels in the hippocampus through a cholinergic-independent mechanism (Segal and Guidotti, 1981). It might be the case that NO is released from these septal projections onto hippocampal neurones and can modulate their activity. The importance of the septal input in affecting the inducibility of LTP is well established (Larson et al., 1986), and it is possible, therefore, that by removing NO, the influence of septal projections on synaptic plasticity is altered. Alternatively, the vascular isoform of NOS may make an important contribution to hippocampal LTP. For example, if NOS inhibitors uncouple cerebral blood flow from metabolic demand (Adachi et al., 1992; Goadsby et al., 1992; Northington et al., 1992; Kelly et al., 1994), it is possible that the vascular system is unable to respond to the extra metabolic demands that accrue following the induction of LTP. Unfortunately, however, the results reported here offer no support for any of these possibilities.

If NO is not required for the induction of LTP, the question remains as to why NOS

inhibitors and haemoglobin prevent LTP induction *in vitro*. One possibility concerns the specificity of many of the compounds used as experimental tools to investigate the role of NO in LTP. For example, haemoglobin is a large molecule with a number of potential binding sites for several different ligands. Consequently, this protein may bind to more than just NO. Similarly, the arginine derivitives which are widely used as inhibitors of NOS, may also have other effects. It has been suggested that certain NOS inhibitors, including L-NMMA and L-NOArg, and haemoglobin, block the induction of LTP by some means other than preventing NO from acting as a retrograde messenger (Musleh et al., 1993). Musleh and colleagues measured the amount of depolarisation that resulted from the 'theta burst stimulation' (TBS) used to induce LTP. They noticed that both the NOS inhibitors and haemoglobin caused a reduction of approximately 35% in the amount of depolarisation summation that occurred during TBS stimulation. The magnitude of this effect is similar to that seen in the presence of D-AP5 and is non-additive with the reduction produced by the NMDA antagonist. The authors reasoned that this reduction in the amount of depolarisation seen during TBS could account for the observed decrease in the magnitude of LTP and may involve either a direct or indirect effect of NOS inhibitors on the NMDA receptor population. They predicted that LTP induced independently of NMDA receptors would be unaffected by NOS inhibitors. In a further experiment, they found that NMDA receptor-independent LTP, induced by application of the K^+ channel blocker, TEA (Aniksztejn and Ben-Ari, 1991), was, indeed, unaffected by NOS inhibitors, a result which seemed to provide some support for this hypothesis. These results are, however, far from conclusive. Firstly, another study has reported that L-NOArg does not reduce the amount of depolarisation occurring during tetanic stimulation (Haley et al., 1993). Secondly, it is not clear whether NMDA receptor-dependent and -independent forms of LTP in CA1 share common mechanisms of expression, although both do appear to be triggered by an increase in intracellular Ca^{2+} levels. Thirdly, even if a common mechanism is involved, it is possible that exposure to TEA is equivalent to delivering a 'strong' tetanus which, as others have shown (Chetkovich et al., 1993; Haley et al., 1993), can overcome NOS inhibition, possibly by recruiting alternative second messenger systems. Although the results of Musleh and colleagues (1993) are inconclusive, the possibility that many of the compounds used to investigate the role of NO in LTP may not be as specific as many people may have hoped requires further consideration. It is not clear, however, quite how a lack of specificity of these compounds could account for the apparent differences between *in vivo* and *in vitro* studies, or for the differential temperature sensitivity found with hippocampal slices.

L-NAME may increase the magnitude of LTP

An intriguing observation from Expt 5.7 was that the level of long-lasting synaptic potentiation in L-NAME treated animals appeared to be greater than in control animals when expressed as a ratio of tetanised to non-tetanised hemispheres and thus accounting for any underlying baseline changes. Although this effect was not quite significant ($p = 0.07$), there is a clear trend suggesting a L-NAME-induced enhancement of LTP.

This would be entirely consistent with a number of studies that have provided evidence that NO can directly influence the activity of the NMDA receptor. The first indication of this came from the observation that the NO donor, sodium nitroprusside (SNP), inhibited NMDA-specific [^{3}H] L-glutamate binding to rat brain synaptic membranes (Fujimori and Pan-Hou, 1991). Subsequent studies have shown that NO and NO donors block NMDA currents during whole-cell patch clamp recording from cultured neurones (Manzoni et al., 1992a), and prevent the increase in intracellular Ca^{2+} levels that accompanies NMDA receptor activation (Manzoni et al., 1992a; Lei et al., 1992; Hoyt et al., 1992). Conversely, NMDA responses are enhanced by pre-treatment with haemoglobin (Manzoni et al., 1992a). Although there is some concern about the specificity of certain NO donors, in particular SNP (East et al., 1991; Kiedrowski et al., 1992b; Manzoni et al., 1992b), the fact that NMDA currents are reduced by a number of structurally different compounds all of which release NO, and by the gas itself, implies that the NO is the active agent. Furthermore, when the donor molecule, sin-1, is depleted of its NO, it is without effect on NMDA currents (Manzoni et al., 1992a). The action of NO on the NMDA receptor is cGMP-independent (East et al., 1991; Manzoni et al., 1992a; Hoyt et al., 1992; Kiedrowski et al., 1992b), and the observation that NO donors reduce NMDA currents in isolated outside-out patches suggests that this effect is not mediated by any second messenger system (Manzoni et al., 1992a). NO is thought to act directly on the NMDA receptor complex, probably by binding to the 'redox' modulatory site (Lei et al., 1992). Oxidizing and reducing agents decrease and increase the magnitude of NMDA currents respectively, by binding to a pair of cysteine residues located on the extracellular surface of the receptor protein (Lazarewicz et al., 1989; Aizenman et al., 1989; Aizenman et al., 1990). NO is thought to modulate NMDA receptor activity by oxidizing these free sulphydryl groups to form S-nitrosothiols (Stamler et al., 1992; Lei et al., 1992).

Because NO can block NMDA receptors (Manzoni et al., 1992; Lei et al., 1992; Izumi et al.,1992b), inhibition of NO synthase may result in more NMDA receptor activation during

the tetanus and thus elicit greater or more long-lasting potentiation. The magnitude of such an effect might vary as a function of the extent of NMDA receptor activation and thus be determined by the size of the tetanus. Interestingly, there was no indication of greater LTP following L-NAME injection in Expt 5.2 in which a strong tetanus was used. The results of Expt 5.2, however, are plotted without taking into account any differential changes in the baseline that might have occurred in saline and L-NAME treated rats (for which there is no data because recordings were made unilaterally and all animals were tetanised). If similar changes in the baseline occurred in Expt 5.2 as those measured in Expt 5.6, the LTP shown by the L-NAME group is likely to have been greater than that of the saline group. The possibility that L-NAME may enhance LTP should perhaps not be overemphasised. The effect did not reach statistical significance and is calculated in the presence of gradually changing baselines in both the L-NAME and saline groups. An intracerebroventricular route of administration, which may avoid changes in baseline field-potentials, could potentially provide an opportunity to investigate this possibility further.

The blockade of NMDA receptors by NO may be more than just a trivial side effect of this molecule. NO, produced following NMDA receptor activation (Garthwaite et al., 1988), can diffuse into the synaptic cleft and limit subsequent NMDA receptor-mediated currents. NO may, therefore, act as a negative feedback system to prevent 'NMDA receptor wind up' and consequent overexcitation leading to cell death. Along similar lines, NO may account for the NMDA receptor-mediated inhibition of LTP induction (Izumi et al., 1992b). Prior synaptic activity (Coan et al., 1989; Huang et al., 1992) and exposure to low concentrations of NMDA (Izumi et al., 1992a) both limit subsequent attempts to induce LTP. The NMDA receptor-mediated inhibition of LTP induction is reversed by NOS inhibitors and haemoglobin, and mimicked by SNP, results which are consistent with the idea that NO is formed following NMDA receptor activation and then prevents further activity (Izumi et al., 1992b).

In the light of these results, the outcome of experiments examining the role of NO in LTP may well depend upon prior levels of synaptic activity and possibly upon the extent to which neurones are exposed to NOS inhibitors prior to induction. It seems likely that NO has more than one action on neurones in the CNS and it is also conceivable, therefore, that NOS inhibitors exert two opposing effects during the induction of LTP. Consequently, the increase in NMDA receptor activity that may occur following L-NAME treatment due to removal of the NO block of NMDA receptors could be offset by an effect of L-NAME on

LTP further downstream. The results obtained in this series of experiments could be due, therefore, to a combination of effects superimposed upon each other.

Implications for behavioural studies

There are several reports in the literature indicating that NO synthase inhibitors impair performance in a variety of learning paradigms including tasks thought to require the integrity of the hippocampus (Chapman et al.,1992; Hölscher and Rose, 1992; Böhme et al., 1993). Although it is by no means certain if, and to what extent, the mechanisms of LTP might also be involved in learning, the lack of an effect of L-NAME on the induction of hippocampal LTP *in vivo* would appear to call into question whether NO synthase inhibition is producing a learning impairment through an effect on LTP-like processes. This does not, however, preclude the possibility that NO synthase inhibition is interfering with some other aspect of hippocampal physiology which might result in a spatial learning impairment, or affects brain function elsewhere which might account for apparent impairments of other types of learning. It is unclear to what extent the L-NAME induced reduction in the size of dentate field epsps would affect the normal function of the hippocampus or other brain areas. A detailed investigation into the effects of NO synthase inhibitors on various forms of learning in the watermaze has been conducted and is described in the following chapter (see Chapter 6).

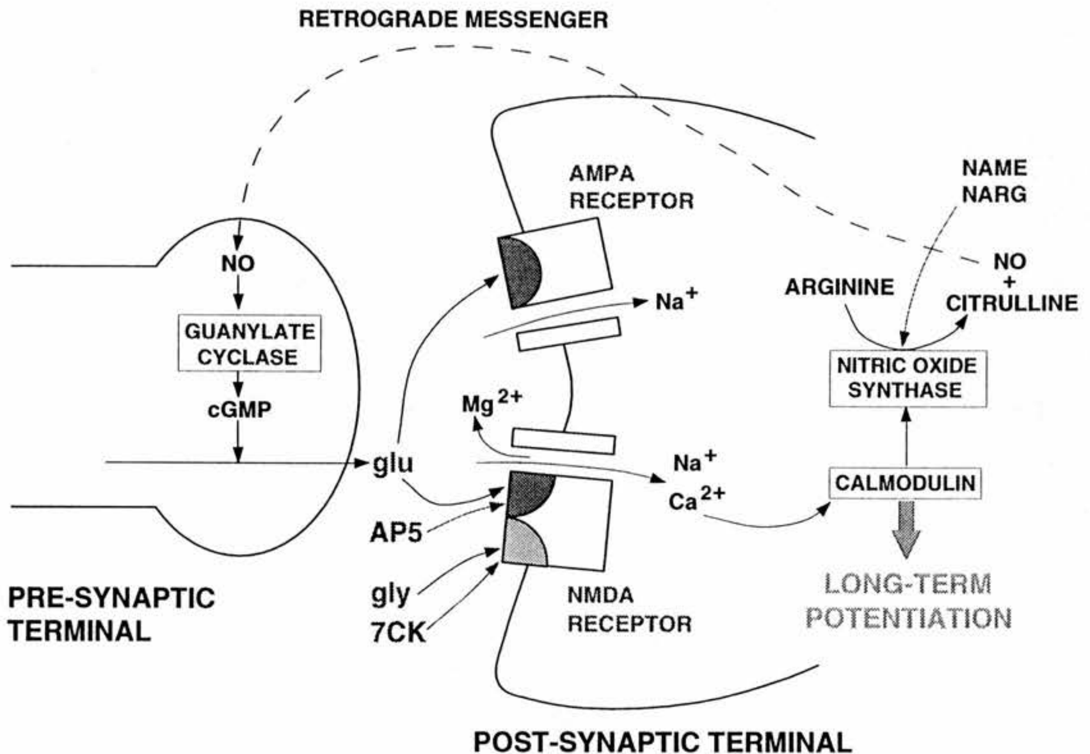


Fig. 5.1

A potential mechanism by which NO, acting as a retrograde messenger, might contribute to the induction of LTP.

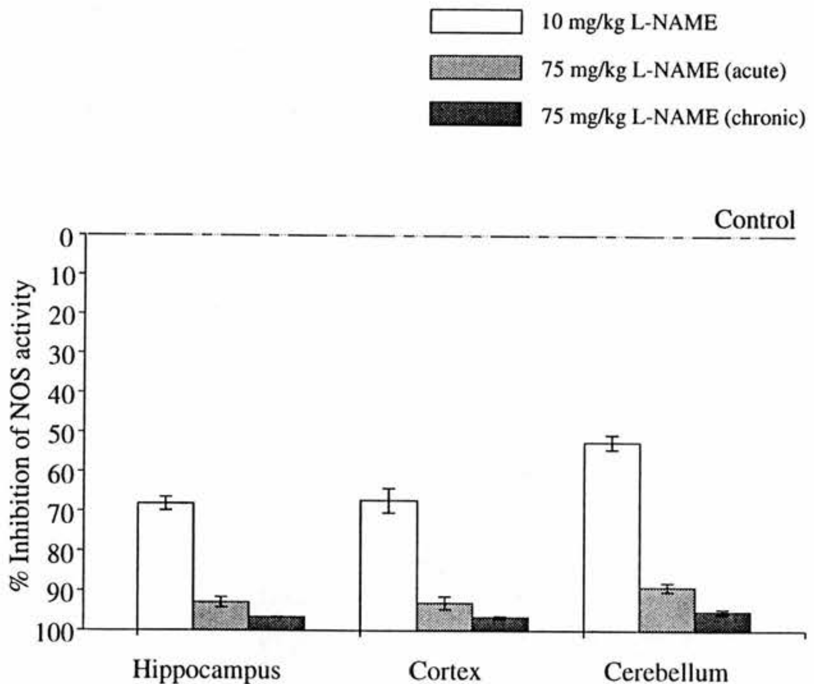


Fig. 5.1.1

NO synthase activity was determined by measuring the conversion of radiolabelled arginine to citrulline. L-NAME produced a dose-related impairment of NO synthase activity in the brain (Expt 5.1).

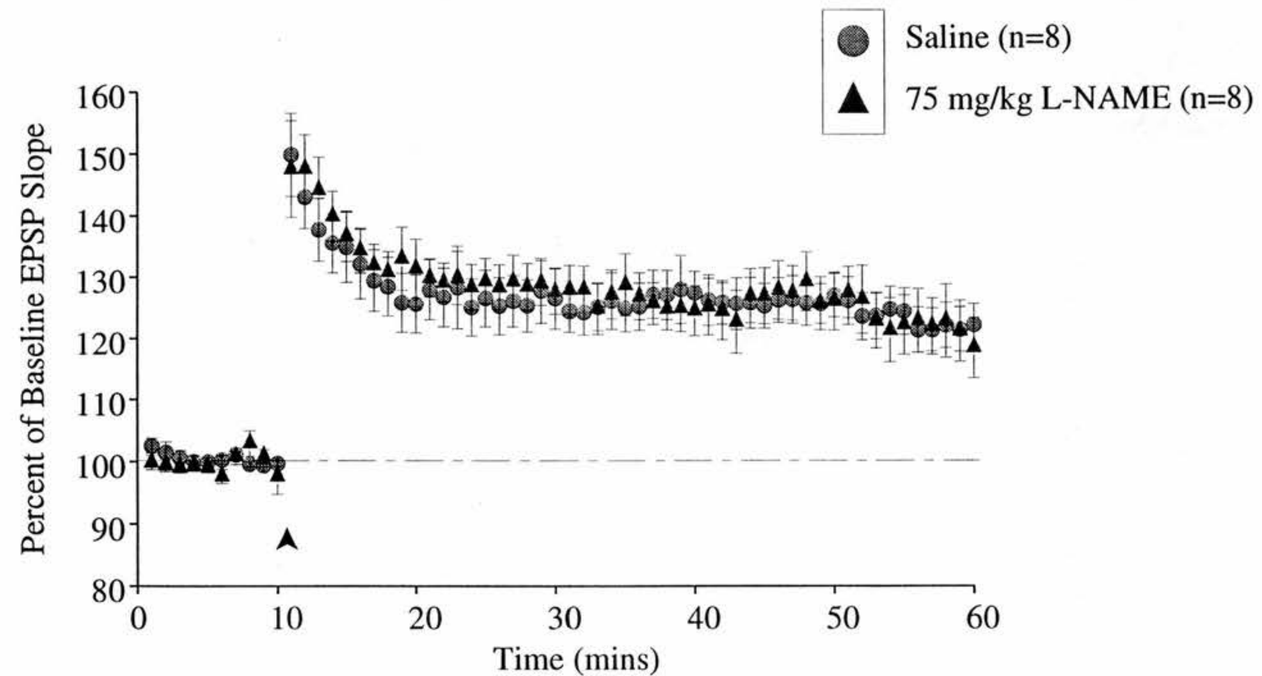


Fig. 5.2.1

L-NAME (75 mg/kg) does not inhibit the induction of LTP induced by a strongly suprathreshold tetanus (Expt 5.2). (▲= tetanus).

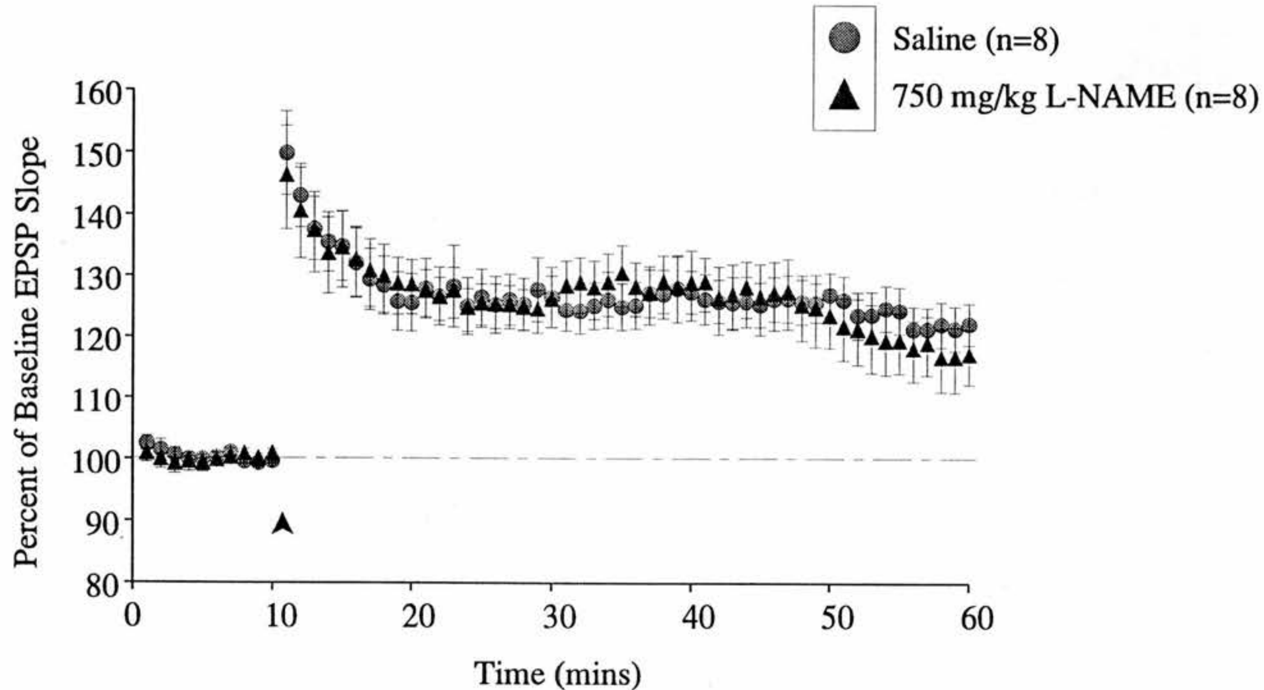


Fig. 5.2.2

L-NAME (750 mg/kg) does not inhibit the induction of LTP induced by a strongly suprathreshold tetanus (Expt 5.2). (▲ = tetanus).

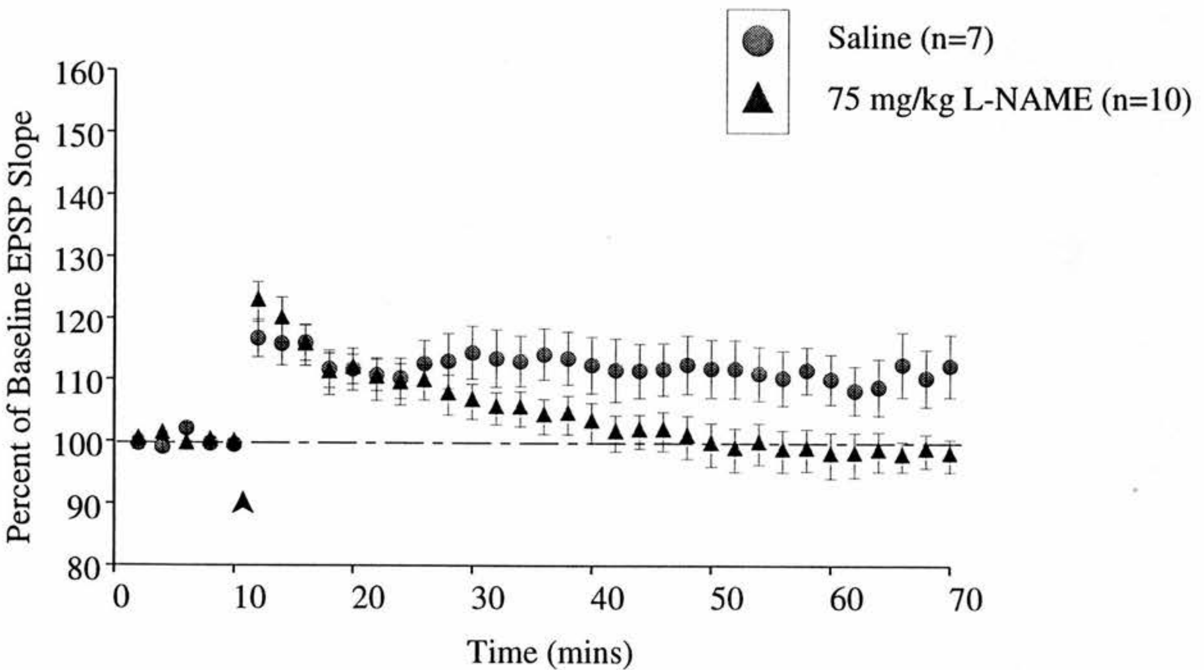


Fig. 5.3.1
L-NAME (75 mg/kg) produces an apparent block of LTP induced by a weak near threshold tetanus (Expt 5.3). (▲ = tetanus).

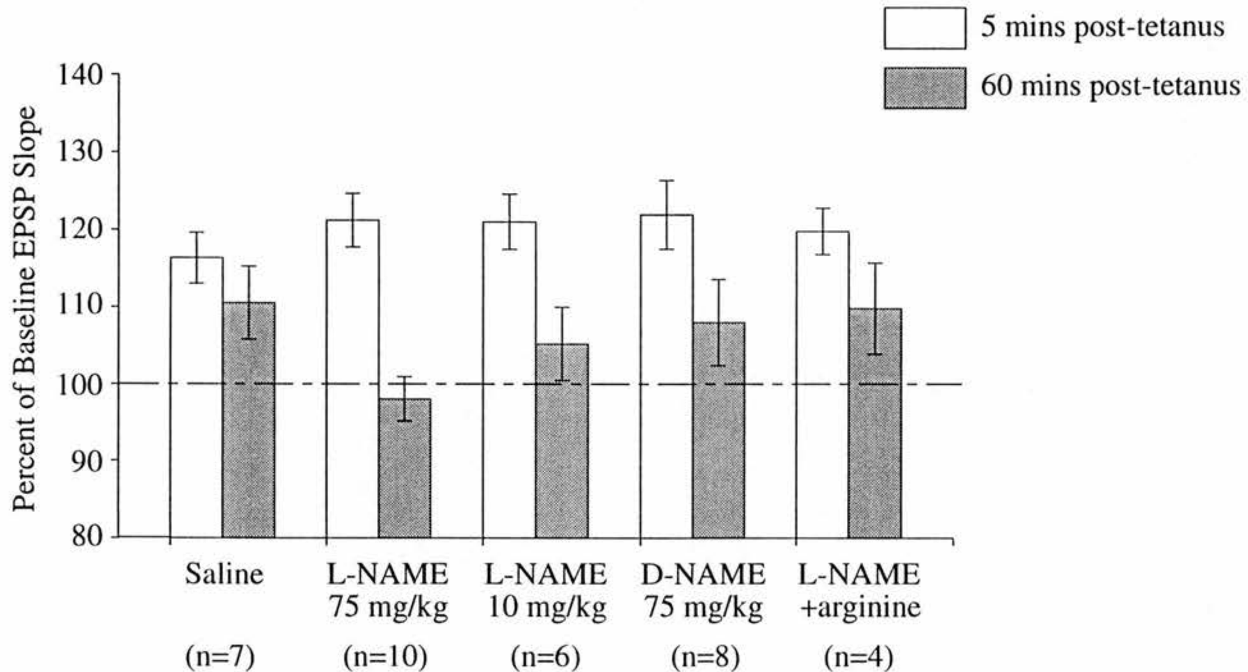


Fig. 5.3.2

L-NAME (75 mg/kg) did not affect the amount of potentiation seen 5 min post-tetanus but there was a significant difference between 75 mg/kg L-NAME and saline controls 60 min post-tetanus. This effect was dose-related, stereoselective and reversible with co-administration of 225 mg/kg L-arginine (Expt 5.3).

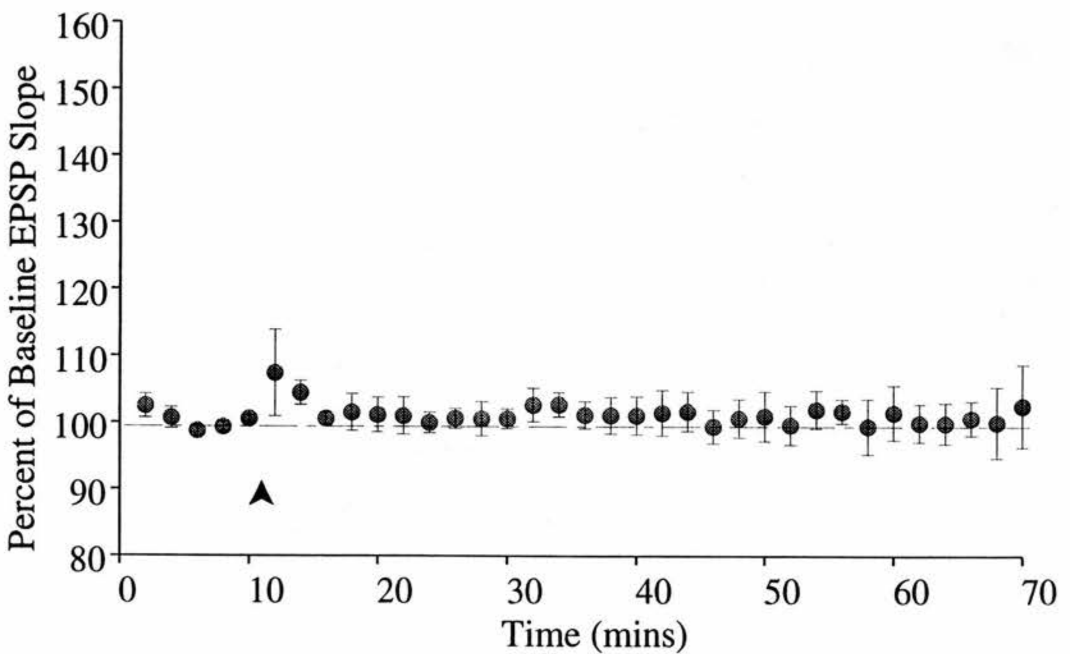


Fig. 5.4.1
LTP induced by the weak tetanus was blocked by D-AP5 (300 nmoles, i.c.v. Expt 5.4). (▲= tetanus).

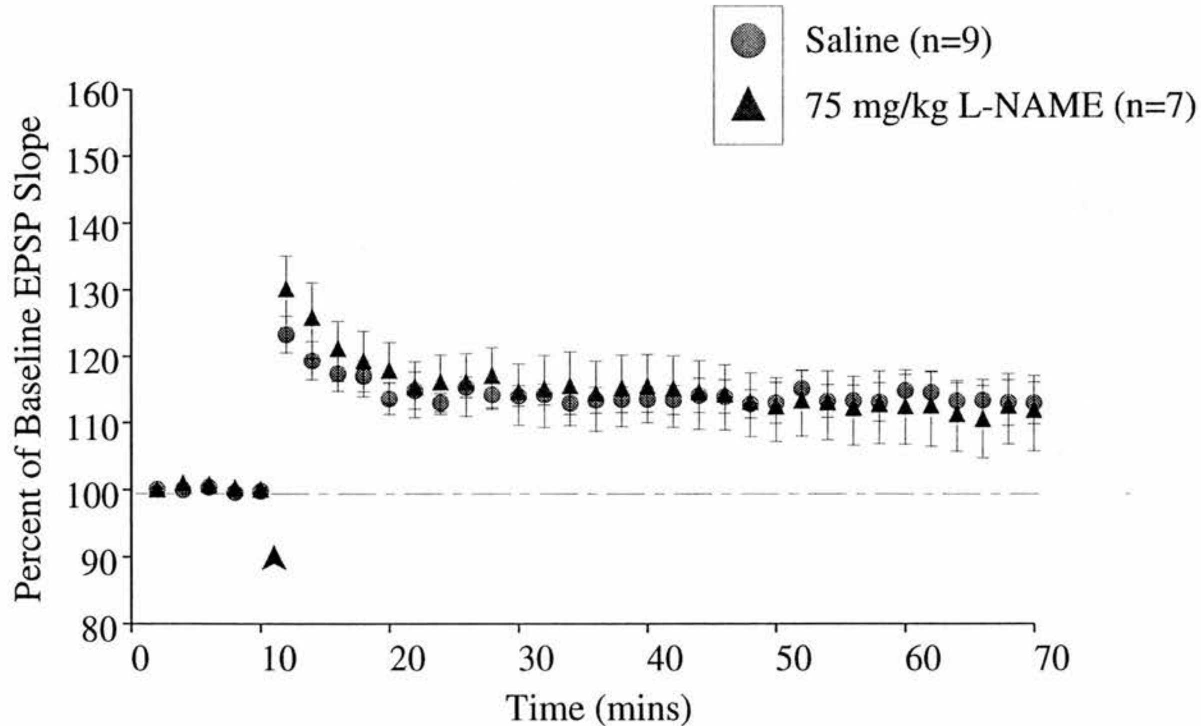


Fig. 5.5.1

Chronic treatment (10 to 20 days) with L-NAME (75 mg/kg) does not block LTP induced by the weak tetanus (Expt 5.5). (▲ = tetanus).

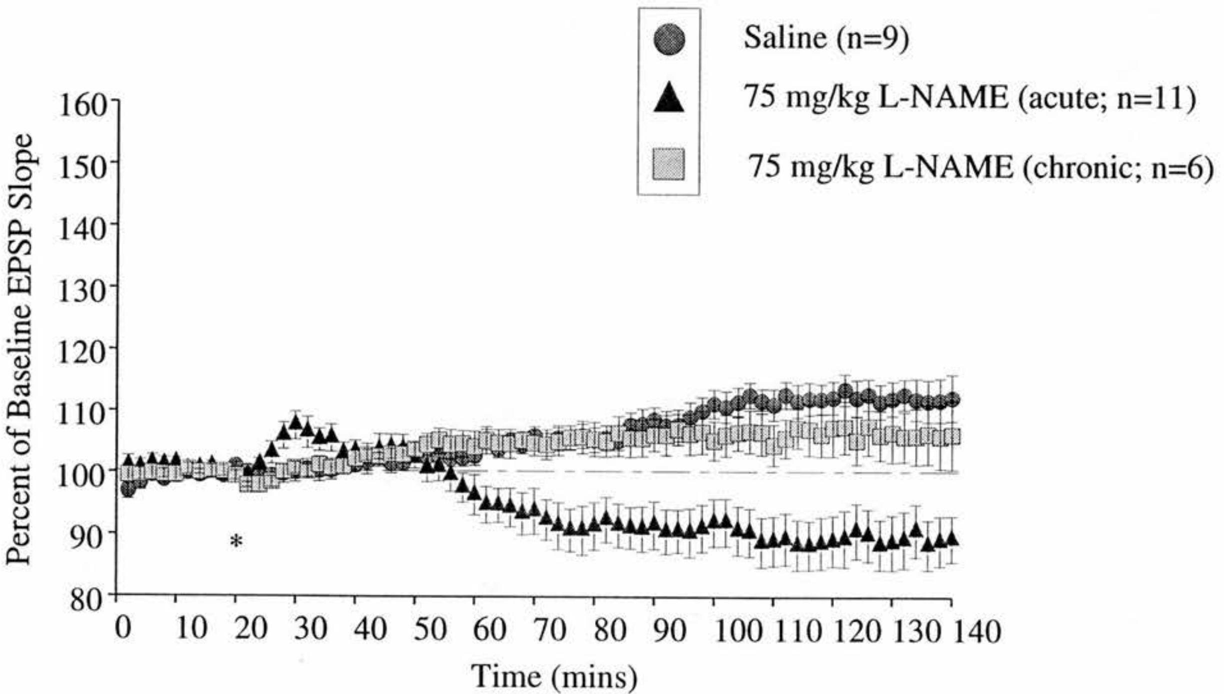


Fig. 5.6.1

Acute L-NAME (75 mg/kg) causes changes in baseline field potentials over an extended 2 hour baseline in the absence of a tetanus. Chronic L-NAME (75 mg/kg) treatment produced a profile of effect similar to saline treated animals (Expt 5.6). (* = injection).

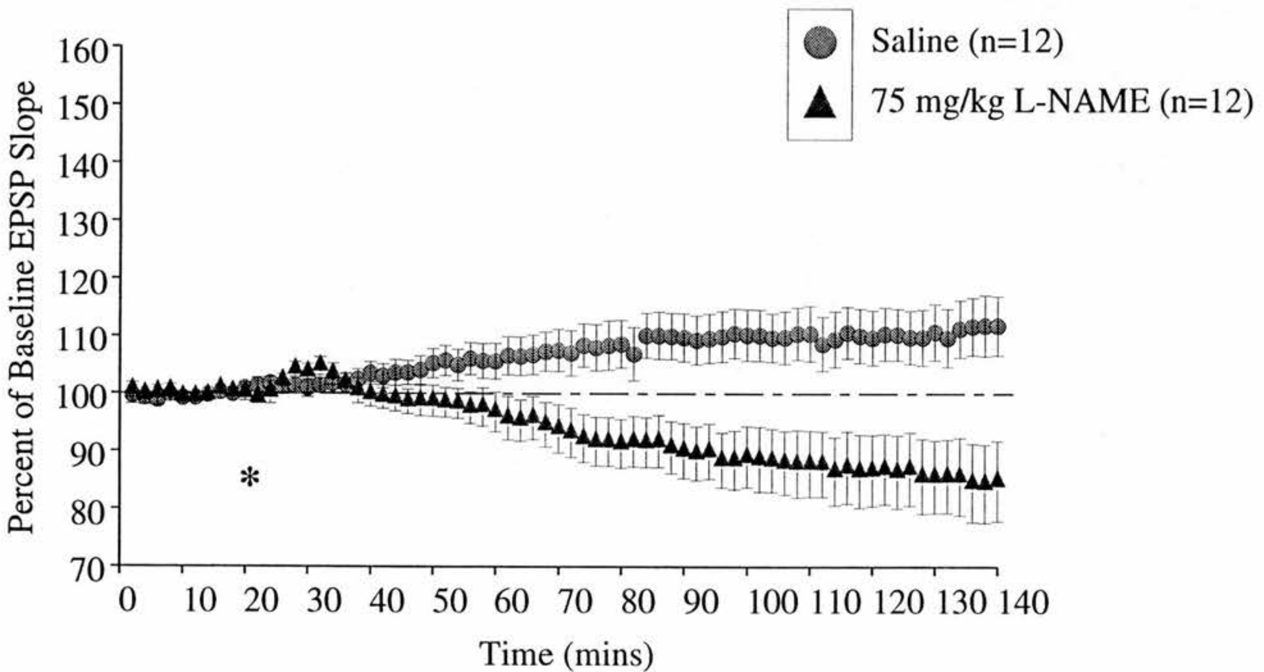


Fig. 5.7.1

The effect of acute L-NAME (75 mg/kg) and saline on the non-tetanised hemisphere in Expt 5.7 (compare with Expt 5.6). (* = injection).

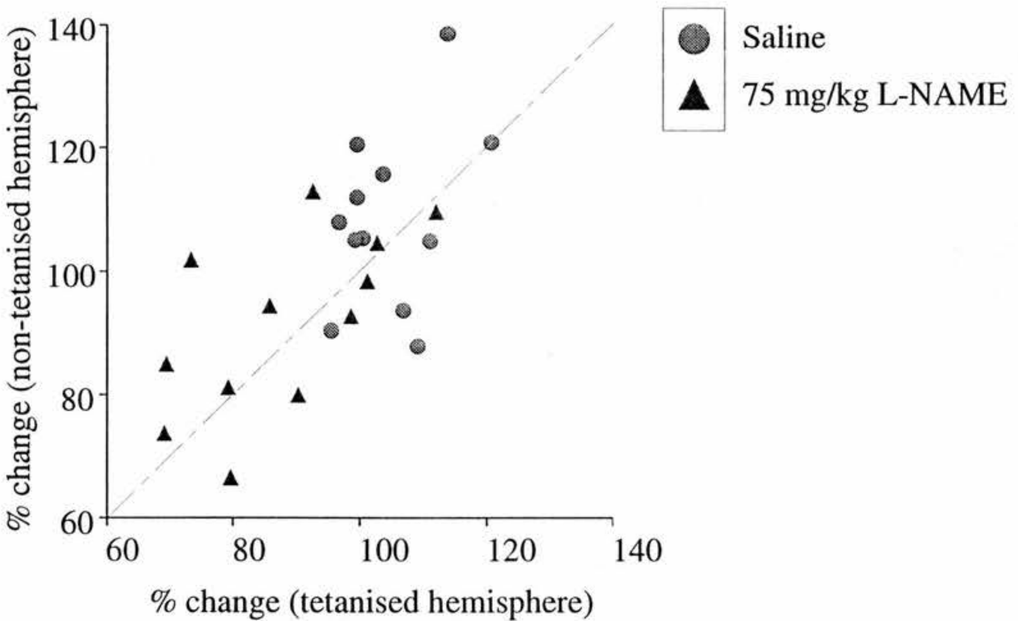


Fig. 5.7.2

The within-subject variation between the tetanised (to be) and non-tetanised (to be) pathways during the one hour period after injection and before tetanus (Expt 5.7). This was highly correlated for the L-NAME injected animals but not for the saline group. If the changes in both hemispheres were perfectly correlated, the points would fall on the identity line shown.

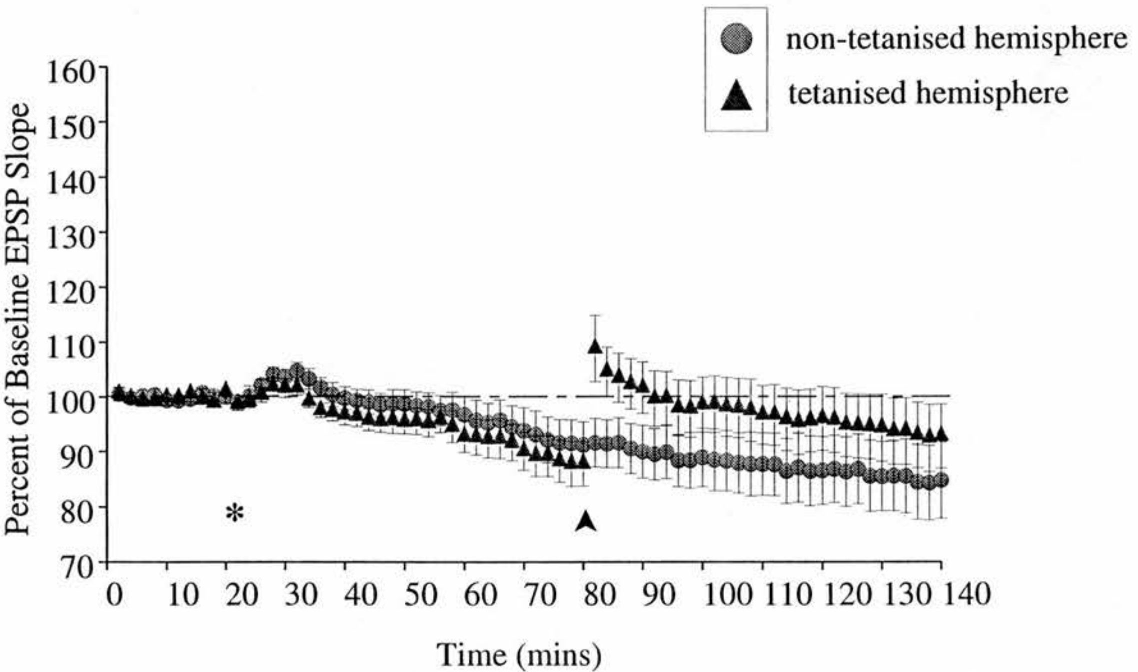


Fig. 5.7.3

The effect of acute L-NAME (75 mg/kg) on the tetanised and non-tetanised hemispheres in Expt 5.7. (* = injection; ▲ = tetanus).

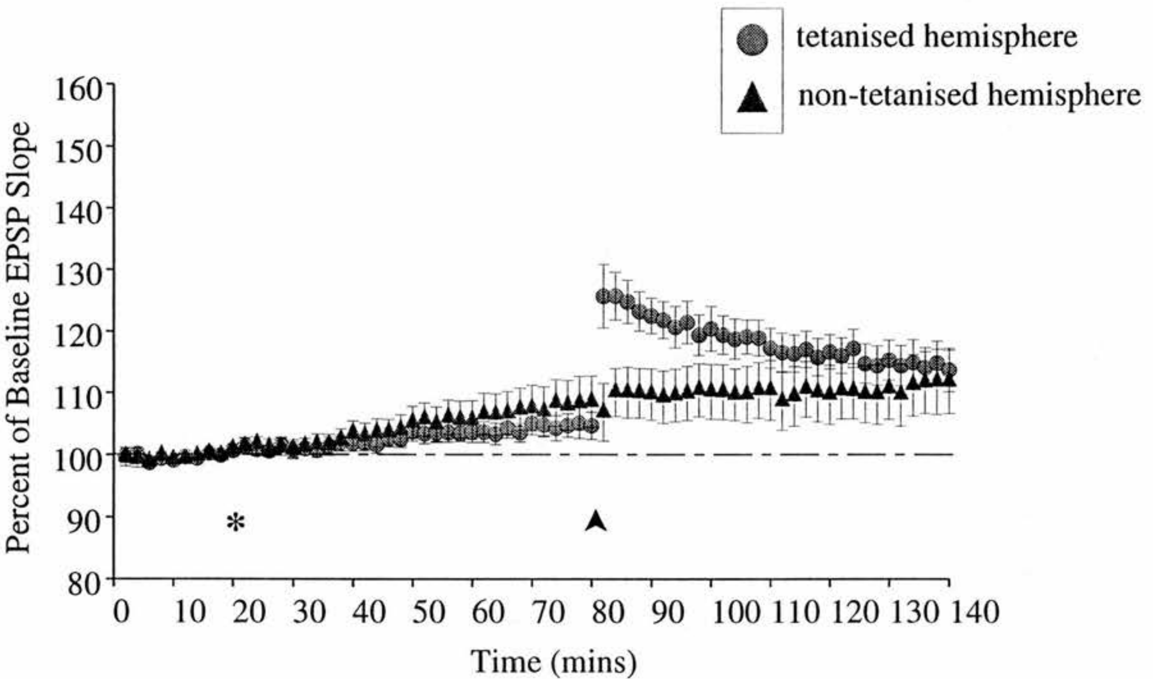


Fig. 5.7.4

The effect of saline on the tetanised and non-tetanised hemispheres in Expt 5.7. (* = injection; ▲ = tetanus).

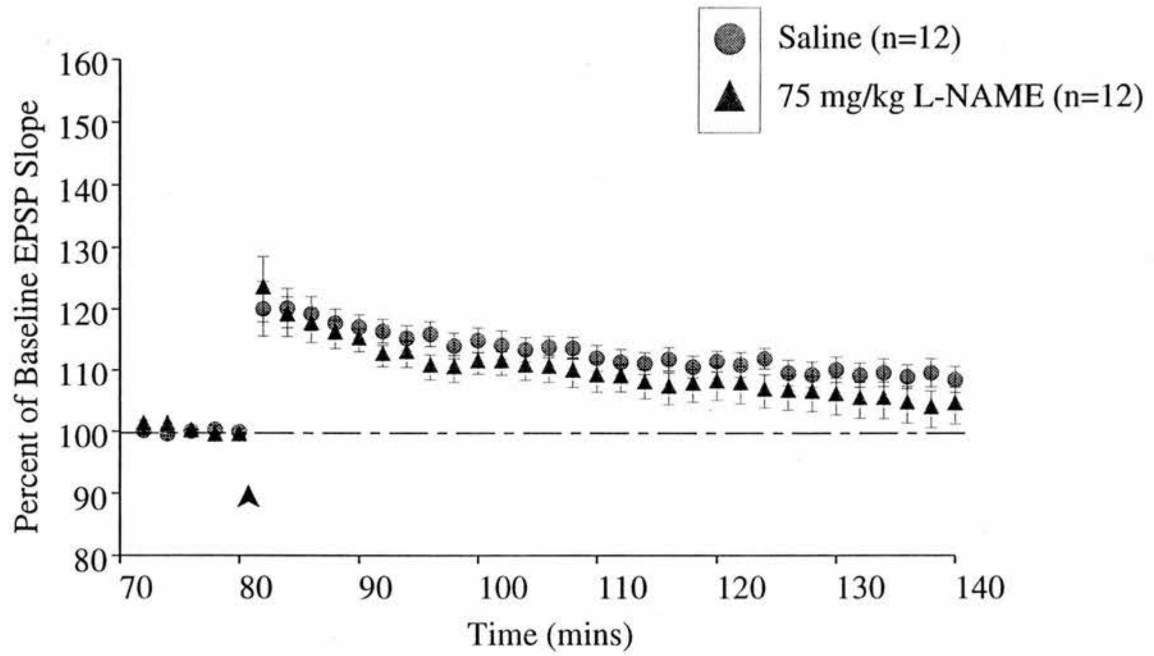


Fig. 5.7.5

Acute L-NAME (75 mg/kg) produces a trend towards less LTP induced by a weak tetanus in Expt 5.7, when the data are plotted relative to a within-hemisphere baseline of 10 min prior to the tetanus (compare with Expt 5.3). (⮝ = tetanus).

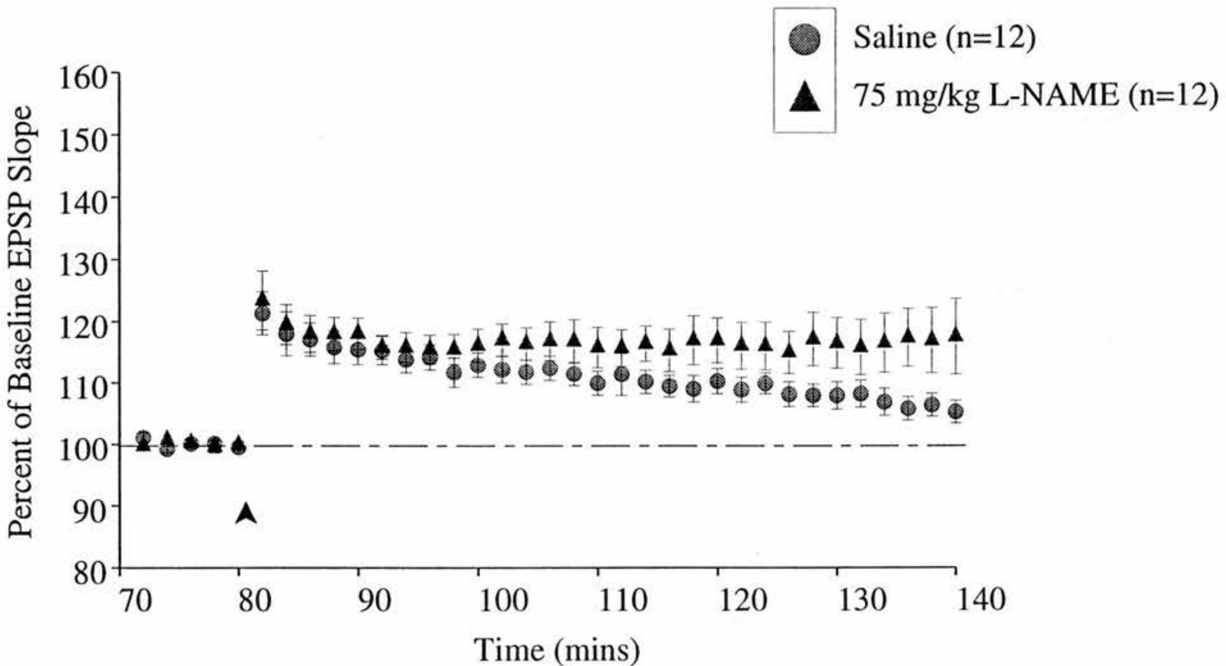


Fig. 5.7.6

Acute L-NAME (75 mg/kg) appears to enhance the amount of potentiation, when the data are plotted using the non-tetanised hemisphere as a within-subject control at each time point throughout the experiment (Expt 5.7).
(⮝ = tetanus).

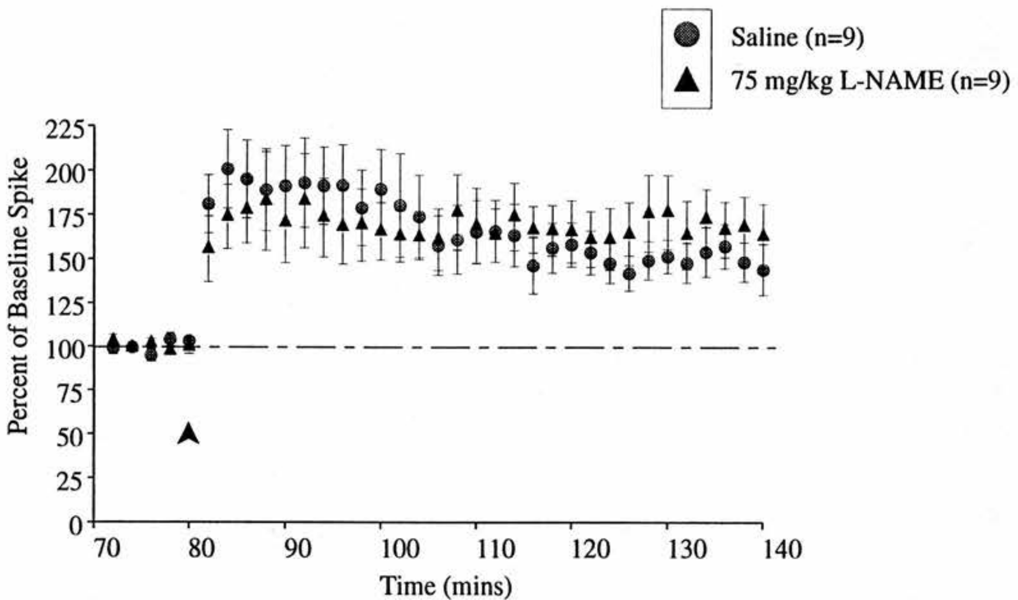


Fig. 5.7.7

Acute L-NAME (75 mg/kg) did not block potentiation of the population spike induced by a weak tetanus (Expt 5.7).

(▲ = tetanus).

Chapter 6 - The effects of the nitric oxide synthase inhibitor, L-NAME, on learning in the watermaze.

There are now several reports in the literature claiming that inhibitors of NOS impair certain forms of learning (Chapman et al., 1992; Hölscher and Rose, 1992; Böhme et al., 1993). For example, Chapman et al., (1992) have reported that i.p. injections of L-NAME (75 mg/kg) impair acquisition of spatial reference memory in the watermaze. Similarly, inhibitors of NOS have also been reported to disrupt performance in a spatial reference memory task in the 8-arm radial maze (Böhme et al., 1993). The effects of NOS inhibitors on learning do not, however, appear to be restricted to hippocampal-dependent tasks such as spatial learning. Deficits in the acquisition of conditioned eyeblink responses (Chapman et al., 1992) and in one trial inhibitory avoidance in the chick (Hölscher and Rose, 1992) following administration of NOS inhibitors have also been reported.

The following series of behavioural experiments was run concurrently with the electrophysiological study outlined in Chapter 5 in an attempt to determine whether or not the spatial learning impairment resulting from L-NAME administration (Chapman et al., 1992) is due to a selective disruption of LTP-like processes in the hippocampus. In terms of behaviour, the key issue to address concerns the specificity of the impairment seen following the administration of an NOS inhibitor. The following series of experiments was conducted to examine, in detail, whether impaired performance in the watermaze results from a specific disruption of spatial learning or derives from an effect upon some other behavioural process (or processes) engaged in this and other kinds of tasks.

Experiment 6.1: The effect of L-NAME on spatial reference memory.

The purpose of the following pilot experiment was to examine whether L-NAME affects the acquisition of a spatial reference memory task in the watermaze using a standard training paradigm.

Procedure

Following one day of drug-free, non-spatial pretraining (6 trials, 60 sec max. swim time), all animals were trained to find a fixed location, hidden escape platform positioned in the centre of either the NE or SW quadrant of the pool (3 days, 6 trials/day, 120 sec max. swim time, ITI of 5-10 min including 30 sec on the platform, random start position across trials). The assignment of animals to groups was counterbalanced with respect to platform position and performance during pre-training. On each day, one hour before the start of behavioural

testing, the rats were briefly anaesthetised with halothane (in order to ensure a successful injection of what is a relatively large volume) and injected i.p. with either saline ($n = 11$) or L-NAME (75 mg/kg; $n = 9$). In this and subsequent experiments reported in this chapter, all drug solutions were made up in 0.9% physiological saline at the appropriate concentration and injected in a volume of 10 ml/kg. Twenty four hours after the final day of spatial training (and one hour after a final injection), the rats' memory of the platform location was assessed in a transfer test during which the platform was removed from the pool and the animals allowed to swim freely for 60 sec.

Results

All animals were capable of swimming around the pool and climbing onto the platform. Both groups showed an improvement in performance, in terms of a reduction in escape latencies, with training, although this was much more appreciable for the saline group (Figure 6.1.1). An ANOVA of escape latencies across the 18 training trials revealed a significant effect of group ($F(1,19) = 15.87$; $p < 0.001$), an effect of trial ($F(17,323) = 9.35$; $p < 0.0001$) and a groups by trials interaction ($F(17,323) = 2.34$; $p < 0.01$). A subsequent analysis of simple main effects showed that there was no significant difference between the groups on trial 1 (saline = 77.6 ± 15.3s; L-NAME = 69.7 ± 13.8s; $F < 1$; $p > 0.20$) or by the end of training (trial 15; $F = 2.27$; $p > 0.10$; trial 16; $F = 1.12$; $p > 0.20$; trial 17; $F = 1.75$; $p > 0.10$; trial 18; $F < 1$; $p > 0.20$; see Figure 6.1.1). A comparison of mean swim speeds on the first day of spatial training (trials 1-6) revealed no difference between the groups (saline = 0.27 ± 0.01 m/s; L-NAME = 0.29 ± 0.01m/s; $F < 1$; $p > 0.20$).

During the transfer test, saline injected rats searched persistently in the vicinity of the former platform location and consequently spent a large proportion of the 60 seconds in the 'training' quadrant (Figure 6.1.2 and 6.1.3). L-NAME injected rats showed a much smaller spatial bias towards the training quadrant (Figure 6.1.2 and 6.1.3). An ANOVA of the amount of time spent in the 4 quadrants of the pool during the transfer test revealed both an effect of quadrant ($F(2,54) = 25.36$; $p < 0.001$) and a groups by quadrants interaction ($F(2,54) = 11.20$; $p < 0.001$). A comparison of the percentage time spent in the training quadrant only, confirmed that the saline group were spending significantly more time than the L-NAME group in this region of the pool during the transfer test (saline = 51.1 ± 3.6%; L-NAME = 30.7 ± 3.3%; $t(18) = 4.05$; $p < 0.001$).

In summary, L-NAME (75 mg/kg) injected rats were impaired on a spatial learning task in the watermaze, relative to saline injected controls, both in terms of escape latencies during training and performance in the transfer test. The results of this pilot study gave good grounds for a further, more detailed investigation of the effects of L-NAME on spatial learning in the watermaze.

Experiment 6.2: A further investigation of the effects of L-NAME on spatial reference memory.

The following experiment was conducted as a replication of Expt 6.1 but with additional groups of animals included in an attempt to determine whether or not any deficit was specifically due to an inhibition of NOS. The dose-dependency and stereoselectivity of the L-NAME-induced behavioural impairment were investigated, as was the possibility that the impairment might be reversed by co-administration of L-arginine.

Procedure

Following one day of drug-free, non-spatial pretraining (6 trials, 60 sec max. swim time), all animals were trained to find a fixed location, hidden escape platform as in Expt 6.1 (3 days, 6 trials/day, 120 sec max., ITI of 5-10 min including 30 sec on the platform, random start position across trials, platform NE or SW). The assignment of animals to groups was counterbalanced with respect to the platform position during spatial training and performance during pre-training. On each day, one hour before the start of behavioural testing, the rats were injected i.p. (without halothane anaesthesia in this, and subsequent, experiments) with either saline, 10 mg/kg L-NAME, 75 mg/kg L-NAME, 75 mg/kg D-NAME, 225 mg/kg L-arginine or 75 mg/kg L-NAME co-administered with 225 mg/kg L-arginine. Twenty four hours after the final day of spatial training (and one hour after injection), the rats' memory of the platform location was assessed in a transfer test.

Immediately following the transfer test, the rats received 6 additional 'cue' trials (120 sec max., 30 sec ITI) during which they were required to escape from the water onto a visible grey platform (extending 1-1.5 cm above the water surface). Both the platform location and the rats' starting position were randomly changed from one trial to the next. Curtains were drawn around the pool to obscure extramaze cues.

Results

All animals were capable of swimming around the pool until the platform was located and then climbing onto it. There was no visible sign of any sensorimotor disturbance either during swimming or when attempting to climb onto the platform. On the first day of spatial training, the 75 mg/kg L-NAME treated animals appeared to be swimming faster than those in the other groups. An ANOVA of the mean swim speeds for each animal on the first day of spatial training revealed a significant difference ($F_{(5,47)} = 3.41$; $p < 0.025$) and subsequent Tukey's HSD pairwise comparisons indicated that only the 75 mg/kg L-NAME group (mean = 0.28 ± 0.01 m/s) differed significantly from the saline control group (mean = 0.23 ± 0.01 m/s).

All animals showed a progressive decline in escape latency with training (Figure 6.2.1). An ANOVA of escape latencies for the three days of spatial training revealed an overall effect of group ($F_{(5,47)} = 9.14$; $p < 0.0001$), trial ($F_{(17,799)} = 28.54$; $p < 0.0001$) and a groups by trials interaction ($F_{(85,799)} = 1.36$; $p < 0.05$). Further analysis, using Tukey's HSD pairwise comparisons, showed that the 75 mg/kg L-NAME group performed significantly worse than the other groups ($p < 0.01$). Apart from the first trial, for which there was no difference in escape latency between groups ($F < 1$; $p > 0.20$), this deficit was more pronounced over the early trials and, by the end of training, the 75 mg/kg L-NAME treated rats were escaping from the pool as quickly as the animals from the other treatment groups, as reflected in the groups by trials interaction.

The rats' memory of the platform location was assessed in a transfer test (Figure 6.2.2.). All groups spent more time searching in the quadrant of the pool in which the platform had previously been located (the 'training' quadrant; $F_{(2,141)} = 149.19$; $p < 0.001$), suggesting that the animals had learned something about the former location of the platform, but the groups differed with respect to the extent of learning (groups by quadrants interaction: $F_{(10,141)} = 2.34$; $p < 0.01$). A second ANOVA of the percentage time spent in the training quadrant only, revealed a significant effect of group ($F_{(5,47)} = 2.96$; $p < 0.05$) and subsequent Tukey's HSD pairwise comparisons showed that the 75 mg/kg L-NAME group was spending significantly less time in the training quadrant than the saline group ($p < 0.05$), and less time than that shown by the 10 mg/kg L-NAME, D-NAME and the L-NAME plus arginine groups considered together ($p < 0.025$). None of these three groups, or the L-arginine alone group, differed significantly from the saline injected controls (saline = $65.9 \pm 4.8\%$; 75 mg/kg L-NAME = $39.8 \pm 5.8\%$; 10 mg/kg L-NAME = $57.4 \pm 5.8\%$; D-NAME =

56.6 ± 4.9%; L-arginine = 63.2 ± 4.7% and L-NAME plus arginine = 52.1 ± 6.1%).

Immediately after the transfer test, the rats were given a 'cue' task. All rats escaped from the pool rapidly (Figure 6.2.3) and there was no significant difference between the groups ($F < 1$; $p > 0.20$), nor a groups by trials interaction ($F < 1$; $p > 0.20$). There was, however, a significant improvement across the 6 training trials ($F(5,235) = 16.68$; $p < 0.0001$).

The result of this study and Expt 6.1 are in agreement with Chapman et al., (1992) in demonstrating that L-NAME causes an impairment in performance during the acquisition of a spatial reference memory task in the watermaze. This effect is dose-related, stereoselective and partially reversed by co-administration of L-arginine, results which are consistent with the idea that the deficit is due to the inhibition of NOS. The absence of an effect of L-NAME on the cue task provides some evidence that the deficit in the spatial task is not due to a gross sensorimotor disturbance or alteration of motivational state.

Experiment 6.3: The effect of L-NAME on the retention of previously learned spatial information.

The blockade of NMDA receptors with D-AP5 prevents the induction of LTP but has no effect on its expression or maintenance (Collingridge et al., 1983). If NMDA receptors are required to initiate changes in synaptic weights in the hippocampus, but not to maintain a specific distribution of synaptic weights, then one might predict that D-AP5 would have no effect on the retrieval of spatial information but, at the same time, impair new learning. Behavioural experiments have shown that the intracerebral infusion of D-AP5, at concentrations sufficient to block the induction of LTP *in vivo*, does not affect the retrieval of a previously acquired platform location in the watermaze (Morris et al.,1990a). In a parallel study, however, the same dose of D-AP5 did impair learning about a novel platform location in the same spatial environment.

The precise role of NO in LTP is not yet fully understood. There is, however, evidence to suggest that NO is involved in initial induction processes but not in the long-term expression of potentiation. Haley et al., (1992) demonstrated that L-NAME does not affect synaptic responses evoked by low frequency stimulation either before or after LTP induction. Furthermore, Schuman and Madison (1991) reported that inhibition of LTP by L-methyl arginine was not reversed by L-arginine applied post-tetanically but was prevented by co-administration during the tetanus. If the expression of LTP requires the continued, persistent

activation of NOS, then the addition of L-arginine should reverse the enzyme blockade and result in the immediate expression of LTP. The fact that arginine only reverses the L-methyl arginine inhibition of LTP if present during the tetanic stimulation supports the idea that NO is involved in induction but not long-term expression. Accordingly, if LTP is a substrate for spatial learning, then one might predict that NOS inhibition would prevent new learning but spare retention, resulting in a parallel behavioural impairment to that obtained with D-AP5.

Procedure

Following one day of non-spatial pretraining (6 trials, 60 sec max.), rats (n = 20) were extensively trained (in the absence of any drug injection) to find a fixed location, hidden escape platform (3 days of 4 trials/day, 3 days of 2 trials/day and 4 days of 1 trial/day; trials 1-22; 120 sec max., 30 sec ITI on the platform, platform NE or SW). Following a 3 day interval (in order to equate the training protocol with that used in the D-AP5 experiment of Morris et al., 1990), they then received another 8 days of training with 1 trial per day to the same platform location. At this stage, the rats were injected with saline or L-NAME (75 mg/kg) each day, one hour prior to the start of behavioural testing. The assignment of animals to groups was counterbalanced with respect to both performance during drug-free spatial training and the platform position during the drug phase. Performance was assessed with a first transfer test, 24 hours after trial 26 (and immediately before trial 28) and again 24 hours after trial 31.

Results

During the initial, drug free, spatial training, all animals learned something about the location of the hidden platform as indicated by direct swim paths and short escape latencies on the later training trials (Figure 6.3.1A). On the first trial after drug administration (trial 23), both groups of animals showed good retention of the platform location and escaped rapidly from the water with no significant difference, in terms of escape latency (saline = 18.5 ± 10s; L-NAME = 6.7 ± 1.0s; $t(18) = 1.18$; $p > 0.20$). All rats continued to escape rapidly from the pool during the 8 training trials of the drug phase (Figure 6.3.1B). An ANOVA revealed neither an effect of group ($F(1,18) = 1.10$; $p > 0.20$), nor an effect of trial ($F(7,126) = 1.04$; $p > 0.20$), nor a groups by trials interaction ($F(7,126) = 1.04$; $p > 0.20$). Inspection of the two transfer tests (trials 27 and 32; Figures 6.3.2 and 6.3.3) showed that

both groups searched persistently in the training quadrant. An ANOVA of performance in the first transfer test revealed a significant effect of quadrant ($F (2,54) = 105.81$; $p < 0.001$) but no groups by quadrants interaction ($F < 1$; $p > 0.20$; % time in training quadrant - saline = 55.5 ± 3.1; L-NAME = 57.0 ± 2.9). Similar results were obtained in the second transfer test (% time in training quadrant - saline = 50.6 ± 3.0; L-NAME = 57.6 ± 3.2; quadrants $F (2,54) = 111.59$; $p < 0.001$; groups by quadrants interaction $F (2,54) = 1.73$; $p > 0.10$).

In summary, L-NAME does not affect the retention of previously acquired spatial information. This result is also in agreement with that reported by Chapman et al., (1992).

Experiment 6.4: - The effect of L-NAME upon visual discrimination learning in the watermaze.

It is possible that the L-NAME induced deficit in Expts 6.1 and 6.2 is due to an effect of the drug on some behavioural process other than spatial learning. For example, L-NAME may impair performance through an effect on some procedural component of the task. No impairment was seen, however, on the 'cue' task and therefore a limited claim can be made, namely that the animals do not exhibit any gross sensorimotor disturbance which prevents them from swimming freely, or seeing and then climbing onto the platform. In addition, the fact that L-NAME has no effect on the retention of spatial information adds further support to this interpretation. A more compelling claim for a specific effect of NOS inhibition on the neural mechanisms of spatial learning could be made if the inhibitor had no detectable effect on visual discrimination learning in the watermaze. Visual discrimination learning does not require the integrity of the hippocampal formation (O'Keefe and Nadel, 1978; Morris et al., 1986a) and is unimpaired by the intracerebroventricular infusion of D-AP5 (Morris et al., 1986b). The fact that this form of learning would appear to occur independently of NMDA-receptor mediated synaptic plasticity provides one suitable control task for spatial learning in the watermaze. If the L-NAME induced deficit in the spatial task is due to a blockade of NMDA receptor mediated synaptic plasticity, then L-NAME should be without effect on the visual discrimination task. On the other hand, a non-specific effect of the drug, acting on a procedural component of behavioural performance, might be expected to have a detrimental effect on performance in both spatial and non-spatial tasks.

Procedure

Separate groups of rats were trained to discriminate between a rigid and a floating visible platform. Injections of L-NAME (75 mg/kg; n = 11) or saline (n = 9) were given daily one hour before testing (as in Expt 6.2). Rats received 10 trials per day, with an ITI of 5-10 minutes including 30 sec on the platform, until a criterion of 90% correct choices had been achieved over 2 consecutive days of training (18 out of 20). On each trial, the rat was placed into the water facing the sidewall at a point across the pool from the two visible platforms; these were moved randomly around the pool between trials in order to vary the spatial location of the rewarded platform. In addition, a pseudorandom sequence was used to alternate the left/right orientation of the rewarded/non-rewarded platforms. In a final test, conducted the day after the criterion score was achieved, the rats were presented with 2 visibly identical platforms, only one of which was rigid and a means of escaping the water. If the rats were attending to the visual appearance of the platforms to solve the task then performance should fall to chance.

Results

After several days of training, the animals began to make a deliberate choice for one platform or the other. By day 8, they were starting to achieve the criterion level of performance and all had reached criterion by day 17. An ANOVA of choice accuracy over the first 8 training sessions revealed no differences between the groups ($F < 1$; $p > 0.20$), nor any groups by sessions interaction ($F (7,133) = 1.25$; $p > 0.20$) but a significant improvement across sessions ($F (7,133) = 7.64$; $p < 0.0001$). In addition, there was no difference between the groups in terms of the number of errors made in achieving the criterion score (saline = 44.3 ± 2.2; L-NAME = 45.6 ± 2.6; $t < 1$; $p > 0.20$; Figure 6.4.1). Performance fell to chance on the day of testing with two identical platforms ($52.9 \pm 2.3\%$).

Closer inspection of the first day of training, however, did reveal differences between the saline and L-NAME treated groups. The L-NAME group took significantly longer to escape from the pool on the early training trials (Figure 6.4.2). An ANOVA of the mean escape latencies for the first 8 training sessions revealed a significant effect of group ($F (1,19) = 10.01$; $p < 0.005$), session ($F (7,133) = 156.75$; $p < 0.0001$) and a groups by sessions interaction ($F (7,133) = 6.90$; $p < 0.0001$). A subsequent analysis of simple main effects showed that the two groups differed significantly on day 1 of training ($F = 55.68$; $p < 0.001$).

On day 2, there was still a trend towards a difference between the groups ($F = 2.95$; $0.05 < p < 0.10$), but the escape latencies of the two groups were indistinguishable on subsequent training sessions.

L-NAME did not affect the rate at which criterion levels of performance were attained in the visual discrimination task. There was, however, a subtle effect of the inhibitor on performance on the first day of training which resulted in prolonged escape latencies and a tendency to fail to climb onto the stable platform within 60 sec. This 'first day' effect of L-NAME in the watermaze would be of more consequence in a more rapidly acquired task such as the spatial reference memory task and thus may contribute towards the deficit observed in Expts 6.1 and 6.2.

Experiment 6.5: The effect of L-NAME on acquisition of a novel platform location in a familiar spatial enviroment.

The results of Expts 6.1 - 6.4 suggest a profile of impairment similar to that reported to occur following chronic intraventricular infusion of AP5, namely, an impairment of spatial learning without any effect upon the retention of spatial information or visual discrimination learning (Morris et al., 1986a; Morris et al., 1990a). The prolonged escape latencies of the L-NAME group on day 1 of the visual discrimination task points to an alternative possibility. Specifically, L-NAME may cause a transient and nonspecific impairment that affects performance in several different types of learning, rather than a direct effect on the mechanisms of spatial learning *per se.*

Further experiments were conducted to distinguish between these two possibilities. The first of these, run in parallel with Expt 6.3, began with the same drug-free initial spatial training but, during the drug phase of the experiment, the platform was moved to the opposite quadrant of the pool (a type of 'reversal' task). As previously mentioned, D-AP5 impairs performance on this reversal task, presumably because new learning is involved (Morris et al., 1990a).

Procedure

Separate groups of rats were trained as in Expt 6.3, with the exception that, during the drug phase, the platform was moved to the opposite quadrant of the pool (ie. rats trained initially

to SW had the platform moved to NE).

Results

As in Expt 6.3, all animals demonstrated a considerable reduction in escape latencies during the initial drug-free spatial training (Figure 6.5.1A). On the first trial during the drug administration phase (trial 23), all rats searched persistently in the vicinity of the former platform location and, consequently, the time taken to escape was considerably prolonged with many rats failing to find the platform within the maximum permitted swim time of 120 sec. There was no difference between groups on this first trial (saline = 83.8 ± 13.5s; L-NAME = 64.5 ± 13.9s; $t < 1$; $p > 0.20$). The rats received 8 training trials with the platform in the new location. Both groups spent progressively less time searching in the vicinity of the former platform location and more time in the new location and, thus, their escape latencies reduced across trials (Figure 6.5.1B). An ANOVA revealed a significant improvement across trials ($F(7,126) = 21.02$; $p < 0.0001$), but no effect of group ($F(1,18) = 2.17$; $p > 0.10$) nor a groups by trials interaction ($F < 1$; $p > 0.20$). Transfer tests conducted 24 hours after trials 26 and 31 also showed that L-NAME had no effect on the acquisition of the new platform location (Figures 6.5.2 and 6.5.3). An ANOVA of the first transfer test revealed a significant effect of quadrant ($F(2,54) = 12.60$; $p < 0.001$) but no groups by quadrants interaction ($F < 1$; $p > 0.20$; % time in training quadrant - saline = 32.6 ± 2.7%; L-NAME = 35.2 ± 3.5%). Similarly, on the second transfer test, there was a significant effect of quadrant ($F(2,54) = 59.93$; $p < 0.001$) but again there was no interaction with the drug treatment ($F(2,54) = 1.83$; $p > 0.10$; % time in training quadrant - saline = 42.1 ± 2.5%; L-NAME = 48.9 ± 3.4%). Therefore, and somewhat surprisingly, L-NAME did not impair the learning of a novel platform location in a familiar spatial environment. This is unlikely to be a 'floor' effect as there was a clear improvement in performance between transfer test 1 (trial 27) and transfer test 2 (trial 32) in both groups.

Experiment 6.6 : The effect of L-NAME on spatial learning in a novel environment by experienced animals previously trained in another watermaze.

The lack of an effect of L-NAME on the 'reversal' task (Expt 6.5) might be accounted for in either of two ways. First, the amount of new learning required to solve the 'reversal' task may be minimal. During the initial drug-free spatial training, a relatively detailed

representation of the environment will already have been formed and so, when the platform is moved, the only modification required of that representation is to encode the new goal location. There is no need to 'reverse' the learned spatial relationships between extramaze cues. Learning a new goal location in a familiar environment may necessitate very little, if any, synaptic plasticity. Alternatively, it is possible that the L-NAME-induced deficit observed in Expts. 6.1 and 6.2 is, as previously suggested, due to an effect of the enzyme inhibitor on some behavioural process other than spatial learning which detrimentally affects performance early in training. To distinguish between these two possibilities, a further experiment was conducted in which the initial watermaze training was carried out in a different spatial environment from that used during the drug phase (Gallagher, 1985; Shapiro and O'Connor, 1992). With this training protocol, the experienced animals then have to learn about a wholly novel spatial environment during the drug phase, in contrast to the 'reversal' task in which they have only to learn a new platform location. In this experiment, the training protocol was otherwise identical to that used in the retention/reversal experiments (Expts 6.3 and 6.5), excepting that precautions were taken to check on the extent of generalisation between the two spatial environments.

Procedure

Following one day of non-spatial pretraining (6 trials, 60 sec max.), the rats (n=24) were given spatial training (drug free) in watermaze 1 (3 days of 4 trials/day, 3 days of 2 trials/day and 4 days of 1 trial/day; trials 1-22; 120 sec max, 30 sec ITI). Following a 3 day interval (in order to equate the training protocol with that used in Expts 6.3 and 6.5), a sub-group of 8 rats (made up of 4 animals from each of the saline and L-NAME groups) was given a transfer test in watermaze 2 (trial 23) in order to assess whether training in watermaze 1 resulted in a spatial bias towards any one quadrant in watermaze 2. Immediately following this transfer test, these and the remaining rats began their 8 trials of spatial training in watermaze 2 (1 trial/day), being injected with saline or L-NAME (75 mg/kg) one hour prior to the start of behavioural testing each day. In watermaze 1, the platform was located in one of the 4 quadrants of the pool (NE, NW, SW or SE). In watermaze 2, the platform was located in either the NE or SW quadrants. For every 4 rats trained to a particular platform position in watermaze 2, each of the 4 platform positions had been used during training in watermaze 1. Assignment of rats to groups was counterbalanced with respect to the platform position in both watermaze 1 and 2, and performance over the last 4 training trials in

watermaze 1 (trials 19 - 22). Performance in watermaze 2 was assessed in terms of escape latency and using a transfer test, 24 hours after trial 27 (and immediately before trial 29) and again 24 hours after trial 32. Finally, the rats were returned to watermaze 1 and retention of the platform location in the original environment was assessed with a transfer test in the absence of any drug injection (trial 34), ie. the conditions of original learning.

Results

During spatial training in watermaze 1, both groups of animals rapidly learned to escape from the water. An ANOVA of escape latencies revealed a highly significant improvement across trials ($F(21,462) = 10.42$; $p < 0.0001$; Figure 6.6.1A).

The first transfer test in watermaze 2 (trial 23), before the start of spatial training, was analysed in two ways. First, the data was expressed in terms of the 'future' training quadrant (Figure 6.6.2A). An ANOVA revealed no significant bias to any one quadrant ($F < 1$; $p > 0.20$; % time in training quadrant = 26.3 ± 3.4). Second, the data was re-analysed in terms of the actual quadrants described in terms of compass directions (Figure 6.6.2B); this showed a small bias towards the NW and SW quadrants ($F(2,21) = 4.43$; $p < 0.025$). This corresponds to a bias towards the door into the watermaze room and the location of the carrying cage.

The rats learned about the new spatial environment extremely rapidly, showing a mean escape latency of less than 20 sec by trial 4. An ANOVA of the escape latencies from the 8 training trials in watermaze 2 (Figure 6.6.1B) revealed that there was a significant improvement across trials ($F(7,54) = 3.87$; $p < 0.001$) but no overall effect of group ($F < 1$; $p > 0.20$). There was, however, a just significant groups by trials interaction ($F(7,54) = 2.10$; $p = 0.05$). A subsequent analysis of simple main effects revealed group differences on trials 26 and 32 ($p < 0.05$), but the fact that on trial 26 the saline group were taking, on average, longer to escape while on trial 32 the opposite was true, suggests that these differences are most likely to be due to random fluctuations in performance rather than to some substantial underlying cause.

An ANOVA of the transfer test (trial 28; Figure 6.6.3), conducted after 4 training trials in watermaze 2, revealed a clear bias towards the training quadrant ($F(2,66) = 18.13$; $p < 0.001$). There was no groups by quadrants interaction ($F < 1$; $p > 0.20$; % time in training quadrant - saline = 35.9 ± 2.9%; L-NAME = 39.6 ± 3.4%). This result was repeated in the

second transfer test (trial 33; Figure 6.6.4; $F(2,66) = 78.34$; $p < 0.001$), with there again being no interaction with drug treatment ($F < 1$; $p > 0.20$; % time in training quadrant - saline = 48.8 ± 3.3%; L-NAME = 52.5 ± 3.7%).

Finally, when the rats were returned to watermaze 1, both groups still showed a significant and equivalent bias towards the appropriate training quadrant reflecting their memory of the original training environment despite the intervening training (trial 34; Figure 6.6.5). An ANOVA showed a significant effect of quadrant ($F(2,66) = 9.01$; $p < 0.001$) with no groups by quadrants interaction ($F < 1$; $p > 0.20$; % time in training quadrant - saline = 32.5 ± 3.6%; L-NAME = 36.7 ± 2.8%). It would appear from this result that there is only a small bias towards the training quadrant when the animals are returned to watermaze 1. Further analysis, however, suggests that these values may be an underestimate of how much the rats remember about the platform location. Analysis of the first 30 seconds of trial 34 showed a slightly stronger bias towards the training quadrant (saline = 34.7 ± 4.4%; L-NAME = 39.6 ± 3.1%; quadrant ($F(2,66) = 10.21$; $p < 0.001$), groups by quadrants interaction ($F < 1$; $p > 0.20$), and of the first 15 seconds, an even stronger bias towards (saline = 45.2 ± 6.4%; L-NAME = 47.6 ± 3.8%; quadrant ($F(2,66) = 15.13$; $p < 0.001$), groups by quadrants interaction ($F < 1$; $p > 0.20$). This suggests that the rats do remember where the platform was located but, as a result of the intervening training (including up to 3 previous transfer tests), are less inclined to maintain searching in the training quadrant once they discover the platform is not present.

In summary, L-NAME did not impair the acquisition of spatial reference memory in experienced animals that had previously been trained on a similar watermaze task in a different spatial environment.

Experiment 6.7: The effect of L-NAME on spatial reference memory (1 trial per day).

Examining the pattern of results obtained so far, it would appear that L-NAME can impair performance but only early in training, and only in tasks (spatial and visual discrimination) which involve the animals receiving multiple trials with a short intertrial interval (ITI) in each training session. Thus, L-NAME treatment resulted in a deficit in a spatial reference memory task in which the animals received a block of 6 trials per day with an ITI of 5-10 min (Expts 6.1 and 6.2). A difference between L-NAME and saline groups was also seen on the first day of training in the visual discrimination task (10 trials per day, ITI = 5 - 10 min;

Expt 6.4). In contrast, the performance of the L-NAME treated animals was indistinguishable from saline injected controls on tasks in which there was only 1 training trial per day (Expts 6.3, 6.5 and 6.6).

The lack of a drug effect in Expts 6.3, 6.5 and 6.6 may, however, be unrelated to the scheduling of multiple trials per day but instead be due to the fact that the animals were experienced in the watermaze prior to testing with L-NAME. An experiment was therefore conducted to investigate whether L-NAME would affect spatial reference memory in experimentally naive animals when only a single training trial was given each day.

Procedure

Following 1 day of non-spatial pretraining (6 trials, 60 sec max.), spatial reference memory was assessed in experimentally naive rats which were given only 1 spatial training trial per day for a total of 12 days (120 sec max., 30 sec on platform, platform NE or SW). The assignment of animals to groups was counterbalanced with respect to both performance during pre-training and the platform position during spatial training. Performance was assessed in terms of escape latency and by means of three transfer tests, 24 hours after trial 4 (and immediately before trial 6), 24 hours after trial 9 (and immediately before trial 11) and, finally, 24 hours after trial 14.

Results

An ANOVA of the escape latencies revealed a significant improvement across the 12 training trials ($F(11,187) = 8.81$; $p < 0.0001$; Figure 6.7.1). There was, however, no signicant effect of group ($F < 1$; $p > 0.20$), nor any groups by trials interaction ($F(11,187) = 1.07$; $p > 0.20$). In addition, L-NAME did not affect the amount of time spent in the training quadrant during the transfer tests. An ANOVA of transfer test 1 (trial 5; Figure 6.7.2) revealed a significant effect of quadrant ($F(2,51) = 22.08$; $p < 0.001$) but no groups by quadrants interaction ($F(2,51) = 1.42$; $p > 0.20$; % time in training quadrant - saline = 34.2 ± 2.4%; L-NAME = 40.1 ± 1.9%). Analysis of transfer test 2 (trial 10; Figure 6.7.3) showed both an effect of quadrant ($F(2,51) = 49.54$; $p < 0.001$) and an interaction with drug treatment ($F(2,51) = 3.35$; $p < 0.05$). A comparison of the time spent solely in the training quadrant did not, however, reveal a difference between the groups ($t(17) = 1.45$; $p > 0.10$; saline = 41.9 ± 2.1%; L-NAME = 48.8 ± 4.5%). An ANOVA of transfer test 3 (trial 15;

Figure 6.7.4) also revealed a significant effect of quadrant ($F(2,51) = 90.04$; $p < 0.001$) but no interaction with drug treatment ($F(2,51) = 1.90$; $p > 0.10$; % time in training quadrant - saline = 49.8 ± 4.2%; L-NAME = 56.6 ± 1.8%). It appears, therefore, that L-NAME treatment does not impair spatial reference memory in the watermaze if the animals receive only one training trial per day. This lack of effect is, statistically, neither a 'ceiling' nor 'floor' effect in that both groups showed a progressive increase in the bias towards the training quadrant over the three transfer tests.

DISCUSSION

The main findings of this series of experiments were that animals injected i.p. with a dose of L-NAME sufficient to bring about a > 90% reduction in hippocampal NOS activity (Expt 5.1) were impaired in both a spatial reference memory and a visual discrimination task on early training trials (when these were conducted with multiple trials per session), but that the same dose of L-NAME was without effect on performance in retention, reversal learning, the learning of a new spatial environment in watermaze-experienced animals and, even, acquisition of the basic watermaze task in experimentally naive animals given one training trial per day.

L-NAME does not selectively impair spatial learning

These results are consistent with previously published reports on the effects of systemic administration of NOS inhibitors on learning in the watermaze (Chapman et al., 1992). L-NAME impairs the acquisition of a spatial reference memory task with multiple trials per session but does not affect the retention of previously learned spatial information. This impairment is dose related, stereoselective and reversed by co-administration of L-arginine. In an attempt to determine the behavioural specificity of this impairment, performance in a visual discrimination task was assessed. The rate at which criterion levels of performance were attained was unaffected by L-NAME. This apparent dissociation between the effects of L-NAME on performance of a spatial task and a visual discrimination task is, in several respects, similar to that obtained with D-AP5 (Morris et al., 1986a), and with selective hippocampal lesions (Morris et al., 1986b). Spared performance on the visual discrimination task suggests that L-NAME treated rats do not exhibit a gross sensorimotor impairment and

can indeed learn some tasks normally.

Closer analysis of the visual discrimination task, however, revealed that the L-NAME animals took significantly longer to escape from the pool during the first training session. This finding suggests that L-NAME may cause a transient but more general disturbance of function extending beyond the domain of spatial learning. Irrespective of the nature of this disturbance, it could account for the apparent impairment of spatial learning in Expts 6.1 and 6.2, bearing in mind that the 18 training trials took place over only 3 days and the deficit appears to be largest over the first 2 days. If L-NAME is disrupting the mechanisms underlying spatial learning, then rats should be impaired relative to controls without regard to intertrial interval or the number of trials per session. The results of the platform reversal task (Expt 6.5) and the two watermaze task (Expt 6.6) would appear to negate this hypothesis and suggest that the initial deficit (Expts 6.1 and 6.2) is due to a subtle but less specific effect. The result of the one trial a day spatial reference memory task (Expt 6.7) further calls into question the hypothesis that L-NAME is disrupting spatial learning.

The implications for the relationship between LTP and learning

Given the fact that NOS inhibitors block the induction of LTP *in vitro,* the apparent lack of an effect on spatial learning would appear to contradict the hypothesis that LTP and certain kinds of learning share a common underlying mechanism. However, the results from the parallel electrophysiological study (see Chapter 5), have shown that the very same dose and route of administration of L-NAME (causing a > 90% inhibition of NOS) does not appear to block the induction of NMDA-dependent LTP in the dentate gyrus *in vivo.* Thus, an accurate description of the two studies is that a compound which does *not* block LTP does *not* impair spatial learning. This result does not contradict the hippocampal LTP/spatial learning hypothesis. L-NAME did, however, cause a number of physiological and behavioural changes, including a reduction in dentate gyrus field-potentials (Expt 5.6), cerebrovascular changes throughout the brain (see Appendix B) and a transient behavioural dysfunction affecting both a hippocampal-independent and a hippocampal-dependent task.

The nature of the functional disturbance induced by L-NAME

In attempting to provide an explanation for the observed pattern of results, two distinct but related questions need to be addressed. First, what is it about the various tasks used in this

study that results in impaired performance with some, but not other, paradigms? Second, what possible physiological actions of L-NAME might be responsible for the effects on performance? It may then be possible to determine how these two factors interact to produce the observed pattern of results.

What is it about the various tasks used in this study that results in impaired performance in some, but not other, paradigms? L-NAME impairs performance in tasks involving training with multiple trials and short ITIs. In contrast, L-NAME treated rats receiving just one training trial per day are unimpaired. One possibility is that the physical demands of a task involving multiple trials are much greater and that, for example, animals become tired following repeated swims. Drug treatment may accentuate this fatigue and this, in turn, could indirectly interfere with the animals' ability to learn. This account predicts a deficit in both the spatial and the visual discrimination tasks for as long as the animals are taking a considerable time to escape from the water. As performance improves, however, the drug induced deficit would be expected to disappear, as indeed it did in both Expts 6.2 and 6.4. There are, however, other differences between the multiple- and single-trial paradigms. With one training trial per day, the rats find the platform and are then removed to their home cage for the rest of the day - a satisfactory escape. In contrast, with multiple trials, having found the platform, the rats are soon after put back into the water for further trials. Thus, the platform may be less rewarding to the rats with multiple trials and thus offer less incentive to escape. It is possible, therefore, that there are incentive differences between the two tasks. A further possibility is that repeated sequential exposure to the aversive elements of the watermaze in the multiple trial paradigm is more anxiogenic. L-NAME treatment could conceivably interact with the altered psychological state of the animal during multiple-trial paradigms in such a way as to interfere, albeit indirectly, with the rat's ability to learn. In the single-trial paradigms, however, locating the platform results in a complete escape from the water for 24 hours which could potentially make the task less stressful. Similarly, as animals become more familiar with the procedural demands of the task during training, they may become less anxious. In more general terms, any drug induced feeling of ill-being could conceivably interact with physiological factors such as fatigue or psychological factors such as motivation or anxiety to produce an apparent learning impairment. Neither account requires, however, that L-NAME interact with, or that NO be involved in, the neural mechanisms underlying the associative processes of spatial or visual discrimination learning.

What physiological actions of L-NAME might be responsible for the impairments in

performance? There is a growing literature regarding putative roles of NO throughout the body including effects on the vasculature, immune system, viscera and spinal cord (Garthwaite, 1991; Moncada, 1992; Schuman and Madison, 1994b). In addition, the NOS enzyme is found in many brain areas (Bredt et al., 1991b; Vincent and Kimura, 1992) and there are numerous reported actions of NOS inhibitors in a wide variety of brain regions. The use of an i.p. injection as the route of administration is likely to result in a near global inhibition of NOS. For example, in the parallel physiological study (see Chapter 5), there is evidence for a near complete enzyme inhibition in at least 3 brain areas (cortex, cerebellum and hippocampus), a global cerebral oligaemia and a dramatic rise in blood pressure (Appendix B). There is also a decrease in the size of the hippocampal field potentials and it is possible that similar reductions in function may occur in other brain areas.

There are, therefore, numerous potential sites of action for L-NAME following an i.p. injection and it is not known how the physiological effects of this inhibitor might interact with the different physical and psychological demands of the tasks that have been used. The fact that there is a deficit on the early training sessions (Expts 6.1, 6.2 and 6.4) which then disappears could be explained by the development of a rapid onset tolerance. At the molecular level this seems unlikely. The results of Expt 5.1 and those of Dwyer et al., (1991) have shown that the level of NOS inhibition actually increases with repeated injections. At the psychological level, however, a tolerance to the effects of the drug remains a possibility.

In addition, the possibility that the synthesis of NO is an essential step in the mechanisms that underlie other forms of learning, cannot be ruled out (eg. conditioned eyeblink responding - Chapman et al., 1992; social interaction - Böhme et al., 1993; and one trial inhibitory avoidance - Hölscher and Rose, 1992). However, the non-specific effects witnessed in these experiments may also influence performance on other behavioural paradigms.

Conclusions

L-NAME causes a behavioural syndrome which is likely to extend beyond the domain of learning *per se*. Furthermore, i.p. injection of L-NAME results in a multiplicity of physiological actions *in vivo* which do not include a block of LTP in the dentate gyrus. These findings neither compromise nor support the hypothesis that the mechanisms of LTP

may also underly certain types of learning, but point to the need for further enquiry into the functional role of NO in the brain. The possibility that a more complete inhibition of NOS may result in a real impairment of spatial learning cannot be ruled out.

The pattern of results obtained with L-NAME, described in Chapters 5 and 6, is similar to that seen with the glycine antagonist, 7CK (see Chapter 4). In both cases, behavioural performance on a spatial learning task is impaired in the absence of an effect on hippocampal LTP *in vivo*. Both studies illustrate the problems associated with using drugs to investigate the mechanisms of learning and memory and, in particular, the difficulties in determining the precise cause of a behavioural deficit. The failure of both studies stems from the inability of both experimental approaches to successfully block the induction of hippocampal LTP *in vivo*. In contrast, D-AP5 infusion produces a relaible block of LTP induction *in vivo*. The LTP/learning hypothesis can only be tested using a manipulation which blocks LTP. Therefore. in the final set of experiments (Chapter 7), D-AP5 infusion was used to ensure a block of hippocampal LTP. The effects of this on spatial learning were assessed using behavioural protocols introduced in this chapter (Expt 6.6).

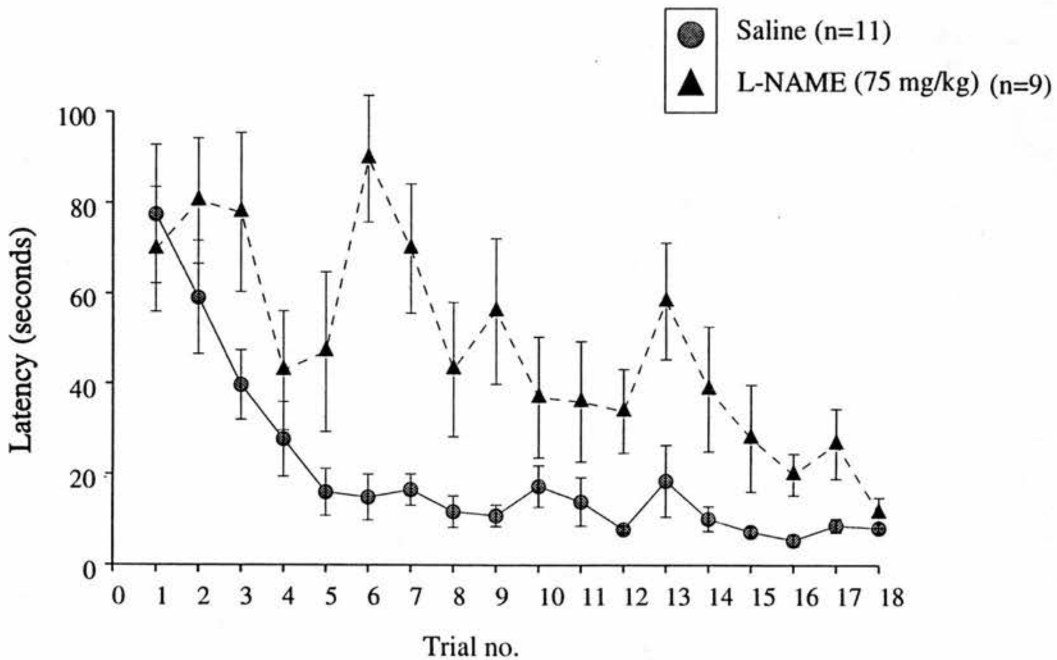
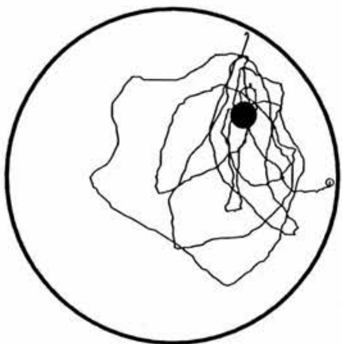
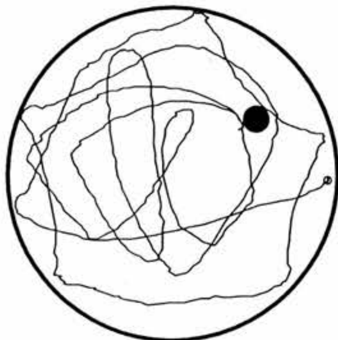


Fig 6.1.1

Mean escape latency (+/- 1 SEM) during acquisition of a standard spatial reference memory task in the watermaze for saline and L-NAME-treated animals (Expt 6.1).



Saline



L-NAME

Fig 6.1.2

During the transfer test the platform is removed from the pool and the rat allowed to swim freely for 60 sec. The above are representative pathways of subjects from both saline and L-NAME (75 mg/kg) groups (Expt 6.1).

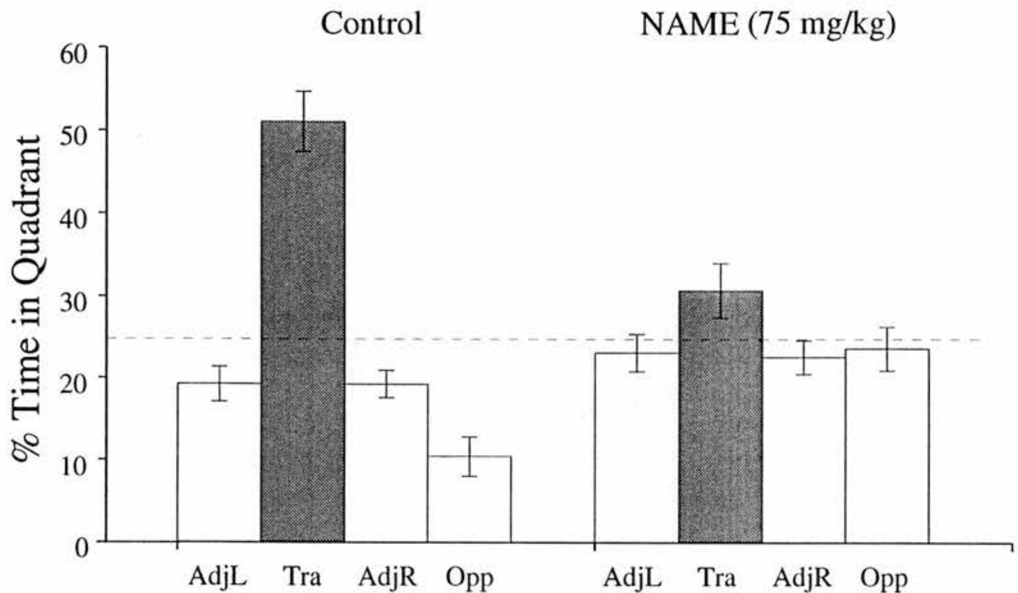


Fig. 6.1.3

The percentage time spent in each quadrant of the pool during the 60 sec transfer test (Expt 6.1). (AdjL = quadrant adjacent to, and to the left of the quadrant to which the animals were trained; Tra = the training quadrant; AdjR = quadrant adjacent to, and to the right of the training quadrant; Opp = the quadrant opposite the training quadrant).

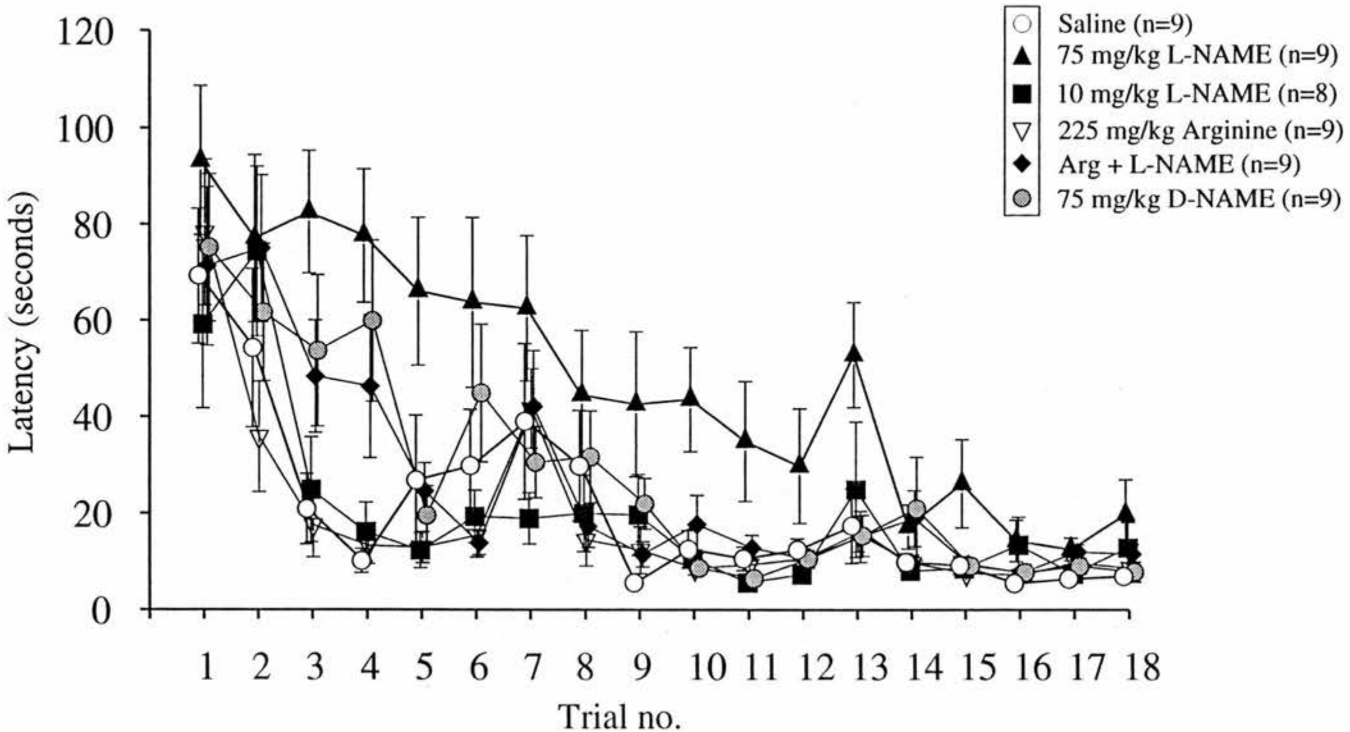


Fig 6.2.1

Mean escape latency (+/- 1 SEM) during acquisition of a standard spatial reference memory task in the watermaze (Expt 6.2).

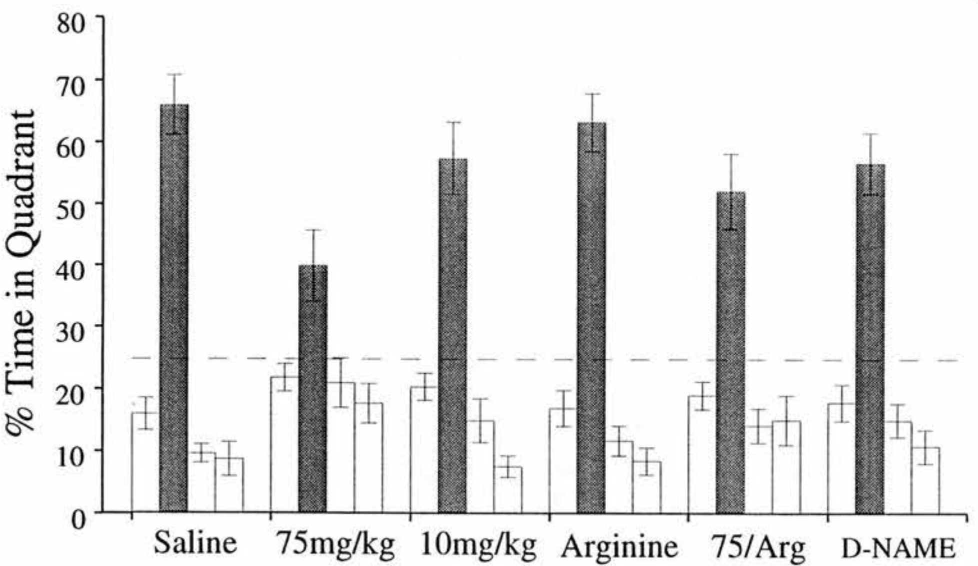


Fig. 6.2.2

The percentage time spent in each quadrant of the pool during the 60 sec transfer test (Expt 6.2). The training quadrant is shaded grey.

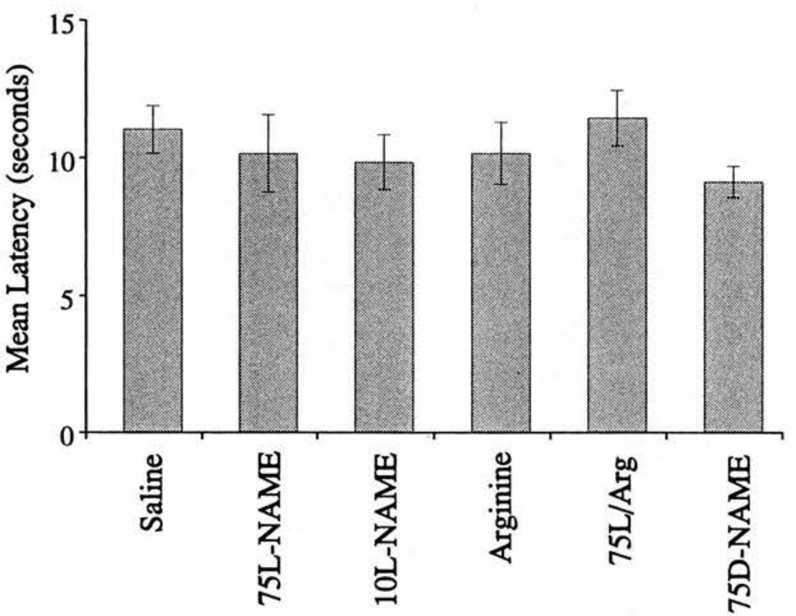


Fig 6.2.3

Mean escape latencies (of 6 trials) during the single visible platform ('cue') task (Expt 6.2).

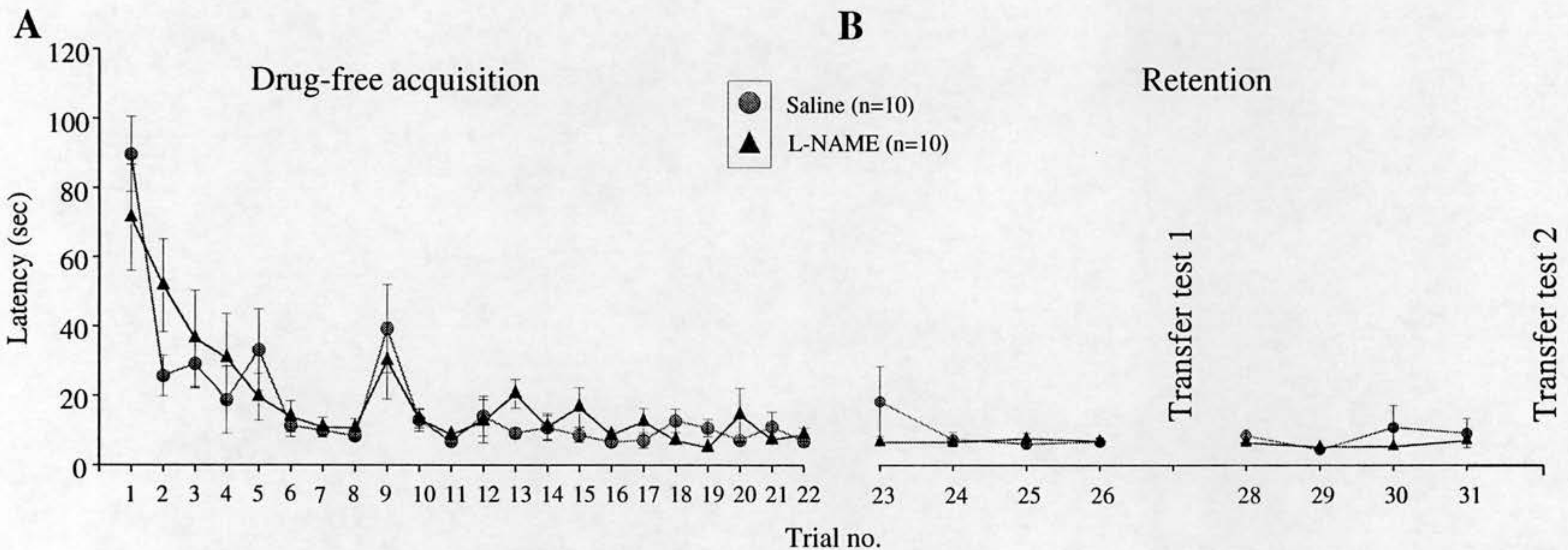


Fig. 6.3.1

L-NAME (75 mg/kg) does not affect the retention of previously learned spatial information (Expt. 6.3). A - Mean escape latency (+/- 1SEM) during the initial, drug free, spatial training. B - Mean escape latency (+/- 1SEM) during retention.

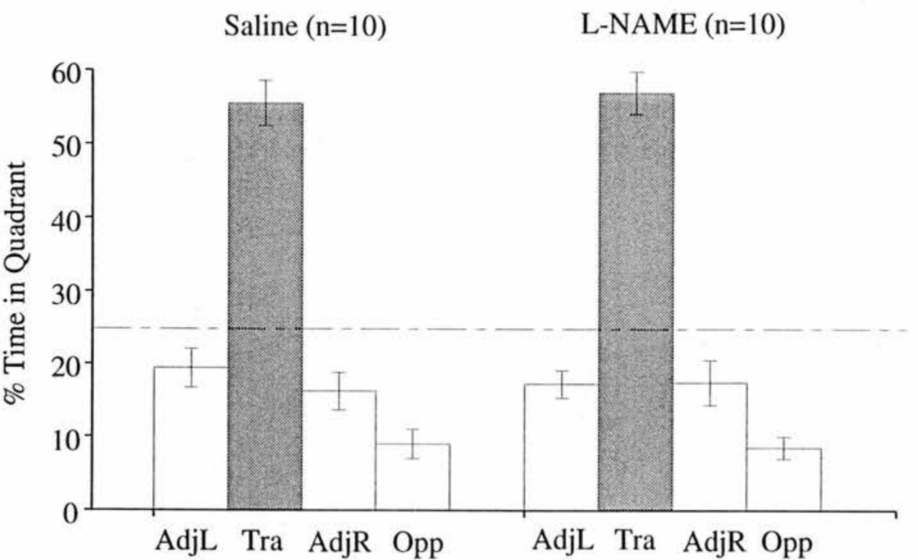


Fig. 6.3.2

The percentage time spent in each quadrant of the pool during the 60 sec transfer test after 4 retention trials (trial 27; Expt. 6.3). (AdjL = quadrant adjacent to, and to the left of the quadrant to which animals were trained; Tra = the training quadrant; AdjR = quadrant adjacent to, and to the right of the training quadrant; Opp = the quadrant opposite the training quadrant).

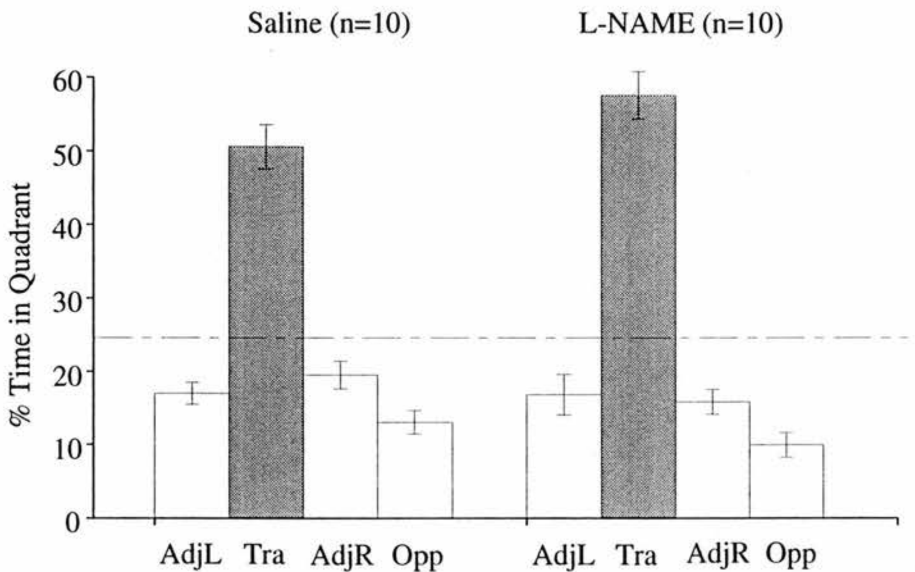


Fig. 6.3.3

The percentage time spent in each quadrant of the pool during the 60 sec transfer test after 8 retention trials (trial 32; Expt. 6.3). (AdjL = quadrant adjacent to, and to the left of the quadrant to which animals were trained; Tra = the training quadrant; AdjR = quadrant adjacent to, and to the right of the training quadrant; Opp = the quadrant opposite the training quadrant).

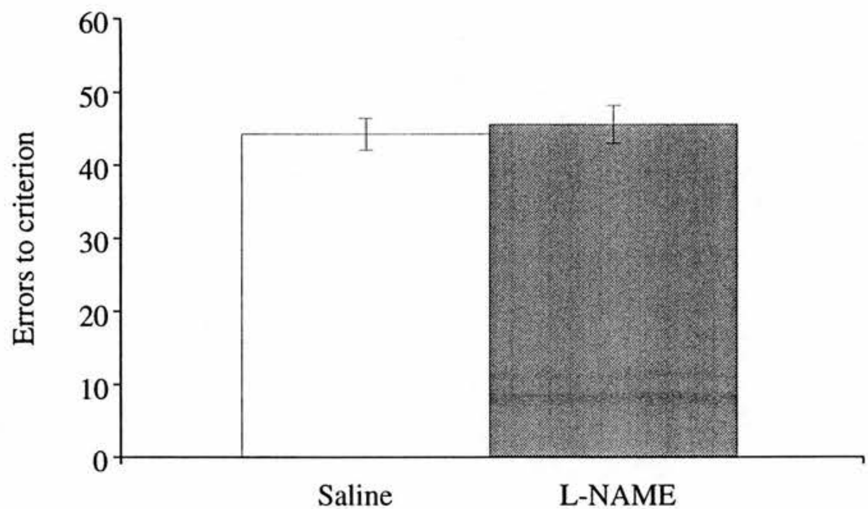


Fig 6.4.1

L-NAME (75 mg/kg) does not impair acquisition of a 2 platform, visual discrimination task in the watermaze. There is no difference between the two groups in terms of errors made in attaining a criterion of 18 out of 20 correct choices (Expt. 6.4).

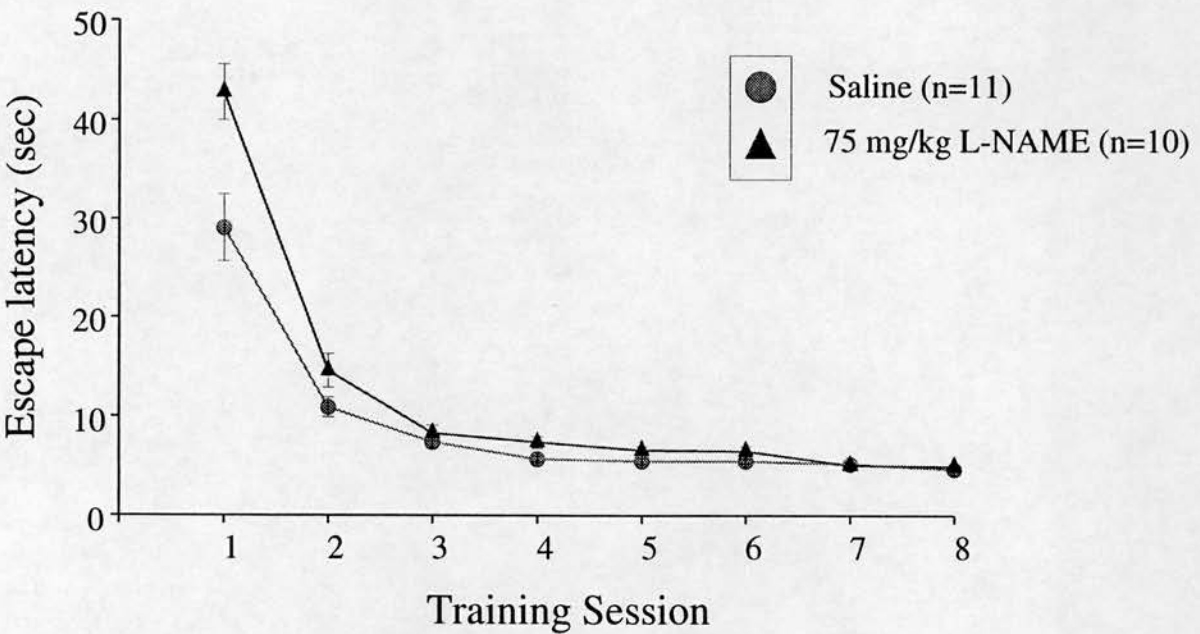


Fig. 6.4.2

Mean escape latencies during the first 8 training sessions of the visual discrimination task. L-NAME (75 mg/kg) treated rats took significantly longer to escape from the pool on the first training session.

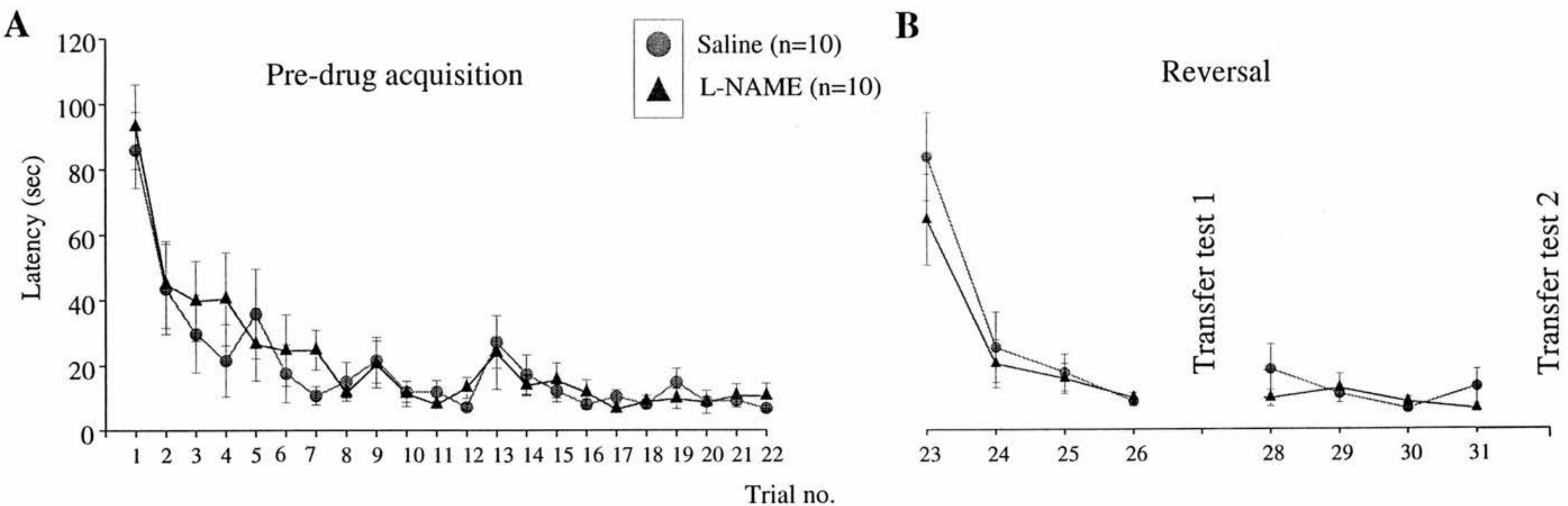


Fig. 6.5.1

L-NAME (75 mg/kg) does not impair acquisition of a novel platform location in a familiar spatial environment (reversal; Expt. 6.5). A - Mean escape latencies (+/- 1SEM) during the initial, drug free, spatial training. B - Mean escape latencies (+/- 1SEM) during reversal.

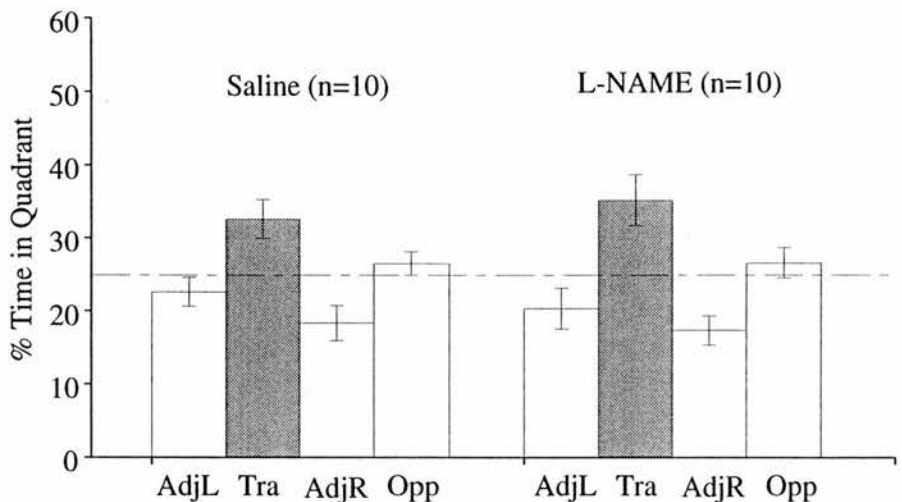


Fig. 6.5.2

The percentage time spent in each quadrant of the pool during the 60 sec transfer test after 4 reversal trials (trial 27; Expt. 6.5). (AdjL = quadrant adjacent to, and to the left of the quadrant to which animals were trained; Tra = the training quadrant; AdjR = quadrant adjacent to, and to the right of the training quadrant; Opp = the quadrant opposite the training quadrant).

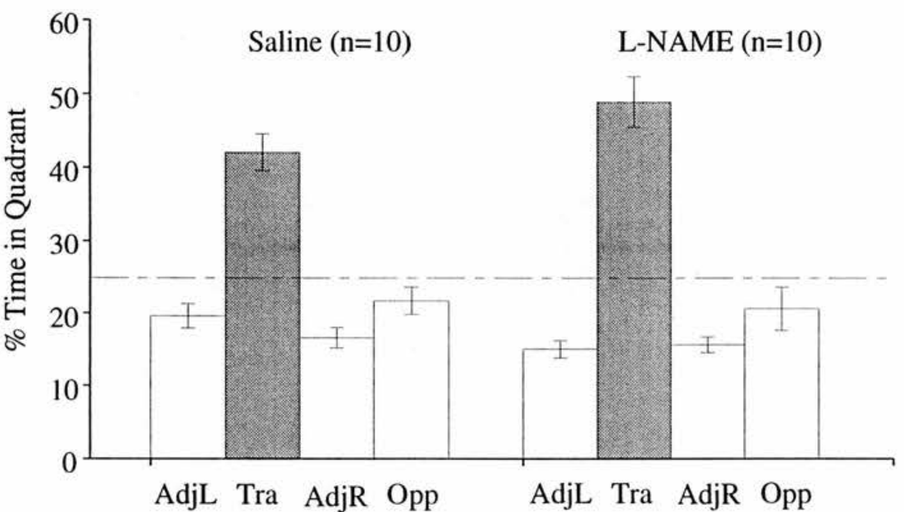


Fig. 6.5.3

The percentage time spent in each quadrant of the pool during the 60 sec transfer test after 8 reversal trials (trial 32; Expt. 6.5). (AdjL = quadrant adjacent to, and to the left of the quadrant to which animals were trained; Tra = the training quadrant; AdjR = quadrant adjacent to, and to the right of the training quadrant; Opp = the quadrant opposite the training quadrant).

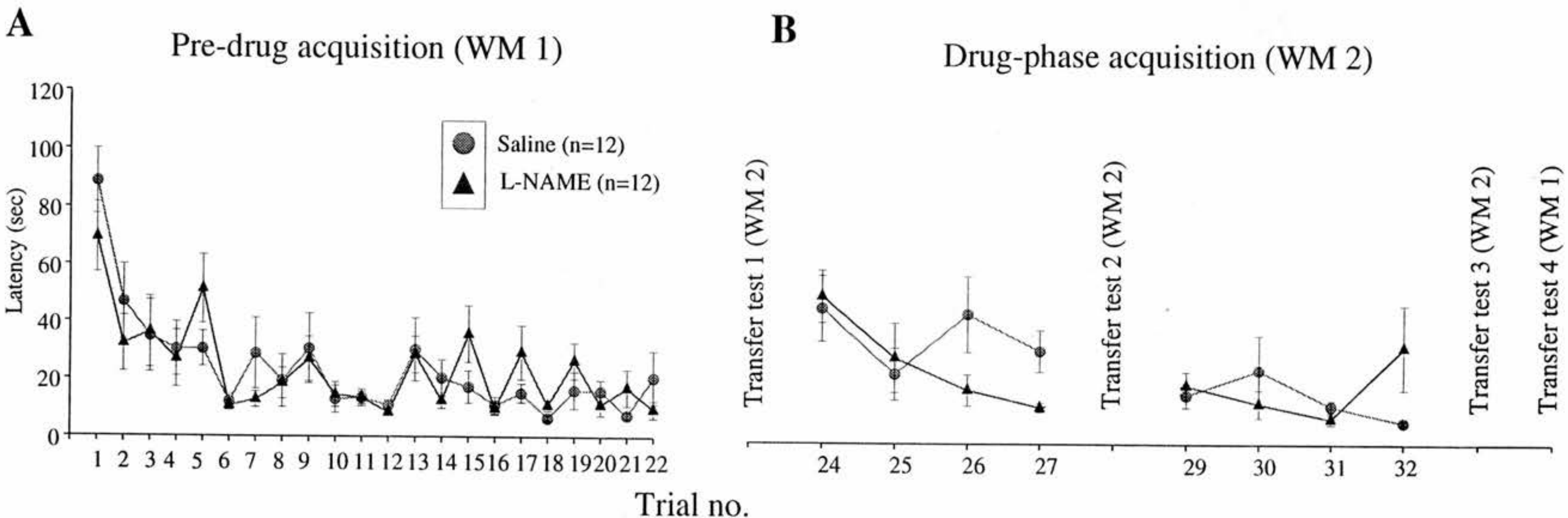


Fig. 6.6.1

L-NAME (75 mg/kg) does not impair spatial learning in a novel environment in experienced animals previously trained in another watermaze. A - Mean escape latency (+/- 1SEM) during the initial, drug free, spatial training in watermaze 1. B - Mean escape latency (+/- 1SEM) during acquisition in a novel spatial environment in watermaze 2 (Expt. 6.6).

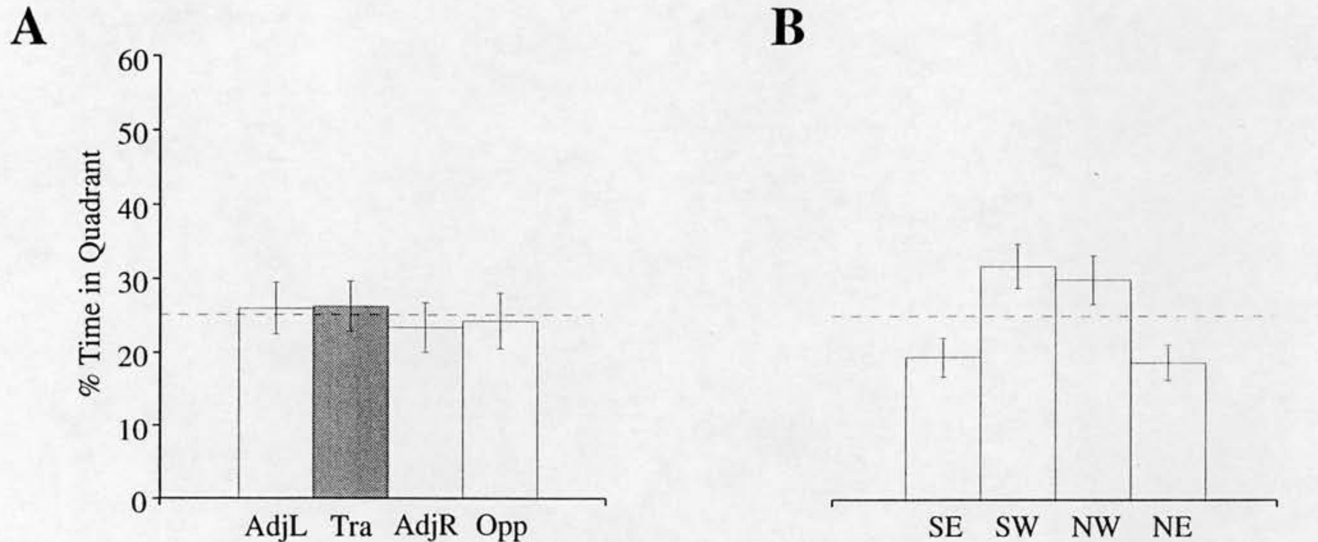


Fig. 6.6.2

The mean percentage time spent in the 4 quadrants ot the pool during the first transfer test in watermaze 2 (trial 23; Expt 6.6). A - Organised with respect to the future training quadrant (AdjL = quadrant adjacent to, and to the left of the quadrant to which the animals were trained; Tra = the training quadrant; AdjR = quadrant adjacent to, and to the right of the training quadrant; Opp = the quadrant opposite the training quadrant). B - Organised in terms of actual quadrants described by compass directions.

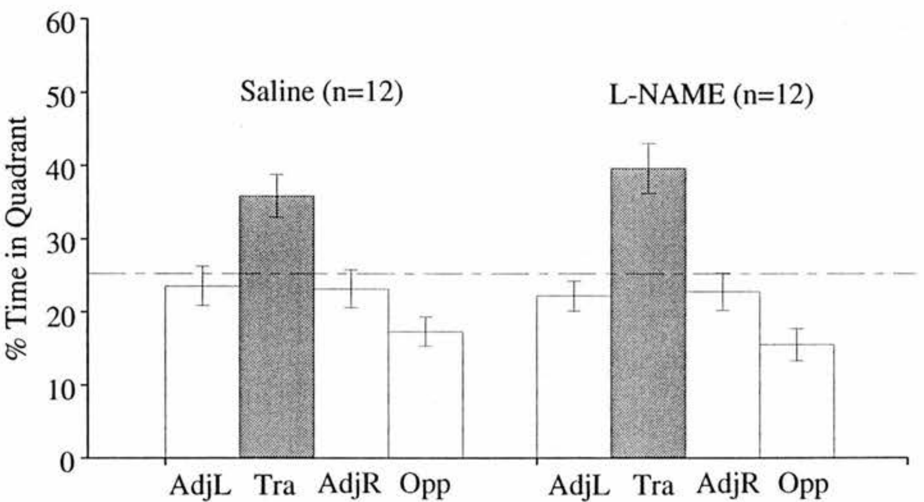


Fig. 6.6.3

The percentage time spent in each quadrant of the pool during the 60 sec transfer test after 4 trials in WM2 (trial 28; Expt. 6.6). (AdjL = quadrant adjacent to, and to the left of the quadrant to which animals were trained; Tra = the training quadrant; AdjR = quadrant adjacent to, and to the right of the training quadrant; Opp = the quadrant opposite the training quadrant).

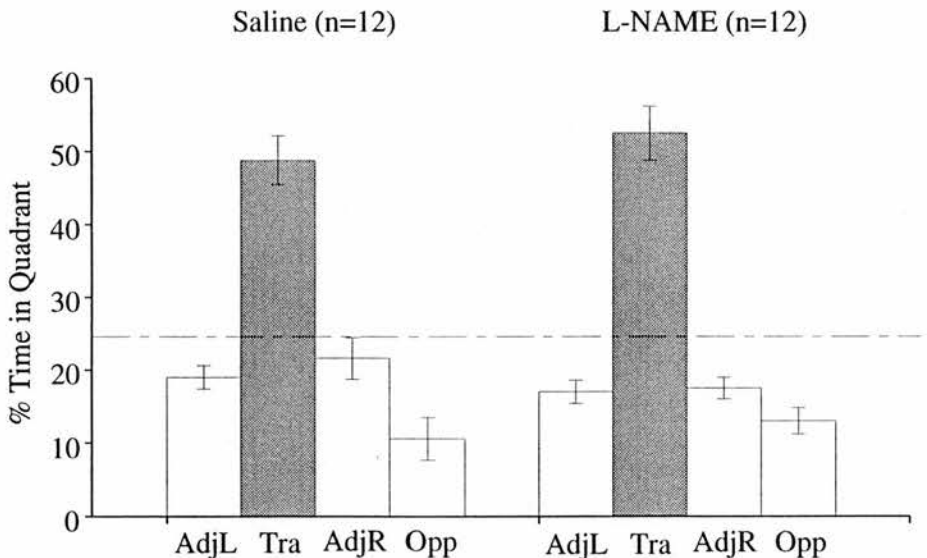


Fig. 6.6.4

The percentage time spent in each quadrant of the pool during the 60 sec transfer test after 8 trials in WM2 (trial 33; Expt. 6.6). (AdjL = quadrant adjacent to, and to the left of the quadrant to which animals were trained; Tra = the training quadrant; AdjR = quadrant adjacent to, and to the right of the training quadrant; Opp = the quadrant opposite the training quadrant).

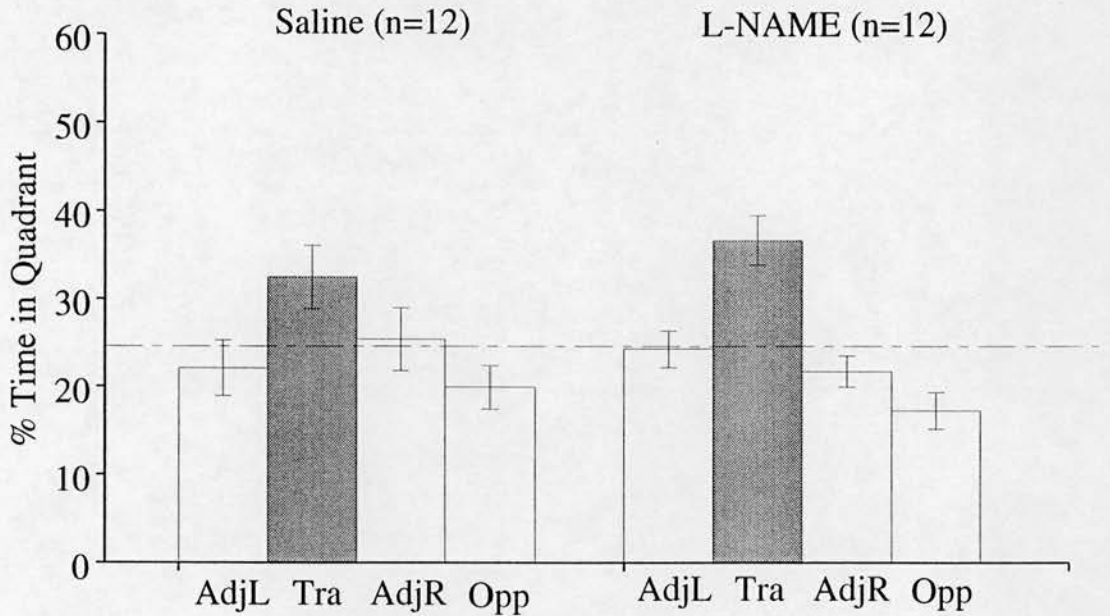


Fig. 6.6.5

The percentage time spent in each quadrant of the pool during the 60 sec transfer test when returned to WM1 (trial 34; Expt. 6.6). (AdjL = quadrant adjacent to, and to the left of the quadrant to which animals were trained; Tra = the training quadrant; AdjR = quadrant adjacent to, and to the right of the training quadrant; Opp = the quadrant opposite the training quadrant).

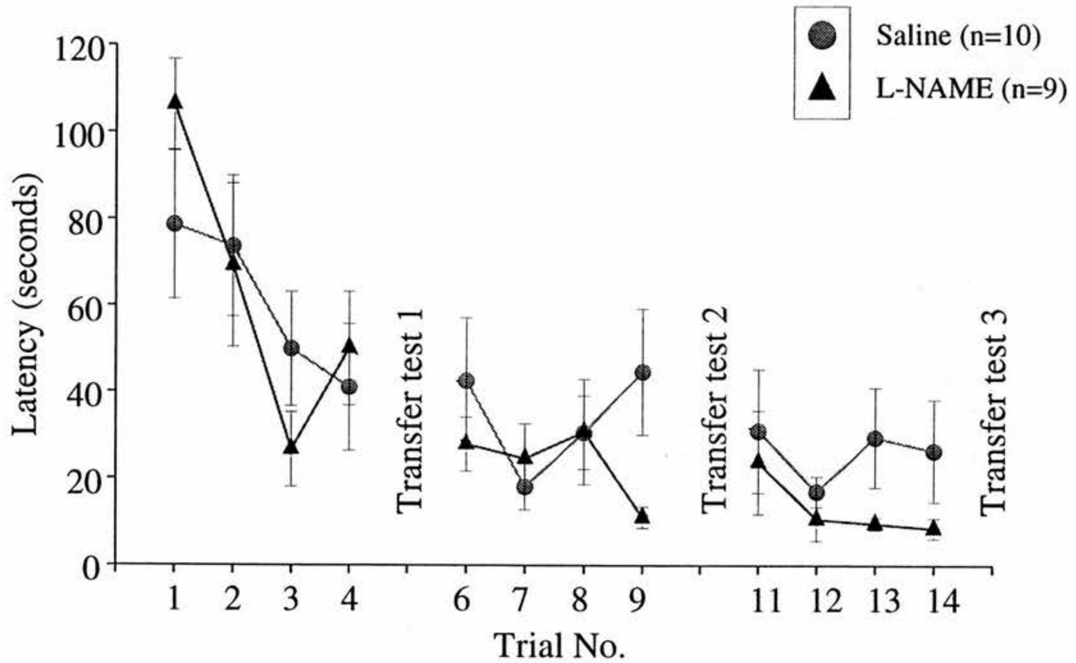


Fig. 6.7.1

Mean escape latency (+/- 1SEM) during acquisition of a standard spatial reference memory task in the watermaze for animals trained with 1 trial/ day (Expt 6.7).

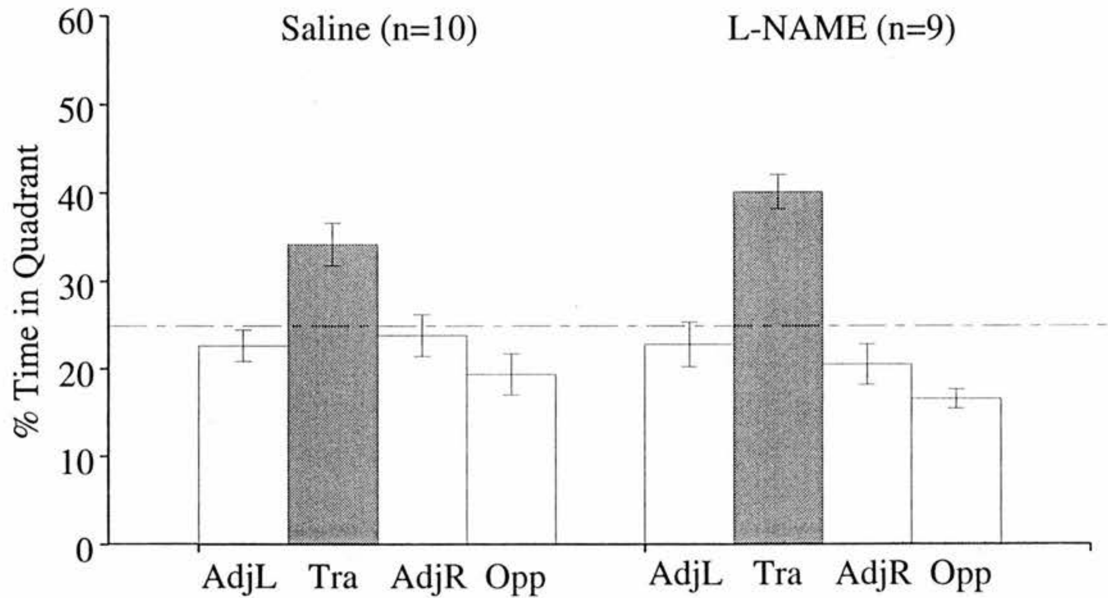


Fig 6.7.2

The percentage time spent in each quadrant of the pool during the 60 sec transfer test, conducted after 4 training trials (trial 5; Expt. 6.7). (AdjL = quadrant adjacent to, and to the left of the quadrant to which animals were trained; Tra = the training quadrant; AdjR = quadrant adjacent to, and to the right of the training quadrant; Opp = the quadrant opposite the training quadrant).

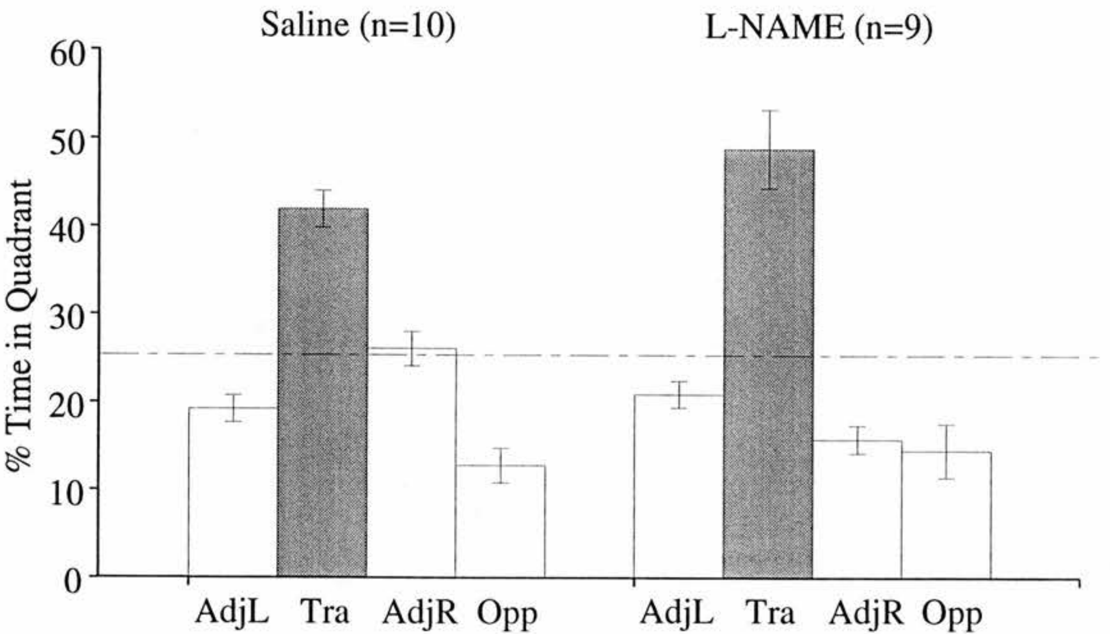


Fig 6.7.3

The percentage time spent in each quadrant of the pool during the 60 sec transfer test, conducted after 8 training trials (trial 10; Expt. 6.7). (AdjL = quadrant adjacent to, and to the left of the quadrant to which animals were trained; Tra = the training quadrant; AdjR = quadrant adjacent to, and to the right of the training quadrant; Opp = the quadrant opposite the training quadrant).

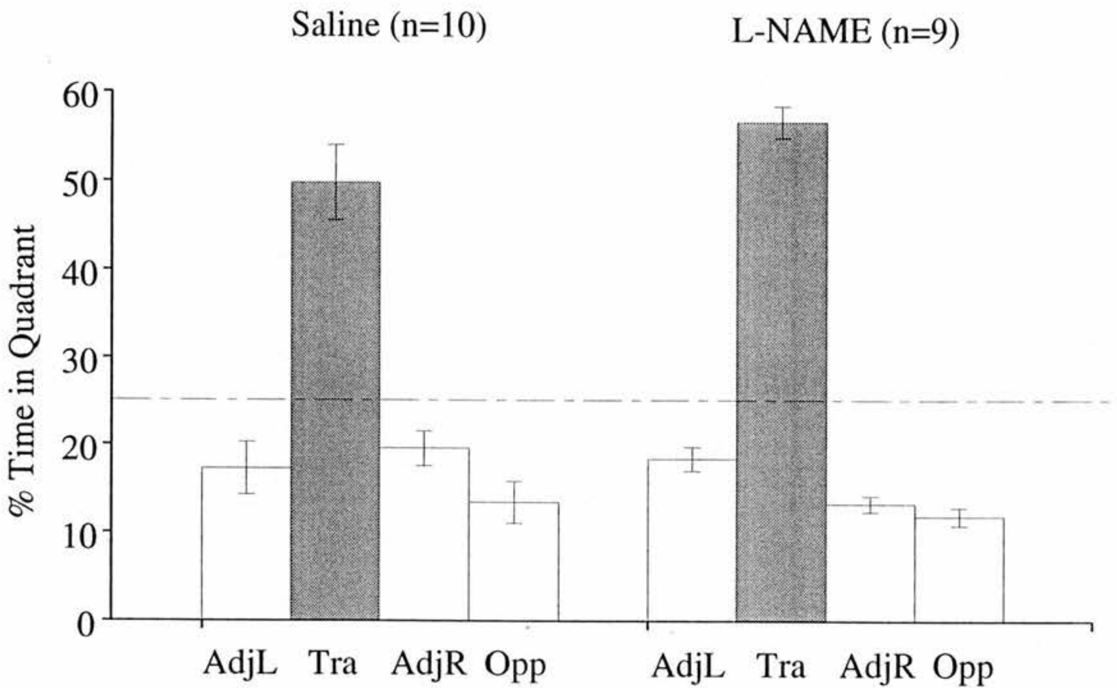


Fig 6.7.4

The percentage time spent in each quadrant of the pool during the 60 sec transfer test, conducted after 12 training trials (trial 15; Expt. 6.7). (AdjL = quadrant adjacent to, and to the left of the quadrant to which animals were trained; Tra = the training quadrant; AdjR = quadrant adjacent to, and to the right of the training quadrant; Opp = the quadrant opposite the training quadrant).

Chapter 7- A further investigation of the effects of D-AP5 on spatial learning and LTP *in vivo*.

In both series of experiments described so far (Chapters 4, 5 and 6), the experimental manipulation chosen to investigate the relationship between hippocampal LTP and spatial learning has failed to result in a block of LTP *in vivo*. Although 7CK and L-NAME have both previously been shown to block LTP in hippocampal slices, the experiments described here have failed to find any effect of these drug treatments on hippocampal LTP *in vivo*. In the absence of an effect on LTP, these manipulations are of no use for investigating the relationship between hippocampal LTP and spatial learning. An alternative approach was therefore sought in which a pharmacological manipulation already known to reliably block the induction of hippocampal LTP *in vivo* was adopted but using a novel behavioural protocol in the watermaze.

As described in Chapter 2, intraventricular infusion of the competitive NMDA antagonist, AP5, prevents the induction of LTP in the dentate gyrus *in vivo* (Morris et al., 1986a; Errington et al., 1987; Morris, 1989). AP5 also impairs the acquisition of spatial learning tasks in the watermaze (Morris et al., 1986a; Morris, 1989). In an elegant and important study, Davis et al., (1992) showed that the AP5 induced spatial learning impairment occurred across a comparable dose range to the inhibition of hippocampal LTP *in vivo*. These AP5 studies have provided possibly the most important piece of evidence in support of the hippocampal LTP/spatial learning hypothesis.

This work is not without its share of criticism, however, and is far from conclusive proof that LTP-like mechanisms are engaged during spatial learning (Keith and Rudy, 1990). As mentioned in Chapter 2, three key issues remain to be addressed. The first question that arises concerns the nature of the behavioural impairment resulting from AP5 infusion. It remains to be determined, with any degree of certainty, whether the AP5-induced watermaze deficit is due to a direct action on the neural mechanisms of spatial learning or whether the drug is affecting some other sensorimotor or motivational process which also contributes to the overall level of performance. Second, it is not clear whether the behavioural deficit is due to an effect of AP5 in hippocampus or whether the drug is acting in some other area of the brain. Third, even if AP5 is causing a selective spatial learning impairment through an action in the hippocampus, the possibility remains that the drug is interfering with hippocampal physiology by some means other than a block of LTP-like processes. Each issue merits careful consideration and will be discussed in turn.

Learning versus Performance

The first key issue is to determine whether AP5 is specifically affecting spatial learning or whether the drug is interfering with some other behavioural process which may also indirectly affect the rate of learning in the watermaze. Performance on complex learning tasks, such as spatial learning in the watermaze, can be disrupted in a number of different ways which are independent of the learning process *per se*. A drug-induced learning impairment may be the result of an effect on sensory, motor or motivational processes, all of which will contribute to the overall level of performance. Distinguishing between 'learning' and 'performance' effects is notoriously difficult and is a potential confound for all pharmacological studies of learning and memory.

The intraventricular infusion of AP5 results in the widespread distribution of the drug across the whole brain (Butcher et al., 1991; Davis et al., 1992). Bearing in mind the similar widespread distribution of NMDA receptors (Monaghan and Cotman, 1985), intraventricular infusion of AP5 is likely to have a number of effects, in addition to any action on synaptic plasticity in the hippocampus. Not surprisingly, in view of this global distribution of NMDA receptors, there is considerable evidence suggesting that NMDA receptor antagonists, including AP5, may affect both sensorimotor and motivational processes.

For example, it is possible that AP5 may interfere with the accumulation of sensory information, or with the subsequent relaying of that information to higher centres in the brain. An extremely important aspect of spatial learning in the watermaze concerns the rats' ability to see clearly the distal extramaze cues, and to use these cues to navigate to the platform. This of course requires an intact visual system. A particular concern, therefore, is that AP5 may interfere in some way with the visual system and thus impair performance. Indeed, NMDA receptors in the lateral geniculate nucleus (LGN) and visual cortex have been implicated in the normal throughput of visual information (Fox et al., 1989; Daw et al., 1990; Sillito et al., 1990). There is also evidence that NMDA receptors contribute to synaptic transmission in the ventrobasal thalamus in response to stimulation of somatosensory afferent fibres, adding further weight to the suggestion that these receptors may be involved in the relaying of certain types of sensory information to the brain (Salt, 1986; 1987; Eaton and Salt, 1989).

NMDA receptors are also likely to play an important role in synaptic transmission in the spinal cord where they are thought to play an important role in both sensory and locomotor processes. Intrathecal injections of AP5 into the spinal cord produce an analgesia to various

painful stimuli and, in addition, cause a locomotor paralysis of the hindquarters (Cahusac et al., 1984). NMDA receptor antagonists have also been shown to depress polysynaptic transmission in the spinal cord following activation of limb nerves (Davies and Watkins, 1982, 1983). It would appear, therefore, that NMDA receptors are involved in neurotransmission in the spinal cord, contributing to the control of movement, posture and nociception.

Furthermore, there is considerable evidence to suggest that NMDA receptors may be involved in the mechanisms of motor control, not only in the spinal cord but also in higher centres (Dale and Roberts, 1984; Klockgether et al., 1986; Dale, 1989). Administration of both competitive and non-competitive NMDA antagonists can cause ataxia (Contreras et al., 1986; Compton et al., 1987; Koek et al., 1987; Contreras et al., 1988; Leung and Desborough, 1988; Mondadori et al., 1989; Tricklebank et al., 1989). This is commonly characterised by a slowing of the righting reflex, an abnormal gait, and a frequent loss of balance which often results in the rat falling over. Indeed, Morris himself reports that during watermaze training there is evidence of a AP5-induced motor dysfunction (Morris et al., 1986a; Morris, 1989). The AP5 treated rats occasionally fell off the platform when attempting the 'wet dog shake' and often displayed a degree of flaccidity. This lack of muscle tone is in agreement with previous studies which have shown that competitive NMDA antagonists, including AP5, cause muscle relaxation (Turski et al., 1985; Turski et al., 1987).

The tendency for AP5-infused animals to fall off the platform early in training might have important consequences for the rate of learning in the watermaze. The inability of these rats to use the platform as an effective refuge may reduce the motivation of these animals to find the platform, and could, in turn, result in them searching for another means of escape from the water. In an attempt to reduce the motor side effects of AP5 treatment, Morris gave rats extensive non-spatial pre-training before drug infusion and the subsequent assessment of spatial learning (Morris, 1989). Non-spatial pre-training ameliorated the AP5-induced motor impairment, reducing the number of times these rats fell off the platform. In contrast, non-spatial pre-training had no significant effect on the magnitude of the spatial learning impairment. This result goes some way to arguing against the possibility that the observed spatial learning impairment is due to a drug-induced motor dysfunction. However, a reduction in the number of times the rats fall off the platform does not rule out the possibility that a more subtle motor system impairment still remains in these animals which could

account for the spatial learning deficit.

In addition to any indirect effect on motivation via a reduction in the reward value of the platform, AP5 infusion may also have a direct effect on the motivational state of the animal. There are indications that chronic intraventricular infusion of AP5 may cause weight loss which in some instances may be indicative of a change in the motivational state of the animal (Tonkiss et al., 1988; Davis et al., 1992). It is not clear, however, how this relates to performance in the watermaze. The observation that NMDA receptor antagonists have anxiolytic properties may be more pertinent with regards to the these experiments (Clineschmidt et al., 1982; Bennet and Amrich, 1986; Dunn et al., 1989). It has been suggested that these anxiolytic effects of AP5 could result in a reduction in incentive to escape from the water, relative to control animals (Keith and Rudy, 1990). The fact that benzodiazepines produce a similar behavioural impairment in the watermaze to that obtained with AP5 has been cited by Keith and Rudy as evidence in favour of this hypothesis (N. McNaughton and Morris, 1987), although the deficit with benzodiazepines could equally well be due to other effects of these compounds (eg. sedative effects).

It is also worth noting that both competitive and non-competitive NMDA antagonists, including AP5, produce stereotypical behaviour at higher doses (Compton et al., 1987; Koek et al., 1987; Contreras et al., 1988; Tricklebank et al., 1989). The stereotypy is characterised by typical repetitive, non-directional movement patterns with excessive sniffing, grooming and head weaving. Stereotypical behaviour occurs at relatively lower doses with non-competitive antagonists such as MK-801 than with competitive antagonists, such as AP5. It is not clear to what extent the psychomimetic effects and other abnormal behavioural traits resulting from the administration of non-competitive NMDA antagonists, such as PCP and MK-801, are mediated by NMDA receptors, and whether or not higher doses of competitive antagonists would result in a similar behavioural profile. There do appear to be subtle qualitative differences between the behavioural profiles of competitive and non-competitive NMDA antagonists, over and above any differences in relative potency. For example, whereas non-competitive antagonists increase locomotor activity (Koek et al., 1987; Tricklebank et al., 1989; Kretschmer et al., 1992), competitive antagonists, including AP5, reduce locomotor activity and can cause a mild sedation (Leung and Desborough, 1988; Kretschmer et al., 1992). This may be in part due to the muscle relaxant properties of these compounds (Turski et al., 1985; Turski et al., 1987).

What is clear, however, is that at high doses of both competitive and non-competitive

antagonists, the animals exhibit a gross sensorimotor impairment and become untestable on behavioural paradigms assessing learning and memory (Mondadori et al., 1989; Keith and Rudy, 1990; Davis et al., 1992). With AP5, the gross sensorimotor disturbances appear to occur at doses well in excess of those required to block LTP. Nevertheless, the concern is that these non-associative behavioural effects are still present in a more subtle form at doses which are used to assess learning, and contribute to the behavioural deficit in the watermaze.

In view of these potential non-associative side effects that might arise from AP5 administration, one must be cautious when interpreting the results of learning and memory experiments. Great efforts have been made in this laboratory in an attempt to determine whether AP5 impairs spatial learning through a selective effect on the mechanisms of learning, or whether the behavioural deficit can be attributed to actions of the drug on sensorimotor or motivational processes which also contribute to the level of performance. Clearly there are sensorimotor and motivational changes as a result of AP5 treatment (eg. muscle flaccidity, falling off the platform, slowed righting reflex) which, potentially, could account for the deficits in the watermaze. What is not clear is the extent to which these non-associative 'performance' effects contribute, if at all, to the spatial learning impairment. For example, as previously mentioned, extensive non-spatial pre-training greatly reduced the frequency with which AP5 infused rats fell off the escape platform but did not significantly affect the magnitude of the spatial learning impairment (Morris, 1989). Nevertheless, the possibility remains that the sensorimotor dysfunction is still present after non-spatial training but in a more subtle form, and may still contribute to the behavioural deficit.

The effects of any drug on learning are only meaningful if, at the same time, one can demonstrate some degree of behavioural selectivity in their action. If one is to claim that the AP5 impairment is due to a selective action on the learning mechanism, it is essential to demonstrate that the drug is without effect on a control task in which the procedural demands are similar to those of the main study, ie. a task in which both the sensorimotor and motivational demands are equated. Common practice with the watermaze is to use a visual discrimination task as a control task for spatial learning.

Morris et al., (1986a) showed that AP5 treated rats were unimpaired on the acquisition of a visual discrimination task in the watermaze. This suggests that these animals are capable of swimming relatively normally, of climbing onto the platform, and of discriminating between the visual appearance of the two platforms. This is an important result and goes some way to arguing against the possibility that the spatial learning impairment is due to a gross

sensorimotor or motivational effect of the drug treatment (eg. impaired visual acuity).

There are, however, a number of problems with using this visual discrimination task as the sole control task for spatial learning in the watermaze. For example, different measures of performance are used to assess learning in the two tasks. In the visual discrimination task choice accuracy and errors to criterion are used to assess performance, whereas during the spatial task escape latencies are recorded during training and transfer tests conducted to assess the extent of any learning. The results of Chapter 6 have already demonstrated (Expts 6.2 and 6.3) that comparing different measures of performance can lead to problems when interpreting results.

In addition, there may be crucial differences in terms of the sensory and motivational demands of the two tasks. For example, in the visual discrimination task the rats can see the platform and hence a means of escape, whereas in the spatial task the platform is hidden below the water surface. Whether or not the rat can see the platform may have important consequences with regard to the motivational state of the animal. It is also likely that the level of visual acuity required in the two tasks is significantly different. The visual discrimination task requires that the animals differentiate between two distinctive platforms which are located just a few feet across the pool from their starting position. Furthermore, the rats are able to swim quite near to the two platforms in order to have a closer look before making a deliberate choice. In contrast, during the spatial task the rat must attend to distant cues in order to find the platform and this may require a higher level of visual acuity. It is possible that AP5 causes a subtle visual impairment which allows the animal to differentiate between two distinctive platforms which are relatively close but, at the same time, results in a partial blindness with respect to more distal cues. As Morris suggests, it is possible that a more subtle visual discrimination between stripes of differing spatial frequency, or of lower contrast discriminability, may reveal an AP5-induced deficit in visual acuity (Morris, 1990). Indeed, the competitive NMDA antagonists AP7 and CPP have been found to impair brightness discrimination in the rat (Tang and Ho, 1988). The possibility remains, therefore, that AP5 is producing a subtle visual impairment which may be detected using a more sophisticated visual discrimination task, and which could account for the behavioural impairment during spatial learning.

It would appear, therefore, that the visual discrimination task, alone, does not represent an adequate control task for spatial learning in the watermaze, and that, despite the behavioural dissociation between the effects of AP5 on spatial learning and visual discrimination

learning, an explanation based on non-specific side effects of AP5 cannot be ruled out. However, the subsequent demonstration that AP5 also has no effect on the retrieval of previously acquired spatial information may have important implications for the 'learning versus performance' debate (Morris, 1989).

Morris showed that rats trained to find a fixed location, hidden escape platform in the absence of AP5, and then subsequently tested on the same platform location in the presence of the drug, do not show a retrieval deficit (Morris, 1989). This result indicates that LTP-like processes are not required for the retrieval of spatial information. On its own, however, the lack of a retrieval deficit following extensive spatial training in the absence of any drug treatment is open to alternative interpretations. For example, this null result may reflect the fact that extensive spatial training in the watermaze, prior to drug treatment, negates the effects of AP5 on procedural, non-spatial aspects of performance, and that an AP5-induced deficit is only seen in relatively naive animals, unfamiliar with the watermaze. In a key experiment (Morris et al., 1990a), Morris trained rats, as normal animals in the absence of any drug treatment, to find a fixed location, hidden escape platform. The animals were then divided into four groups. Two groups were implanted with minipumps infusing 30 mM D-AP5 (a dose sufficient to block LTP) and the other two groups were implanted with minipumps delivering aCSF. Two groups, one from each treatment condition, were then trained to the same platform location, to test for retention of spatial information. The other two groups were trained to a novel platform location in the opposite quadrant of the pool (a type of 'reversal' which presumably involves some new learning). Both the AP5 and aCSF treated rats showed effective retrieval and retention of the original platform location and there was no significant difference between the two groups. In contrast, the AP5 infused animals were impaired on learning the new platform location (the 'reversal' task). Taken together, these results suggest that NMDA receptor activation is required for the initial storage of new information, but that these receptors are not recruited during the retrieval of previously acquired spatial information. Furthermore, these results supply a parallel to observations from electrophysiological studies showing that NMDA receptors are required for the induction, but not the expression, of LTP (Davies and Collingridge, 1989). The deficit in performance exhibited by the AP5-treated animals when the platform was moved to a novel location in the pool demonstrates that new learning is still impaired in watermaze-experienced animals and suggests that the sparing of retrieval is not simply due to prior watermaze training.

Furthermore, these results make an important contribution to the 'learning versus performance' debate. Under conditions where sensorimotor and motivational demands appear to be equated (ie. 'same' versus 'different' platform location), AP5 impairs new learning but spares retrieval. One might argue that if the AP5-induced watermaze impairment is due to non-associative side effects of the drug, then performance on the retention task ('same' platform position) should be equally affected. The successful retrieval displayed by AP5 treated animals suggests that there is no gross sensorimotor or motivational deficit in these animals, and that these animals are able to see, and effectively make use of, distal cues in order to find the hidden platform. Although not conclusive, this result goes some way towards arguing against a sensorimotor or motivational account of the AP5-induced behavioural deficit during spatial learning.

The dissociation between the AP5/same and AP5/different groups does not completely rule out the possibility that a subtle visual impairment may be responsible for any spatial learning deficit. It may be that during the retrieval of a familiar platform location, the spatial layout of the relevant distal cues is already well known to the animal and, therefore, a slight disturbance of the visual system (eg. partial blindness) may not seriously impair performance. In contrast, when trying to learn a new platform position, the rat needs to learn about the spatial relationships between a group of different distal cues, and it is not inconceivable that a subtle visual impairment may indeed hinder this new learning in a less familiar part of the environment. An analogy may be drawn with our own experience. It is easy, for example, to find the light switch in a poorly lit room if one is familiar with the layout of that room (eg. your own bedroom). In contrast, it is much harder to find one's way around in near darkness in a strange and unfamiliar environment (eg. a hotel bedroom). Nevertheless, the findings of the retention/reversal experiment make an important contribution to the 'learning versus performance' debate, and may go some way towards eliminating a sensorimotor or motivational account of the AP5-induced behavioural deficit during spatial learning.

Hippocampal versus non-hippocampal effects of AP5

Irrespective of whether the AP5-induced behavioural deficit is due to an effect on learning or due to an effect on performance, an independent but related question remains as to the site of action of the drug. As previously mentioned, intraventricular infusion of AP5 results in a widespread distribution of the drug throughout the forebrain (Butcher et al., 1991; Davis et

al., 1992). A similarly global distribution of NMDA receptors suggests that AP5 could be acting at any one of several potential sites (Monaghan and Cotman, 1985). It is possible, for example, that AP5 infusion is indeed causing a selective impairment of spatial learning but rather than due to a blockade of LTP-like events in hippocampus, the deficit is the result of NMDA receptor blockade in some other area of the brain (eg. prefrontal, parietal or cingulate cortex). Only limited support for a hippocampal site of action may be derived from the significant correlation between whole tissue drug levels in this brain area and learning ability, because, this may simply reflect a more general correlation between whole tissue levels of the drug throughout the brain and performance.

In an attempt to address this issue, Morris and colleagues infused AP5 directly into the hippocampus (Morris et al., 1989). Acute injections of the drug or vehicle solution were given 15 min prior to behavioural testing on each day of training. The rats were trained to find a fixed location, hidden escape platform according to a standard spatial reference memory procedure (3 days/8 trials per day), and their memory of the platform location assessed by means of a transfer test immediately after the final training trial. The infusion of 2 μl of 50 mM D,L-AP5 (100 nmol) impaired performance both in terms of escape latencies during training and on the transfer test. The same infusion protocol also prevented the induction of LTP in the dentate gyrus *in vivo*. Using radiolabelled [^{3}H] D,L-AP4 or [^{3}H] D,L-AP7 (which are similar but not identical to AP5), Morris and colleagues showed that with this direct intrahippocampal infusion (as used in the behavioural and electrophysiological parts of the study), the diffusion of the drug was reasonably well confined to within the hippocampus. On the basis of this result the authors concluded that the D-AP5-induced spatial learning impairment is indeed due to the blockade of NMDA receptors in the hippocampus and that this occurs at comparable doses to those required to block LTP *in vivo*.

Several points should be made, however, regarding these local microinfusion studies. First, although similar, AP4 and AP7 may behave differently from AP5 in terms of their diffusion from the injection site. Second, although the density of the radiolabel fell off sharply at the boundaries of the hippocampus, significant amounts of the drug did diffuse outside the hippocampus. Indeed, less than 50% of the total radiolabel infused was found within the hippocampus. It is not clear, therefore, whether or not the drug diffusion is truly restricted to within the hippocampus. Third, it remains to be established whether local microinfusion of AP5 into the hippocampus impairs performance through a selective effect on learning or

whether the AP5-induced deficit can be attributed to a sensorimotor or motivational effect of the drug treatment. No control task was run in this study to determine the behavioural specificity of this manipulation. Somewhat surprisingly, there was still some evidence of a sensorimotor dysfunction in the AP5 treated animals on early training trials, with rats often displaying difficulty in remaining on the escape platform. It is not evident whether this is due to an effect of the drug in hippocampus or in some other brain area. If the drug diffusion is truly restricted to the hippocampus following a local microinfusion, then this would suggest that blockade of NMDA receptors in the hippocampus may have effects on sensorimotor systems, although it is not clear how this could be rationalised in view of the absence of any sensorimotor impairment in hippocampal lesioned animals (Morris et al., 1982). The alternative is that there is indeed some diffusion of the drug out of the hippocampus into areas of the brain that can influence other aspects of performance. Nevertheless, the demonstration that intrahippocampal infusion of the drug also disrupts performance in the watermaze is consistent with the hypothesis that D-AP5 is acting on hippocampal processes to impair spatial learning.

LTP versus other aspects of hippocampal physiology

From the studies presented so far, there is some evidence, although not conclusive, to suggest that the spatial learning deficit resulting from AP5 infusion may be a specific spatial learning impairment, and may be the result of a blockade of NMDA receptors in the hippocampus. The possibility remains, however, that AP5 is exerting its behavioural effects through a hippocampal dependent mechanism other than a blockade of LTP. There is evidence that NMDA receptor antagonists can influence other aspects of hippocampal physiology. For example, AP5 causes a decrease in hippocampal theta (Leung and Desborough, 1988). The importance of hippocampal theta for spatial learning has been well documented (Winson, 1978; O'Keefe and Nadel, 1978). It follows, therefore, that the observed spatial learning impairment could be due to a disruption of hippocampal theta and not to a blockade of LTP-like processes. In defence of an LTP account, the effects of AP5 on hippocampal theta are only seen at higher doses and are accompanied by pronounced sensorimotor side effects, including severe ataxia and walking difficulties. This would suggest that the effect on theta probably occurs at much higher doses than those required to see a significant spatial learning impairment and a block of hippocampal LTP. Similarly, the non-competitive NMDA antagonist, MK-801, has only a small effect on hippocampal theta

at higher doses which are also normally associated with pronounced behavioural side effects (Wishaw and Auer, 1989). It seems unlikely, therefore, that the observed behavioural impairment is due to an effect of the NMDA receptor antagonist on hippocampal theta rhythm. Nevertheless, this illustrates the interpretive difficulties associated with these studies.

AP5 has also been shown to affect low frequency neurotransmission in the hippocampus but only at very high concentrations (Hablitz and Langmoen, 1986). At more moderate concentrations, however, NMDA antagonists have been found to reduce cell excitability (Errington et al., 1987; Abraham and Mason, 1988) and to cut short normal complex-spike firing in the hippocampus (Abraham and Kairiss, 1988). There is now a growing belief that NMDA receptors play a more widespread role in 'normal' synaptic transmission and that these receptors may contribute to more than just the recruitment of LTP-like processes (Bekkers and Stevens, 1990b). This belief is derived, at least in part, from the observation that AP5 blocked responses in the thalamus evoked by natural sensory stimulation but was without effect on electrically-evoked responses (Salt, 1986). This calls into question the idea that NMDA receptors are not recruited during normal synaptic transmission and suggests that AP5 may well have more widespread effects on the system properties of the hippocampus. If this is the case, and AP5 is causing a general disruption of hippocampal activity, then one might expect a pattern of behavioural impairment similar to that obtained with a hippocampal lesion. The possibility that these other hippocampal effects of AP5 infusion are responsible for the behavioural impairment cannot be ruled out.

A need for further investigation

Experiments using the competitive NMDA antagonist, D-AP5, provide a major source of evidence in support of the hippocampal LTP/spatial learning hypothesis. AP5 impairs spatial learning in the watermaze at doses which also block hippocampal LTP in the same animals. Questions remain, however, regarding (i) the specificity of the behavioural impairment, (ii) the site of action of AP5, and (iii) whether or not the impairment is due specifically to a block of LTP-like mechanisms. The final series of experiments in this thesis is concerned, primarily, with the first of these issues, and will attempt to further investigate the nature of the AP5-induced behavioural impairment in the watermaze.

Much of the introduction to this particular chapter has been concerned with whether AP5

impairs spatial learning in the watermaze through a specific effect on learning, or whether the drug is disrupting some non-associative, procedural aspect of watermaze performance. A cornerstone of the argument favouring a specific effect on the mechanisms of spatial learning is that AP5 impairs the acquisition of a spatial learning task in the watermaze but spares both the retrieval of previously acquired spatial information and visual discrimination learning. In combination, the absence of an effect of AP5 on both the retrieval task and the visual discrimination task provides some evidence against a sensorimotor or motivational account. A key result for this argument is the demonstration that AP5 spares the retrieval and retention of a previously acquired platform location but impairs learning about a novel platform position in the same familiar environment under conditions in which the sensorimotor and motivational demands of the two tasks are equated (the retention/reversal study; Morris et al., 1990a).

Although this retention/reversal study makes an important contribution to our understanding of the relationship between hippocampal LTP and spatial learning, a number of questions remain. First, it is not clear how much new learning is actually required to solve the 'reversal' task and, second, whether or not any new learning would require hippocampal-dependent mechanisms. One might argue, for example, that during the initial drug-free spatial training, a detailed representation of the environment will already have been formed and, therefore, when the platform is moved to a different part of the pool, the only modification required of that representation is to encode a new goal location. This new learning may necessitate little, if any, synaptic plasticity, and, may or may not occur in the hippocampus. One cannot rule out the possibility that the deficit seen with D-AP5 in the reversal task is due to an action of the drug on some behavioural process other than a block of new spatial learning. Ideally, new spatial learning needs to be assessed in an entirely novel spatial environment in these watermaze-experienced animals. Furthermore, in the Morris et al. study (1990a) the effects of D-AP5 treatment on acquisition and retrieval were assessed in separate groups of animals. A more attractive experimental design would allow both the retrieval of previously acquired spatial information and new learning to be assessed within the same subjects. The two-watermaze experiment described in Chapter 6 (Expt 6.6) provides such an opportunity. In Expt 6.6, the initial drug-free watermaze training was carried out in a different spatial environment from that used during testing in the presence of the drug. With this behavioural protocol, the watermaze-experienced animals have to learn about an entirely novel spatial environment during the drug phase of the study. This is in contrast to the 'reversal' task in which the rats have only to learn that the goal location has

changed while the drug is present.

The experiments described in this chapter of the thesis involve the use of two watermazes, located in two distinct spatial environments, using the behavioural protocol previously described in Expt 6.6. Intuitively, one would expect that learning about the spatial layout of the second watermaze will require the hippocampus. Nevertheless, the first experiment described in this chapter examines the effect of lesioning the hippocampus on performance in the two-watermaze task, in order to determine whether or not new spatial learning in watermaze-experienced animals still requires the hippocampus.

Following on from this, a second study has investigated the effects of D-AP5 infusion on performance during this task. The two-watermaze design should allow both acquisition and retrieval to be tested, in the presence of D-AP5, in the same animals. Furthermore, it is possible that the extensive spatial training in the watermaze, prior to testing in the presence of the drug, may have important consequences for the magnitude of any sensorimotor or motivational side effects resulting from D-AP5 treatment. By using animals that are already well practised with the procedural elements of the task (eg. knowing that there is a means of escape via a submerged platform, climbing onto and remaining upon the platform), this design should allow a more pure assessment of the effects of AP5 on the spatial component of the watermaze task.

In order to ensure that LTP was indeed blocked in these animals, electrophysiological recordings were also made from each animal at the end of the behavioural experiment. In addition, tissue levels of D-AP5 were quantified using HPLC with fluorescence detection. Two doses of D-AP5 were used in this study. Initially, a dose of 30 mM D-AP5 was used. This dose is known to block the induction of dentate gyrus LTP *in vivo* and has previously been used in a number of watermaze studies, including the aforementioned retention/reversal study (Morris et al., 1990a). As the experiment proceeded, it became clear that the infusion of 30 mM D-AP5 was resulting in an almost complete block of LTP and yet was causing only a mild behavioural disturbance. The possibility arose, therefore, that a slightly lower dose of D-AP5 would also block LTP but have no significant effect on watermaze performance. Consequently, a group of animals infused with 25 mM D-AP5 was also included.

Experiment 7.1 - The effects of hippocampal lesions on spatial learning in a novel environment in experienced animals previously trained in another watermaze.

Procedure

The rats (n = 14) were trained according to the behavioural protocol used in Expt 6.6. Following 1 day of non-spatial pre-training (6 trials, 60 sec max.), the rats were given 10 days of spatial training in watermaze 1 (3 days of 4 trials/day, 3 days of 2 trials/day and 4 days of 1 trial/day; trials 1-22; 120 sec max., 30 sec ITI). Six animals were then given ibotenic acid lesions of the hippocampus (see Chapter 3). The rats were allowed 10 days to recover from surgery before commencing training in watermaze 2. The control group (n=8) contained both unoperated (n=4) and sham operated animals (n=4).

In watermaze 2, all rats were first given a transfer test (trial 23) in order to assess whether prior training in watermaze 1 resulted in a spatial bias in watermaze 2. Immediately after the transfer test, the rats received their first spatial training trial in watermaze 2. In total, the rats were given 8 spatial training trials in watermaze 2 (1 trial/day), with the platform located in either the NE or SW quadrants. In watermaze 1, the platform was located in one of the 4 quadrants of the pool (NE, NW ,SW or SE). For every 4 rats trained to a particular platform position in watermaze 2, each of the 4 platform positions had been used during training in watermaze 1. The assignment of animals to groups was counterbalanced with respect to platform positions in both watermazes 1 and 2 and with respect to performance over the last 4 training trials in watermaze 1 (trials 19-22). In watermaze 2, the rats were also given another 2 transfer tests, 24 hours after trial 27 (and immediately before trial 29) and 24 hours after trial 32. Finally, the rats were returned to watermaze 1 and their memory of the platform location assessed with a transfer test (trial 34). On completion of behavioural testing, the rats were perfused and their brains removed for histological analysis.

Results

All animals learned to escape rapidly from the water during training in watermaze 1. An ANOVA of escape latencies revealed a highly significant improvement across trials ($F_{(21,252)} = 4.77$; $p < 0.0001$; Figure 7.1.1A).

Analysis of the first transfer test (trial 23), before the start of spatial training in watermaze 2, revealed no spatial bias when the results were analysed in terms of the 'future' training quadrant ($F < 1$; $p > 0.20$; Figure 7.1.2). There was, however, an overall bias towards the SW quadrant of watermaze 2 which was shown by both the control and hippocampal lesioned animals (% time in SW - controls = 38.8 ± 2.3; HPC = 33.9 ± 2.1; a significant effect of quadrant, $F (2,36) = 19.26$; $p < 0.0001$; no groups by quadrants interaction, $F < 1$; $p > 0.20$; Figure 7.1.3). A similar bias was also seen in Expt 6.6 and may correspond to the location of the carrying cage.

During spatial training in watermaze 2, there was a trend for hippocampal-lesioned rats to take longer to find the platform than controls ($F (1,12) = 3.41$; $0.05 < p < 0.10$; Figure 7.1.1B). The ANOVA also revealed a significant improvement across the 8 training trials ($F (7,84) = 2.92$; $p < 0.01$) but no groups by trials interaction ($F < 1$; $p > 0.20$).

After 4 training trials in watermaze 2, the control rats were spending significantly more time in the training quadrant compared with hippocampal lesioned animals (Figure 7.1.4). An ANOVA of the transfer test conducted after 4 trials (trial 28) revealed a trend towards an effect of quadrant ($F (2,36) = 2.65$; $0.05 < p < 0.10$) and a significant groups by quadrants interaction ($F (2,36) = 3.76$; $p < 0.05$). A subsequent comparison of the amount of time spent in the training quadrant only, showed that the controls were indeed spending more time searching there than the HPC-lesioned group (controls = 42.7 ± 5.8%; HPC = 22.6 ± 4.3%: $t (12) = 2.59$; $p < 0.05$).

A similar result was obtained in the transfer test after 8 training trials in watermaze 2 (Figure 7.1.5). An ANOVA revealed a significant effect of quadrant ($F (2,36) = 10.79$; $p < 0.001$) and a trend towards a groups by quadrants interaction ($F (2,36) = 3.10$; $0.05 < p < 0.10$). Comparison of the training quadrant times only, confirmed that there was indeed a stronger bias towards this quadrant in the control group (control = 55.5 ± 6.2%; HPC = 33.6 ± 6.6%; $t (12) = 2.41$; $p < 0.05$).

Finally, when the animals were returned to watermaze 1 and given a transfer test (trial 34), there was little retention, if any, of the former platform location. Analysis of the full 60 seconds of trial 34 showed no mean bias towards the former training quadrant (controls = 29.5 ± 4.1%; HPC = 25.1 ± 3.6%; no effect of quadrant $F < 1$; $p > 0.20$; Figure 7.1.6). If only the first 15 seconds of this transfer test were examined, then the controls do show a greater bias towards the former training quadrant, although there was still no significant effect of quadrant (controls = 38.7 ± 6.6%; HPC = 23.8 ± 8.1%; quadrant $F (2,36) = 1.40$; p

> 0.20; Figure 7.1.7). It is worth noting, however, that within the control group, some animals did show a considerable bias towards the former training quadrant during the 60 sec probe trial (Figure 7.1.8).

The extent of damage in hippocampal lesioned animals was assessed and used to determine whether or not an animal was accepted into the study. In order to be included, the hippocampal damage had to fulfill certain criteria which were assessed without any knowledge of the behavioural performance of that subject. Briefly, for an animal to be included in the study it was necessary for almost all of the CA1 - CA4 pyramidal cells and most of the granule cells in the dentate gyrus to have been removed with no, or little, damage to adjacent areas (see Figure 4.2.7).

Experiment 7.2 - The effects of D-AP5 on spatial learning in a novel environment in experienced animals previously trained in another watermaze in a different spatial environment.

Drugs

All drug solutions were made up in pyrogen free water. A 100 mM stock solution of D-2-amino-5-phosphopentanoate (D-AP5; Tocris Neuramin) was made up in equimolar NaOH. As with 7CK, spiking with small aliquots (1-2 μl) of 5M NaOH was sometimes required to ensure that the drug fully dissolved. This was then diluted in an appropriate volume of aCSF to give a final concentration of either 25 or 30 mM.

Procedure

As in Expts 6.6 and 7.1, a group of naive rats (n = 31) were given 1 day of non-spatial pre-training in watermaze 2 (6 trials, 60 sec max.), followed by 10 days of spatial training in watermaze 1 (3 days of 4 trials/day, 3 days of 2 trials/day and 4 days of 1 trial/day; trials 1-22; 120 sec max., 30 sec ITI). 24 hours after the last spatial training trial, the rats were anaesthetised with Avertin (0.29 g/kg) and Alzet osmotic minipumps, infusing either 25 mM D-AP5 (n = 6), 30 mM D-AP5 (n = 12) or aCSF (n = 13), were implanted into the right lateral cerebral ventricle (day 0; see Chapter 3). During surgery the positions for stimulating and recording electrodes were marked on the skull. The rats were allowed 2 days (days 1-2)

to recover from the acute effects of surgery, which also allowed intracranial drug concentrations to reach a steady state level. On day 3 after implantation, all rats were first given a transfer test in watermaze 2 (trial 23) in order to assess whether prior training in watermaze 1 resulted in a spatial bias in watermaze 2. Immediately after this transfer test, the rats received their first spatial training trial in watermaze 2. As in Expts 6.6 and 7.1, the assignment of animals to groups was counterbalanced with respect to platform positions in both watermazes 1 and 2 and performance over the last 4 training trials in watermaze 1 (trials 19-22). The rats were given 8 spatial training trials (1 trial/day) in watermaze 2, with the platform located in either the NE or SW quadrants. In watermaze 1, the platform was located in one of the 4 quadrants of the pool (NE, NW, SW or SE). For every 4 rats trained to a particular platform position in watermaze 2, each of the 4 platform positions had been used during training in watermaze 1. The rats were given a further 2 transfer tests in watermaze 2, 24 hours after trial 27 (and immediately before trial 29; day 7) and 24 hours after trial 32 (day 11). On day 12, the rats were returned to watermaze 1 and their memory of the platform location in the original training environment assessed with a final transfer test (trial 34).

Immediately following trial 34, the animals were anaesthetised with urethane and an attempt made to induce LTP. The animals were prepared as described in Chapter 3 (Electrophysiological methodology), with recording and stimulating electrodes implanted in the hippocampus contralateral to the infusion cannula. The rat was then left for an hour, without stimulating, to allow the tissue to recover from the implantation and ensure stable baseline field epsps. Following a 20 min baseline period of low frequency stimulation (0.05 Hz, 700μA biphasic pulses of 100μs half pulse duration), a high frequency tetanus (4 trains of 33 pulses at 250 Hz, intertrain interval of 20 sec, 700μA biphasic pulses of 100μs half pulse duration) was delivered in an attempt to induce LTP. Low frequency test pulses were then continued for a further hour. Immediately before and after this main series, an input/output function was recorded.

The rats were then sacrificed and their brains removed on ice. A coronal wedge of brain tissue, in the region of the cannula, was removed for histological analysis (see Chapter 3). In addition, in order to determine D-AP5 tissue levels, samples from right and left hippocampus were dissected out and stored at -20°C.

As in Expts 4.1 and 4.2, there was a limitation in terms of the number of animals in which electrophysiological recordings could be made from in a single day (maximum of 3) and,

therefore, the experiment was staggered with only 2 or 3 animals beginning the experiment each day.

Determination of D-AP5 tissue levels.

D-AP5 was separated from the other tissue constituents by high performance liquid chromotography (HPLC) and quantified by fluorescence detection (Lindroth and Mopper, 1979). Tissue samples were thawed and then extracted in 0.6 M (7%) perchloric acid (PCA). Briefly, the samples were homogenised in PCA and left for 10 minutes. They were then centrifuged (16,000 rpm for 2 min.) and the supernatant decanted off and neutralised with 2M $KHCO_3$. The samples were then recentrifuged (16,000 rpm for 2 min) and the resulting supernatant diluted tenfold in distilled water.

D-AP5 was then separated from other primary amines (derivatised amino acids) by reverse phase HPLC on a 5μm Nucleosil C-18 column (250 x 4.6 mm). Separation was acheived using a gradient elution system. The mobile phases (Buffer A - 50 mM phosphate buffer (pH 5.14), 2.5% tetrahydrofuran; Buffer B - methanol, 1.25% tetrahydrofuran) were pumped at 1 ml/min according to the following gradient profile: time (min), %B - 0,0; 5,0; 7,25; 15,50; 23,60; 25,90; 28,100; 32,100; 42,0) by means of a Varian Vista 5500 pumping system (with a 9090 automatic column injector).

D-AP5 was detected by fluorescence, using an Applied Biosystems 980 fluorescence detector (excitation wavelength = 230 μm; emission wavelength > 398 μm), following pre-column derivatisation with *o*-pthaldialdehyde. The peak sizes were quantified using a microcomputer-based integration package (Maxima 820) and standardised relative to a sample containing a known amount of D-AP5. Figure 7.1 shows a representative chromatogram obtained from a 50 pmoles standard sample containing of a number of amino acids and D-AP5.

Results - D-AP5 Tissue Levels

The mean hippocampal whole-tissue concentration of D-AP5 in animals infused with a 30 mM drug solution was 0.77 $\pm$ 0.07 nmol/mg wet weight. D-AP5 tissue levels were slightly higher in the right hippocampus (ipsilateral to the infusion cannula; 0.96 $\pm$ 0.010 nmol/mg wet weight) compared with the left hippocampus (0.57 $\pm$ 0.05 nmol/mg wet weight). Infusion of 25 mM D-AP5 resulted in a mean hippocampal whole tissue concentration of

0.59 ± 0.03 nmol/mg wet weight (right hippocampus = 0.75 ± 0.05 nmol/mg wet weight; left hippocampus = 0.41 ± 0.03 nmol mg/wet weight.

Tissue levels of endogenous amino acids were comparable to those reported elsewhere for both control and D-AP5 infused animals (see Butcher and Hamberger, 1987; Butcher et al., 1991; Davis et al., 1992). The mean hippocampal whole-tissue concentrations of selected endogenous amino acids were as follows: glutamate = 6.68 ± 0.19 nmol mg/wet weight; aspartate = 1.39 ± 0.04 nmol mg/wet weight; phenylalanine = 0.09 ± 0.003 nmol mg/wet weight).

In this study, animals in the experimental group were infused with either 25 or 30 mM D-AP5 (n = 6 and n = 12 respectively). Both infusion doses resulted in D-AP5 tissue levels well in excess of the drug concentration required to block the induction of LTP in the dentate gyrus *in vivo* (approx. 0.27 nmol/mg wet weight; see Davis et al., 1992). Inspection of the data revealed no differences between the 25 and 30 mM D-AP5 groups, in terms of either behavioural or electrophysiological measurements. Therefore, for most of the analyses, these two groups are combined to form one experimental D-AP5 group (n = 18). Statistical comparisons of the 25 and 30 mM D-AP5 groups are also reported where appropriate.

Behaviour

During spatial training in watermaze 1 (pre-drug phase), all animals showed a progressive improvement in terms of reduced escape latencies across trials ($F(21,609) = 11.51$; $p < 0.0001$; Figure 7.2.1A).

Infusion of D-AP5 did not grossly affect the behaviour of the animals. Nevertheless, the drug treated animals did display a prolonged righting reflex and on a number of occasions had difficulty remaining on the platform, especially on the early training trials. There was also evidence of some muscle flaccidity.

Following minipump implantation, all rats were given a transfer test (trial 23) before the start of spatial training in watermaze 2. The results of trial 23 were analysed in three ways. Firstly, the data was expressed in terms of the 'future' training quadrant and an ANOVA showed no significant effect of quadrant ($F(2,87) = 1.15$; $p > 0.20$; Figure 7.2.2). Second, the data was re-analysed in terms of the actual quadrants as described by compass points. Interestingly, there was no bias to the SW quadrant in these animals, an ANOVA revealing

no significant bias towards any one quadrant ($F(2,87) = 1.41$; $p > 0.20$; Figure 7.2.3). Finally, all the animals involved in the study ($n = 31$) were divided into four groups according to the location of the platform during training in watermaze 1 (eg. NE, NW, SE, SW). These four groups were then compared with respect to the amount of time spent in the four quadrants of watermaze 2 (as described by the compass points; Figure 7.2.4). An ANOVA showed that there was no interaction between the training quadrant in watermaze 1 and the amount of time spent in the 4 quadrants of the pool during trial 23 in watermaze 2 ($F < 1$; $p > 0.20$). The results of trial 23 suggest that training in watermaze 1 does not result in a spatial bias in watermaze 2.

The rats were then given 8 spatial training trials in watermaze 2 (Figure 7.2.1B). Although both groups showed some improvement in terms of escape latency, on average, the aCSF rats escaped from the pool more rapidly than the D-AP5 rats. An ANOVA confirmed that there was a significant effect of group ($F(1,29) = 4.68$; $p < 0.05$). In addition, the ANOVA revealed a significant effect of trial ($F(7,203) = 19.62$; $p < 0.0001$) but no groups by trials interaction ($F < 1$; $p > 0.20$). Comparison of the 25 and 30 mM D-AP5 groups revealed neither an effect of group ($F < 1$; $p > 0.20$) nor a groups by trials interaction ($F < 1$; $p > 0.20$).

One possible explanation for the prolonged escape latencies shown by the D-AP5 group is that these animals are actually swimming slower than the control rats. A comparison of the swim speeds of the two groups, however, did not reveal any such difference, either during trial 23 (the transfer test before spatial training in watermaze 2; aCSF = 0.31 ± 0.01m/s; D=AP5 = 0.32 ± 0.01m/s; $t < 1$; $p > 0.20$), or in terms of the mean swim speed averaged over the entire drug phase of the experiment (mean swim speed for both groups = 0.29 ± 0.01m/s; $t < 1$; $p > 0.20$).

Analysis of the transfer test, conducted after 4 spatial training trials in watermaze 2 (trial 28; Figure 7.2.5), revealed a significant effect of quadrant ($F(2,87) = 41.45$; $p < 0.001$) but no groups by quadrants interaction ($F(2,87) = 1.18$; $p > 0.20$: % time in training quadrant - aCSF = 48.0 ± 3.6; D-AP5 = 41.4 ± 3.5). There was no difference between the 25 and 30 mM D-AP5 groups ($F < 1$; $p > 0.20$; % time in training quadrant - 30mM = 43.8 ± 4.6; 25mM = 36.7 ± 4.9). Similarly, analysis of the transfer test after 8 training trials (trial 33; Figures 7.2.6 and 7.2.7), also showed a significant effect of quadrant ($F(2,87) = 51.34$; $p < 0.001$) but no groups by quadrants interaction ($F(2,87) = 2.06$; $0.10 < p < 0.20$; % time in training quadrant - aCSF = 54.9 ± 3.4; D-AP5 = 46.2 ± 4.2). Again there was no significant

difference between the 25 and 30 mM D-AP5 groups ($F < 1$; $p > 0.20$; % time in training quadrant - 30 mM = 47.6 ± 5.4; 25 mM = 43.4 ± 6.7).

In the light of previous work examining the effects of D-AP5 on performance in the watermaze (Morris et al., 1986a; Morris 1989; Davis et al., 1992), the lack of a significant effect of the drug on transfer test performance during trials 28 and 33 was somewhat surprising. D-AP5 infused animals did, however, take longer to escape from the pool during training, a result which indicates a dissocation between the effects of the drug treatment on these two different measures of spatial learning. Consequently, an alternative means of assessing performance during the transfer tests was also used. The computer software used (Watermaze), can count the number of times the rat passes through a circle of specified diameter centred on the platform position (annulus crossings). The number of annulus crossings made in the training quadrant can then be compared with the number of crossings made of similar positions in the other three quadrants of the pool. This may provide a more sensitive measure of the animals' knowledge of the exact platform location. During both transfer tests (trials 28 and 33), the rats made significantly more annulus crossings in the training quadrant than in the other three quadrants of the pool. An ANOVA of annulus crossings during trial 28, revealed a significant effect of quadrant ($F(3,87) = 18.64$; $p < 0.0001$), but no effect of drug treatment ($F < 1$; $p > 0.20$), nor a groups by quadrants interaction ($F < 1$; $p > 0.20$; Figure 7.2.8). Similarly, analysis of trial 33 also showed an effect of quadrant ($F(3,87) = 30.80$; $p < 0.0001$), but no effect of group ($F < 1$; $p > 0.20$), nor a groups by quadrants interaction ($F < 1$; $p > 0.20$; Figure 7.2.9). It would appear, therefore, that there is no effect of D-AP5 treatment during the transfer tests, using this measure of performance.

Because trials 28 and 33 are the second and third transfer test the rats have been given in watermaze 2, it is possible that, as a result of previous extinction trials (transfer tests), the animals are less inclined to persist searching in the training quadrant, having discovered that the platform is not present. This may result in an underestimate of the rats' knowledge of the platform location when using the quadrant times analysis for the full 60 seconds of the transfer test (eg. Expt 4.4). This in turn, may mask any drug induced impairment by reducing the amount of time spent in the training quadrant by the control animals. Therefore, the two transfer tests (trials 28 and 33) were re-analysed looking at just the first 15 or 30 seconds of the probe trial. Re-analysis of trial 28 did not show any difference between the groups across quadrants during either the first 15, or the first 30 seconds, of the

transfer test ($F < 1$; $p > 0.20$ for both analyses). An ANOVA of the time spent in the four quadrants of the pool during the first 30 seconds of trial 33 revealed a significant effect of quadrant ($F (2,87) = 59.59$; $p < 0.001$) and a trend towards a groups by quadrants interaction but this did not quite reach significance ($F (2,87) = 3.07$; $p = 0.05$; Figure 7.2.10). A comparison of the amount of time spent in the training quadrant only during trial 33 showed that D-AP5 treated rats spent significantly less time in the training quadrant during the first 30 seconds of the transfer test than controls ($t (29) = 2.10$; $p < 0.05$; aCSF = 65.8 ± 5.1%; D-AP5 = 50.7 ± 4.9%). A similar pattern of results was obtained by analysing just the first 15 seconds of trial 33. An ANOVA revealed a significant effect of quadrant ($F (2,87) = 52.28$; $p < 0.001$) and a non-significant trend towards a group by quadrants interaction ($F (2,87) = 2.68$; $0.05 < p < 0.10$). Analysis of the training quadrant times only also revealed a non-significant trend towards better performance in control animals ($t (29) = 1.98$; $0.05 < p < 0.10$; aCSF = 71.1 ± 3.6%; D-AP5 = 54.2 ± 6.2%; Figure 7.2.11).

Finally, when the rats were returned to watermaze 1, both the aCSF and D-AP5 infused rats still showed a significant and equivalent bias towards the appropriate training quadrant (trial 34; Figure 7.2.12). This suggests that the rats show some retention of the original training environment despite the intervening training, and that this is not affected by D-AP5 infusion. An ANOVA of the time spent in the 4 quadrants of the pool during trial 34, revealed a significant effect of quadrant ($F (2,87) = 11.23$; $p < 0.001$), with no groups by quadrants interaction ($F < 1$; $p > 0.20$; % time in training quadrant - aCSF = 33.7 ± 3.0; D-AP5 = 39.4 ± 3.4). There was no difference between the 25 and 30 mM D-AP5 groups in terms of performance during trial 34 ($F < 1$; $p > 0.20$: % time in training quadrant - 30 mM = 38.0 ± 4.2; 25 mM = 42.2 ± 6.3). Analysis of either the first 15, or first 30, seconds of trial 34 also failed to reveal a difference between the two groups across quadrants ($F < 1$; $p > 0.20$ for both analyses).

In summary, D-AP5 infusion resulted in prolonged escape latencies during acquisiton in the second watermaze. There was, however, no difference between the groups during the transfer test conducted after 4 training trials in this novel environment, when analysed either in terms of quadrant times or in terms of annulus crossings. Similarly, in a transfer test conducted after 8 spatial training trials in this novel environment, there was no difference between D-AP5 and aCSF infused rats when the full 60 seconds of the probe trial was analysed. During the initial stages of this transfer test (the first 15 or 30 seconds), however, the control rats did tend to spend more time in the training quadrant than the D-AP5 treated

rats. It appears that, possibly as a result of two previous transfer tests, the control rats were less inclined to persist searching in the training quadrant having found that the platform was not present. This extinction effect may result in an underestimate of the knowledge of the platform location in control animals which in turn may mask any drug induced impairment in performance. D-AP5 did not affect the retrieval of previously acquired spatial information in watermaze 1.

Electrophysiology

Comparison of baseline field epsps, resulting from low frequency stimulation of the perforant path (700 μA, 0.05 Hz), and averaged over the 10 minute period immediately prior to the tetanus, revealed that the mean slope of the field epsp was significantly larger in the D-AP5 group than in the aCSF infused controls (aCSF = 3.96 ± 0.32 mV/ms; n = 13; D-AP5 = 5.24 ± 0.47 mV/ms; n = 18; $t(29) = 2.10$; $p < 0.05$). There was no difference between the 25 and 30 mM D-AP5 groups (25 mM = 4.81 ± 0.77 mV/ms; n = 6; 30 mM = 5.46 ± 0.60 mV/ms; n = 12; $t < 1$; $p > 0.20$). In a subset of the animals participating in the study, the population spike of the field epsp was also monitored. Only animals with a population spike of > 1 mV pre-tetanus were included in the population spike analysis (aCSF - 9 out of 13; D-AP5 - 16 out of 18). The pre-tetanus population spike was also significantly greater in the D-AP5 group (aCSF = 3.33 ± 0.47 mV; D-AP5 = 7.32 ± 1.18 mV; $t(23) = 2.45$; $p < 0.05$). Again there was no difference between the 25 and 30 mM D-AP5 groups (25 mM = 5.41 ± 1.07 mV; n = 5; 30 mM = 8.18 ±1.61 mV/ms; n = 11; $t(14) = 1.09$; $p > 0.20$).

For each animal, the mean absolute slope value was then used as a within-subject baseline (= 100%) and the data normalised with respect to this value. The chronic infusion of D-AP5 (25 or 30 mM) resulted in an almost complete blockade of both LTP and STP in the dentate gyrus (Figure 7.2.13). During the first 4 minutes after the tetanus there was little if any potentiation in the D-AP5 infused rats (aCSF = 144.6 ± 3.4%; D-AP5 = 104.9 ± 0.9%; $t(29) = 13.01$; $p < 0.0001$). There was no difference between the 25 and 30 mM D-AP5 groups (25 mM = 104.5 ± 1.1%; n = 6; 30 mM = 105.1 ± 1.2%; n = 12; $t < 1$; $p > 0.20$). Examination of the data 10 minutes after high frequency stimulation (6-10 min post-tetanus) revealed that whereas there was considerable potentiation in the aCSF infused animals, there was virtually no potentiation exhibited by the D-AP5 treated animals (aCSF = 135.0 ± 2.3%; D-AP5 = 104.2 ± 1.1%; $t(29) = 13.28$; $p < 0.0001$). Similarly, after an hour (50-60 min post-tetanus), there was much less potentiation in the D-AP5 rats, relative to controls

(aCSF = 133.8 ± 2.4%; D-AP5 = 107.9 ± 1.2%; $t (29) = 10.42$; $p < 0.0001$). Again there was no difference between the 25 and 30 mM D-AP5 groups (25 mM = 107.4 ± 2.0%; 30 mM = 108.1 ± 1.5%; $t < 1$; $p > 0.20$).

D-AP5 infusion also resulted in a complete blockade of spike potentiation (Figure 7.2.14). Analysis of the first 4 minutes after the tetanus showed that there was no spike potentiation in the D-AP5 group (aCSF = 272.3 ± 55.1%; D-AP5 = 95.3 ± 2.9%; $t (23) = 4.34$; $p < 0.0005$). A similar result was obtained one hour after the tetanus (aCSF = 217.8 ± 45.1%; D-AP5 = 95.4 ± 5.6%; $t (23) = 3.59$; $p < 0.005$). There was no difference between the 25 and 30 mM D-AP5 groups, in terms of spike potentaion, either 4 minutes or one hour after high frequency stimulation (0 - 4 min: 25 mM = 93.0 ± 6.8%; n = 5; 30 mM = 95.9 ± 3.1%; n = 11; $t < 1$; $p > 0.20$; 50 - 60 min: 25 mM = 104.4 ± 1.4%; n = 5; 30 mM = 91.3 ± 6.8%; n = 11; $t (14) = 1.20$; $p > 0.20$).

Clearly there is very little, if any, LTP in the D-AP5-infused rats. This result is complicated, however, by the fact that the pre-tetanus field epsp is significantly larger in the D-AP5 group than in the aCSF group, both in terms of the slope of the field epsp and the population spike. It is possible that the failure to see LTP in the D-AP5-treated rats is due to the fact that the field epsp evoked in drug-treated animals is saturated, and is already at, or near, its asymptotic level. A similar result has been reported elsewhere by Davis et al., (1992). They found that the mean slope value of the 'high concentration' D-AP5 group was significantly greater than that of the controls. In an attempt to determine whether or not such a ceiling effect might account for the apparent blockade of LTP following D-AP5 infusion, analyses were performed to investigate whether there was any correlation between the size of the pre-tetanus field epsp and the amount of potentiation induced. There was no significant correlation between the mean absolute slope value for the 10 minute period immediately prior to tetanus and the amount of LTP seen one hour post-tetanus, within either the aCSF group ($r (12) = 0.01$; $p = 0.98$) or the D-AP5 group ($r (17) = 0.16$; $p = 0.52$). There was also no correlation between the size of the population spike and the amount of slope LTP that could be induced (aCSF - $r (8) = 0.19$; $p = 0.62$; D-AP5 - $r (15) = 0.27$; $p = 0.31$). For the aCSF group, the size of the pre-tetanus population spike did not affect the amount of spike potentiation induced ($r (8) = 0.32$; $p = 0.40$), although there was a trend towards a correlation in the D-AP5 group ($r (15) = 0.44$; $p = 0.09$). It, therefore, appears unlikely that the failure to induce LTP in animals infused with D-AP5 was due to the field epsp in these rats being already at, or near, its asymptotic level.

Histology

Examination of the tissue wedges taken from implanted animals revealed that the cannula was correctly located in the right lateral ventricle. On a limited number of occasions there was a small amount of damage to the overlying cortex and some enlargement of the ventricle. In a small number of animals there was some damage to the fornix immediately below the ventricle. The histology was comparable to that obtained in Expt 4.1 (see Figure 4.1.6).

DISCUSSION

The experiments described in this chapter have investigated the effects of two manipulations, both of which are known to affect spatial learning in the watermaze in experimentally naive subjects, on the acquisition of a spatial reference memory task in watermaze-experienced animals. By using rats that are already well practised with the procedural aspects of the task, this design should allow a purer assessment of the effects of these manipulations on the spatial component of the watermaze task.

Animals with ibotenic acid lesions of the hippocampus were markedly impaired on the acquisition of a spatial learning task in the watermaze despite extensive pre-operative training in a different spatial environment. Neither control nor lesioned animals showed any significant retention of the platform location acquired during pre-operative training in watermaze 1 when tested post-operatively (Expt 7.1).

The effects of D-AP5 on this task were less clear cut (Expt 7.2). D-AP5 infusion (25 or 30 mM) resulted in prolonged escape latencies during acquisiton in the second watermaze. In contrast, however, these animals showed little impairment when performance was assessed by means of two transfer tests conducted after 4 and 8 training trials in the novel environment. Both aCSF and D-AP5 infused rats demonstrated effective retrieval of the pre-operatively acquired platform location in watermaze 1. There was no significant difference between the two groups on this retention trial. Subsequent electrophysiological investigations revealed that these doses of D-AP5 were sufficent to block dentate gyrus LTP *in vivo* in these same animals.

Spatial learning in watermaze-experienced animals is still hippocampal-dependent

In view of the vast literature documenting the effects of hippocampal lesions on spatial learning, one would expect that learning about a novel spatial environment should require an intact hippocampus, irrespective of whether or not the animals had received watermaze training prior to surgery. Not surprisingly, therefore, hippocampal lesioned animals were impaired on the acquisition of a spatial learning task in watermaze 2, despite extensive pre-operative training in watermaze 1. During training in watermaze 2, there was a trend for the lesioned animals to take longer to find the escape platform, although this did not quite reach statistical significance. This lack of statistical significance may be attributable to the degree of variability in escape latencies, especially in the lesion group, and may reflect the relatively small group sizes. There was, however, a marked impairment in terms of transfer test performance after both 4 and 8 training trials in the novel environment. This deficit comes as little surprise in that hippocampal lesioned animals are also impaired when trained to a novel platform location in a familiar spatial environment (the 'reversal' task; Good and Morris, personal communication). The lesion impairment confirms that proficient performance on the two watermaze task is dependent upon the hippocampus.

In the absence of any significant retention of the pre-operatively acquired platform location (watermaze 1) in control animals, it is impossible to assess in this study whether or not hippocampal damage disrupts the retrieval of previously acquired spatial information. A number of other studies have, however, found that hippocampal damage results in a retrograde amnesia and impairs the retrieval of pre-operatively acquired spatial information (Schenk and Morris, 1985; Morris et al., 1990b; Bolhuis et al., 1994). The poor retention of spatial information in these control animals is in contrast to the performance of control subjects in both Expts 6.6 and 7.2. This may be explained by the longer delay interposed between training in watermaze 1 and the start of training in watermaze 2 in the lesion study. In this experiment there is an 11 day delay between training in watermaze 1 and the commencement of training in watermaze 2 (1 day surgery and 10 days post-operative recovery) whereas in both Expts 6.6 and 7.2 there is only 3 days between training in watermaze 1 and the start of training in watermaze 2 (eg. Expt 7.2: 1 day surgery and 2 days post-operative recovery). It is worth noting that a subset of the control animals in Expt 7.1 do show a strong spatial bias to the training quadrant when returned to watermaze 1 (Figure 7.1.8). A replication of this study seems warranted.

Does D-AP5 impair spatial learning in this task?

The aim of this thesis is to test the hypothesis that LTP-like mechanisms in the hippocampus underlie spatial learning. Expt 7.1 establishes that learning in the two watermaze paradigm does indeed require the hippocampus. In the light of previous studies (Morris et al., 1990a), one would predict that experimental manipulations which prevent the induction of LTP should impair new spatial learning in watermaze 2 but allow efficient retrieval of spatial information acquired during pre-drug training in watermaze 1. The results of Expt 7.2 clearly bear out the second of these predictions, namely that infusion of the NMDA antagonist, D-AP5, does not affect the retrieval of previously acquired spatial information. In contrast, the effects of D-AP5 on new spatial learning in watermaze 2 were somewhat ambiguous. Although D-AP5 infused rats took longer to escape from the water during spatial training in watermaze 2, there was little indication of a spatial learning impairment when performance was assessed by means of transfer tests. This dissociation between these two measures of performance is quite different from the pattern of results obtained with hippocampal lesions. If anything, the lesioned animals showed a greater impairment on the transfer tests than during training where the difference between the groups, in terms of escape latencies, did not quite reach statistical significance. These results highlight crucial differences between the behavioural effects of the two manipulations (see also Morris et al., 1990a), and reaffirm the point that, although the hippocampal lesioned animal provides some kind of baseline with which these subsequent drug studies may be compared, the effects of D-AP5 treatment cannot simply be regarded as producing a 'temporary hippocampal lesion'.

The apparent discrepancy between the effects of D-AP5 on escape latencies and transfer test performance poses a problem, both in terms of providing a concise account of these results, and with regard to assessing whether or not LTP-like processes are required for spatial learning. As previously mentioned in Chapter 2, there are potential problems with both measures of performance. Generally speaking, the transfer test is thought to provide the better measure of the rats' memory for the platform location. Escape latencies are widely considered to provide a less direct measure of spatial learning. For example, on any one trial, there is always the possibility that a rat, with little knowledge of the goal location, might bump into the platform by chance after only a few seconds. In addition, escape latencies may be influenced by non-spatial factors such as swim speed, although, in this particular case, the longer escape latencies of the D-AP5 treated animals cannot be explained

in this way. Nevertheless, the possibility that a more subtle sensorimotor or motivational disturbance is responsible for the prolonged escape latencies cannot be ruled out. In support of this possibility, observation of the D-AP5 animals did reveal a degree of sensorimotor impairment. These animals displayed a prolonged righting reflex in their home cages, a degree of muscle flaccidity, and on a number of occasions had difficulties in remaining on the platform, especially on the early training trials.

Bearing in mind the obvious problems associated with using escape latency as a measure of performance, one might be led to conclude that D-AP5 does not impair spatial learning, on the basis that these animals are not significantly impaired relative to control subjects when the extent of any learning is assessed by two 60 second transfer tests (trials 28 and 33). However, the results of the transfer tests may also be misleading. The transfer test can be considered as an extinction trial, in that the rat is not rewarded for going to the previously correct location. After a certain amount of time searching in the vicinity of the former platform location, the rat may well begin to search elsewhere for an alternative means of escape. This will result in an underestimate of the rat's knowledge of the platform location. The significance of this increases the more often the animal is given a transfer test and can result in rats spending progressively less time in the training quadrant with each transfer test (see Expt 4.4). Indeed, there is some evidence from the analysis of trial 33 to support this possibility. Trial 33 is the third transfer test that these animals have been given in watermaze 2. Maybe not surprisingly, therefore, control animals tend to spend less time searching in the training quadrant as the probe trial progresses (proportion of time in training quadrant during first 15 seconds = 71.1%; 30 seconds = 65.8%; 60 seconds = 54.9%). Therefore, analysis of the full 60 seconds of the probe trial may underestimate the amount of spatial learning in the control group. Interestingly, analysis of the first 30 seconds of trial 33 did reveal a significant difference between the aCSF and D-AP5 groups, with the control animals spending significantly more time in the former training quadrant. One certainly cannot rule out the possibility, therefore, that D-AP5 infusion is causing a subtle spatial learning impairment and that a 60 seconds transfer test analysis is not sufficiently sensitive to pick up this small deficit. On the other hand, the strong spatial bias displayed by the D-AP5 group during both transfer tests raises a question as to whether or not D-AP5 does in fact disrupt the mechanisms of spatial learning.

It is extremely difficult to formulate a concise account which can explain both the differences between the groups in terms of escape latencies and the apparent lack of an

impairment during the two transfer tests. One might argue that the D-AP5 animals are able to navigate to roughly the right vicinity of the pool but are then unable to fine tune their search for the platform. This could be due to less efficient learning about the platform location or to a subtle sensory effect on visual acuity, and could conceivably result in prolonged escape latencies but no difference in terms of the quadrant times analysis during the transfer tests. This account seems unlikely, however, in that when annulus crossings are used as the measure of performance during the probe trials, there was still no difference between the groups.

Whatever the explanation for the dissociation between escape latency and transfer test measures of performance, it is clear that, despite taking consistently longer to locate the escape platform during training, the D-AP5 animals are capable of considerable spatial learning and do show a strong bias to the correct quadrant of the pool during the transfer tests. Indeed, using conventional measures of performance (annulus crossings and quadrant times analysis during 60 second probe trial), the D-AP5 group are indistinguishable from controls. This result is somewhat surprising and, when combined with the electrophysiological data derived from the animals in this study, the extent to which the D-AP5 animals learn about the spatial location of the platform poses a serious challenge for the hippocampal LTP/spatial learning hypothesis.

Does D-AP5 block LTP in these animals?

The infusion of either 25 or 30 mM D-AP5 resulted in a hugely significant reduction in the amount of LTP that could be induced, in terms of both the slope of the field epsp (Figure 7.2.13) and the population spike (Figure 7.2.14). The absence of LTP in these animals is unlikely to be due to the fact that the pre-tetanus evoked response is significantly greater in D-AP5 treated animals and, therefore, nearer its asymptotic level. The effects of D-AP5 on dentate gyrus LTP *in vivo* are well documented, including studies in which there was no difference between groups in terms of the size of the pre-tetanus response (Morris et al., 1986a; Errington et al., 1987; Morris et al., 1989) In addition, correlation analyses provided no indication that the amount of LTP that could be induced was related to the size of the pre-tetanus evoked response.

The reason for the greater magnitude of the responses obtained in D-AP5 treated animals is not immediately apparent. One possible explanation for this finding is that the implantation

of the electrodes causes some tissue damage which results in a reduction in the size of the field potential. Because D-AP5 has neuroprotective properties (Meldrum, 1989; Meldrum and Garthwaite, 1990), it is possible that the drug treatment may reduce the extent of any tissue damage and thus preserve the magnitude of the evoked response.

How much synaptic plasticity is available to these animals?

The extent to which rats in the D-AP5 group learned about the spatial location of the platform was somewhat surprising. Indeed, according to conventional transfer test measures of performance there was little evidence of a spatial learning impairment in these animals. One possible explanation for the lack of a robust learning deficit is that although the D-AP5 rats show significantly less potentiation than the controls, they may still have sufficient residual synaptic plasticity to support normal learning. When considering the results of this experiment as a whole, it is important to know just how much potentiation, if any, the D-AP5 infused rats exhibit.

Previous experiments, reported in an earlier part of this thesis (Expts 5.6 and 5.7), have shown that, on occasions, there is a slow gradual increase in the size of the field epsp in non-tetanised pathways. It is evident from Figure 7.2.13 that there may be a similar rise during the post-tetanus one hour period in this experiment, resulting in apparently greater % LTP in both groups. This suggests that the % LTP seen one hour after the tetanus in the D-AP5 group may be an overestimate of the actual amount of potentiation induced. Examination of the data revealed that 10 minutes after the tetanus there was only 4% potentiation in the D-AP5 group (compared with 8% after an hour). It is possible that, in the absence of a rising baseline, this value of 4% potentiation seen 10 min after tetanisation, may have decayed even further back to baseline after one hour.

These results suggest, therefore, that the infusion of 30 mM D-AP5 produces an almost, if not, complete blockade of LTP in these animals. In agreement with this suggestion, the whole tissue levels of D-AP5 present in hippocampal tissue taken from these rats were found to be well in excess of the drug concentration previously found to cause a complete blockade of dentate LTP *in vivo* (Davis et al., 1992). This indicates that a lower dose of D-AP5 could be used to achieve the same block of LTP but with the possibility of reduced sensorimotor side effects (eg. a decrease in the number of times the AP5 treated rats fell off the platform). Indeed, the rationale for including some 25 mM animals was to determine whether or not a

lower dose of D-AP5 (< 30 mM) would still block LTP but leave spatial learning intact. Examination of the data revealed that rats infused with 25 mM D-AP5 were indistinguishable from animals infused with 30 mM D-AP5, both in terms of the behavioural and electrophysiological analyses. Whole tissue levels of D-AP5 obtained from the 25 mM group were also found to be in excess of those required to block LTP *in vivo* (Davis et al., 1992). The possibility remains, therefore, that a dose of less than 25 mM D-AP5 may block LTP and yet, at the same time, spare spatial learning, both in terms of transfer test performance and escape latencies during training. In view of the potential influence of non-associative side effects of the drug treatment on watermaze performance, it is surely important to use the minimal dose of AP5 necessary to block LTP.

The counter argument is that a different set of stimulation parameters may reveal more residual plasticity in these AP5 treated animals. As discussed earlier in this thesis (Chapter 5), the stimulation parameters used to induce LTP will determine not only the amount of LTP obtained in controls but may also influence the degree of LTP inhibition produced by a drug treatment. The possibility cannot be ruled out that a different set of stimulation parameters may result in slightly, but significantly, more LTP in D-AP5 infused animals. More importantly, the patterns of activity that occur naturally during learning may be less susceptible to this level of NMDA receptor blockade.

The precise relationship between LTP, which is induced artificially by electrical stimulation, and any potential changes in synaptic efficacy that might occur naturally during learning is not well defined. One might expect that potentiation induced by high frequency electrical stimulation is likely to represent a crude form of endogenous plasticity. The hypothesis under investigation is not that LTP *is* the physiological process underlying learning, but rather that the mechanisms recruited during the induction and expression of LTP may share common features with the mechanisms of learning. Nevertheless, the question remains as to how far an analogy between tetanus-induced LTP and endogenous changes in synaptic efficacy can be taken. The stimulation parameters chosen to induce LTP and the physiological relevance of these parameters may be of considerable importance when evaluating the results of experiments such as this.

Implications for the hippocampal LTP/spatial learning hypothesis

Two distinctly opposite conclusions can be drawn from these data depending on one's

viewpoint. One could argue, for example, that because the D-AP5 treated animals show a deficit during training, in terms of escape latencies, and a block of LTP, these results support the hippocampal LTP/spatial learning hypothesis. Alternatively, the fact that these animals demonstrate considerable learning, and may even be comparable with control animals in terms of transfer test performance, despite a near-complete block of LTP, may be taken as evidence against a requirement for LTP-like mechanisms during spatial learning, and thus used to refute the hypothesis.

The fact that these D-AP5 animals are capable of some spatial learning is not inconsistent with previous watermaze studies conducted in this laboratory. For example, in the original study (Morris et al., 1986a), the performance of D-AP5 treated rats on the 'reversal' phase of the experiment, during which the platform position was moved to the opposite quadrant of the pool, suggests that these animals had in fact learned something about the original platform location. During the reversal phase, an animal that has learned about the original platform location will begin by searching persistently in that location before searching elsewhere. This will result in prolonged escape latencies compared with the last few trials to the original platform position. In contrast, a rat with little knowledge of the former platform location will not be biased against the correct goal position during reversal. If the animal is using a non-spatial strategy, then escape latencies during reversal should be similar to those during the later training trials to the original platform location. Hippocampal lesioned animals adopt a non-spatial strategy and there is little difference in escape latencies between the end of the original training and the start of reversal training (Sutherland et al., 1983). The fact that the D-AP5 animals show a marked increase in escape latencies at the start of the reversal phase suggests that these animals do have some spatial knowledge of the previous platform location.

The fact that D-AP5 animals are capable of some spatial learning may also be directly illustrated by examining the results of the dose response study (Davis et al., 1992). These data show that even the 'high' concentration group (which display a total block of LTP) show a considerable and highly significant bias to the training quadrant during the transfer test (41.4% of the 60 sec trial). Davis et al., (1992) found that only at 'very high concentrations' of D-AP5, which were clearly having gross sensorimotor side effects, was there no evidence of any spatial bias during the probe trial. These results clearly show that, even in the presence of a complete block of LTP, D-AP5 treated animals are capable of some spatial learning. One qualifier for this result is that the transfer test was conducted a mere 30 sec

after the last spatial training trial. It may be that NMDA receptor-independent mechanisms may be able to support some short-term memory for the platform location between the end of training and the probe trial. It is possible, therefore, that these transfer test scores may overestimate any long-term memory for the platform location which may be NMDA receptor-dependent. Nevertheless, it certainly appears that D-AP5 infusion, at doses which block LTP, does not result in a complete block of spatial learning. D-AP5 infused rats learn considerably more than hippocampal lesioned animals and, using certain experimental paradigms, may be capable of nearly as much spatial learning as controls (Expt 7.2).

Although there seems little doubt that D-AP5 treated rats are capable of some spatial learning, there is considerable debate regarding the interpretation of this result. One viewpoint is that because these animals learn something in the presence of a block of LTP, then LTP-like processes cannot be required for spatial learning (Keith and Rudy, 1990). The alternative viewpoint is that because these animals are impaired relative to controls, this result therefore supports the LTP/learning hypothesis. The argument between these two contrasting viewpoints has been fully documented in an issue of *Psychobiology* (Vol. 3; p 250 - 272). In their contribution to this issue, Keith and Rudy (1990) use the fact that the AP5 animals show some learning as grounds to refute the LTP/learning hypothesis. Clearly this goes too far. As Morris (1990) rightly points out, it is extremely unlikely that a pharmacological manipulation which possibly affects just one aspect of hippocampal physiology will reproduce all the effects of a neurotoxic lesion, which is likely to destroy all the cells in that structure. As mentioned previously, there are obvious and important differences between the behavioural syndromes resulting from D-AP5 treatment and a hippocampal lesion. For example, D-AP5 spares the retrieval of previously acquired spatial information, a finding which is in direct contrast to the effect of a hippocampal lesion in the watermaze. It might be the case, therefore, that D-AP5 slows down the rate of spatial learning rather than preventing new learning completely. It is, therefore, premature to dismiss the LTP/learning hypothesis on the basis that D-AP5 treated animals do not behave exactly like hippocampal lesioned animals. Furthermore, it is also worth noting that even hippocampal lesioned animals, after extensive training, show some learning about the platform location and display a degree of spatial bias to the training quadrant in a transfer test (Morris et al., 1990b). It is not unreasonable, therefore, to expect some spatial learning in the D-AP5 group.

A question remains, however, as to how much learning to expect in these animals. While it

may be wrong to reject the hypothesis on the basis that D-AP5 animals are not as impaired as hippocampal lesioned animals, it may be equally inappropriate to use the results of Expt 7.2, where, although showing some impairment, they clearly learn a considerable amount about the spatial location of the platform, as support for the hypothesis. It is difficult to predict the extent to which D-AP5 might impair learning in these studies. Nevertheless, the amount that the D-AP5 treated animals learn about the platform location in Expt 7.2 is certainly surprising. For example, intuitively, one would predict that there is more new learning required in the two watermaze experiment, during which the rat has to learn about a whole new spatial environment, than in the 'reversal' experiment (Morris et al., 1990a) during which the animal, being already familiar with the spatial layout of the room, has only to learn about the new goal location. Hence, one would predict that the two watermaze experiment should be more sensitive to the D-AP5 manipulation than the 'reversal' task. It would appear that this is not the case. D-AP5 produces a deficit on the reversal task, in terms of both escape latencies and transfer test performance. In contrast, although the D-AP5 treated animals show a latency impairment during training in Expt 7.2, there is little evidence of a deficit in the two transfer tests conducted in watermaze 2. This result appears to be at odds with what one would predict on the basis of the hippocampal LTP/spatial learning hypothesis.

Learning versus Performance - Can the pattern of results obtained with D-AP5 in the watermaze be explained by a sensorimotor or motivational account?

In the introduction to this chapter, a considerable amount of work was reviewed indicating that the administration of NMDA receptor antagonists, such as D-AP5, may well result in a number of sensorimotor and motivational side effects, in addition to any effects on synaptic plasticity in the hippocampus. The difficulty in separating specific drug effects on the mechanisms of learning from possible effects on sensorimotor or motivational processes has already been discussed at length. Indeed, this whole thesis has been dominated by the 'learning versus performance' debate. In view of the potential influence of non-associative side effects on performance in the watermaze, combined with the extent to which the D-AP5 animals learn about the platform location, one possible explanation for the pattern of results obtained in Expt 7.2 is that D-AP5 does not interfere directly with the mechanisms of spatial learning but impairs performance through an effect on some sensorimotor or motivational process which can also influence the overall level of performance. Such an account may

also, of course, explain the deficits obtained with D-AP5 in other watermaze studies. It may indeed be the case that D-AP5 has no direct effect on the mechanisms of spatial learning but reduces the level of performance by affecting, for example, the motivational state of the animal. Alternatively, D-AP5 could be disrupting the rat's locomotor systems or affecting its ability to accumulate sensory information. D-AP5 may be creating a feeling of ill-being which in itself could retard learning. These animals *will* learn the task more slowly but not because of any direct effect on the neural mechanisms responsible for information storage. It is certainly possible, therefore, that the mechanisms of spatial learning are essentially intact in the presence of D-AP5, and that the extent of any deficit is dependent on the sensorimotor or motivational demands of the particular behavioural paradigm used to assess learning.

The results of Expt 7.2 are not inconsistent with such an account. The strong spatial bias shown by these animals in the transfer test may reflect the fact that the mechanisms of spatial learning are working normally in these rats. It is not inconceivable that what deficit there is with these animals, in terms of escape latencies during training, could be due to non-associative, sensorimotor or motivational effects of the drug administration. After all, the D-AP5 rats did display signs of a sensorimotor impairment. They often fell off the platform during the drug phase of training in watermaze 2 and displayed both a degree of muscle flaccidity and a prolonged righting reflex. Repeatedly falling off the platform is likely to reduce the incentive value of the goal location and may have important consequences for the motivational state of the animal. It is not unreasonable, therefore, to suggest that a 'performance' effect could account for these results.

The question remains, however, as to why performance is spared to a greater extent in Expt 7.2 compared with previous watermaze studies. An obvious possibility is that the extensive spatial training in watermaze 1, prior to the drug phase of the experiment in watermaze 2, may have a profound effect on the magnitude of any drug-induced sensorimotor or motivational disturbance. By the time the rats are given D-AP5 and begin training in watermaze 2, they are already well practised with the procedural aspects of the watermaze task. They are aware that there is a means of escape from the water and that this involves locating a hidden platform. It is quite possible, therefore, that to these rats the watermaze is a much less stressful environment than it would be to a naive animal. It may be that D-AP5 interacts with the heightened state of anxiety in naive animals to retard learning, but in more experienced animals the effects of D-AP5 on the psychological state of the animal are less telling. The watermaze experienced animals in Expt 7.2 are also well practised with the

sensorimotor demands of the task prior to any drug treatment. Although there was still some evidence of a sensorimotor disturbance in these animals, it is possible that the extensive pre-training in watermaze 1 may have ameliorated any drug-induced effects on performance. One certainly cannot rule out an account whereby D-AP5 impairs performance in the watermaze through some non-associative effect on sensorimotor or motivational processes which is greatly reduced in watermaze-experienced animals. It was hoped that the 2-watermaze paradigm would provide a purer test of the spatial component of the watermaze task, under conditions where little or no procedural learning is required. The fact that D-AP5 treated animals display considerable spatial learning during Expt 7.2 indicate that deficits observed using other watermaze paradigms may be due, at least in part, to the drug interfering with procedural aspects of performance. Under conditions which reduce the possibility that the drug treatment may disrupt non-spatial aspects of performance, D-AP5 treated animals do seem capable of considerable spatial learning.

Another potentially important variable between this and a number of other watermaze studies is the inter-trial interval (ITI). As the results described in Chapter 6 have already illustrated, it is possible that the design of the training schedule may have an important bearing on whether or not a drug treatment impairs performance in the watermaze. For example, L-NAME impaired watermaze performance on paradigms which involved the animals receiving blocks of multiple trials with a short ITI but was without effect when animals were given just a single training trial per day. Although L-NAME and D-AP5 are chemically unrelated, and there is no reason to assume that an NMDA antagonist will produce similar behavioural side effects to an NOS inhibitor, the possibility that the effects of D-AP5 on watermaze performance may also be dependent on the type of training schedule cannot be ruled out. Examination of the literature provides evidence both for and against this possibility. Davis et al., (1992) found that D-AP5 impaired performance on a spatial learning task which involved training the animals for 5 days with 6 training trials per day, separated by a 30 sec ITI. The authors noted that there was a strong tendency for the performance of animals receiving higher concentrations of D-AP5 to deteriorate across the 6 trials of a session, suggesting that the animals may become tired and that this, in turn, may exacerbate any drug-induced impairment. Against this, however, 40 mM D,L-AP5 has been found to impair spatial learning when trials were separated by a 4 hr ITI (Morris et al., 1986a). This would seem to be sufficient time for animals to recover between trials from fatigue, although one cannot rule out the possibility that the rats had not recovered fully within 4 hr. In addition, in the same study, the authors found that visual discrimination

learning in the watermaze was unaffected by D-AP5 when rats were trained with 10 training trials per day. This could be taken as evidence against the importance of the ITI in determining whether or not there is a drug-induced deficit. These animals were unimpaired despite receiving 10 trials per day with a short ITI, a training schedule which might be expected to be quite tiring. However, there are additional differences between the sensorimotor and motivational demands of the two tasks which could also be important. Furthermore, as only errors to criterion were reported for the visual discrimination study, it remains possible that D-AP5 could have had subtle effects on other measures of performance. For example, as with L-NAME (see Expt 6.4), although D-AP5 did not affect the rate at which the criterion score was acheived, it is possible that the drug treatment may have resulted in prolonged escape latencies during training.

The importance of the training schedule, with regard to the effects of D-AP5 on spatial learning in the watermaze, remains unresolved. A systematic comparison of the effects of D-AP5 on spatial learning in the watermaze using both single trial and multiple trial paradigms is required (as in Chapter 6). If NMDA receptor-dependent LTP-like mechanisms are indeed responsible for spatial learning, then D-AP5 treated rats should be impaired irrespective of the ITI or the number of trials per session.

In the absence of these studies, one can only speculate as to the importance of the training schedule in determining whether or not D-AP5 impairs performance on spatial learning tasks in the watermaze. A detailed discussion of how a drug treatment (eg. L-NAME) may affect performance on multiple trial paradigms but spare performance on single trial paradigms is provided in Chapter 6. Many of these arguments may also apply to D-AP5. It may be, for example, that D-AP5 animals tire much more rapidly than control animals when blocks of multiple trials are used, and that this retards the rate of learning. Alternatively, there may be differences in incentive between single and multiple trial paradigms which may interact with D-AP5 treatment to produce the observed pattern of results. A single trial per day training schedule could be less stressful to the animal compared with multiple trial paradigms. D-AP5 may interact with the increased level of anxiety that results from being repeatedly put back in the water during a multiple trial paradigm to retard learning. It could be argued that a single trial paradigm with a 24 hr ITI represents a purer test of spatial learning and is less succeptable to influence by non-associative drug effects such as fatigue. It is possible that multiple trial paradigms accentuate any drug-induced differences in performance so that any small sensorimotor disturbance could be exacerbated to produce a significant learning

impairment.

The extent to which the experimental subjects have prior experience in the watermaze, and the design of the training schedule (eg. single versus multiple trial paradigms), may both be important factors in determining the magnitude of any drug-induced, sensorimotor or motivational impairment. Consequently, both factors may also influence the size of any spatial learning deficit obtained with D-AP5. Such an account, however, has great difficulty in explaining the D-AP5-induced deficit on the 'reversal' task (Morris et al., 1990a). D-AP5 treated rats were impaired relative to controls when trained to a new platform location in a familiar spatial environment (AP5 'different' versus aCSF 'different'). In contrast, a similar group of D-AP5 treated rats were unaffected when retention of the pre-operatively acquired platform location was assessed using the same experimental design (AP5 'same' versus aCSF 'same'). All the experimental subjects had extensive spatial training in the watermaze prior to drug administration and testing during the drug phase of the experiment involved just one training trial per day with an ITI of 24 hr. The pattern of results obtained with D-AP5 in this study is at odds with an account based on either watermaze experience, the number of training trials per session, or the length of the ITI. It is not clear why the sensorimotor or motivational side effects of D-AP5 infusion would be of more significance on the reversal task than during either the retention task or the two watermaze task. If one is to pursue a sensorimotor/motivational account of the effects of D-AP5 in the watermaze, then clearly other factors must be considered in order to explain the pattern of results obtained with D-AP5.

As previously mentioned, the fact that the deficit on the 'reversal' task was greater than that obtained during the two watermaze task is somewhat surprising. One possibility is that if the deficit is due to a drug-induced enhancement of fatigue, then the magnitude of the impairment may be related to the amount of time the rats spend in the water. During the reversal experiment the rats will begin by searching in the vicinity of the former platform location before searching elsewhere. Consequently, these animals may take longer to find the platform than animals for which this spatial environment is entirely novel. As a result of prior training, these animals have a spatial bias which will effectively hinder their search for the new platform location, resulting in them spending more time in the water. This may result in increased fatigue which, in turn, may then retard learning about the new platform location. In the two-watermaze task, watermaze experienced animals are placed in an entirely novel spatial environment during the drug phase of the experiment, and do not have

a strong spatial bias against the platform location. They, therefore, spend less time in the water on the early training trials and, consequently, the D-AP5 induced effects on fatigue may be less marked. This, in turn, could explain why D-AP5 animals appear less impaired on the two-watermaze task than on the 'reversal' task.

There are other possible explanations as to why D-AP5 might impair performance on the reversal task to a greater extent than during the two watermaze task. For example, it may be that finding that the platform is no longer where expected in a familiar spatial environment is more stressful than being placed in an unfamiliar spatial environment. It could be that D-AP5 treatment interacts with differences in the levels of anxiety arising from the two tasks to produce a bigger impairment on the reversal task than on the two-watermaze task. It is also worth pointing out that although in both the reversal study (Morris et al., 1990a) and the two-watermaze study (Expt 7.2), D-AP5 was infused at a dose of 30 mM, because no tissue levels were reported for the reversal study, it is possible that differences in the hippocampal whole tissue levels of D-AP5 obtained in these animals could account for the pattern of results.

It is certainly not impossible to explain the pattern of results obtained with D-AP5 treated animals, both in Expt 7.2 and other watermaze studies, in terms of non-mnemonic drug effects on sensorimotor and/or motivational processes. Furthermore, the considerable spatial learning displayed by the drug treated animals in Expt 7.2 may reflect the fact that the mechanisms of spatial learning remain relatively intact in D-AP5 treated animals, a conclusion which, of course, is in direct conflict with the hippocampal LTP/spatial learning hypothesis. However, such a conclusion may be somewhat premature and, in view of the ambiguous nature of the results of Expt 7.2, rather precarious.

Can support for the hippocampal LTP/spatial learning hypothesis be derived from this study?

The extent to which the D-AP5 group are able to learn about the spatial location of the platform, as measured by transfer test performance, combined with the absence of LTP in these animals, is certainly a result which represents a challenge for the hippocampal LTP/spatial learning hypothesis. In the extreme, this result could be taken as evidence to refute the hypothesis. However, to reject a hypothesis on the basis of a negative result is, at the best of times, premature. In this particular case, the result in question cannot even be

considered as negative in that the D-AP5 treated animals *were* impaired relative to controls on certain measures of performance. One should not forget that the D-AP5 treated animals took significantly longer to escape from the pool during training in watermaze 2, an impairment which was consistent across the 8 spatial training trials. Therefore, despite the fact that the D-AP5 treated animals do learn a considerable amount about the spatial location of the platform, they are still significantly impaired relative to controls. Surely to refute the hypothesis on the basis of these results would be grossly inappropriate.

Indeed, from a different viewpoint, the results of Expt 7.2 could be interpreted as supporting the hippocampal LTP/spatial learning hypothesis. Infusion of D-AP5 blocks LTP in the dentate gyrus of the hippocampal formation and produces a significant latency impairment when watermaze-experienced rats are trained to find an escape platform in a novel spatial environment. This result suggests that although prior watermaze training may result in a reduction in the magnitude of any spatial learning deficit, the D-AP5 treated animals are, nevertheless, still impaired during acquisition in a novel spatial environment. As reported in previous studies, D-AP5 appears to slow down the rate of learning without preventing new learning altogether (Davis et al., 1992). It may be the case that the effects of D-AP5 in retarding learning are more subtle than previously imagined and, as acquisition in watermaze 2 appears to be extremely rapid, the magnitude of any impairment obtained with D-AP5 is much smaller than expected. It is also possible that while this impairment shows up in terms of prolonged escape latencies during training, the 60 second probe trial analysis may be insufficiently sensitive to detect a more subtle spatial learning impairment. Some support for this intuition may be derived from the analysis of only the early part of trial 33 which did reveal a significant difference between the two groups in terms of the spatial bias towards the training quadrant.

One may, therefore, be able to derive some support for the hippocampal LTP/spatial learning hypothesis from these studies. Despite displaying considerable spatial learning, D-AP5 treated animals may still learn slower than controls in a novel spatial environment. In contrast, these animals do not show any deficit when retrieval is assessed with a final transfer test in the original pre-drug training environment (watermaze 1). These results could be interpreted as demonstrating impaired acquisition and spared retrieval in the same animals. This is an attractive result which would certainly make an invaluable contribution to the 'learning versus performance' debate. However, one criticism of this conclusion is that whereas the acquisition impairment is measured in terms of prolonged escape latencies

during training, the evidence for a sparing of retrieval is derived from a transfer test measure of performance. Indeed, when novel acquisition is assessed using transfer tests, there is little evidence of an impairment. One cannot rule out the possibility, therefore, that the dissociation between acquisition and retrieval simply reflects the use of different measures of performance.

Conclusions

The experiments described in this chapter of the thesis have involved the use of a two-watermaze task to examine the role of NMDA receptor-dependent LTP-like mechanisms in the hippocampus during spatial learning. The results of Expt 7.1 confirmed the intuition that acquisition in watermaze 2 is indeed hippocampal dependent. If the hippocampal LTP/spatial learning hypothesis is correct then blocking hippocampal LTP-like processes should prevent new spatial learning in watermaze 2. The results of Expt 7.2 were, however, somewhat ambiguous. The deficit in the D-AP5 treated animals was much smaller than might have been expected from previous watermaze studies (Morris et al., 1986; Morris et al., 1990; Davis et al., 1992). Although the D-AP5 treated animals consistently displayed prolonged escape latencies during training, they did learn a great deal about the spatial location of the platform as demonstrated by their performance in the transfer tests. There are two distinctly opposing interpretations of this data. One could argue that because D-AP5 treated animals are significantly impaired in terms of escape latencies, and, at the same time, show a block of LTP, that this result supports the hippocampal LTP/spatial learning hypothesis. Equally, however, because the D-AP5 group display considerable spatial learning in the absence of LTP, these results could be said to contradict the hypothesis. What is clear, is that the effects of D-AP5 infusion on spatial learning are much less severe than the effects of a hippocampal lesion. A comparison of the results obtained with hippocampal lesions (Expt 7.1), and those obtained with D-AP5 (Expt 7.2), demonstrates that infusion of the NMDA antagonist does not reproduce all the behavioural effects of a hippocampal lesion, and is likely to retard the rate of learning rather than prevent new learning completely.

The extent to which animals infused with D-AP5, at a dose sufficient to block LTP, were capable of learning about the spatial location of the platform was certainly surprising, and it could be argued that this reflects the fact that the mechanisms of spatial learning remain intact in these animals. Furthermore, it could also be argued that the subtle but detectable

sensorimotor impairment (the D-AP5 rats did display a prolonged righting reflex and often fell off the platform on early training trials) observed in these animals may account for what deficit there was. The possibility that the effects of D-AP5 on spatial learning in the watermaze, both in Expt 7.2 and previous studies, are due to a disruption of non-associative sensorimotor or motivational processes certainly cannot be ruled out. On the other hand, the presence of non-associative side effects in no way precludes the possibility that D-AP5 also directly disrupts the mechanisms of spatial learning, and that an effect on LTP-like processes in the hippocampus is responsible for the subtle behavioural impairment seen in Expt 7.2. It is also worth noting that the whole tissue levels of D-AP5 in the hippocampus resulting from the infusion of a 25 or 30 mM solution were well in excess of the concentrations required to block LTP (Davis et al., 1992). It remains to be seen whether or not a lower dose of D-AP5 can be found that still blocks LTP but spares spatial learning completely in terms of both escape latency and transfer test measures of performance

The two-watermaze task should, in theory, provide a purer assessment of spatial learning in the watermaze in that the rats are already extremely familiar with procedural aspects of performance. It was hoped that by using watermaze experienced animals, the possibility that the drug treatment might interfere with some non-spatial aspect of performance would be reduced. Indeed, the surprisingly small deficit seen with D-AP5 in Expt 7.2, relative to previous watermaze studies, may reflect the fact that extensive prior training in a different watermaze may have ameliorated any drug-induced effects on performance. This behavioural paradigm may, therefore, be of some importance for assessing the effects of drugs on spatial learning and memory in the watermaze.

Nevertheless, these experiments have failed to resolve fully the two major issues which they were designed to address. The results described in this chapter still do not allow us to establish whether the D-AP5 watermaze impairment is due to effects on learning or performance and, consequently, they do not provide a conclusive verdict as to the validity of the hippocampal LTP/spatial learning hypothesis.

In summary, the series of investigations that have been conducted in this laboratory investigating the effects of D-AP5 on a variety of behavioural tasks in the watermaze (including the experiments described in this chapter of the thesis) have, so far at least, failed to conclusively determine whether or not hippocampal NMDA receptor-dependent LTP-like processes are required for spatial learning. It is important, therefore, to investigate other experimental approaches as a means of testing the hippocampal LTP/spatial learning

hypothesis. Chapter 8 will examine other potential lines of evidence with the hope that they may elucidate the relationship between hippocampal LTP and spatial learning.

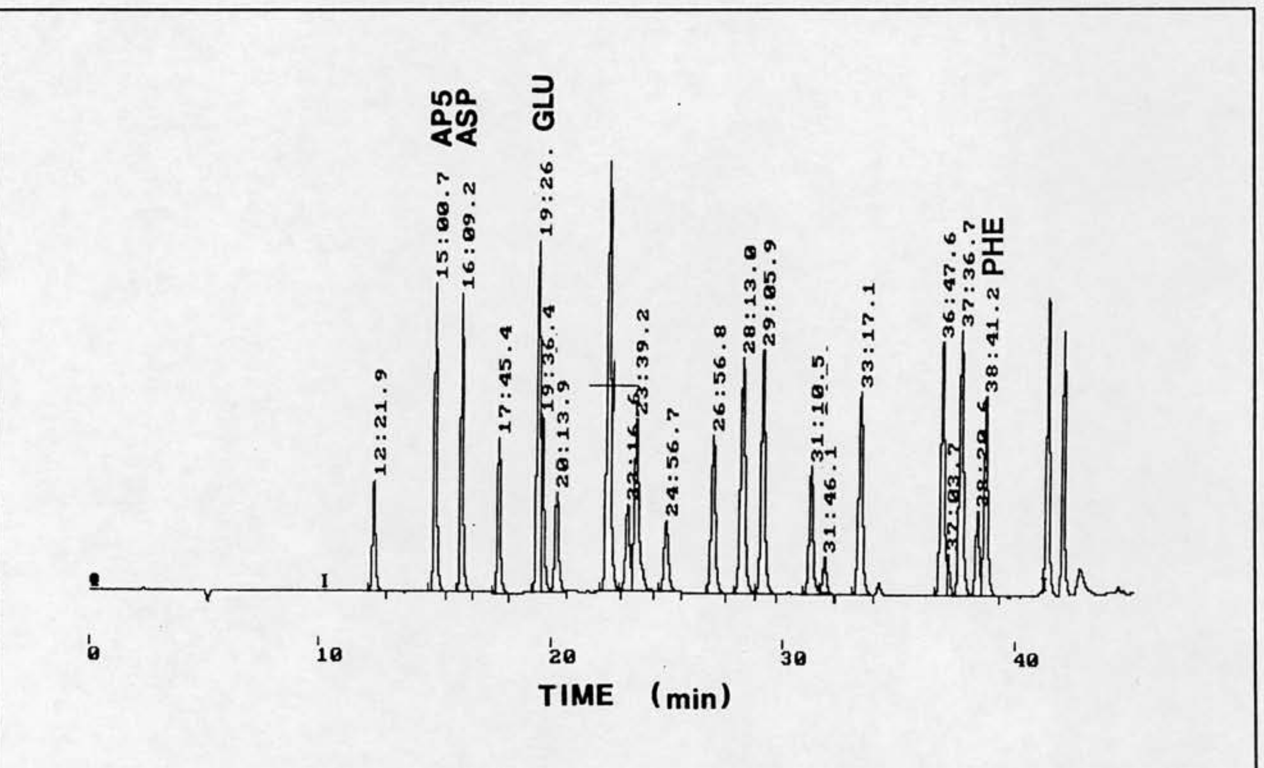


Fig. 7.1
The representative chromatogram above shows that AP5 can be separated easily from various amino acids (all compounds at 50 pmol).

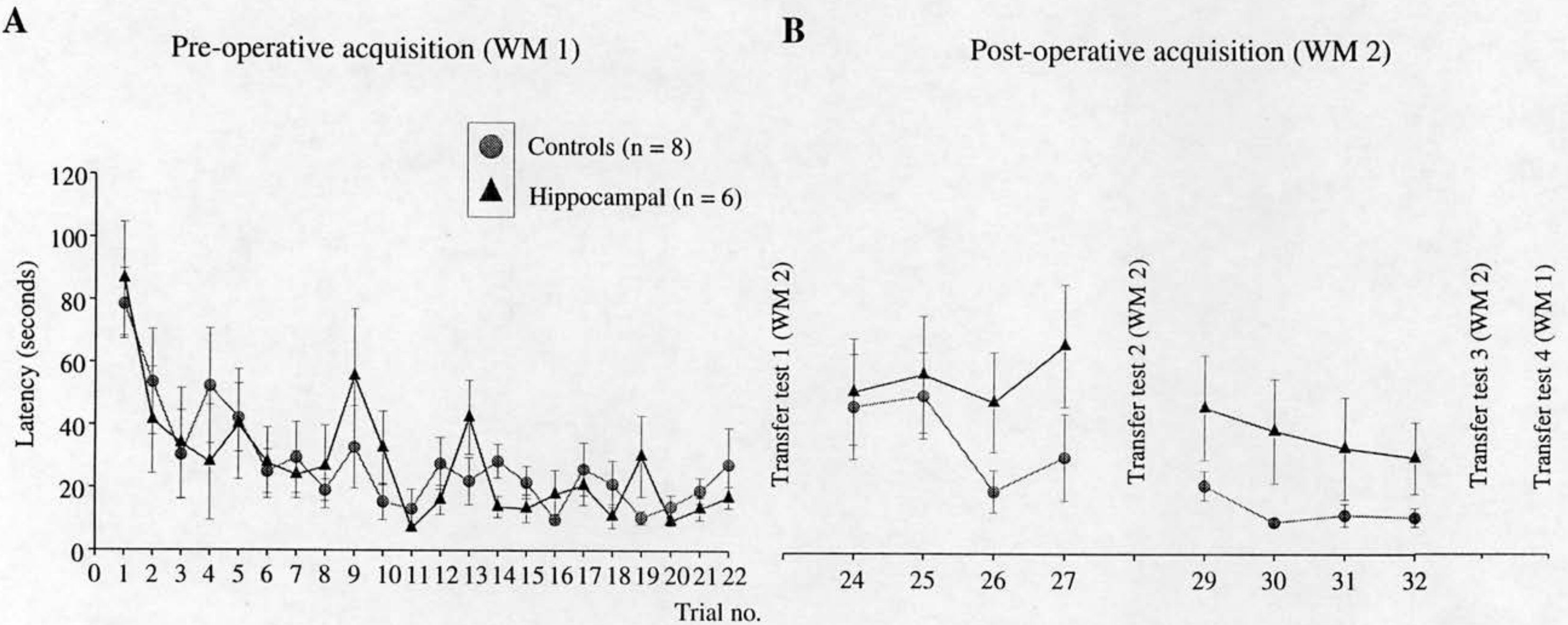


Fig. 7.1.1

Hippocampal lesions impair spatial learning in a novel environment in experienced animals previously trained in another watermaze. A - Mean escape latency (+/- 1SEM) during the initial spatial training in watermaze 1, prior to surgery. B - Mean escape latency (+/- 1SEM) during acquisition in watermaze 2, after surgery (Expt. 7.1).

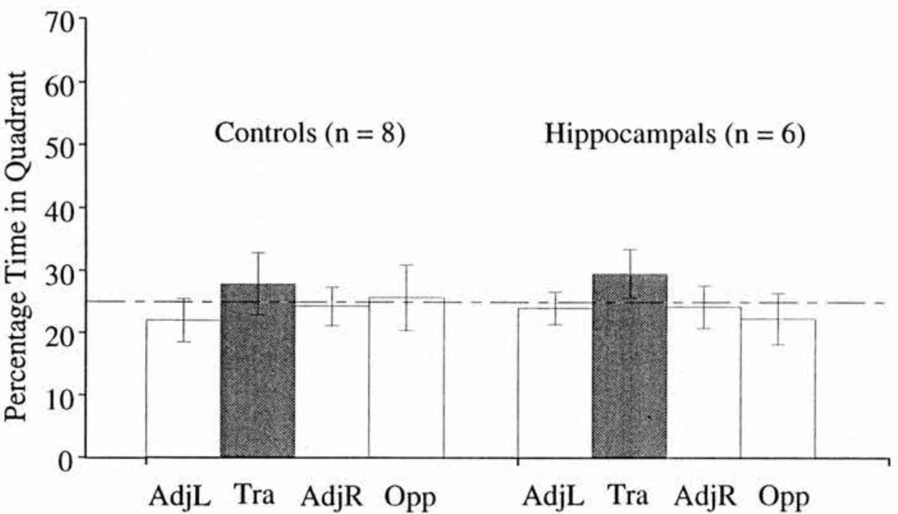


Fig. 7.1.2

The percentage time spent in each quadrant of the pool during the full 60 sec of the first transfer test in watermaze 2 (trial 23; Expt 7.1). (AdjL = quadrant adjacent to, and to the left of the quadrant to which the animals were trained; Tra = the training quadrant; AdjR = quadrant adjacent to, and to the right of the training quadrant; Opp = the quadrant opposite the training quadrant). The data was analysed with respect to the 'future' training quadrant in watermaze 2.

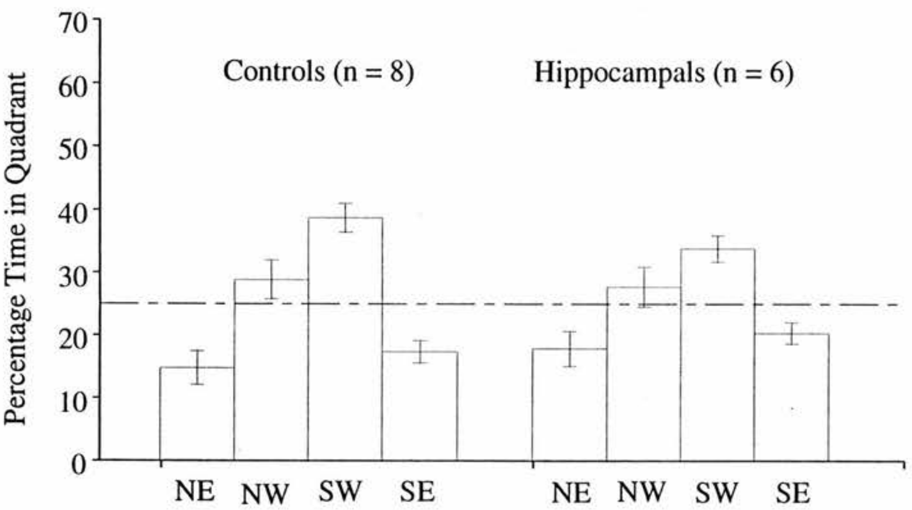


Fig 7.1.3

The percentage time spent in each quadrant of the pool during the full 60 seconds of the first transfer test in watermaze 2 (trial 23; Expt 7.1). The data was analysed with respect to compass directions, with no regard to any 'future' training quadrant. Both groups show a bias to the SW quadrant.

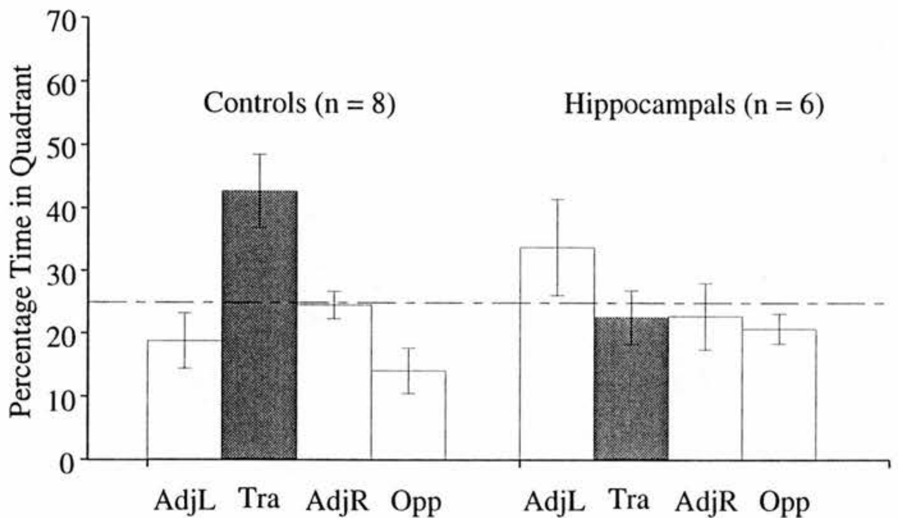


Fig. 7.1.4

The percentage time spent in each quadrant of the pool during the 60 sec transfer test, conducted after 4 training trials in watermaze 2 (trial 28; Expt 7.1). (AdjL = quadrant adjacent to, and to the left of the quadrant to which the animals were trained; Tra = the training quadrant; AdjR = quadrant adjacent to, and to the right of the training quadrant; Opp = the quadrant opposite the training quadrant).

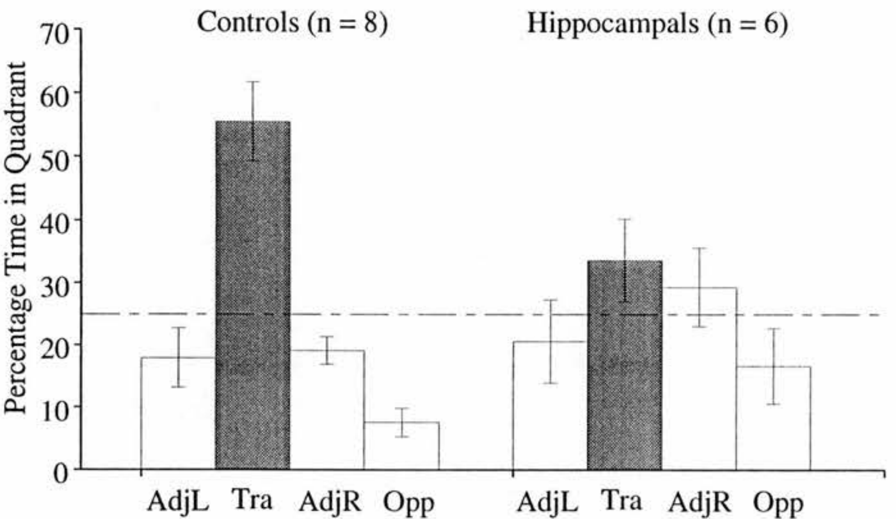


Fig. 7.1.5

The percentage time spent in each quadrant of the pool during the 60 sec transfer test, conducted after 8 training trials in watermaze 2 (trial 33; Expt 7.1). (AdjL = quadrant adjacent to, and to the left of the quadrant to which the animals were trained; Tra = the training quadrant; AdjR = quadrant adjacent to, and to the right of the training quadrant; Opp = the quadrant opposite the training quadrant).

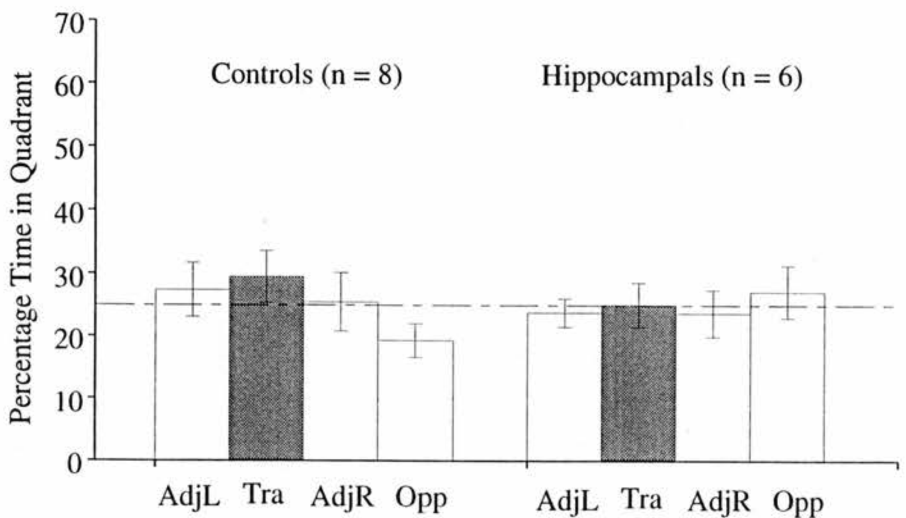


Fig. 7.1.6

The percentage time spent in each quadrant of the pool during the full 60 sec of the transfer test in watermaze 1, conducted after training in watermaze 2 (trial 34; Expt 7.1). (AdjL = quadrant adjacent to, and to the left of the quadrant to which the animals were trained; Tra = the training quadrant; AdjR = quadrant adjacent to, and to the right of the training quadrant; Opp = the quadrant opposite the training quadrant).

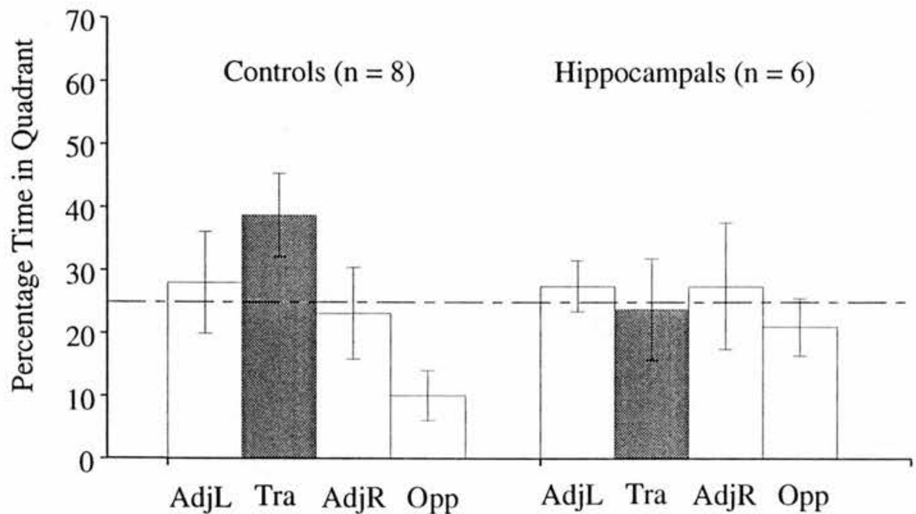


Fig. 7.1.7

The percentage time spent in each quadrant of the pool during the first 15 sec of the transfer test in watermaze 1, conducted after training in watermaze 2 (trial 34; Expt 7.1). (AdjL = quadrant adjacent to, and to the left of the quadrant to which the animals were trained; Tra = the training quadrant; AdjR = quadrant adjacent to, and to the right of the training quadrant; Opp = the quadrant opposite the training quadrant).

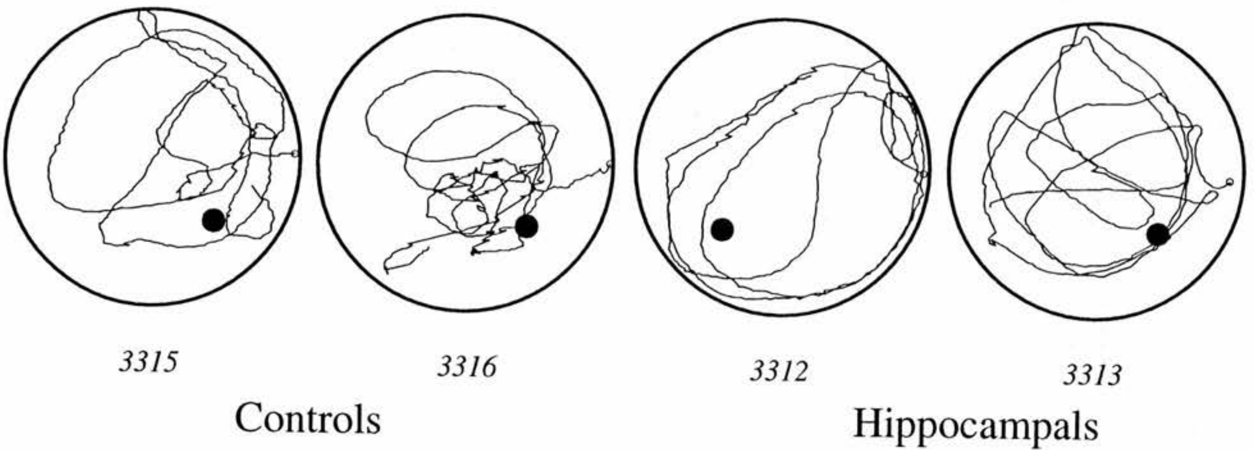


Fig. 7.1.8

During the transfer test the platform is removed from the pool and the rat allowed to swim freely for 60 seconds. The above are representative pathways of subjects taken from both experimental groups (Expt 7.1). The swim path taken by rat no. 3316 suggests that some of the control rats do show some recall of the platform position in watermaze 1 (trial 34).

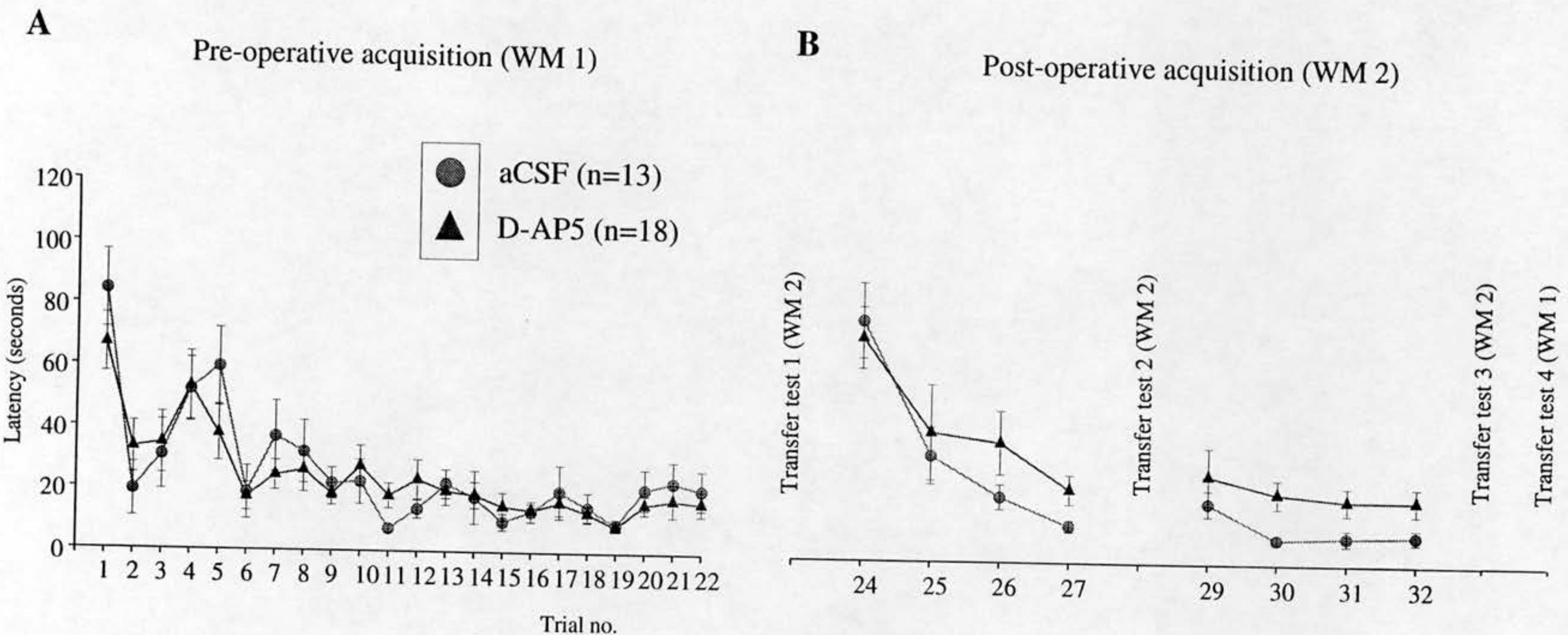


Fig. 7.2.1

The effects of D-AP5 on spatial learning in a novel environment in experienced animals previously trained in another watermaze. A - Mean escape latency (+/- 1SEM) during the initial spatial training in watermaze 1, prior to surgery. B - Mean escape latency (+/- 1SEM) during acquisition in watermaze 2, after surgery (Expt. 7.2).

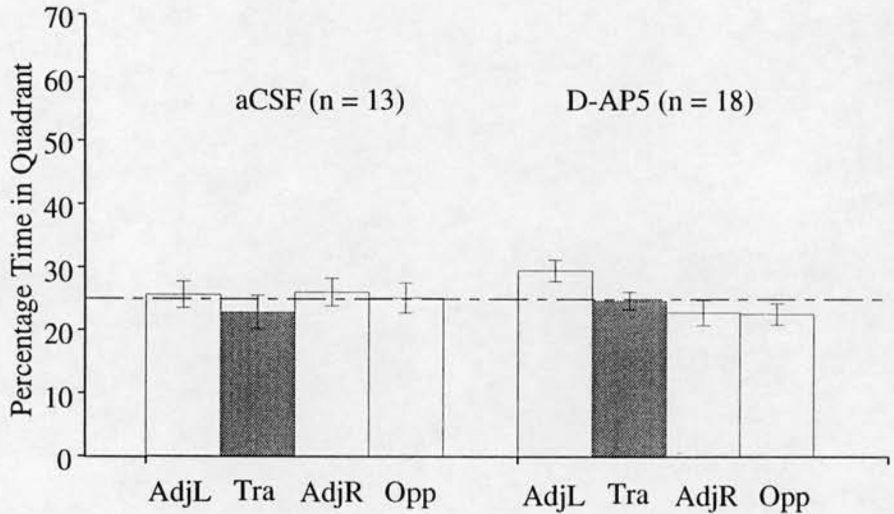


Fig. 7.2.2

The percentage time spent in each quadrant of the pool during the full 60 sec of the first transfer test in watermaze 2 (trial 23; Expt 7.2). (AdjL = quadrant adjacent to, and to the left of the quadrant to which the animals were trained; Tra = the training quadrant; AdjR = quadrant adjacent to, and to the right of the training quadrant; Opp = the quadrant opposite the training quadrant). The data was analysed with respect to the 'future' training quadrant in watermaze 2.

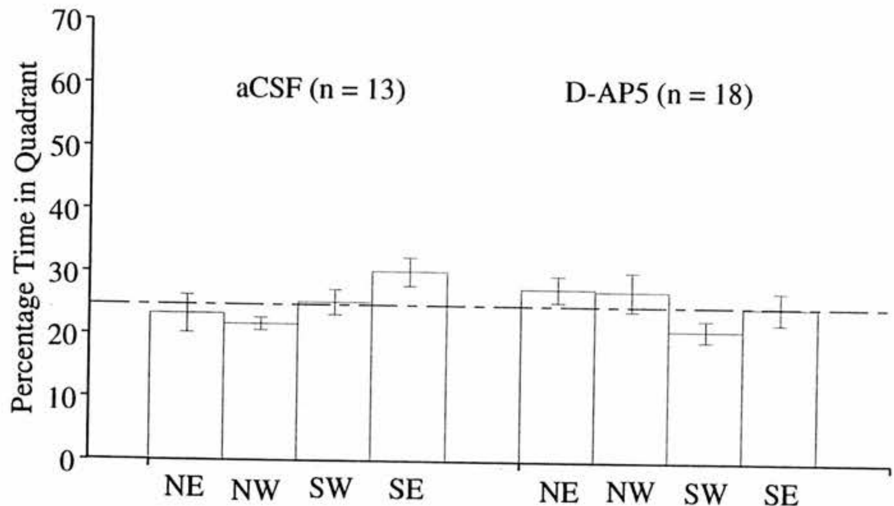


Fig 7.2.3

The percentage time spent in each quadrant of the pool during the full 60 seconds of the first transfer test in watermaze 2 (trial 23; Expt 7.2). The data was analysed with respect to compass directions, with no regard to any 'future' training quadrant.

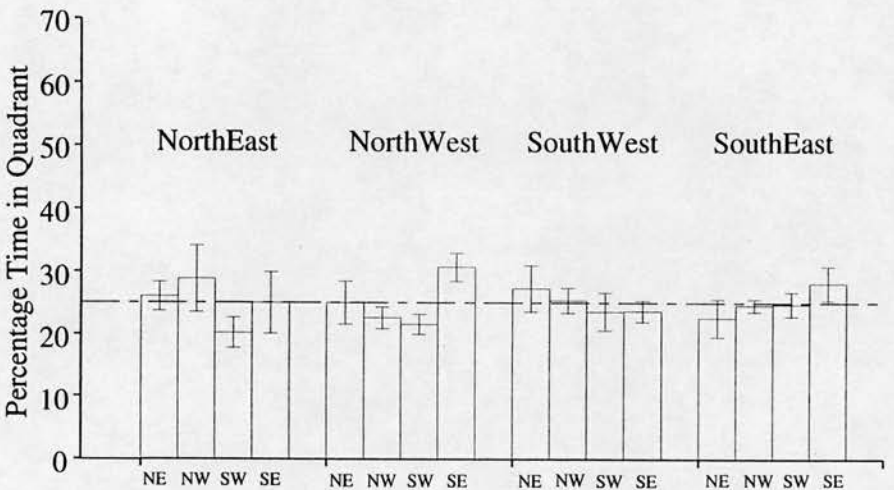


Fig. 7.2.4

All the animals involved in this study (Expt 7.2; n = 31) were divided into 4 groups according to the location of the platform during training in watermaze 1 (the 4 groups shown above). These four groups were then compared with respect to the amount of time spent in the 4 quadrants of watermaze 2 during trial 23 (x-axis above).

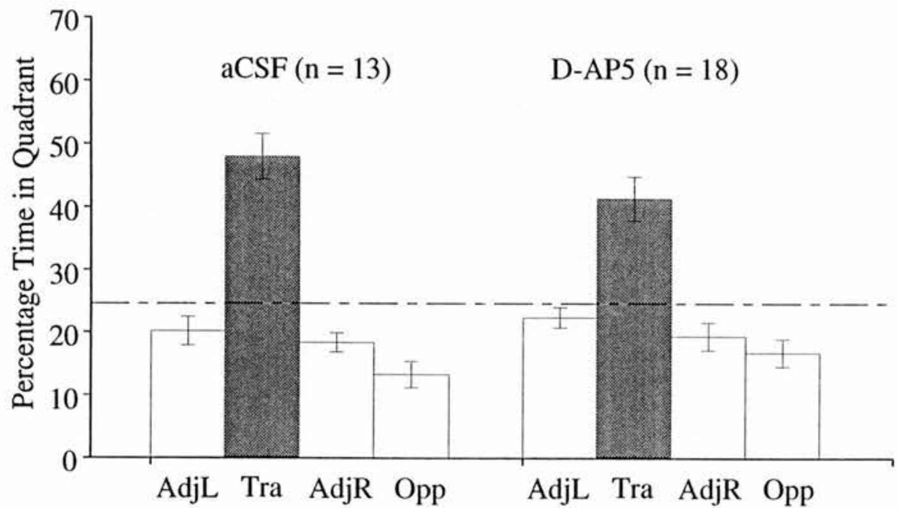


Fig. 7.2.5

The percentage time spent in each quadrant of the pool during the 60 sec transfer test, conducted after 4 training trials in watermaze 2 (trial 28; Expt 7.2). (AdjL = quadrant adjacent to, and to the left of the quadrant to which the animals were trained; Tra = the training quadrant; AdjR = quadrant adjacent to, and to the right of the training quadrant; Opp = the quadrant opposite the training quadrant).

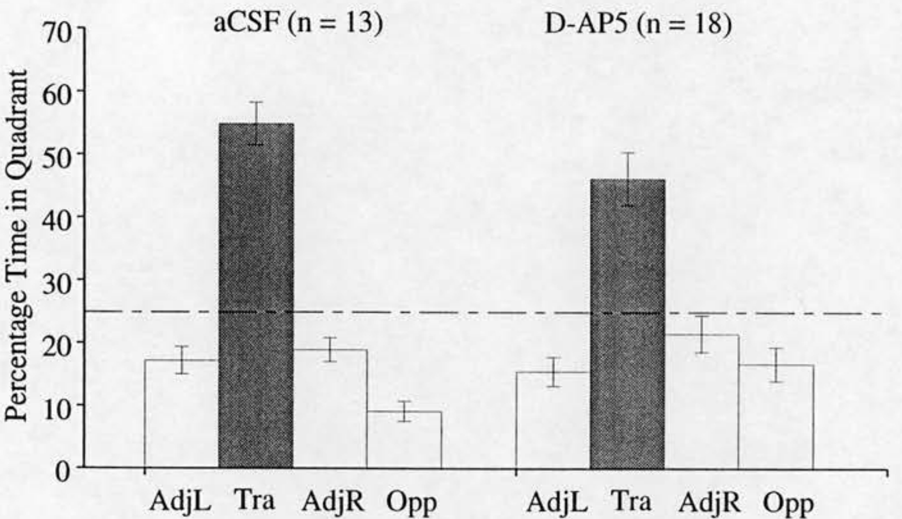


Fig. 7.2.6

The percentage time spent in each quadrant of the pool during the 60 sec transfer test, conducted after 8 training trials in watermaze 2 (trial 33; Expt 7.2). (AdjL = quadrant adjacent to, and to the left of the quadrant to which the animals were trained; Tra = the training quadrant; AdjR = quadrant adjacent to, and to the right of the training quadrant; Opp = the quadrant opposite the training quadrant).

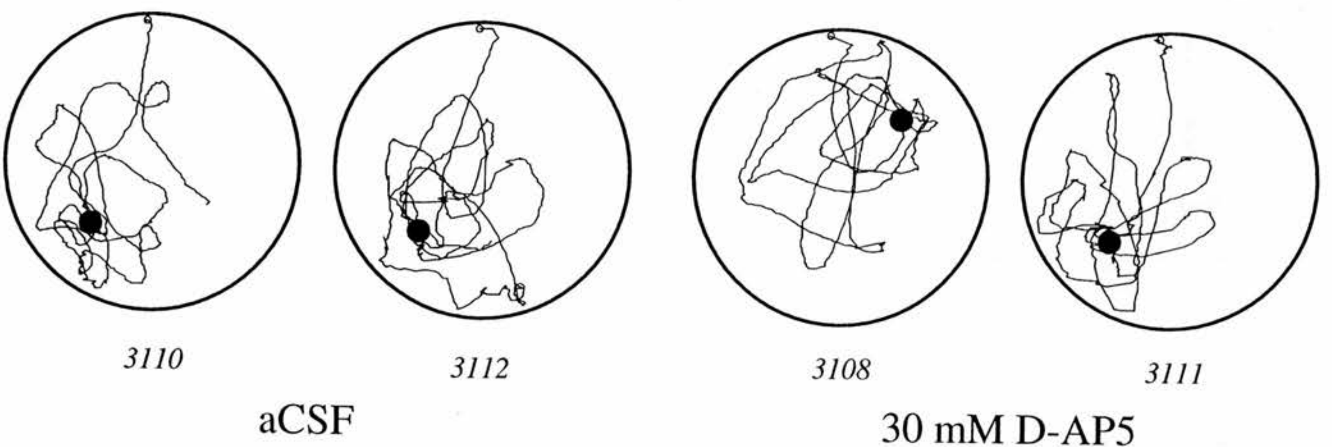


Fig. 7.2.7

The above are representative pathways of subjects from both groups during the 60 transfer test, conducted after 8 trials in watermaze 2 (trial 33; Expt 7.2).

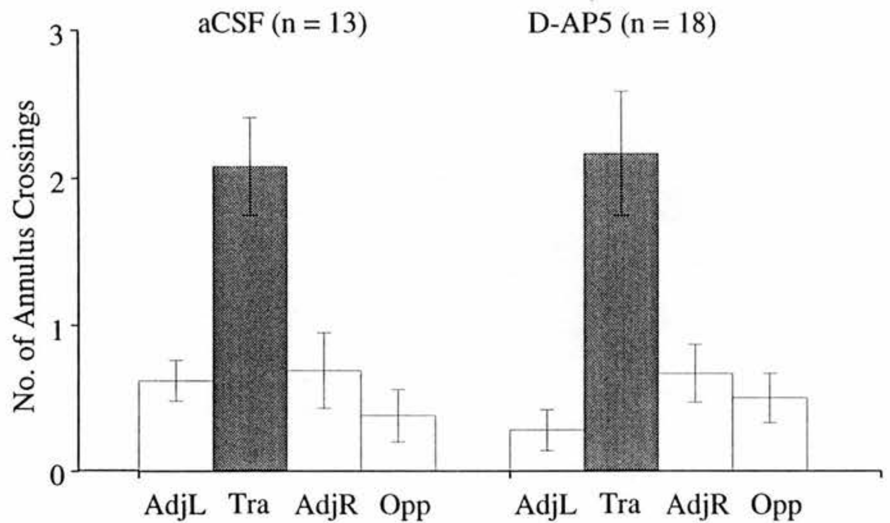


Fig 7.2.8

The number of annulus crossings made in each quadrant of the pool during the transfer test, conducted after 4 training trials in watermaze 2 (trial 28; Expt 7.2). (AdjL = quadrant adjacent to, and to the left of the quadrant to which the animals were trained; Tra = the training quadrant; AdjR = quadrant adjacent to, and to the right of the training quadrant; Opp = the quadrant opposite the training quadrant).

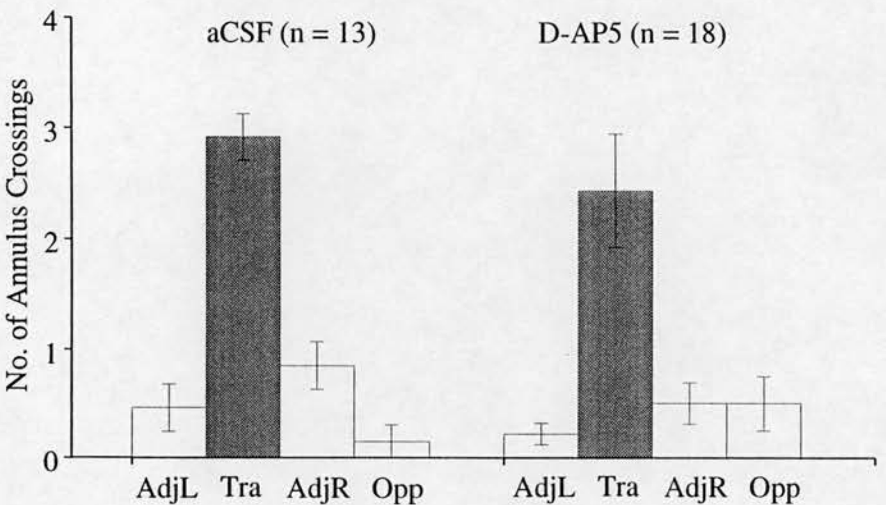


Fig 7.2.9

The number of annulus crossings made in each quadrant of the pool during the transfer test, conducted after 8 training trials in watermaze 2 (trial 33; Expt 7.2). (AdjL = quadrant adjacent to, and to the left of the quadrant to which the animals were trained; Tra = the training quadrant; AdjR = quadrant adjacent to, and to the right of the training quadrant; Opp = the quadrant opposite the training quadrant).

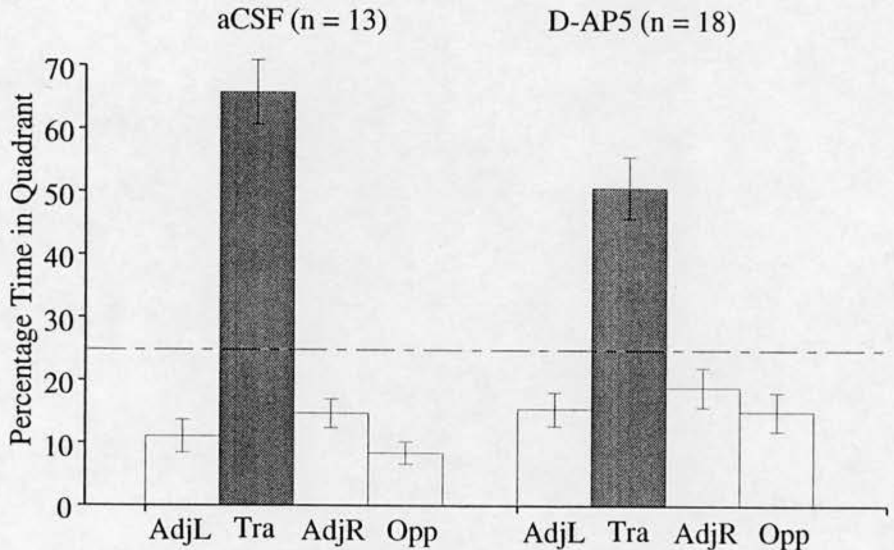


Fig. 7.2.10

The percentage time spent in each quadrant of the pool during the first 30 sec of the transfer test, conducted after 8 training trials in watermaze 2 (trial 33; Expt 7.2). (AdjL = quadrant adjacent to, and to the left of the quadrant to which the animals were trained; Tra = the training quadrant; AdjR = quadrant adjacent to, and to the right of the training quadrant; Opp = the quadrant opposite the training quadrant).

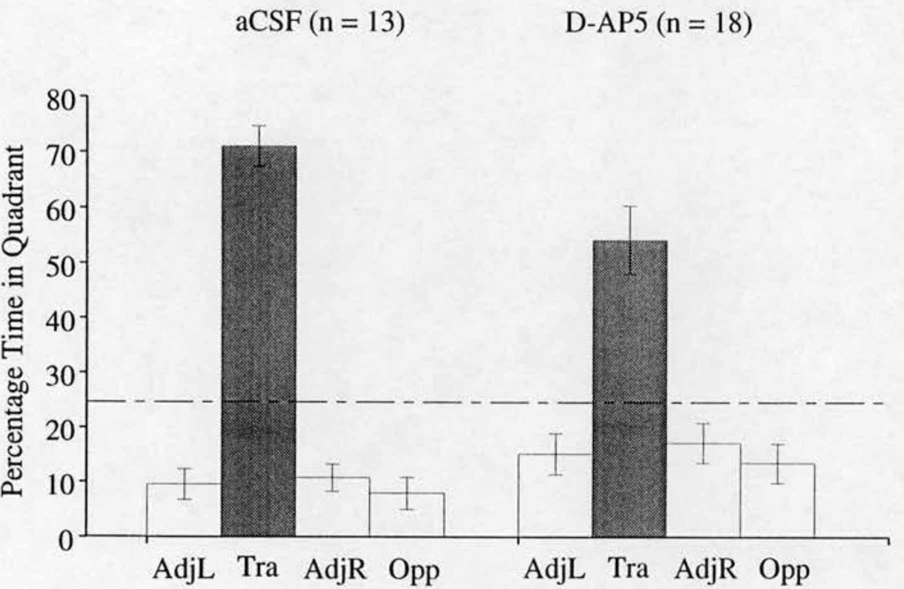


Fig. 7.2.11

The percentage time spent in each quadrant of the pool during the first 15 sec of the transfer test, conducted after 8 training trials in watermaze 2 (trial 33; Expt 7.2). (AdjL = quadrant adjacent to, and to the left of the quadrant to which the animals were trained; Tra = the training quadrant; AdjR = quadrant adjacent to, and to the right of the training quadrant; Opp = the quadrant opposite the training quadrant).

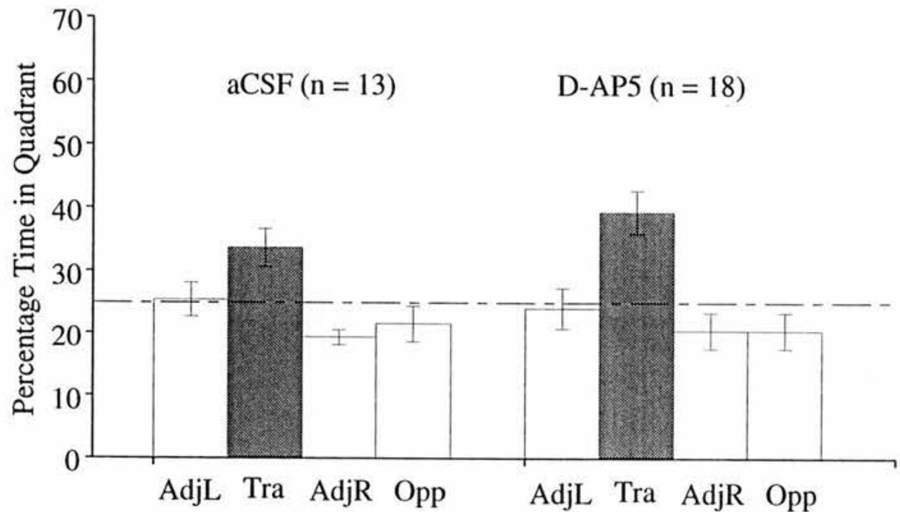


Fig. 7.2.12

The percentage time spent in each quadrant of the pool during the full 60 sec of the transfer test in watermaze 1, conducted after training in watermaze 2 (trial 34; Expt 7.2). (AdjL = quadrant adjacent to, and to the left of the quadrant to which the animals were trained; Tra = the training quadrant; AdjR = quadrant adjacent to, and to the right of the training quadrant; Opp = the quadrant opposite the training quadrant).

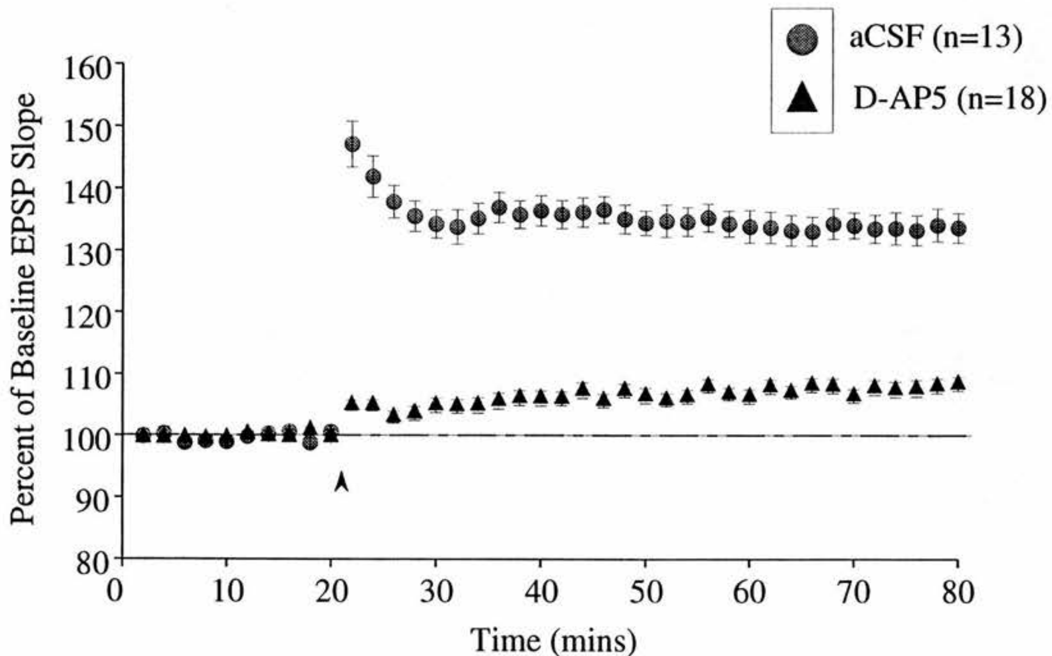


Fig. 7.2.13

The chronic infusion of D-AP5 blocks LTP of the field EPSP slope in the dentate gyrus *in vivo* (Expt 7.2). (⋏ = tetanus).

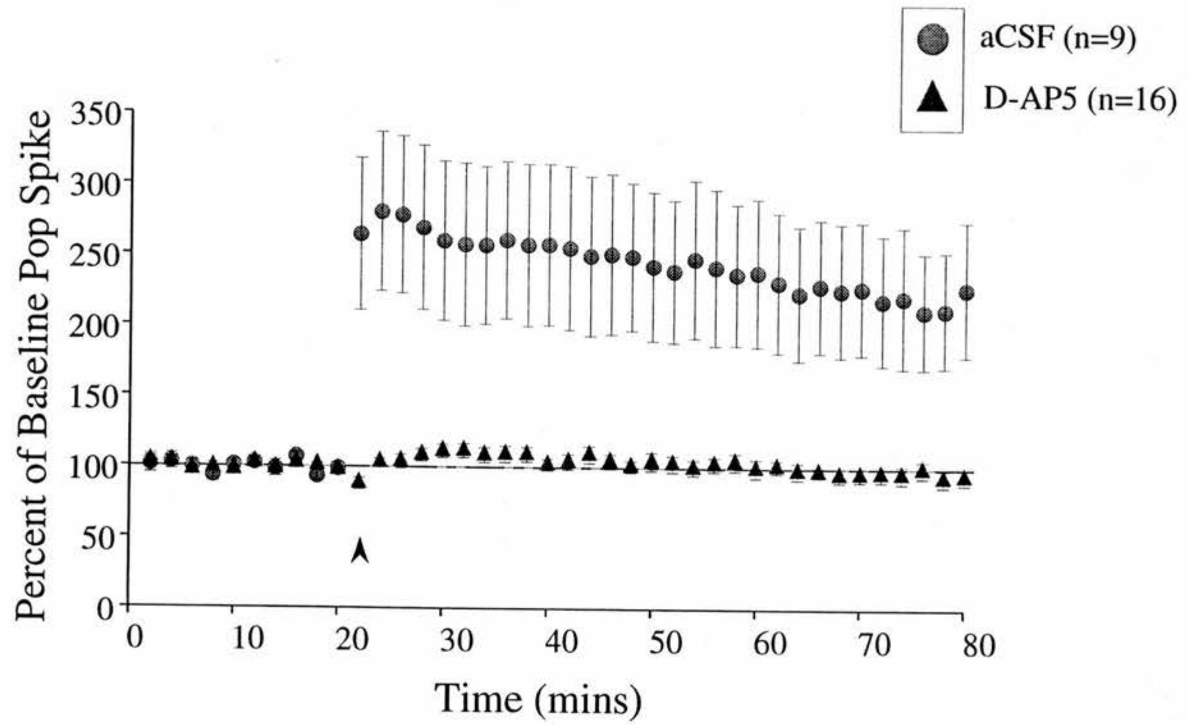


Fig. 7.2.14

The chronic infusion of D-AP5 blocks LTP of the population spike in the dentate gyrus *in vivo* (Expt 7.2). (⋏= tetanus).

Chapter 8 - General Discussion

The aim of this thesis was to investigate the relationship between hippocampal LTP and spatial learning. The experiments were designed to test the hypothesis that LTP-like events in the hippocampus contribute to the mechanisms of spatial learning by assessing (i) the effects on spatial learning of manipulating the inducability of LTP through a site on the NMDA receptor complex (the glycine site), (ii) the effects on spatial learning of preventing the development and expression of LTP at a site downstream from the NMDA receptor (the synthesis of the putative retrograde messenger, NO), and (iii) the effects of the competitive NMDA antagonist, D-AP5, on spatial learning using a different watermaze protocol from those adopted in previous studies.

The first set of experiments (Chapter 4) investigated the effects of manipulating the NMDA receptor-associated glycine site on spatial learning in the watermaze. Chronic intraventricular infusion of the glycine receptor antagonist, 7-Chlorokynurenate (7CK), impaired performance during the acquisition of a standard spatial reference memory task but failed to affect the induction of LTP at perforant path-granule cell synapses *in vivo* in the same animals. This dissociation does not compromise the hippocampal LTP/spatial learning hypothesis. There are a number of ways in which a drug might influence performance on a spatial learning task in the watermaze without disrupting the learning mechanism *per se*. These results in no way exclude a role for hippocampal LTP-like events in the mechanisms of spatial learning. The failure of this infusion protocol to block LTP in the hippocampus means that these experiments are of no use for assessing the importance of LTP-like processes in this brain area with regard to spatial learning.

In the final experiment of Chapter 4, D-cycloserine (DCS) caused a subtle enhancement of spatial learning in the watermaze. This result is consistent with an involvement of NMDA receptor-mediated activity during spatial learning. Further studies are required, however, to determine (i) whether or not this facilitation is due to an effect on LTP-like processes, and (ii) whether the effects of DCS are specific to hippocampal-dependent forms of learning or whether this facilitation is part of a more global performance enhancement.

The experiments described in Chapters 5 and 6 showed that inhibition of NO synthase (NOS) neither prevented the induction of LTP in the dentate gyrus *in vivo* nor resulted in a selective spatial learning impairment. The i.p. injection of an NOS inhibitor (L-NAME) did have profound effects on the cerebrovascular system (see Appendix B) and resulted in a reduction in the size of evoked field potentials recorded in the dentate gyrus. In addition, L-NAME caused a transient impairment of performance on both hippocampal-dependent and

-independent learning tasks in the watermaze early in training when training schedules with blocks of multiple trials were used. Under conditions where the animals received just one training trial per day, however, spatial learning proceeded unimpaired in L-NAME-injected rats. This pattern of results suggests that any deficit is unlikely to be due to a direct effect on the learning mechanism. As in the previous experiments with 7CK, the failure of L-NAME to prevent the development of LTP precludes any investigation into the relationship between hippocampal LTP and spatial learning.

In a final series of experiments (Chapter 7), a manipulation was chosen which is known to block the induction of hippocampal LTP *in vivo*. A further investigation of the effects of the competitive NMDA antagonist, D-AP5, on spatial learning in the watermaze was conducted using a behavioural task involving two separate watermazes in two distinct spatial environments. The effects of D-AP5 on spatial learning were assessed in watermaze experienced animals that had previously received extensive training, in the absence of any drug treatment, on a standard spatial reference memory task in watermaze 1. These animals were already well practised with the procedural aspects of the watermaze task before being exposed to the drug treatment. D-AP5 treatment resulted in prolonged escape latencies during training in a novel spatial environment (watermaze 2). However, these animals displayed considerable knowledge of the platform location when learning was assessed by means of two 60 sec probe trials. Indeed, the performance of the D-AP5 group was not significantly different from the aCSF group during the two 60 sec transfer tests conducted in watermaze 2. Both the aCSF and D-AP5 groups showed equivalent retrieval of the platform location in the original spatial environment used during pre-drug training (watermaze 1).

The results of Chapter 7 are somewhat ambiguous and could be interpreted as providing evidence either for or against the hippocampal LTP/spatial learning hypothesis. On the one hand, because D-AP5 impairs performance during spatial learning in watermaze 2, and prevents the induction of LTP in the dentate gyrus in these same animals, this result could be supportive. It may well be that D-AP5 retards the rate of learning through a blockade of LTP-like processes, but does not prevent spatial learning completely, a result consistent with previous studies (Morris et al., 1986a; Davis et al., 1992). On the other hand, although the D-AP5 treated animals took longer to escape from the water during training, the extent to which these animals learned about the spatial location of the platform was both considerable and surprising, and could reflect the fact that the mechanisms responsible for spatial learning remain essentially intact in these animals. In addition, there was some evidence of a

sensorimotor impairment in these rats. It is possible that this could contribute in some way to the prolonged escape latencies during training in watermaze 2.

Studies conducted using the competitive NMDA antagonist, AP5, constitute a central part of the investigation into the putative role of LTP-like processes during spatial learning. The potential problems associated with these studies have been discussed at length. The results described in Chapter 7, while making an important contribution to our knowledge of the behavioural syndrome that results from AP5 infusion, do not, however, allow us to separate possible effects of the drug treatment on learning from possible effects on performance. Consequently, these studies do not allow us to determine conclusively, one way or the other, whether or not NMDA receptor-dependent LTP-like processes in hippocampus constitute the neural mechanisms of spatial learning. In light of this, alternative experimental approaches to test the hippocampal LTP/spatial learning hypothesis must be sought. D-AP5 infusion is by no means the only experimental manipulation that has been used to investigate the relationship between hippocampal LTP and spatial learning. This final chapter will examine other potential lines of evidence that may provide support for or against the hippocampal LTP/spatial learning hypothesis. Both pharmacological and non-pharmacological studies will be discussed in an attempt to evaluate the current standing of the hippocampal LTP/spatial learning hypothesis.

Non-competitive NMDA antagonists and spatial learning

The NMDA receptor complex is a large protein with several distinct ligand binding sites. There are, therefore, a number of possible ways in which the activity of this receptor may be manipulated. Attempts to modulate the inducibility of LTP by means of glycine receptor ligands have already been discussed (see Chapter 4). An obvious alternative is to block NMDA receptor activity via the PCP binding site which is located deep in the ion channel of the NMDA receptor complex (Lodge and Anis, 1982; Kemp et al., 1987a). As previously mentioned, non-competitive NMDA receptor antagonists, including PCP and MK-801 (dizoclipine), have been shown to block the induction of LTP in the hippocampus, both *in vitro* (Stringer et al., 1984; Swartzwelder et al., 1989) and *in vivo* (Stringer and Guyenet, 1983; Abraham and Mason, 1988). These compounds potentially provide an attractive means of assessing the effects of blocking NMDA receptor activity on spatial learning in that they readily cross the blood brain barrier and thus can be administered systemically.

The non-competitive NMDA antagonist, MK-801, causes a striking and reliable impairment of performance on spatial learning tasks in the watermaze (Halliwell and Morris, 1987; Mondadori et al., 1989; Robinson et al., 1989; Wishaw and Auer, 1989; Heale and Harley, 1990). Taken at face value, these results are consistent with the hippocampal LTP/spatial learning hypothesis. There is considerable debate, however, concerning (i) whether or not the performance deficit is due to a selective effect on learning, and (ii) whether or not this impairment occurs at doses which also block the induction of LTP (see Keith and Rudy, 1990; Gallagher, 1990; Morris, 1990). A key issue is whether or not doses of non-competitive NMDA antagonists can be found which block LTP *in vivo* but, at the same time, cause only a minimal sensorimotor disturbance, and thus allow the animal to behave 'normally' during testing. Of particular concern are the marked sensorimotor impairments and stereotypical behaviour which accompany the administration of non-competitive NMDA antagonists (Contreras et al., 1986; Compton et al., 1987; Koek et al., 1987; Contreras et al., 1988; Mondadori et al., 1989; Tricklebank et al., 1989). In general, these unwanted side effects appear to be more pronounced for non-competitive NMDA antagonists than for competitive antagonists such as AP5. As with the AP5 studies, it is important to determine whether the MK-801 induced behavioural impairment in the watermaze is due to an effect on 'learning' or 'performance'.

A number of laboratories have investigated the effects of systemic administration of MK-801 on performance in the watermaze. For example, Mondadori et al. (1989) tested the effects of three doses of MK-801 (administered i.p. 30 min prior to testing) on spatial learning in the watermaze in gerbils. They found that a 0.1 mg/kg dose had no obvious behavioural side effects but did not produce a significant spatial learning impairment. A 0.3 mg/kg dose did impair spatial learning but also produced hyperactivity and a moderate ataxia. Animals given a 1.0 mg/kg dose of MK-801 were severely ataxic and untestable in the watermaze. The results of this study indicate that performance in the watermaze is only impaired at doses of MK-801 which also cause sensorimotor dysfunction. However, in a separate study, Robinson and colleagues (Robinson et al., 1989) found that MK-801 (0.05 - 0.08 mg/kg administered subcutaneously 20 min prior to testing) impaired spatial learning in rats in the watermaze but was without effect on the retrieval of previously acquired spatial information. The higher dose of MK-801 (0.08 mg/kg) also impaired performance on a visible platform task ('cue' task) and resulted in unusual swimming patterns, suggesting that at this dose, MK-801 may cause a more general impairment in performance. Nevertheless, the 0.05 mg/kg dose of MK-801 impaired performance on the spatial learning task while leaving

performance on the cue task intact in the same animals. It should be noted that the behavioural dissociation obtained in this study may simply reflect the order in which the tasks were run. The cue task was always run after the spatial task and the lack of an effect on the non-spatial paradigm may simply be a consequence of prior watermaze training. However, Wishaw and Auer (1989) have also found that MK-801 (0.05 - 0.10 mg/kg, intravenous) impairs the acquisiton of a new platform location in a familiar environment but is without effect on retrieval of a well learned platform location, or on a cue task. Similarly, Heale and Harley (1990) observed a deficit in acquisition but not on retrieval using a comparable dose of MK-801 (0.07 mg/kg i.p., approx 35 min prior to testing). It should be pointed out that there was some evidence of a sensorimotor impairment, even with the lowest doses of the drug (0.05 - 0.10 mg/kg; Wishaw and Auer, 1989).

In summary, MK-801 disrupts spatial learning in the watermaze but also causes pronounced sensorimotor impairments. It is possible, therefore, that the deficits in the watermaze observed with MK-801 may be due to non-mnemonic effects of the drug treatment. There is some evidence, however, that a dose of MK-801 (approx 0.05 mg/kg) may be found which impairs spatial learning but leaves cue learning and the retrieval of previously acquired spatial information unaffected. This pattern of results is similar to that obtained with the competitive NMDA antagonist, D-AP5, and goes some way to arguing against a gross sensorimotor or motivational account. Nevertheless, as is also the case with D-AP5, dissociating effects on learning from effects on sensorimotor or motivational aspects of performance following the systemic administration of non-competitive NMDA antagonists would appear to be extremely difficult.

Irrespective of whether the MK-801 induced watermaze impairment is due to an effect on learning or performance, it seems unlikely that these lower doses of MK-801 (eg. 0.05 mg/kg i.p., 30 min prior to testing), that are free from gross sensorimotor side effects, and that allow the animals to be tested on learning and memory tasks, are sufficient to prevent the induction of LTP *in vivo*. Abraham and Mason (1988) have shown that a much higher dose of MK-801 (1.0 mg/kg) is required to prevent the induction of LTP in the dentate gyrus in anaesthetised rats and that, even then, a blockade of LTP is only seen some 150 min after drug administration. Furthermore, at these doses, MK-801 also affects low-frequency neurotransmission. The slow onset of this effect on LTP can be attributed to the use-dependent nature of the NMDA receptor channel block seen with these compounds (Kemp et al., 1987a). MK-801 can only gain access to the ion channel after the Mg^{2+} block has been

removed following a period of activity. These near lethal, higher doses of MK-801, which are required to block hippocampal LTP, are accompanied by a pronounced sensorimotor disturbance and stereotypical behaviour which renders the animal untestable. Conversely, it seems extremely unlikely that the lower doses (0.05 mg/kg) used in some of the behavioural studies are sufficient to block LTP (see also Halliwell and Morris, 1987).

There is, however, one proviso. Both Abraham and Mason (1988), and Halliwell and Morris (1987), examined the effects of MK-801 on LTP induction in anaesthetised animals. It has been suggested that the block of LTP may develop faster in unanaesthetised animals (Gallagher, 1990). This is not an unreasonable suggestion bearing in mind that there will be considerably more neuronal activity in an awake animal, which could potentially facilitate the rate at which MK-801 can gain access to the ion channel. To date, there is only one published report investigating the effects of MK-801 on LTP induction in awake animals (Gilbert and Mack, 1990). Gilbert and Mack delivered a high frequency tetanus just 20 min after administering 0.1 or 1.0 mg/kg MK-801 i.p. to unanaesthetised rats. Both doses blocked potentiation of the population spike suggesting that lower doses might indeed be more effective in awake animals. Unfortunately, no LTP of the EPSP slope was obtained in the control rats, and so it remains to be seen whether or not this result holds true for potentiation of the synaptic component of the evoked response. It is also worth noting that the higher dose of MK-801 also produced a decrease in the size of the baseline EPSP slope values. Further studies are required to determine whether or not a 0.05 mg/kg dose of MK-801 will prevent the induction of slope LTP in unanaesthetised rats.

In summary, the use of non-competitive NMDA antagonists, as a means of testing the hippocampal LTP/spatial learning hypothesis, adds little, if anything, to what is known as a result of using the competitive NMDA antagonist, AP5. Studies in which MK-801 and PCP have been used, are complicated by the use-dependent nature of the NMDA receptor blockade and by the sensorimotor side effects of these drugs at doses required to block LTP. It appears that the sensorimotor side effects associated with doses of AP5 required to block LTP are less pronounced than those associated with doses of MK-801 required to achieve the same result. Indeed, concerns have been voiced that it is unlikely that a dose of MK-801 can be found which blocks LTP and also permits the animal to behave 'normally' during testing (Keith and Rudy, 1990; Morris, 1990).

It is not clear whether the more pronounced sensorimotor impairments that occur with non-competitive NMDA antagonists, relative to those obtained with AP5, are due to (i) the route

of administration commonly used (i.p. versus i.c.v), (ii) a difference in the functional consequence between competitive and non-competitive NMDA receptor antagonism, or (iii) NMDA receptor-independent side effects of these compounds. With regard to the first possibility, it is possible that by giving these compounds i.p., NMDA receptors in the spinal cord are being affected, resulting in a more pronounced sensorimotor disturbance. It may be that delivering the drug directly into the brain reduces the magnitude of any sensorimotor dysfunction.

NMDA antagonists and other tests of spatial learning

So far the discussion in this thesis has been limited to studies in which spatial learning has been assessed using the open field watermaze. The other behavioural paradigm commonly used for assessing spatial learning is the radial maze (Olton and Samuelson, 1976). The radial maze consists of a number of arms (commonly 8 or 12) radiating out from a central area like spokes in a wheel. The aim of the task, as far as the rat is concerned, is to collect the hidden food rewards which are located at the ends of the arms. The rats are able to choose the correct arm by using the distal extramaze cues around the laboratory. As the food rewards are not replaced, the rat has to adopt a 'win-shift' strategy and remember which arms he has already visited. This provides a test of working memory. The radial maze apparatus also allows several other different types of memory to be examined simultaneously. For example, a common approach is to bait only certain arms but to bait always the same arms. This provides a test of reference memory in that the rat also has to learn which arms are always rewarded. Non-spatial versions of the working and reference memory tests can also be conducted. Visual and tactile cues, contained within the arms themselves, can be used to distinguish baited from non-baited arms, providing a non-spatial (or 'cue') control task with similar sensorimotor and motivational demands.

Aspects of radial maze performance are sensitive to hippocampal damage (see Jarrard, 1993). Although there is some debate as to the precise pattern of impairments in the radial maze that arise following a hippocampal lesion, it is generally the case that both spatial working memory and spatial reference memory are impaired while both variants of the cue task survive relatively intact (Jarrard, 1986). The radial maze therefore provides a suitable means of assessing hippocampal-dependent spatial learning. This is of particular importance in that it provides an alternative to the watermaze, with different motivational ('appetitive' versus 'aversive') and motor system demands (eg. 'running' versus 'swimming'). In addition,

the non-spatial 'cue' task provides an appropriate control task for determining whether drug-induced behavioural impairments are due to effects on learning or performance.

The effects of both competitive (Danysz et al., 1988; Ward et al., 1990; Lyford and Jarrard, 1991; Bolhuis and Reid, 1992) and non-competitive NMDA antagonists (Danysz et al., 1988; Butelman, 1989; Shapiro and Caramanos, 1990; Ward et al., 1990; Wozniak et al., 1990; Shapiro and O'Connor, 1992) on performance in the radial maze have been assessed. In view of the problems associated with non-competitive NMDA antagonists, this section will concentrate on studies using competitive NMDA antagonists. The competitive NMDA antagonists AP5 and CPP have both been shown to impair certain aspects of performance in the radial maze.

For example, Ward and co-workers investigated the effects of CPP on both spatial working memory and spatial reference memory in the 8 arm radial maze (Ward et al., 1990). The rats were trained to a high level of performance in the maze before any drug administration. CPP (1 - 30 mg/kg, i.p.) caused a dose-dependent increase in the number of errors made by rats required to sample all 8 arms in a simple working memory task. A 30 mg/kg dose of CPP resulted in severely abnormal behaviour and often left the rats untestable. The 10 mg/kg dose of CPP significantly impaired performance and produced no stereotypical behaviour, although there was evidence of a mild ataxia. Subsequent experiments showed that a 10 mg/kg dose of CPP impaired performance on the 8 choice task when a delay of 1 hr was interposed between trials 4 and 5, and also impaired the working memory component of a radial maze task in which 4 out of 8 arms were baited. There was a trend for CPP injected animals to do worse than controls on the reference memory component of this task but this did not reach significance. A previous study has shown that a 10 mg/kg dose of CPP is sufficient to block the induction of LTP in the dentate gyrus *in vivo* (Abraham and Mason, 1988).

Ward et al., did not, however, test rats on a non-spatial version of the radial maze task and therefore have no control procedure to determine whether the behavioural impairment is due to a selective effect on spatial learning or whether the observed deficit is due to a more general effect of CPP on sensorimotor or motivational components of performance. The observation that a 10 mg/kg dose of the drug caused some degree of ataxia suggests that this may be an important consideration. Lyford and Jarrard (1991) have conducted a similar radial maze study but also included a non-spatial 'cue' version of the task. In agreement with Ward et al. (1990), they found that CPP (8 or 16 mg/kg, i.p., 45 min prior to testing)

impaired both the working memory and reference memory components of spatial learning in the radial maze. However, they also found that these doses of CPP had a comparable effect on both the 'place' (spatial) and 'cue' (non-spatial) versions of the task. The lack of a dissociation between the spatial and non-spatial radial maze tasks following CPP treatment is in contrast to the effects of hippocampal lesions which cause an impairment on the spatial version of the task but have little effect on the non-spatial 'cue' task (Jarrard, 1986). The pattern of results obtained with CPP in the radial maze also differs from results obtained with AP5 in the watermaze. AP5 treated animals were found to be impaired on a spatial task but were unimpaired on a non-spatial visual discrimination task (Morris et al., 1986). This suggests that i.p. injections of CPP may result in a more general impairment of performance in the radial maze, which may well involve drug effects on behavioural processes other than learning, and probably extends to brain areas outwith the hippocampus.

This lack of behavioural selectivity following CPP administration may simply reflect the i.p. route of administration which is likely to result in a more widespread distribution of the drug compared with an i.c.v infusion. An obvious alternative strategy is to deliver the drug directly into the brain. Danysz and co-workers found that acute i.c.v injections of AP5 impaired the acquisition of a spatial working memory task in the radial maze (Danysz et al., 1988). AP5 treatment increased the number of errors made during testing but had no effect on the overall time taken to complete the task. In a separate study, Bolhuis and Reid (1992) found that chronic infusion of 30 mM D-AP5, a dose which is known to block the induction of dentate gyrus LTP *in vivo* (see Chapter 7), resulted in a delay-dependent impairment of spatial working memory in rats that had previously been trained to asymptotic performance in the maze prior to drug treatment. This impairment only became apparent after continued radial maze training with delays, and there was no impairment at all when no delay was interposed during testing. Both studies, therefore, report that i.c.v infusion of AP5 impairs performance on spatial tasks in the radial maze. However, in neither study were AP5 treated animals tested on a non-spatial 'cue' version of the task to determine whether the behavioural deficit was a selective spatial learning impairment or was due to a more general disruption of performance. It remains to be seen what pattern of results would be obtained with AP5 infusion if performance on both place and cue versions of the radial maze task were assessed in the same animals (Lyford and Jarrard, 1991).

It is also worth noting that, with the exception of the Danysz et al. (1988) study which involved testing the effects of AP5 in experimentally naive subjects, these radial maze

studies have assessed performance in rats that have been trained extensively in the same radial maze prior to drug testing and are, therefore, already familiar with the spatial layout of the testing environment. It is not clear how much new spatial learning is required during the drug phase of these experiments. In a recent study, Caramanos and Shapiro (1994) have shown that chronic i.c.v infusion of AP5 (20 or 30 mM D,L-AP5) impairs spatial working memory in an unfamiliar environment but not in a familiar environment. The same pattern of results has also been obtained with the non-competitive NMDA antagonist, MK-801 (Shapiro and O'Connor, 1992). These studies involve the use of two radial mazes located in two different spatial environments, and are similar to the two-watermaze experiments described in this thesis (Expts 6.6, 7.1 and 7.2). As in the two-watermaze experiments, the rats were extensively trained, in the absence of any drug treatment, in one radial maze. These animals were, therefore, well practised with the procedural elements of the radial maze task prior to receiving the drug. Spatial working memory was then tested in both the same and a different spatial environment. NMDA receptor antagonists impaired spatial working memory in the unfamiliar but not the familiar environment. These results are consistent with the hypothesis that NMDA receptor-dependent mechanisms are required during the formation of a spatial representation in an unfamiliar environment but are not required for the utilisation of a pre-formed spatial representation during working memory procedures in a familiar environment.

Despite the fact there are important differences between the two studies (radial maze vs. watermaze, deficit on working memory task vs. deficit on reference memory task), the pattern of results obtained by Caramanos and Shapiro (1994) with AP5 in their two maze study is, in some respects, similar to the results obtained in Expt 7.2. In both studies, in animals experienced with the procedural demands of the behavioural tasks, AP5 causes some impairment of performance in the novel spatial environment but does not affect performance in the familiar environment. The absence of a drug effect on working memory in the familiar environment goes some way to arguing against a non-specific sensorimotor or motivational account of the AP5-induced behavioural impairment in the radial maze study. The authors argue that because the procedural demands of the spatial working memory task are the same in both the familiar and unfamiliar environments, the AP5 induced deficit in the novel environment cannot be explained by an impairment of these non-spatial elements of performance. This is not entirely true, however, in that the dissociation between the effects of AP5 in the familiar and novel environments could, for example, be explained by a subtle visual impairment. It may be that during a spatial working memory task in a familiar

environment, the spatial layout of the relevant distal cues is already well known to the animal and, therefore, a slight disturbance of the visual system (eg. partial blindness) may not seriously compromise performance. In contrast, during working memory in an unfamiliar environment, the animal is required to learn *de novo* about the spatial relationships between cues, and it may be that under these conditions any subtle visual disturbance may be crucial and disrupt performance.

The possibility cannot be ruled out, therefore, that the behavioural impairments seen in both the two radial maze study (Caramanos and Shapiro, 1994), and the two watermaze study (Expt 7.2), are due to a non-specific effect of AP5 on sensorimotor or motivational aspects of performance. Interestingly, Caramanos and Shapiro (1994) reported that a slightly higher dose of AP5 (33 mM D,L-AP5) did infact impair spatial working memory in a familiar spatial environment. This dose of AP5 was, however, also associated with hyperactivity and a degree of motor impairment, including hind limb flaccidity. The authors suggest that these non-mnemonic effects of AP5 infusion may be responsible for, or at least contribute to, the spatial working memory impairment obtained in the familiar environment with higher doses of AP5. They claim that these non-associative motor side effects were not present at the lower doses of AP5 (20 or 30 D,L-AP5) and argue that the impairment in a novel spatial environment is due to an effect of the drug on 'learning' rather than on 'performance'. However, the possibility cannot be ruled out that a subtle, cryptic sensorimotor impairment remains even at these lower doses, and that non-spatial, 'performance' effects are in fact responsible for the learning impairment.

In summary, radial maze studies have made an important contribution to the investigation of the relationship between hippocampal LTP and spatial learning. Because of the different sensorimotor and motivational demands of radial maze and watemaze studies, a combination of data derived from both behavioural paradigms should provide a powerful analysis of the hippocampal LTP/spatial learning hypothesis. However, the difficulties in dissociating drug effects on learning from drug effects on performance are as problematic in the radial maze as they are in the watermaze. In general, few radial maze studies have incorporated a non-spatial, 'cue' version of the task to control for sensorimotor or motivational side effects of the drug treatment (Lyford and Jarrard, 1991).

Are there other lines of evidence supporting the hippocampal LTP/spatial learning hypothesis?

Pharmacological studies constitute a central part of the investigation into the possible role of LTP-like processes in spatial learning. Nevertheless, the use of drug studies to test the hippocampal LTP/spatial learning hypothesis has, so far at least, proved inconclusive. With many pharmacological studies there is the concern that the drug may be diffusing to a number of brain regions (or into the spinal cord or periphary following systemic administration), and that any behavioural impairment may be due to effects outwith the hippocampus. A number of alternative approaches have been adopted in an attempt to determine whether or not LTP-like processeses in the hippocampus underlie spatial learning. These were outlined briefly in Chapter 2 (p46-47). What support can be derived from these other lines of enquiry?

Do changes in synaptic strength occur during learning?

The idea that learning is accompanied by an increase in synaptic efficacy forms the basis of the hypothesis under investigation in this thesis. The prospect of actually observing such changes in the intact mammalian nervous system during complex forms of learning, however, may well prove to be a fanciful notion which lies beyond the range of the experimental techniques currently available. In contrast, in simple systems such as *Aplysia*, changes in the magnitude of synaptic responses have been observed as a result of simple learning events (Kandel et al., 1983; Kandel, 1991). These observations suggest that synaptic plasticity may, indeed, be a plausible mechanism for information storage, and have fuelled attempts to provide a similar demonstration in the mammalian CNS. Clearly, the complexity of the mammalian CNS will present problems, both in terms of reliably measuring the strength of synaptic connections *in vivo*, and for interpreting the behavioural significance of any changes in the level of synaptic transmission. Nevertheless, this approach is an important component of the overall investigation into the relationship between LTP and learning.

There are now a number of reports demonstrating that classical conditioning can be accompanied by an increase in the activity of hippocampal neurones (Berger et al., 1976; Berger and Thompson, 1978; Thompson et al., 1980; Rutherich et al., 1982; Weisz et al., 1984; Berger et al., 1986; Skelton et al., 1987). Quite how these changes are related to the

hippocampal LTP/spatial learning hypothesis is not entirely clear, in that, for the most part, simple forms of learning, such as classical conditioning, do not require the hippocampus. Other researchers have observed changes in hippocampal neuronal activity when rats are exposed to novel and/or complex environments, or during exploration (Sharp et al., 1985; Green and Greenough, 1986; Sharp et al., 1987; Sharp et al., 1989; Green et al., 1990). Intuitively, these results seem to be more relevant to the hypothesis that synaptic changes in the hippocampus are involved in encoding spatial or contextual information.

There are, however, a number of problems associated with these studies. For example, the precise nature of the changes in hippocampal neurotransmission that have been reported to accompany exposure to a novel or complex environment are not entirely consistent across studies. They include an increase in both the epsp and the population spike (Sharp et al., 1985; Green and Greenough, 1986), an increase in the population spike with no change in the epsp (Sharp et al., 1987), and an increase in the epsp accompanied by a decrease in the size of the population spike (Sharp et al., 1989; Green et al., 1990). In addition, although changes have been observed following exposure to a novel or complex environment, somewhat surprisingly, corresponding changes in hippocampal neuronal activity have not been observed during spatial learning. For example, acquisition of a spatial task in the radial maze was not accompanied by measurable changes in hippocampal synaptic transmission (Hargreaves et al., 1990). Furthermore, the examination of low-frequency control groups from studies investigating the effects of electrical saturation on spatial learning, has provided no evidence of any potentiation of hippocampal neuronal activity as a result of spatial training in the watermaze (McNaughton et al., 1986; Castro et al., 1989; Robinson, 1992; Cain et al., 1993).

The absence of any change in synaptic transmission during spatial learning is difficult to reconcile with the observation that hippocampal neural activity increases with exploration or exposure to a novel environment. In their radial maze study, Hargreaves and colleagues did notice that the size of the evoked response in the hippocampus was particularly sensitive to whether or not the rats were moving when the responses were sampled (Hargeaves et al., 1990). In their paper, the authors stressed the importance of controlling for ongoing behaviour, and in particular movement, when sampling evoked responses in this way. Subsequently, in a recent paper, evidence has been presented which indicates that the increase in the size of evoked field potentials in the dentate gyrus, and the concomitant reduction in the population spike, which accompany exploration (Sharp et al., 1989; Green et

al., 1990) are due, in part, to an increase in brain temperature as a result of increased muscle activity, and may not be entirely the result of a behaviour-dependent modification of synaptic plasticity (Moser et al., 1993a). This result calls into question the evidence that exposure to a novel environment results in the modification of synaptic transmission in the hippocampus. However, in a further study, having controlled for any concomitant behaviourally induced temperature change, Moser et al. (1993b) did in fact find some evidence of a temperature-independent short-term increase in both the magnitude of the field epsp and the population spike during exploration of a novel environment. Furthermore, it seems unlikely that a rise in brain temperature during exploration could account for the observation that both the field epsp and the population spike of evoked responses were significantly larger in hippocampal slices prepared from rats which were reared for 3-4 weeks in an enriched environment (Green and Greenough, 1986). It is possible, therefore, that exposure to a novel or complex environment may still result in some underlying modification of synaptic transmission.

In summary, it is questionable how much support for the hippocampal LTP/spatial learning hypothesis has yet been derived from this avenue of approach. Despite there being some indication that exposure to a complex or novel environment may lead to some modification of hippocampal synaptic transmission (Green and Greenough, 1986; Moser et al., 1993b), the absence of any changes in synaptic efficacy during spatial learning itself remains a problem. There are a number of possible explanations as to why researchers have failed to observe potentiation of synaptic transmission in the hippocampus following spatial learning. It is possible, for example, that the changes in synaptic weights that accompany learning are limited to a small number of cells. Consequently, they may be undetectable when recording field potentials from a population of neurones with extracellular electrodes. An alternative explanation is that the changes in synaptic transmission that occur during learning involve both increases and decreases in synaptic efficacy, with the result that there is little overall change in the size of evoked field potentials (Morris and Willshaw, 1989).

The behavioural significance of any change in the level of neurotransmission that accompanies learning will be open to different interpretations. For example, it is essential to establish that any changes in neural activity associated with a learning event do not just reflect a generalised increase in forebrain excitability, possibly as a consequence of an enhanced state of arousal. There is a need to demonstrate some degree of synapse specificity with these behaviour-induced changes in neurotransmission. Ultimately some form of

demonstration that LTP-like events actually occur during learning will be required if one is to prove, unequivocally, that hippocampal synaptic plasticity is the neuronal mechanism underlying spatial learning.

Do LTP parameters correlate with learning?

In the absence of a direct demonstration that LTP-like events occur naturally during learning, some experimental support for the hippocampal LTP/spatial learning hypothesis has been derived from studies which demonstrate correlations between parameters associated with LTP (eg. induction threshold, magnitude of increase, persistence, rate of decay) and aspects of behavioural performance such as the acquisition and retention of spatial information. For example, Barnes (1979) discovered a strong positive correlation between the percentage increase in the amplitude of dentate gyrus field epsps, following three sessions of high frequency stimulation of the perforant path, and performance on a spatial 'circlular platform' task. In this task the rats are placed on a brightly illuminated table top from which they can escape by finding a goal tunnel which leads below the surface. The entrance to the tunnel is one of 18 holes, located around the outer circumference of the table, and can be found using the extramaze cues around the room. Significant correlations existed both within and between groups of young and old rats. Repeated high frequency stimulation resulted in a cumulative increase in the magnitude of LTP which eventually reached an asymptote. In older rats the rate at which LTP reached this asymptote was slower and its subsequent decay was faster (Barnes and McNaughton, 1980). This paralleled the rates of acquisition and forgetting of the circular platform task in similar groups of young and old rats (Barnes and McNaughton, 1985). More recently, Jeffery and Morris (1993) have found a strong positive correlation in normal adult rats between the cumulative level of LTP in the dentate gyrus following repeated tetanisation and performance (measured as % time in the training quadrant) on a spatial learning task in the watermaze.

Although these results are suggestive, a number of concerns remain. For example, although both Barnes (1979) and, Jeffery and Morris (1993), report positive correlations between LTP and spatial learning, they disagree as to the precise LTP parameter which correlates best with learning ability. Whereas Jeffery and Morris report a positive correlation between spatial learning ability and the cumulative level of LTP observed after numerous sessions of high frequency stimulation, Barnes found a difference between young and old rats in terms of the amount of LTP seen after just 3 days of tetanisation, but not after 12 days. Furthermore,

other researchers have failed to observe any positive correlation between LTP parameters and spatial learning ability, either in the radial maze (Robinson, 1992) or in the watermaze (Cain et al., 1993).

It is also important to note that a positive correlation between LTP and spatial learning does not provide conclusive evidence for a causal link. As Jeffery and Morris (1993) point out, there are a number of possible alternative explanations which could account for this positive correlation. For example, it is possible that some related aspect of hippocampal physiology, such as dentate gyrus excitability, is the actual determinant of learning ability and, at the same time, also influences the magnitude of LTP that can be induced. Consequently, a correlation between dentate gyrus excitability and learning could result in a 'secondary association' between LTP and learning. Alternatively, both LTP and learning could be dependent upon non-specific brain states such as arousal, or be affected by the amount of stress experienced by the animal (see Foy et al., 1987; Shors et al., 1989). It is also possible that hippocampal plasticity merely reflects the general plasticity of neurones all across the forebrain and that, although spatial learning is dependent on changes in synaptic efficacy, these changes may occur outwith the hippocampus.

In summary, attempts to determine whether or not LTP parameters correlate with learning have proved inconclusive. There is disagreement as to whether or not any reliable correlation exists between hippocampal LTP and spatial learning (see Robinson, 1992; Cain et al., 1993), and in cases where correlations have been identified, there is disagreement as to which LTP parameter correlates best with learning ability. Furthermore, without evidence that LTP-like changes actually occur as a consequence of learning, causality cannot be conclusively established. Taken on its own, correlational data merely provides circumstantial evidence for a causal link between spatial learning and hippocampal LTP.

Does electrical saturation of synaptic plasticity impair spatial learning?

If memories are formed and stored through the strengthening of cell-cell connections, then it should be possible to disrupt the learning process by artificially driving the synapses to their upper limit, thus preventing any further increase in synaptic efficacy. This hypothesis formed the basis of an experiment conducted by McNaughton and colleagues, in which repeated bilateral high-frequency electrical stimulation was delivered to the perforant path in an attempt to 'saturate' the learning mechanism (McNaughton et al., 1986). Rats were

anaesthetised, chronically implanted with bilateral recording and stimulating electrodes, and allowed to recover. The recording electrodes were lowered into the dentate hilus and the stimulating electrodes positioned in the angular bundle of the perforant path. The perforant path is the major input to the hippocampus and dentate gyrus, and it was hoped that locating the stimulating electrodes in the angular bundle would achieve maximal activation of all the afferent fibres entering the hippocampal formation. A course of repeated high frequency stimulation was then given with the result that the responses, recorded in the dentate gyrus, increased until an asymptote was reached. Spatial learning was then assessed using the circular platform task (Barnes, 1979). The rats which were given repeated tetanisation prior to testing were impaired on the acquisition of this task in a novel spatial environment, relative to control animals receiving just low frequency stimulation. Saturation also disrupted the retention of recently acquired spatial information and learning about a new goal location in a familiar environment. There was no effect, however, on the retention of well learned goal locations in the circle maze, or on short term working memory in the radial maze, under conditions where the reference memory component of the task was well learned. These results suggested that saturation of perforant path-granule cell synapses disrupts new spatial learning while leaving well-established memories intact.

The saturation impairment was considered as strong support for the idea that information is stored as changes in synaptic efficacy and, in many respects, this result was seen as a cornerstone of the hippocampal LTP/spatial learning hypothesis. The beauty of this approach lay in the specificity of the experimental manipulation. The hope was that, in contrast to drug studies in which the distribution of the drug could not be restricted to the hippocampus, electrical saturation may be limited to a single set of synapses in the hippocampal formation. Whereas there is always the possibility that a drug-induced learning impairment is due to unwanted side-effects of the drug on some process other than hippocampal LTP, electrical saturation appears to greatly reduce (although not completely) the likelihood that the observed deficit is due to an extra-hippocampal effect.

In a subsequent study conducted in McNaughton's laboratory, repeated bilateral high frequency stimulation resulted in an impairment in the acquisition of a standard spatial reference memory task in the watermaze (Castro et al., 1989). The same animals were unimpaired on a single visible platform task which argues against the possibility that the observed spatial learning deficit is due to a gross sensorimotor or motivational disturbance. An additional group of rats, given the same course of high frequency stimulation, were only

tested once LTP had decayed back to near-baseline levels. These rats were unimpaired, suggesting that the spatial learning deficit is recoverable and unlikely to be due to permanent damage caused by high frequency stimulation.

The work of McNaughton and colleagues was seen by some as particularly convincing evidence supporting the hypothesis that LTP-like events in the hippocampus contribute to the mechanisms responsible for spatial learning. Subsequent attempts to replicate this effect, however, have proved unsuccessful. A number of research groups, including McNaughton's own laboratory, have failed to find any effect of saturation on spatial learning in either the radial maze (Robinson, 1992) or in the watermaze (McNamara et al., 1992; Cain et al., 1993; Jeffery and Morris, 1993; Korol et al., 1993; Sutherland et al., 1993). In the watermaze studies, a variety of paradigms have been adopted, including attempts to replicate exactly the original experiment (Castro et al., 1989), but all without success. The failure of a number of laboratories to observe an effect of saturation on spatial learning in the watermaze has resulted in the wide spread belief that the original study of Castro et al. yielded a spurious positive result. A spatial learning deficit was observed in one study but only after the induction of seizure activity following repeated kindling (McNamara et al., 1992). Although abnormal hippocampal activity of this type could account for the deficit reported by Castro et al., it seems more likely that the experiment was subject to a type 1 statistical error (a 'false positive'). The fact that only four animals were included in each group could have contributed to this.

The question remains as to why saturation of perforant path synapses does not disrupt spatial learning in the watermaze. It is, of course, possible that the hippocampal LTP/spatial learning hypothesis is incorrect and that increases in synaptic efficacy in the hippocampus are not required for spatial learning. To refute the hypothesis on the basis of a negative result, however, would be to ignore a number of other possible explanations. A possible alternative explanation as to why spatial learning remains unimpaired in these experiments, is that the stimulation protocols that have been used fail to saturate all of the perforant path-granule cell synapses in the dentate gyrus. There is reason to believe that a single stimulating electrode, positioned in the angular bundle, is unlikely to activate the more ventral regions of the dentate gyrus (Gall et al.,1984; Cole et al., 1989; Barnes et al., 1993, 1994b), although, at the same time, it is also unclear what contribution, if any, the ventral hippocampus makes to spatial learning (Moser et al., 1990). Alternatively, it is possible that although an asymptote is reached in terms of the size of the evoked field epsp, this may not

reflect the true maximal level of activation obtainable in these synapses. Indeed, there is some indication that different tetanic stimulation parameters can obtain different amounts of LTP at asymptote (Jeffery and Morris, 1993). If, on the other hand, we assume that saturation of synapses in the dentate gyrus has been achieved, it may be that other synapses in the hippocampus proper, or in other brain areas, can compensate for the loss of plasticity in the dentate gyrus. It is also not clear what contribution long-term depression (LTD), or LTD-like events, make to spatial learning. Presumably reductions in synaptic efficacy are still possible after electrical saturation and may contribute to the mechanisms of spatial learning.

In summary, it now seems that, contrary to initial reports (McNaughton et al., 1986; Castro et al., 1989), saturation of perforant path-granule cell synapses in the dentate gyrus does not impair spatial learning. Although this finding does not preclude a role for LTP-like events in spatial learning, the theory "has been deprived of one of its major lines of experimental support," (Korol et al., 1993). Clearly further research is required to determine whether the failure to observe a spatial learning deficit in these experiments is due to an inability to fully saturate perforant path-granule cell synapses in both hemispheres, or whether this null result truly reflects the fact that changes in the efficacy of these synapses are not required for spatial learning.

There are a number of experimental approaches which may represent a means of achieving a more complete saturation of synapses in the dentate gyrus. For example, multiple stimulating electrodes may provide such an opportunity. An alternative approach, which has been adopted with some success, is to lesion partially the hippocampus and thus reduce the area of tissue that can support learning before saturating (Mumby et al., 1993).

Some comfort for proponents of the LTP/learning hypothesis may also be derived from reports that electroconvulsive therapy (ECT), which is normally used for the treatment of depressive illness, prevents the induction of hippocampal LTP (Hesse and Tyler, 1976; Anwyl et al., 1987; Stewart and Reid, 1993a) and impairs performance on a number of learning tasks including spatial learning in the watermaze (Holzhauer and Bures, 1986; Stewart and Reid,1993b; Barnes et al., 1994b). It has been suggested that the repeated seizure activity induced by ECT may result in the prior induction of LTP and subsequent saturation of synapses. Although ECT may represent a more effective way of ensuring that all perforant path-granule cell synapses (and other intrahippocampal connections) are maximally activated, other brain areas will, of course, be affected by the global application

of current to the brain. Therefore, the specificity of the manipulation, which was the attractive feature of the original studies, will be lost. The same will be true of attempts to saturate LTP chemically using drugs which have been shown to enhance synaptic transmission in the hippocampus and occlude subsequent attempts to induce potentiation (eg. sodium nitroprusside - Böhme et al., 1991; Bon et al., 1992; eg. 1S,3R aminocylopentane dicarboxylate (trans ACPD) - Bortolotto and Collingridge, 1993). The use of pharmacological agents to saturate LTP would provide a means by which a larger number of synapses could be accessed but, at the same time, such an approach would also be subject to many of the problems encountered when using NMDA antagonists, or any other drug for that matter, to investigate the relationship between LTP and learning. Nevertheless, the suggestion from the ECT studies is that a protocol which may achieve full saturation may also impair spatial learning and this should encourage researchers to further investigate the possibility that a saturation-induced learning impairment may be obtainable by a more discrete means, under the appropriate experimental conditions.

Do genetically engineered mutant mice deficient in LTP show a specific spatial learning impairment?

In recent years two research groups have pioneered a novel approach for investigating the relationship between hippocampal LTP and spatial learning, founded on the striking advances that have been made in the field of molecular biology (Silva et al., 1992a; Silva et al., 1992b; Grant et al., 1992). Genetically engineered mutant mice, lacking in a specific enzyme required for the development of LTP, were created by transfecting embryonic stem cells with a plasmid vector containing a defective copy of the gene encoding for this protein. The defective gene is incorporated, following homologous recombination, into the genetic material of the stem cell, which is then used to generate the mutant mice.

Using this gene knockout technique, Silva and co-workers (Silva et al., 1992a; Silva et al., 1992b) targeted the α-isoform of Ca^{2+}-calmodulin-dependent multifunctional protein kinase II (CaMKII), an enzyme thought to be required for the generation of LTP (Malenka et al., 1989; Malinow et al., 1989). The CaMKII enzyme is only expressed in the later stages of development and is found in appreciable amounts in forebrain neurones. CaMKII constitutes 2% of the total protein content of the hippocampus, 1% in the cerebral cortex, and is also found to a lesser extent in the striatum and amygdala. The mutant mice exhibited a 45% reduction in the levels of this protein compared to the wild type. Attempts to induce LTP in

hippocampal slices prepared from these mice were generally unsuccessful. On a small number of occasions, however, normal LTP was elicited. Normal synaptic transmission appeared unaltered, although there was a reduction in the magnitude of paired-pulse facilitation. In terms of behaviour, the mutant animals showed a degree of hyperexcitability but otherwise appeared normal. The mice were first trained to escape from the watermaze by means of a single visible platform. Initially, the mutant mice took consistently longer to escape from the pool than did the wild type mice, but by the fifth block of training trials the two groups were indistinguishable. In a subsequent study, the same mice were also impaired on the acquisition of a standard spatial reference memory task in the watermaze. Finally, the mice were trained to find a hidden escape platform, located in one arm of a plus maze which was placed in the same watermaze, in the same spatial environment. On this task, the mutant mice were not significantly impaired.

Grant et al. (1992) have used a similar approach to generate a variety of mutant mice, deficient in a number of non-receptor-dependent tyrosine kinases, a group of enzymes previously implicated in LTP (O'Dell et al., 1991b). One particular mutant, containing a defective copy of the *fyn* gene, has proved to be of particular interest. The *fyn* mutants exhibited no LTP in response to a weak tetanus, reduced LTP following a much stronger high frequency stimulus, and normal LTP when induced by pairing post-synaptic depolarisation with pre-synaptic activation. Taken together, these results suggest that this enzyme might play a role in setting the threshold for LTP induction. In all other respects, synaptic transmission appeared normal. A behavioural analysis of these mice has been conducted in the watermaze. The mutant mice were grossly impaired on a spatial reference memory version of the task, showing little, if any, improvement over the 7 days of training. The animals were then also trained on a single visible platform task and again, the mutant animals were impaired relative to the wild type, although they did appear to have caught up by the seventh training session on this task.

Taken at face value, both studies appear to provide results which are consistent with the possibility that a mechanism, similar to that responsible for the generation of LTP, may be required for certain forms of learning (Silva et al., 1992a; Silva et al., 1992b; Grant et al., 1992). Both the CaMKII and *fyn* mutants show a reduction in the inducibility of LTP and are impaired on a spatial learning task in the watermaze. Questions remain, however, regarding the specificity of both the behavioural and electrophysiological deficits. In terms of the behaviour, both mutants exhibited a striking, if transient, impairment on the single

visible platform task, in addition to the spatial learning deficits. The fact that other hippocampal-independent forms of learning may also be affected in these mice raises questions as to the underlying nature of the impairment. Silva and colleagues did show that the CaMKII mutants were unimpaired on a plus maze version of the watermaze task. A dissociation between performance in the plus maze task and the conventional watermaze task would go some way to arguing against a gross sensorimotor or motivational deficit. However, it is possible that the behavioural dissociation observed in this study simply reflects the order in which the tasks were run. The lack of a deficit on the cross maze task may be a consequence of prior training in the watermaze, whereas the impairment observed the conventional spatial task could be the result of testing animals which are experimentally naive. It remains to be seen whether or not the same pattern of results is obtained when the order in which the tasks are run is reversed.

Similar concerns have been voiced regarding the electrophysiological results. For example, the CaMKII enzyme is likely to be involved in a number of cellular processes, in addition to any role in synaptic plasticity. The reduction of paired-pulse facilitation would appear to support this contention and confirms the belief that this enzyme may be involved in the regulation of transmitter release from the pre-synaptic terminal. It is, of course, distinctly possible that such an effect on transmitter release could disrupt the mechanisms of spatial learning, independently of any effect on LTP.

Although it was hoped that gene knockout experiments of this type would improve the specificity of the manipulation used to investigate the LTP/learning hypothesis, it remains highly questionable as to whether or not this is the case. Both of the mutations described are not exclusively restricted to the hippocampus, with the enzymes being found in significant amounts in other areas of the forebrain in wild type mice. It is not implausible that a deficiency in these enzymes in the cortex could account for the spatial learning impairment. Another potential problem with this approach concerns the possible effects that an absence of these enzymes would have upon the development of the CNS. In the case of the α -CaMKII mutants, this may be only a minor problem, as this isoform of the enzyme is only expressed in the later stages of development, and there are no visible indications, to date, that the absence of this protein has compromised the anatomical organisation of the brain. In contrast, there are striking developmental abnormalities in the hippocampus of *fyn* mutant mice. These animals possess an increased number of granule cells and pyramidal cells in the dentate gyrus and CA3 subfields respectively, resulting in the irregular undulated appearance

of the principal cell layer. The consequences of these abnormalities for the normal functioning of the hippocampus and, consequently, for spatial learning are unknown, but the possibility remains that the lack of these genes during development could disrupt the formation of normal patterns of neuronal connectivity.

More recently, the same gene knockout technique has been used to create a strain of mutant mice which are deficient in the γ isoform of the enzyme Ca^{2+}-phospholipid-dependent protein kinase (PKCγ; Abeliovich et al., 1993a; Abeliovich et al., 1993b). Behavioural and electrophysiological investigations of these mutant mice have proved extremely interesting. Synaptic transmission has been studied in the CA1 subfield of hippocampal slices taken from both mutant and wild type animals. The PKCγ mutants exhibited normal hippocampal synaptic transmission, but when attempts were made to induce LTP using a conventional high frequency tetanus (5 trains of 100 Hz stimulation), there was an almost complete block of LTP in these slices (Abeliovich et al., 1993a). Behavioural analysis in the watermaze revealed that the PKCγ mutants were indistinguishable from the wild type mice on a standard spatial reference memory task, both in terms of escape latencies during training and in terms of the amount of time spent in the training quadrant during a transfer test conducted at the end of spatial training (Abeliovich et al., 1993b).

Taken together, these results demonstrate apparently normal spatial learning in animals which show a complete block of LTP using a conventional high frequency tetanus. These results would appear, therefore, to provide evidence which could be used to refute the hippocampal LTP/spatial learning hypothesis. However, further investigations revealed that the inducibility of LTP in slices obtained from the mutant animals was dependent on the prior history of the synapses (Abeliovich et al., 1993a). The authors found that if the conventional high frequency tetanus used to induce LTP in wild type animals was preceded by a 'priming' stimulus, consisting of a period of low frequency stimulation which is commonly used to induce LTD (900 pulses at 1 Hz; Dudek and Bear, 1992), then normal LTP could be induced in slices from the PKCγ mutants. The period of low frequency stimulation is somehow enabling LTP induction in the mutant slices, although the mechanism by which this occurs is unknown. Although the stimulation parameters used to 'prime' the mutant slices and allow LTP induction were those which induce LTD, the actual generation of LTD did not appear to be necessary for the subsequent induction of LTP. Furthermore, closer examination of the behavioural data revealed that the PKCγ mutants did, in fact, show a mild deficit during the transfer test at the end of spatial training in terms of

the number of annulus crossings made during the 60 sec. Therefore, following further investigation, an accurate description of the results obtained with PKCγ mutants is that LTP can in fact be obtained in these animals under certain conditions, and these animals also exhibit a mild spatial learning impairment in the watermaze.

These results are difficult to interpret with regard to the hippocampal LTP/spatial learning hypothesis. They certainly do not give sufficient grounds to refute the hypothesis. They do, however, illustrate the problems associated with investigating the relationship between an artificially induced experimental phenomenon, namely LTP, and a complex form of behaviour such as spatial learning. The results of the electrophysiological study suggest that a conventional high frequency tetanus may not always accurately assess the capacity of synapses to exhibit plasticity. It should also be pointed out that it remains to be seen whether or not LTP can be induced in the PKCγ mutants using conventional tetanus parameters *in vivo*. Nevertheless, this result raises an important issue concerning the type of stimulation parameters that should be used to assess whether or not, or indeed the extent to which, synaptic plasticity can be induced in a particular group of animals. It is still far from clear how the patterns of electrical stimulation which are commonly used to induce LTP in the laboratory relate to the patterns of activity that might occur naturally during normal hippocampal function.

The pattern of results obtained with the PKCγ mutants is, in some respects, not too disimilar to the results obtained with D-AP5 in Expt 7.2. Both the PKCγ mutants and the D-AP5 infused rats show a near complete block of LTP using a conventional tetanus, and display only a mild spatial learning impairment in the watermaze (although the exact nature of the impairment differs between the two sets of animals). Both groups of animals show considerable spatial learning and are unimpaired on certain measures of performance despite an apparent block of LTP. One interpretation of these results is that LTP-like processes in the hippocampus are not required for spatial learning. Nevertheless, both groups of animals do exhibit a subtle but significant impairment, relative to the appropriate controls, on some measure of performance in the watermaze. Both studies suggest that if blocking LTP does infact causally disrupt spatial learning then any impairment is likely to be small. It is clear that blocking LTP in the hippocampus does not produce as dramatic an effect on learning as a complete hippocampal lesion. Manipulations such as AP5 infusion retard the rate of acquisition without preventing learning completely and may often result in only a subtle behavioural impairment. These results illustrate the importance of using several different

measures of performance to assess spatial learning in the watermaze. Furthermore, in the case of the PKCγ mutants, it is in fact possible to induce normal LTP if the tetanus is preceded by a period of low frequency stimulation. It is extremely unlikely that LTP could be rescued in a similar way in AP5 treated animals. One would expect that the NMDA receptor dependent nature of LTP induction should remain the same, irrespective of the history of the synapses. Nevertheless, this result illustates the point that conventional methods used to assess the presence/absence of synaptic plasticity may need to be rethought.

In summary, despite the problems associated with a genetic approach, including possible developmental abnormalities and the inability to localise the gene knockout specifically to the hippocampus, the importance of these studies should not be undermined. They highlight an exciting and novel approach, the potential of which may not yet fully be appreciated. Ideally, what is required is a gene, which is necessary for the induction of LTP, which can be switched on and off, and which can be attatched to a hippocampal-specific promoter. The speed at which recent advances have been made in molecular biology, provides hope that such a valuable experimental tool may not be too far away.

Are LTP-like processes in the hippocampus required for other forms of learning?

In this thesis, the possibility that LTP-like events might be engaged during learning has been evaluated exclusively with respect to the hippocampus and hippocampal-dependent forms of learning. The behavioural baseline to which these experiments are compared is the performance of hippocampal lesioned animals. The hypothesis is that by preventing changes in synaptic efficacy in the hippocampus, one should disrupt those forms of learning which are sensitive to hippocampal lesions. So far, this thesis has been concerned specifically with spatial learning. Hippocampal lesioned animals do, however, also exhibit reliable learning deficits on several behavioural tasks which appear, at least intuitively, to be non-spatial. For example, lesioned animals are impaired on an operant 'differential reinforcement of low rates' (DRL) task (Sinden et al., 1986) and on the acquisition of taste-potentiated odour aversion (Miller et al., 1986; Bermudez-Rattoni et al., 1987). Performance on these tasks is also disrupted by NMDA antagonists. For example, intraventricular infusion of AP5 impairs performance on the DRL task (Tonkiss et al., 1988). Similarly, both AP5 (Willner et al., 1992) and MK-801 (Robinson et al., 1989) have been found to disrupt taste-potentiated odour aversion. These results are consistent with a role for NMDA receptor-dependent mechanisms in a wider range of types of learning.

As outlined in Chapter 2, one prominent theory of hippocampal function concerns the possible role of this structure in the processing of contextual information (Hirsh, 1974; Good and Honey, 1991). Hippocampal lesions have been found to impair contextual fear conditioning (Kim and Fanselow, 1992; Phillips and Le Doux, 1992; Kim et al., 1993) and the contextual retrieval of CS-US associations formed during appetitive Pavlovian conditioning (Honey and Good, 1993). The effects of AP5 on performance on these tasks have been assessed with mixed results. The infusion of AP5, either i.c.v (Kim et al., 1991; Kim et al., 1992), or directly into the hippocampus (Young et al., 1994), has been found to block contextual fear conditioning in rats. AP5 impairs the acquisition of contextual fear conditioning but does not affect the subsequent expression or retrieval of this learning (Kim et al., 1991). This result is similar to that obtained with AP5 in the watermaze (Morris et al., 1990a), and parallels the observation in electrophysiological studies that AP5 blocks the induction, but not the expression, of LTP (Collingridge et al., 1983). These results would appear to support a role for NMDA receptor mediated processes, such as LTP, in a hippocampal-dependent form of learning. In contrast, however, preliminary studies conducted in this laboratory by myself in collaboration with Dr. Mark Good (unpublished observations), investigating the role of LTP-like processes in the contextual retrieval of Pavlovian associations, have so far yielded negative results. Intraventricular infusion of D-AP5 (15-30 mM) did not impair the contextual specificity of conditioning, using a training protocol known to be sensitive to ibotenate hippocampal lesions (Honey and Good, 1993). Subsequent electrophysiological investigations have revealed that there was a near complete block of LTP in these animals at both doses of AP5. These results are in contrast to those obtained using the contextual fear conditioning paradigm, and indicate that NMDA receptor mediated events in hippocampus may not be required for all forms of hippocampal-dependent learning.

It appears, therefore, that although AP5 infusion mimics some of the behavioural effects resulting from a neurotoxic lesion of the hippocampus, the drug treatment does not reproduce every manifestation of the hippocampal lesion syndrome. For example, although AP5 infusion produces a similar impairment to a hippocampal lesion on the operant DRL task, drug infusion does not reproduce the effects of the lesion on the contextual specificity of Pavlovian conditioning. Similarly, whereas a hippocampal lesion impairs both the acquisition and retrieval of spatial information, administration of the NMDA receptor antagonist does not cause a retrograde amnesia. The results of Chapter 7 further illustrate important differences between the two experimental manipulations. It is obvious from these

studies that the effects of AP5 on spatial learning are much less severe than those of a neurotoxic lesion. One possible explanation for this is that NMDA receptor-dependent mechanisms in the hippocampus are not required for spatial learning. However, if one adopts the alternative viewpoint that NMDA receptor-mediated processes are required for spatial learning in some way or another, then it seems that NMDA receptor blockade retards the rate of learning without preventing new learning completely.

The obvious differences between the two experimental manipulations raises an important issue. The experimental strategy that has been adopted in most quarters is to use the hippocampal lesion syndrome as the model system with which to test the putative role of LTP-like mechanisms in learning. The hypothesis is that LTP-like events in the hippocampus provide the neural substrate for hippocampal dependent forms of learning and, therefore, manipulations which prevent LTP in the hippocampus should produce similar behavioural effects to those resulting from a hippocampal lesion. It is clearly the case, however, that there are important differences between the effects of a hippocampal lesion and, for example, AP5 infusion. This illustrates a fundamental difficulty with these studies. Although the lesioned animal provides the model system, it may be unreasonable and wholly inappropriate to expect AP5 infusion, or any other manipulation which prevents changes in hippocampal synaptic efficacy, to reproduce the same pattern of behavioural impairments. The converse is also true, of course, in that NMDA receptors in various brain areas will be involved in a number of behavioural processes. Consequently, intraventricular infusion of a drug such as AP5 is likely to affect a great deal more than just hippocampal dependent spatial learning. This could potentially lead to problems when trying to choose the appropriate control task(s) for spatial learning in the watermaze and, furthermore, may create difficulties in interpreting the results of drug studies. For example, it is quite clear from the results of Chapter 7 that AP5 does not produce as pronounced an effect on spatial learning as a hippocampal lesion. The drug treated animals learn a great deal more about the platform location than the lesion group. There is, therefore, a dissociation between the two behavioural syndromes. As previously discussed, the fact that AP5 treated animals learn something about the platform position should not be taken as grounds to refute the hippocampal LTP/spatial learning hypothesis. Nevertheless, the extent of the spatial learning demonstrated by the AP5 treated animals in Expt. 7.2 may equally reflect the fact that the neural mechanisms responsible for spatial learning remain essentially intact in these animals. The difficulty is in choosing the correct interpretation.

Consequences for the universal LTP/learning hypothesis

This thesis has primarily been concerned with addressing the possibility that LTP-like processes in the hippocampus may contribute to the mechanisms of spatial learning. This, of course, is part of a more general hypothesis, namely that LTP-like events at synapses in the CNS may underlie certain forms of learning. Irrespective of whether or not NMDA receptor-mediated events in the hippocampus are specifically involved in spatial learning, there is still a wide body of evidence supporting a role for NMDA receptors in other brain areas, in other forms of learning. Although the results presented in Chapter 7 of this thesis raise serious questions over the putative role of hippocampal LTP-like processes during spatial learning, they only partly compromise the more general form of the LTP/learning hypothesis.

Although not exclusively involved in learning (see *Conclusions*), the NMDA receptor provides an ideal mechanism by which associations can be detected and subsequently stored as long-term changes within the neural circuitry of the brain. The precise function carried out by the NMDA receptor will depend upon the neural circuitry in which that receptor is embedded. More specifically, the type of learning mediated by NMDA receptor-dependent mechanisms in a particular region of the brain will be dictated by the type of information received by that particular brain area. As far as investigating the LTP/learning hypothesis is concerned, having identified a specific neural circuit which is thought to be required for a particular form of learning, one is then in a position to determine whether or not NMDA receptor-mediated events, such as LTP, are required for that learning to proceed. The discovery that the hippocampus, a brain region with a high density of NMDA receptors, was required for spatial learning appeared to provide such an opportunity. However, the lack of a precise psychological account of the type of operation performed by the hippocampus, and a failure to fully understand how this brain structure contributes to spatial learning, has led to difficulties in interpreting the effects of AP5 treatment on performance on tasks such as the watermaze and radial maze. The lack of any kind of cue control in the watermaze has led to a situation where we have little inclination as to what cues are important for spatial learning, or for how and when these cues may be used. Consequently, it is very difficult to control for non-associative side effects of drugs which could potentially interfere with performance by disrupting sensorimotor or motivational processes.

More progress has been made by studying the involvement of NMDA receptors in types of behaviour for which there is a better psychological understanding and for which a specific

neural circuit has been identified (see Morris and Davis, 1994). The study of brain systems for which the link between behaviour and neuronal circuitry is more precisely defined has provided considerable evidence that NMDA receptor-mediated processes may be required for certain forms of learning. For example, there are strong grounds for believing that NMDA receptors may play an important part in the mechanisms of 'filial imprinting' (Bateson, 1966). Filial imprinting describes the behavioural process that occurs during the initial post-natal period, whereby visually naive chicks learn to approach the first striking object they encounter. Under normal conditions, of course, this will be the mother hen. The region of the avian brain that is responsible for imprinting is the 'intermediate extent of the hyperstriatum ventrale' (IMHV) of the left cerebral hemisphere (Horn, 1991). The observation that imprinting is associated with an increase in NMDA-sensitive [^{3}H] glutamate binding in the left IMHV provided the first indication that NMDA receptors might contribute to this form of behaviour (McCabe and Horn, 1988). A subsequent study has shown that the infusion of nM quantities of AP5 into the left IMHV results in a dose-related impairment of imprinting (McCabe et al., 1992). Although it is not known whether the physiological mechanism underlying imprinting involves changes in synaptic efficacy, there is certainly strong support for the involvement of NMDA receptors in this process.

Another form of learning which seems to require NMDA receptor-dependent mechanisms is fear conditioning (Davis, 1992; Morris and Davis, 1994). Fear conditioning is a form of Pavlovian conditioning which occurs when a neutral stimulus, such as a tone, is paired with an aversive stimulus, such as a footshock. After a number of pairings, presentation of the tone will elicit a number of behavioural responses associated with fear, including immobility or freezing, a change in heart rate, vocalisation and a hyper-reactivity to other stimuli. In contrast to the watermaze, where there is little, if any, experimental control over the cues which are used during learning, with fear conditioning, the delivery of both the conditioned stimulus (CS) and the unconditioned stimulus (US) can be precisely controlled. Consequently, the learning episode is more precisely defined and it is, therefore, much easier to control for non-associative side effects of the drug treatment, such as effects on the sensory processing of the CS, or a reduction in the magnitude of the aversive stimulus due to a drug-induced analgesia (see Morris and Davis, 1994).

There is strong evidence to suggest that the neuronal circuitry underlying fear conditioning resides in the amygdala (Davis, 1992). For example, electrical stimulation of the amygdala elicits a behavioural response which appears identical to that resulting from conditioned fear.

In addition, lesions of the amygdala abolish both innate and conditioned forms of fear. These results, coupled with the observation that there is a reasonably high concentration of NMDA receptors present in the amygdala (Monaghan and Cotman, 1985), have led to suggestions that NMDA receptor-dependent mechanisms in this brain area might provide the neural substrate for fear conditioning.

The effects of AP5 on fear conditioning have been assessed using three different behavioural paradigms; fear-potentiated startle (Miserendino et al., 1990; Campeau et al., 1992; Falls et al., 1992), context-specific conditioned freezing (Kim et al., 1991; Kim et al., 1992; see previous section) and step down/through inhibitory avoidance (Kim and McGaugh, 1992; Izquierdo et al., 1993). Local infusion of AP5 into the amygdala blocks the acquisition of fear conditioning at comparable doses in all three behavioural paradigms. AP5 has no effect on the expression of fear conditioning in animals that have previously been trained in the absence of any drug treatment. In contrast, local intra-amygdalar infusions of the AMPA receptor antagonist, CNQX, block the expression of fear conditioning in all three behavioural paradigms (Liang, 1991; Campeau et al., 1992; Izquierdo et al., 1993; Kim et al., 1993).

These results suggest that NMDA receptors in the amygdala may be required for fear conditioning, and are consistent with the hypothesis that NMDA receptor-dependent mechanisms in other brain areas may support other forms of learning. The effects of AP5 and CNQX on the acquisition and expression of fear conditioning parallel the effects of these compounds on the induction and expression of LTP. However, although both NMDA receptor-dependent (Gean et al., 1993) and -independent forms (Chapman and Bellevance, 1992) of LTP have been demonstrated in the amygdala, the relationship between synaptic plasticity in the amygdala and amygdala-dependent learning has not been investigated. The possibility remains that AP5 is disrupting amygdala function through a blockade of NMDA receptors but independently of any effect on NMDA receptor-dependent LTP. It may be, for example, that as a result of infusing the drug directly into the amygdala, the concentrations of AP5 present in this brain structure are well in excess of those required to block LTP. The impairment of fear conditioning may, therefore, be due to a more general effect of AP5 on the system properties of the amygdala. In the absence of tissue levels or electrophysiological analysis, the possibility that intra-amygdalar infusion of AP5 is producing a 'temporary lesion' effect cannot be ruled out.

Conclusions

The aim of this thesis was to provide a rigorous pharmacological assessment of the hippocampal LTP/spatial learning hypothesis. In view of the experimental data presented in this thesis, and the numerous other pharmacological studies reviewed in this section, it is still unclear as to whether or not LTP-like mechanisms in the hippocampus underlie spatial learning. The use of both competitive and non-competitive NMDA antagonists, in a number of behavioural paradigms, has failed to resolve fully the nature of the relationship, if indeed there is one, between hippocampal LTP and spatial learning.

The studies described in this thesis have highlighted the problems associated with a pharmacological investigation of complex forms of behaviour such as learning and memory. The difficulties in separating drug effects on learning from effects on performance have been well-documented. The importance of using appropriate control tasks to establish whether a manipulation is directly affecting the mechanisms underlying learning, or disrupting sensorimotor or motivational processes, has been discussed at length. The problem is in determining what task, or group of tasks, provides an appropriate control for hippocampal-dependent learning. This choice is not helped by a failure to comprehend fully the nature of hippocampal function. This lack of understanding makes it that much harder to predict how NMDA receptor-dependent mechanisms might contribute to the operations performed by this brain structure. A more precise psychological account of hippocampal function would no doubt prove helpful in this respect. The experiments described in this thesis also illustrate the importance of a multidisciplinary approach for testing the hippocampal LTP/spatial learning hypothesis. An experimental design incorporating behavioural, electrophysiological and biochemical analyses represents a powerful approach, and is essential if one is to properly evaluate the relationship between cellular processes and behaviour.

In addition to pharmacological studies, a number of other experimental strategies have been pursued in an attempt to determine the relationship between hippocampal LTP and spatial learning. Attempts to actually observe changes in hippocampal synaptic transmission during spatial learning have proved unsuccessful (McNaughton et al., 1986; Castro et al., 1989; Hargreaves et al., 1990; Robinson., 1992; Cain et al., 1993). There is, however, some indication that exposure to a novel environment may result in a short-term increase in the level of hippocampal synaptic transmission (Moser et al., 1993b). How this finding relates to the hippocampal LTP/spatial learning hypothesis is far from clear. Similarly, although there is some evidence suggesting that LTP parameters may correlate with behavioural

measures of spatial learning and memory (Barnes, 1979; Barnes and McNaughton, 1980; 1985; Jeffery and Morris, 1993), this avenue of approach has proved equally inconclusive. There is disagreement as to whether or not any reliable correlation exists between hippocampal LTP and spatial learning (see Robinson, 1992; Cain et al., 1993), and in cases where correlations have been identified, there is disagreement as to which LTP parameter correlates best with learning ability. The use of gene knockout techniques has provided yet another means by which to test the LTP/learning hypothesis, but although this approach promises a great deal for the future, at present, an inability to restrict the knockout to a particular region of the brain, combined with the concern that developmental abnormalities may contribute to any behavioural impairment, has created difficulties with regard to the interpretation of these studies (Silva et al., 1992a; Silva et al., 1992b; Grant et al., 1992). In summary, while several of these results are consistent with the hippocampal LTP/spatial learning hypothesis, these experimental approaches have proved far from conclusive, and it is doubtful whether the evidence provided by these studies, alone, is sufficient to support the hypothesis.

Until recently, considerable support for the hypothesis had been derived from the demonstration that prior electrical saturation of perforant path-granule cell synapses resulted in a spatial learning impairment, both in the circle maze (McNaughton et al., 1986) and the watermaze (Castro et al., 1989). However, the failure of several groups, including the authors themselves, to successfully replicate these effects of electrical saturation on performance in the watermaze has seriously undermined this contribution. In the absence of a saturation effect, pharmacological studies using NMDA receptor antagonists, such as AP5, will surely take on more significance.

In view of this, it is surely essential to ascertain whether or not AP5 does in fact directly disrupt the neural mechanisms responsible for spatial learning. Although the results of Expt 7.2 are somewhat ambiguous, and open to different interpretations, the extent to which AP5 treated animals learned about the spatial location of the platform in the novel environment during training in watermaze 2 was somewhat surprising, and represents a serious challenge to the hippocampal LTP/spatial learning hypothesis. This result, combined with the failures to replicate the saturation effect, is certainly a cause for concern amongst proponents of the hippocampal LTP/spatial learning hypothesis. One viewpoint is that both these results are simply indicative of the fact that NMDA receptor-dependent LTP-like processes in the hippocampus are not required for spatial learning. Although neither the saturation studies,

nor the results of Expt 7.2, should be used as grounds to reject the hippocampal LTP/spatial learning hypothesis out of hand, they, nevertheless, raise questions over the two major sources of evidence supporting of this theory. Following their failure to replicate the Castro et al. (1989) study, Korol et al. (1993) stated in their discussion that although this finding does not preclude a role for LTP-like events in spatial learning, the theory "has been deprived of one of its major lines of experimental support". Similarly, although the results of Expt 7.2 could be interpreted in a manner that is not inconsistent with a role for LTP-like mechanisms in spatial learning, the extent to which these animals learned about the platform location certainly calls into question the tenet that NMDA receptor antagonists are directly disrupting the mechanisms responsible for spatial learning. Together, the saturation studies from McNaughton's laboratory and studies using the competitive NMDA receptor antagonist, AP5, have provided the bedrock of support for the hippocampal LTP/spatial learning hypothesis. Although observations of behaviourally-induced changes in hippocampal synaptic transmission during exploration (Moser et al., 1993b), correlation studies (Barnes, 1979; Jeffery and Morris, 1993), and gene knockout experiments (Silva et al., 1992a; Silva et al., 1992b; Grant et al., 1992) may provide data which is consistent with the hypothesis, it is questionable as to whether or not these studies are sufficient, on their own, to uphold such a theory. If the theory were to be deprived of both major cornerstones of support, it would surely be a major blow for proponents of a role for hippocampal LTP-like events in spatial learning. The hypothesis cannot survive on theoretical attractiveness alone.

The view that NMDA receptor-dependent mechanisms are not required for spatial learning, although not inconsistent with the data, may, however, be somewhat premature. There are a number of possible reasons why these experimental manipulations may have failed to produce a robust spatial learning impairment that do not require the hypothesis to be refuted. However, if there is a substantive link between hippocampal LTP and spatial learning, then it is quite clear that there is a great deal we do not fully understand about this relationship. Despite the many reservations, there are still a considerable number of studies which *are* consistent with a role for LTP-like mechanisms in spatial learning. As outlined earlier in this chapter, there are a number of possible reasons why the attempted electrical saturation of perforant path-granule cell synapses may have failed to result in a spatial learning impairment. It should also be remembered that the results obtained with AP5 in the two-watermaze study (Expt 7.2) were ambiguous and could be interpreted in favour of the hippocampal LTP/spatial learning hypothesis. After all, these animals did show a consistent

and significantly reliable deficit in terms of escape latencies during training in watermaze 2. Therefore, although the deficit may be a great deal more subtle than that obtained with hippocampal lesions (Expt 7.1), there was an impairment nonetheless. It may be that we have to re-evaluate the relationship between hippocampal LTP and spatial learning, and reassess the way in which we test the hypothesis.

For example, the results of Abeliovich et al. (1993a) suggest that conventional methods of inducing LTP, using a brief period of high frequency electrical stimulation, may not always be sufficient to gauge the capacity for synaptic change. Tetanic stimulation is an extremely artificial form of neuronal activation and it could be that the resulting LTP is merely an experimental phenomenom that bears only a limited resemblance to the sorts of endogenous plasticity that might occur naturally during learning. Similarly, from the point of view of the behavioural analysis, it is quite clear that if synaptic plasticity in the hippocampus is required for hippocampal dependent forms of learning, then preventing changes in synaptic efficacy does not produce as pronounced, or indeed the same pattern of, behavioural effects as a hippocampal lesion. The standard methods of both behavioural and electrophysiological analysis may need to be rethought.

The extent to which the AP5 treated animals learned about the spatial location of the platform in watermaze 2 (Expt 7.2), and the lack of a spatial learning impairment in the various saturation studies, is in marked contrast to the profound deficits obtained with hippocampal lesions. As previously mentioned, it may be unreasonable to expect the AP5 manipulation to mirror precisely the pattern of behavioural effects resulting from a hippocampal lesion. It is, after all, a big step to go from a neurotoxic lesion of the entire brain structure to a pharmacological blockade of just one receptor subtype. It remains to be seen what effects a more complete, but temporary, pharmacological disruption of hippocampal neurotansmission, without destroying the cells within this structure, would have on learning. Direct intrahippocampal infusion of local anaesthetics (eg. lignocaine), $GABA_A$ receptor agonsists (eg. muscimol) or AMPA receptor antagonists (eg. CNQX) may provide an opportunity whereby the hippocampus can be switched off temporarily, thus possibly avoiding the secondary effects associated with a hippocampal lesion.

Although it seems likely that the hippocampus plays some role in spatial learning, we still have little intuition as to the specifics of how the neuronal circuitry within the hippocampus might be involved. It may be that all that is required of the hippocampus is the throughput of neural activity, with there being no requirement for NMDA receptor-dependent changes in

synaptic efficacy within this structure. A lesion will, of course, by destroying all the cells within this structure, completely disrupt the flow of any information passing through the hippocampus. It may be, however, that in the presence of AP5, for example, that NMDA receptor-independent neurotransmission within the hippocampus is sufficient to support spatial learning. It is possible that the site of synaptic change associated with spatial learning is in another part(s) of the brain. A number of other brain regions have been implicated in spatial learning, including the prefrontal (Kolb et al., 1983; Kolb, 1986), cingulate (Sutherland et al., 1988) and parietal areas of the cortex (DiMattia and Kessner, 1988). It may be that memories are laid down and stored in these areas of cortex and NMDA receptor-dependent changes in synaptic efficacy in these brain regions provide the neural basis of spatial learning. It should be remembered, however, that an i.c.v infusion of AP5 was found to result in a fairly uniform distribution of the drug across the forebrain, a point that might argue against a role for NMDA receptor-dependent mechanisms in other brain areas during spatial learning (Butcher et al., 1991).

The results presented in Chapter 7 do not rule out the possibility that NMDA receptor-dependent mechanisms contribute to spatial learning under normal conditions, in the absence of AP5. It is widely believed that when information is stored in the nervous system it is distributed across networks of neurones all over the brain, and that the same information may be stored more than once, in more than one location. This parallel processing could occur at any level of organisation within the brain, and is likely to result in a degree of redundancy within the system. For example, in a recent study conducted in this laboratory, the effects of partial ibotenate hippocampal lesions, of varying sizes, on spatial learning in the watermaze have been investigated. It would appear from the preliminary results of this study that at least 70% of the hippocampus must be removed in order to see a reliable spatial learning deficit (Moser M-B, pers. comm.). This redundancy could also result in learning being spared following experimental manipulations, such as AP5 infusion, which disrupt only a part of the system. In the presence of AP5, other mechanisms, or other synapses, or other networks of neurones, or indeed other brain areas, might be able to compensate and support learning. At a mechanistic level, for example, it is possible that NMDA receptor-independent forms of LTP may play an important role in the hippocampus during spatial learning (Harris and Cotman, 1986; Zalutsky and Nicoll, 1990; Grover and Teyler, 1990; Anikstejn and Ben-Ari, 1991; Johnston, 1992; Bortolotto and Collingridge, 1993). These forms of synaptic plasticity are, in general, less well understood. Little is known about the behavioural significance of these NMDA receptor-independent forms of LTP, and it is far

from clear what contribution, if any, they make to spatial learning.

The possibility remains that LTP does not provide an accurate experimental model of the sort of neural change that accompanies learning. However, even if there is no direct relationship between LTP in the hippocampus and spatial learning, one would surely expect a role, of some form or another, for hippocampal NMDA receptors. Yet the results of Expt 7.2 could be interpreted as indicating otherwise. It is, of course, possible that LTP induction does not provide an accurate indication of hippocampal NMDA receptor activity. It may be that following the infusion of 30 mM D-AP5, there is sufficient blockade of NMDA receptors to prevent LTP induction, but at the same time, there is still a sufficient number of active NMDA receptors to support learning. Nevertheless, the possibility remains that NMDA receptors in the hippocampus are not essential for spatial learning. This then begs the question as to what these receptors are doing in the adult brain. NMDA receptors have been strongly implicated in the experience-dependent self-organization of the nervous system during development (Cline et al., 1987; Kleinschmidt et al., 1987). They are also thought to contribute to the pathology associated with various clinical conditions such as epilepsy (Croucher et al., 1982), ischemia (Rothman and Olney, 1987), and a number of neurodegenerative diseases (Meldrum, 1989; Meldrum and Garthwaite, 1990). The question remains, however, that if these receptors are not involved, at some level, in the processes underlying of spatial learning, then what role, if any, are they performing in the adult hippocampus? Presumably, NMDA receptors represent an important component of neurotransmission in the hippocampus. Indeed, the general consensus of opinion now is that NMDA receptors play a more widespread role in the system properties of the hippocampus (Bekkers and Stevens, 1990b), and are likely to be involved in other aspects of neurotransmission, in addition to any role in synaptic plasticity. The surprise would be, therefore, if such a disruption of hippocampal neurotransmission did not, in some way, affect spatial learning. It would surely be extremely surprising if the only contribution that NMDA receptors make to the adult nervous system is neuropathological, and that they just "lurk malevolently in the surface membrane, waiting to cause epilepsy," (Dingledine, 1986).

To conclude, it would appear that we are still along way from understanding the relationship, if indeed there is one, between hippocampal LTP and spatial learning. In view of both the literature reviewed in this discussion, and the results presented in Chapter 7 of this thesis, it is still far from clear whether or not LTP-like processes in the hippocampus form the neural basis of, or indeed make any contribution to, spatial learning. Although NMDA receptor-

dependent LTP provides an extremely attractive model of the type of neural activity that might underlie certain forms of learning, including spatial learning, the evidence in favour of this hypothesis would now seem somewhat limited. The failure of several groups to find a spatial learning impairment following electrical saturation of perforant path-granule cell synapses has deprived the hypothesis of one major line of support. In the absence of a saturation effect, pharmacological studies demonstrating that NMDA receptor antagonists, such as AP5, impair performance on spatial learning tasks take on more significance. The problems associated with studies investigating the effect of drugs on learning and memory have been well documented in this thesis. It has proved particularly difficult to separate drug effects on learning from effects on performance, an inability that has led to reservations regarding the amount of support that can be derived for the hippocampal LTP/spatial learning hypothesis from these studies. A further concern is that the more experimental manipulations that are found to affect performance on spatial learning tasks in the watermaze (McNamara and Skelton, 1993), and the more manipulations that are found to block or reduce LTP (Bliss and Collingridge, 1993), the weaker the impact of a correlation between the two. The danger is that as the two subsets increase in size, the probability that a positive correlation between the effects of any drug on LTP and learning is due to coincidence rather than causality increases. Potentially more troublesome for the hypothesis, however, is the extent to which the AP5 treated animals learned about the spatial location of the platform in a novel environment during Expt 7.2. Using conventional transfer test measures of performance these rats were unimpaired relative to aCSF infused controls. This result, combined with the observation that LTP could not be induced in these animals, represents a serious challenge to the hippocampal LTP/spatial learning hypothesis. If there is indeed some truth to this hypothesis, then it is quite clear that we are a long way from fully understanding the relationship between hippocampal LTP and spatial learning. Both the electrophysiological and behavioural analyses commonly used to test the hypothesis require careful consideration. It should be stressed that these results do not in any way harm the wider hypothesis that NMDA receptor-dependent mechanisms in other brain areas may contribute to other forms of learning. They do, however, raise serious questions over the two major sources of evidence supporting one specific form of the hypothesis. Finally, if it turns out to be the case that LTP bears little or no resemblance to the neural mechanisms underlying learning, it is worth noting that there is a distinct lack of plausible alternatives. This, of course, does not provide grounds for persisting with the status quo.

Appendices

APPENDIX A - Development of a novel analytical technique to quantify 7CK tissue levels in brain tissue.

The need for some analytical means of quantifying the levels of 7CK present in the brain has already been outlined in Chapter 4. The following section provides an outline of the steps taken during the development of a suitable method.

High performance liquid chromatography (HPLC) is one of the most widely used analytical techniques in science and industry today. Application of this technique involves two distinct processes, a separation event which utilises chromatography and then a subsequent detection process. HPLC is a technique used to separate the individual components (solutes) from a mixture and is achieved by passing a mobile phase, containing the sample, under pressure through a stationary (bulk) phase which is packed in a narrow bore column. Although the basis of the separation process is still not fully understood, it is thought that the components of the mixture (solutes) are adsorbed onto the stationary phase for varying lengths of time according to the equilibrium distribution of each solute between the two phases. This is in turn dependent on the nature of the two phases and the physical properties of the solutes (eg. size, ionic charge, hydrophobicity and hydrophilicity). Separation is thus determined by the degree of retention of each solute by the stationary phase and results in the various components of the mixture eluting from the column at different times. Some means of detection is then required in order to quantify the specific component(s) of interest (eg. electrochemical, fluorescence, UV, refractive index).

Reversed phase chromatography is a particularly versatile and commonly used form of HPLC. Both the amino acid (including D-AP5 - see Chapter 7) and kynurenine (7CK) separation methods involve a reversed phase system. Reversed phase HPLC obtains separation of the solutes between a "polar" mobile phase and a "non-polar" stationary phase. The non-polar stationary phase consists of a slurry of micro silica particles (3-10 μm diameter) which are coated in long-chain hydrocarbons, commonly 8 or 18 carbon atoms long. This creates a hydrophobic layer on the surface of the stationary phase which will attract and retain hydrophobic molecules in the sample. Selectivity, as far as separation is concerned, is determined mainly by the properties of the polar mobile phase. The mobile phase can be aqueous or organic and the separation process aided by altering its composition in any of several ways. In addition to improving the resolution of separation, the composition of the mobile phase will also determine the running time which is an important

consideration when developing an efficient analytical system. There are numerous ways in which altering the mobile phase will affect the retention of the solutes. For example, the ionic composition, concentration, pH and the flow rate of the mobile phase will all influence retention. The choice of buffer system for the mobile phase also needs to be considered. In addition, an ion pair agent can be added to the mobile phase in order to improve the separation of ionisable solutes. Another alternative is to use an organic modifier such as methanol or acetonitrile, which alters the retention of the samples without changing the relative elution order.

Having achieved separation, a high precision, high sensitivity, high stability detection method is required. Many compounds, including kynurenic acid, absorb UV light and then subsequently emit a fraction of this radiation at a longer wavelength. This provides a very selective detection technique, in that only compounds with a high degree of conjugation will fluoresce. As a consequence of the difference in excitation and emission wavelengths, the irradiating light can be filtered out while monitoring the emission wavelength.

Original methodology

A Varian Vista 5500 pumping system with a 9090 automatic column injector and an Applied Biosystems 980 Fluorescence detector were used throughout. Initially, the method used was based on a protocol suggested by John Kemp (pers. comm.). The mobile phase consisted of 20 mM KH_2PO_4, 5mM tetrabutylammonium dihydrogen phosphate (ion pair agent) and 18% acetonitrile (MeCN; organic modifier), with the pH adjusted to 3.7 with concentrated H_3PO_4. This was pumped at 2 ml/min through a RP-C8 Spherisorb column (250 x 4.6 mm; 5μm particle size; Jones Chromatography). The optimum excitation wavelength (331nm) was determined by finding the optimum UV absorption wavelength of 7CK by means of a spectrophotometer. Emissions of > 370nm were monitored.

Optimisation of the detector

The voltage applied to the fluorimeter lamp was systematically altered (820 - 850V) in order to establish the setting that provided the optimum signal to noise ratio. For each run, a 10μl sample of 20μM 7CK (0.2nmol) was injected onto the column and the peak height and the noise level recorded. The optimum voltage setting for the fluorimeter lamp was 835V.

Dose Response function

With the fluorimeter lamp set at a constant voltage of 835V, the peak sizes resulting from a range of 7CK concentrations (10-1000μM) were determined. There was a linear relationship between the signal size and the concentration of the drug. The retention times were approximately 6 and 30 min for kynurenic acid and 7CK respectively.

The Extraction Procedure

7CK is particularly insoluble at neutral or acidic pH. The drug was made up in equimolar NaOH and usually required additional spiking with 5M NaOH in order to fully dissolve. Bearing this in mind, a particular concern is that exposure to an acidic environment during the tissue extraction may cause the precipitation of 7CK out of solution. There is also the possibility that the acidity may have a quenching effect on the 7CK signal. An investigation comparing a 1mM standard prepared from a 100mM stock solution by diluting tenfold in water and then tenfold in 7% PCA with a 1mM standard diluted 100 fold in water showed that the acid dilution step resulted in approximately a 50% reduction in the size of the 7CK signal. However, diluting to 1mM in water followed by a tenfold dilution step in 7% PCA did not reduce the size of the signal relative to a 100μM standard diluted entirely with water. These results suggest that the HPLC signal produced by tissue concentrations of less than 1mM will not be reduced by the acid extraction. In addition, the concentration of PCA used for extraction was reduced from 7% to 1.1% (0.1M) as reported by Swartz et al., (1990). All drug standards were diluted to 500μM in distilled water and then further diluted in 0.1M PCA as appropriate.

Post- column application of zinc acetate

Following a report by Iinuma and co-workers (1985) that the endogenous fluorescence of kynurenic acid is enhanced in the presence of Zn^{2+} ions, several groups have developed methods for the determination of kynurenic acid in biological fluids. Using HPLC with fluorescence detection, the endogenous fluorescence is enhanced 30-50 fold by the post-column application of zinc acetate (Shibata, 1988; Swartz et al.,1990). Many of the following changes in the methodology are taken from the method described by Swartz et al.,(1990). The post-column application of 0.5M zinc acetate at a flow rate of 1ml/min resulted in an enormous enhancement of both the kynurenic acid and 7CK signals. Zinc

application produced, approximately, a 25 fold enhancement of the kynurenic acid signal and a greater than 50 fold enhancement of the 7CK signal. The range of emission wavelengths monitored was also changed to > 398nm (Swartz et al.,1990).

The Mobile Phase

As previously described, there are various ways of improving the resolution of the separation and reducing the running time by altering the composition of the mobile phase. The original method outlined above (before post-column zinc acetate) gave retention times of 6 and 30 min for kynurenic acid and 7CK respectively. Such a high degree of resolution is not required and results in some peak flattening. Removing the ion pair agent (tetrabutylammonium dihydrogen phosphate) resulted in a considerable reduction in retention times. The ion pair agent is normally a mainly hydrophobic molecule with a polar head (similar to soap) and is added to the mobile phase in order to improve the separation of ionisable solutes. The hydrophobic region of the molecule allows the ion pair agent to be adsorbed onto the non-polar stationary phase, leaving the hydrophilic region of the molecule exposed to the polar mobile phase, providing an ion-exchange surface for the solute. The effect is to increase the retention time and improve separation. Consequently, removal of the ion pair agent reduces retention times. Indeed, removing the ion pair agent resulted in too great a reduction in retention time which compromised the separation process. This was countered, in part, by reducing the flow rate to 1ml/min which also had the added advantage that it was better for the column and provided the opportunity for post-column application of zinc acetate. The anionic composition of the mobile phase was changed from phosphate to acetate in order to be compatible with the post-column application. Indeed, Swartz et al., use a 50mM sodium acetate (pH6.2) solution as their mobile phase. Although altering neither the anionic composition nor the pH of the mobile phase had any great effect on retention times or peak sizes, these parameters were slightly preferable. Two organic modifiers were tried - methanol and acetonitrile. An organic modifier alters the retention time of the samples without changing the relative elution order. Acetonitrile is a strong organic modifier which caused a reduction in retention time with increasing concentration but also gave a considerable quench with some indication of peak splitting. Methanol proved to be the better alternative. Although producing less of a reduction in retention time there was only minimal quench of the peaks. Increasing the concentration of methanol reduced the retention times with the optimal concentration being 3 parts sodium acetate to 1 part

methanol (25% MeOH by volume).

The final method

The methodology that was finally arrived at has already been outlined in Chapter 4. Briefly, a mobile phase (3 parts 50mM sodium acetate, pH6.2; 1 part methanol) was pumped through a C8 Spherisorb column at 1ml/min. The endogenous fluorescence (excitation wavelength 331nm) is enhanced by the post-column application of 0.5M zinc acetate and detected at > 398nm. Figure A.1 shows a representative chromatogram for a standard sample containing 100 pmoles of both kynurenic acid and 7CK. The retention times (sec) were 280 and 723 for kynurenic acid and 7CK respectively. For kynurenic acid, this is comparable with the retention time of 270 sec reported by Swartz et al.,(1990). The linear relationship between the drug concentration and the peak size was maintained following the various changes in methodology.

Specificity of the peaks

Swartz et al., (1990) ran standard solutions containing an extensive list of compounds that were fluorescent at an excitation wavelength of 344 nm and an emission wavelength of 398 nm. Of all the fluorescently active compounds that they examined, only kynurenic acid, xanthurenic acid and quinaldic acid had retention times of longer than two minutes. In agreement with Swartz et al., the endogenous fluorescence of xanthurenic acid and quinaldic acid was not enhanced by post-column application of zinc acetate and neither of these compounds co-eluted with 7CK. In addition, this investigation and that of Swartz et al., suggest that neither of these compounds, as detected by fluorimetric methods, are found at detectable levels in brain tissue samples and are therefore unlikely to co-elute and interfere with the 7CK peak in brain tissue samples. The identity of the 7CK peak in brain tissue samples was confirmed by comparison of retention times with a 7CK standard, by spiking with a standard 7CK sample and by the absence of such a peak in brain tissue samples from an aCSF-infused rat.

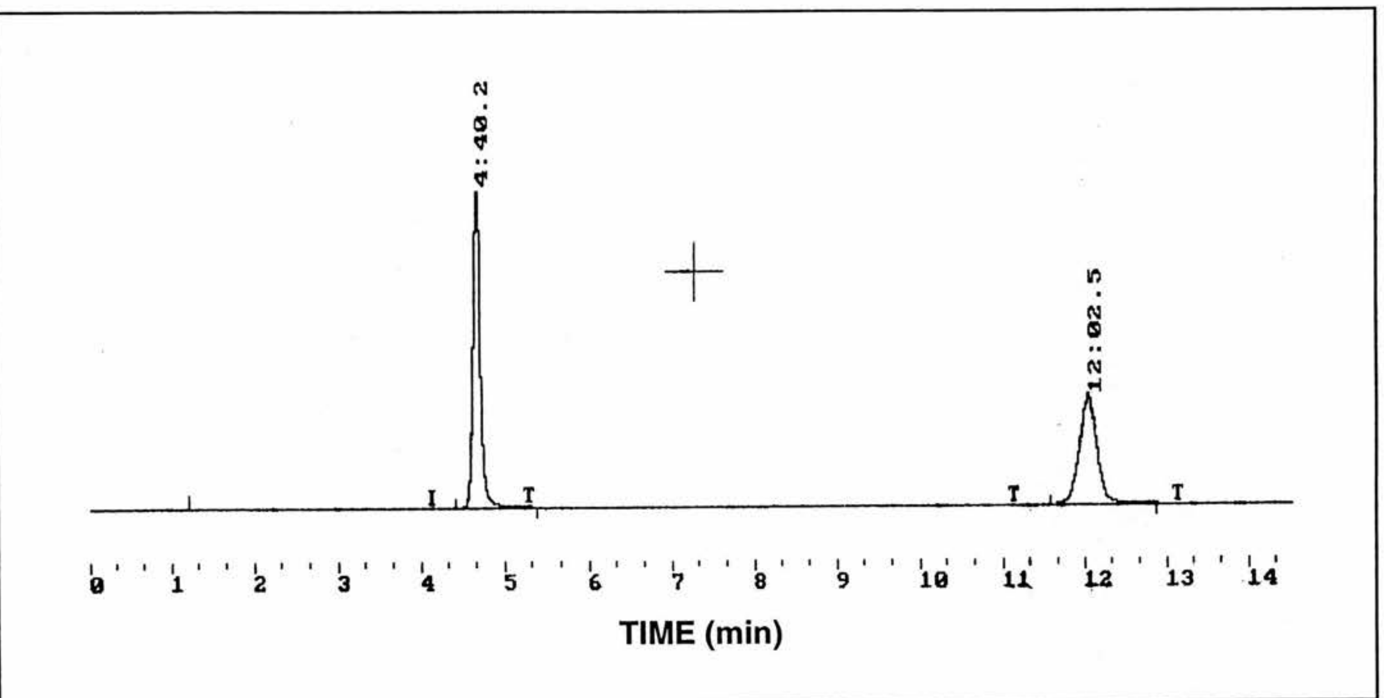


Fig. A.1
The representative chromatogram above shows a standard sample containing 100 pmoles of both kynurenate (4:40.2) and 7-chlorokynurenate (12:02.5).

APPENDIX B - The effects of L-NAME upon cerebrovascular function in the awake rat.

Because NO is known to be involved in a distinct signalling pathway in the vasculature (Moncada, 1992), any investigation of the physiological effects of inhibition of NOS *in vivo* should also include an examination of its effects upon cerebrovascular function. It is worth considering the possibility that physiological changes resulting from L-NAME administration, unrelated to synaptic plasticity, may affect behavioural performance (see Chapter 6). In addition, it is possible that changes in cardiovascular function may contribute to the reduction in the size of hippocampal field epsps following L-NAME administration (Expt 5.6). Accordingly, the effects of inhibition of NOS upon blood pressure, local cerebral glucose utilisation (LCGU) and local cerebral blood flow (LCBF) were investigated. These experiments were performed by Dr. Paul Kelly.

Procedure

A separate group of rats (n=24) were prepared for the measurement of either local cerebral glucose utilization (LCGU) or local cerebral blood flow (LCBF). On the day of the experiment, the animals were anaesthetized with halothane (2%) in a mixture of nitrous oxide (70%) and oxygen (30%). Cannulae were inserted into both femoral arteries for the subsequent measurement of arterial blood pressure and the sampling of arterial blood, and into both femoral veins to allow intravenous fluid replacement and injection of radiolabelled tracers for the measurement of either LCGU or LCBF. The incision sites were infiltrated with local anaesthetic and the wounds sutured closed. A loose-fitting pelvic plaster cast was moulded around the hindquarters of the animal, leaving free access to the abdomen for subsequent i.p. injections. The plaster was taped to a brick and thus restrained and supported. The animals were allowed to recover from anaesthesia for at least two hours before any further experimental manipulation was conducted.

Measurement of LCBF (n=12) and LCGU (n=12) in hippocampus and cerebellum were performed using fully quantitative [^{14}C]-iodoantipyrine (Sakurada et al., 1978) and [^{14}C]-2-deoxyglucose (Sokoloff et al., 1977) autoradiographic techniques, respectively. The protocols were identical to the methodologies as originally described in detail by Sharkey et al., (1991b). The measurement of LCGU was initiated 60 minutes after the i.p. injection of

L-NAME (10 or 75 mg/kg; n=4 at each dose), and the measurement of LCBF after 70 minutes (n=4 at each dose). The relative timing of the two measures was in order to ensure that the blood flow measurement (taken over 45 seconds) coincided with the point of median integrated 2-deoxyglucose specific activity in brain tissue (Sharkey et al., 1991a). Control rats were injected with physiological saline (n=8) and equal numbers used for the measurement of LCGU and LCBF. Arterial blood pressure and rectal temperature were monitored continuously in each animal throughout. Samples of arterial blood were withdrawn before the injection of L-NAME or saline, and again after 70 min, for the measurement of pH, pCO2, pO2 and plasma glucose. At the end of the experimental period, the rats were sacrificed with an overdose of barbiturate, and the brains dissected intact. Autoradiograms were prepared from coronal sections cut in a cryostat, and a quantitative densitometric analysis of the resultant images performed using a computer-based image analysis system (Quantimet 970, Cambridge Instruments).

Results

The i.p. injection of both doses of L-NAME resulted in significant increases in mean arterial blood pressure which was relatively rapid in onset (within 5 minutes) and maintained throughout the experimental period ((mm Hg) saline = 131 ± 5 before vs. 139 ± 4 after; 10 mg/kg L-NAME = 132 ± 2 before vs. 160 ± 4 after; 75 mg/kg L-NAME = 133 ± 4 before vs. 173 ± 8 after). Although the higher dose of L-NAME (75 mg/kg) produced a slightly larger hypertensive response, there were no significant differences in MAPB between the two treatment groups. There were no significant changes in any of the other physiological variables measured following L-NAME injection (eg. plasma glucose concentrations, blood pH, blood gas tensions (Table B.1).

Following L-NAME treatment, there were no significant changes in glucose utilization in any of the hippocampal subfields analysed in this study. Only in the copula pyramis of the cerebellum was a small but statistically significant decrease in LCGU observed (-18%) following both doses of L-NAME. In contrast, decreases in cerebral blood flow were observed in all hippocampal fields and throughout the cerebellum (-25-40%), 70 minutes after the injection of L-NAME, although in two areas, the cerebellar white matter and the CA1 cell field of the caudal hippocampus, decreases were not statistically significant. Increasing the dose of L-NAME to 75 mg/kg had no further effect on LCBF, and there was no significant difference in response between the two doses. Therefore, at both doses of L-

NAME, and in every area of hippocampus and cerebellum examined in this study (19 brain areas), the ratio of mean cereberal blood flow to mean cerebral glucose utilization was decreased following L-NAME treatment (eg. 75 mg/kg; Figure B.1). Such decreases in LCBF in the absence of any changes in LCGU indicate an uncoupling of the fundamental relationship which normally exists between cerebral blood flow and the metabolic demands of brain tissue. These results are discussed in relation to the electrophysiological and behavioural experiments studying the effects of L-NAME in chapters 5 and 6 respectively.

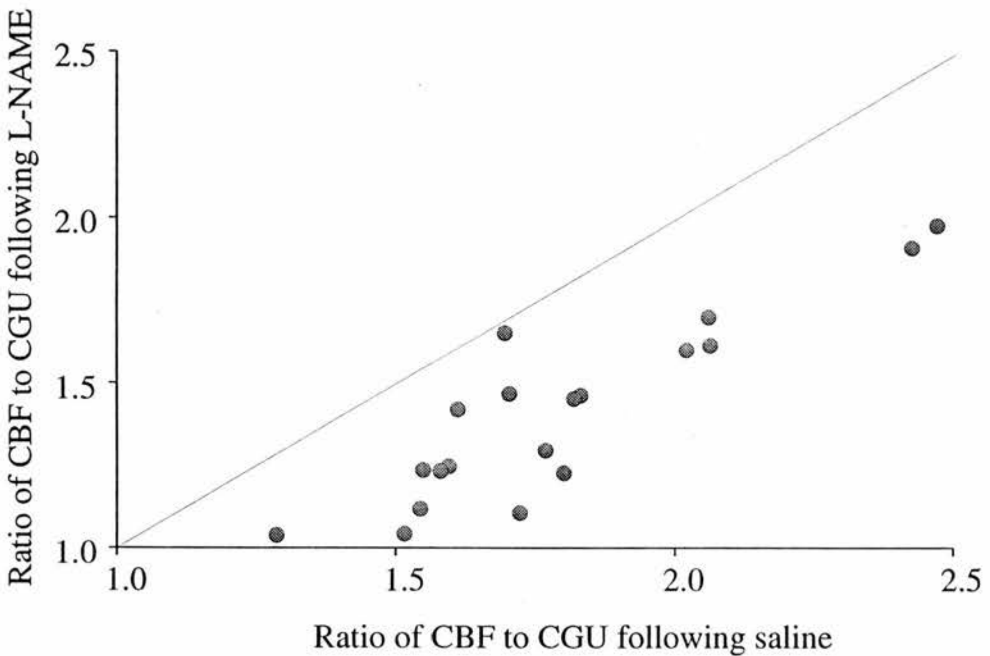


Fig. B.1

L-NAME uncouples cerebral blood flow and metabolism. The ratio of local cerebral blood flow to local cerebral glucose metabolism is lower for animals given a single acute injection of L-NAME (75 mg/kg) than for saline controls. If the flow-metabolism relationship was unchanged by L-NAME, all points (each representing measurements from different subregions of the cerebellum or hippocampus) would fall along the identity line (App. B).

	Saline		L-NAME (10 mg/kg)		L-NAME (75 mg/kg)	
Parameters	Before	After 1 hr	Before	After 1 hr	Before	After 1 hr
Plasma Glucose (mg/l)	1.57±0.10	1.53±0.10	1.52±0.20	1.42±0.10	1.55±0.10	1.31±0.09
pH	7.38±0.01	7.38±0.01	7.35±0.06	7.39±0.04	7.41±0.02	7.41±0.02
pCO_2 (mmHg)	40.2±1.0	39.0±1.0	39.1±2.0	41.7±2.1	42.4±2.0	38.5±2.9
pO_2 (mmHg)	87.2±2.2	88.8±3.3	91.7±3.2	89.8±2.9	89.4±3.0	92.6±2.8
Mean Arterial BP	131±5	139±4	133±2	160±4*†	133±4	173±8*†

Table B.2

The effects of L-NAME upon cerebrovascular function in the awake rat. There were no significant differences between groups in the physiological status of the animals prior to injection. One hour after injection of either 10 or 75 mg/kg L-NAME, mean arterial blood pressure was significantly increased above pre-injection levels, and was significantly higher than saline injected controls. No other physiological variables were significantly altered. Data are presented as mean ± SEM for (n = 4 in each group) measured immediately before the i.p. injection of either saline or L-NAME, and again 1 hour later (App. B).
(* significantly different from pre-injection value; † significantly different from saline control value (Scheffé test)).

APPENDIX C

ıhibition of Nitric Oxide Synthase Does Not Impair Spatial Learning

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ric oxide (NO), a putative intercellular messenger in the IS, may be involved in certain forms of synaptic plasticity d learning. This article reports a series of experiments estigating the effects of N_ω-nitro-L-arginine methyl ester NAME) upon various forms of learning and memory in the termaze. L-NAME (75 mg/kg, i.p., sufficient to bring about 0% inhibition of NO synthesis in brain) produced an aprent impairment in spatial learning when given to naive s during acquisition (3 d, six training trials per day). This pairment was dose related, stereoselective, and attenued by coadministration of L-arginine. A second study owed that L-NAME did not affect the retention of a preusly learned spatial task. In addition, in a visual discrimtion task, the rate at which criterion levels of performance re reached was unaffected by L-NAME. Thus, inhibition NO synthase may cause a selective impairment of spatial rning without effect upon retention. However, analysis of early training trials of the visual discrimination task realed significantly elevated escape latencies in the IAME–treated rats, suggesting that inhibition of NO synse may have more general effects. As normal rats learn spatial task very rapidly, the possibility arises that the parent deficit in learning is due to a disruption of some cess other than learning per se. A further series of eximents investigated this possibility. L-NAME was found to impair the learning of a new platform position in the ne spatial environment. Surprisingly, L-NAME also had no ect on spatial learning in a second watermaze located in ovel spatial environment by rats well practiced with all pects of watermaze training. Finally, L-NAME had no effect spatial learning in naive rats trained with just one trial per /. Thus, systemic injection of an NO synthase inhibitor pairs behavioral performance in two tasks during their ial acquisition, but the basis of this functional disruption unlikely to be due to any direct effect upon the mechams of spatial learning.

Key words: nitric oxide, long-term potentiation, hippompus, rat, watermaze, spatial learning]

ꞏociative long-term potentiation (LTP) is a form of activityendent synaptic plasticity that is widely believed to particte in learning and memory (Bliss and Lømo, 1973; Morris et al., 1990; Bliss and Collingridge, 1993). Evidence supporting a role for the mechanisms of LTP in learning is based, in part, on comparable effects of drugs on LTP and on learning. For example, the induction of LTP requires the activation of NMDA receptors (Collingridge et al., 1983) and it has been shown that the NMDA antagonist D-AP5 impairs spatial learning across a dose range comparable to the inhibition of hippocampal LTP *in vivo* (Davis et al., 1992). Along similar lines, several studies have shown that inhibitors of NO synthase impair certain forms of learning (Chapman et al., 1992; Hölscher and Rose, 1992; Böhme et al., 1993), and block the induction of LTP in the *in vitro* hippocampal slice (Böhme et al., 1991; O'Dell et al., 1991; Schuman and Madison, 1991; Haley et al., 1992).

Although our own observations, made in a parallel study (see following companion article, Bannerman et al., 1994), call into question whether L- nitro-arginine methyl ester (L-NAME) blocks the induction of hippocampal LTP *in vivo,* the possibility that inhibition of NO synthase may limit its induction suggests a novel means to investigate the relationship between LTP and learning. To date, there have been several reports indicating that blocking NO synthase impairs performance in a variety of both spatial and nonspatial learning tasks. Chapman et al. (1992) have shown that intraperitoneal injections of L-NAME (75 mg/kg) impair acquisition of spatial reference memory in the watermaze but do not affect the retention of previously acquired spatial information. Inhibitors of NO synthase have also been reported to impair performance in a spatial reference memory task in the eight-arm radial maze (Böhme et al., 1993). However, the effects of NO synthase inhibitors on learning do not appear to be restricted to hippocampal-dependent tasks such as spatial learning. Deficits in the acquisition of conditioned eyeblink responses (Chapman et al., 1992) and in one trial inhibitory avoidance in the chick (Hölscher and Rose, 1992) following administration of NO synthase inhibitors have also been reported.

A specific issue to address in investigating the action of NO concerns the specificity of the behavioral impairments seen following the administration of an NO synthase inhibitor. Accordingly, a series of experiments were conducted to examine in detail whether impaired performance in the watermaze results from a specific effect on spatial learning or derives from action upon some other process (or processes) engaged in this and other kinds of tasks.

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Materials and Methods

Subjects. Male hooded Lister rats (250–450 gm; n = 157) were used in all experiments. They were individually housed with ad libitum access to food and water. A 12 hr light/dark cycle was maintained (0700–1900) with all testing carried out during the "light" phase.

pparatus. All behavioral testing was carried out in one of two open-d watermazes (Morris, 1981, 1984), each consisting of a large circular k (diameter, 2.0 m; depth, 0.6 m) containing water at 25°C to a depth 0.3 m. The water was made opaque by the addition of powdered k, which not only prevents the animals from seeing the platform but allows efficient tracking of swim paths. In the spatial learning tasks, rats were trained to find a hidden escape platform (diameter, 10 cm) merged 1–1.5 cm below the water surface. Both pools were in the ter of a room containing various prominent cues (wall posters, wall boards, a rig of electrophysiological equipment, and a metal frame). two rooms contained distinguishable extramaze cues. The paths en by the animals in these pools were monitored by a video camera unted in the ceiling. The resulting video signal was relayed to a video order, allowing both on- and off-line analysis, and from there to an ge analyzer (HVS VP112). The x- and y-coordinates of the rats' ition were sampled at 10 Hz by an Archimedes computer (using a gram called WATERMAZE) and stored on disk. This software can pro-e measures of latency, pathlength, swim speed, and so on, and the ribution of time spent in defined regions of the pool (e.g., quadrant, ulus from side wall, etc.). In the visual discrimination task, the rats e trained to escape onto one of two visible and distinguishable plat-ns (diameter, 10 cm) whose visible surfaces were 1–1.5 cm above water. One of these platforms (gray or black-and-white stripes) was d and provided escape from the water; the other (black-and-white pes or gray) was floating and offered insufficient bouyancy to support t. A set of white curtains surrounded the pool occluding extramaze s.

ehavioral pretraining. All rats (with the exception of those partici-ng in the visual discrimination study) received 1 d of nonspatial training consisting of six trials during which the extramaze cues were cured from view by pulling the white curtains around the pool. The form was moved randomly to a different position between trials. s that failed to find the platform after 60 sec were guided to its ition. The rats were allowed to stay on the platform for 30 sec between ls. This pretraining is intended to allow the animals to become ustomed to the apparatus under conditions that prevent any spatial ning but allow them to learn that there is a means of escape from water via the platform. Pretraining was conducted in the absence ny drug treatment, the day before the start of spatial training.

rugs. L-NAME and L-arginine were obtained from Sigma, and NAME was obtained from Bachem. All drug solutions were made n 0.9% physiological saline at an appropriate concentration such an injection volume of 1 ml/100 gm resulted in the final dosages d below.

sults

periment 1: the effect of L-NAME on spatial reference mory (six trials per day)

purpose of the following experiment was to examine wheth-L-NAME affects the acquisition of spatial reference memory he watermaze using a standard training paradigm in which receive a block of six training trials on each day over a iod of 3 d. Additional groups of animals were also run to ermine whether any deficit, resulting from L-NAME admin-ation, was dose related, stereoselective, and/or reversible h coadministration of L- arginine.

cedure

lowing 1 d of drug-free, nonspatial pretraining (trials 1–6), nimals ($n = 53$) were trained to find a fixed location, hidden pe platform (3 d, six trials/day, ITI of 5–10 min, random t position across trials). Rats that failed to find the platform r 120 sec were guided to its location. On each day, 1 hr re the start of behavioral testing, they were injected intra-toneally with either saline ($n = 9$), 10 mg/kg L-NAME (n), 75 mg/kg L-NAME ($n = 9$), 75 mg/kg D-NAME ($n = 9$), mg/kg L-arginine ($n = 9$), or 75 mg/kg L-NAME coadministered with 225 mg/kg L-arginine ($n = 9$). Twenty-four hours after the final day of spatial training (and 1 hr after injection), the rats' memory of the platform location was assessed in a transfer test during which the platform was removed from the pool and the rats allowed to swim freely for 60 sec.

Immediately following the transfer test, the rats received six additional "cue" trials during which they were required to escape onto to a visible gray platform (extending to 1–1.5 cm above the water surface). Both the platform location and the rats' start position were randomly changed from one trial to the next.

Results

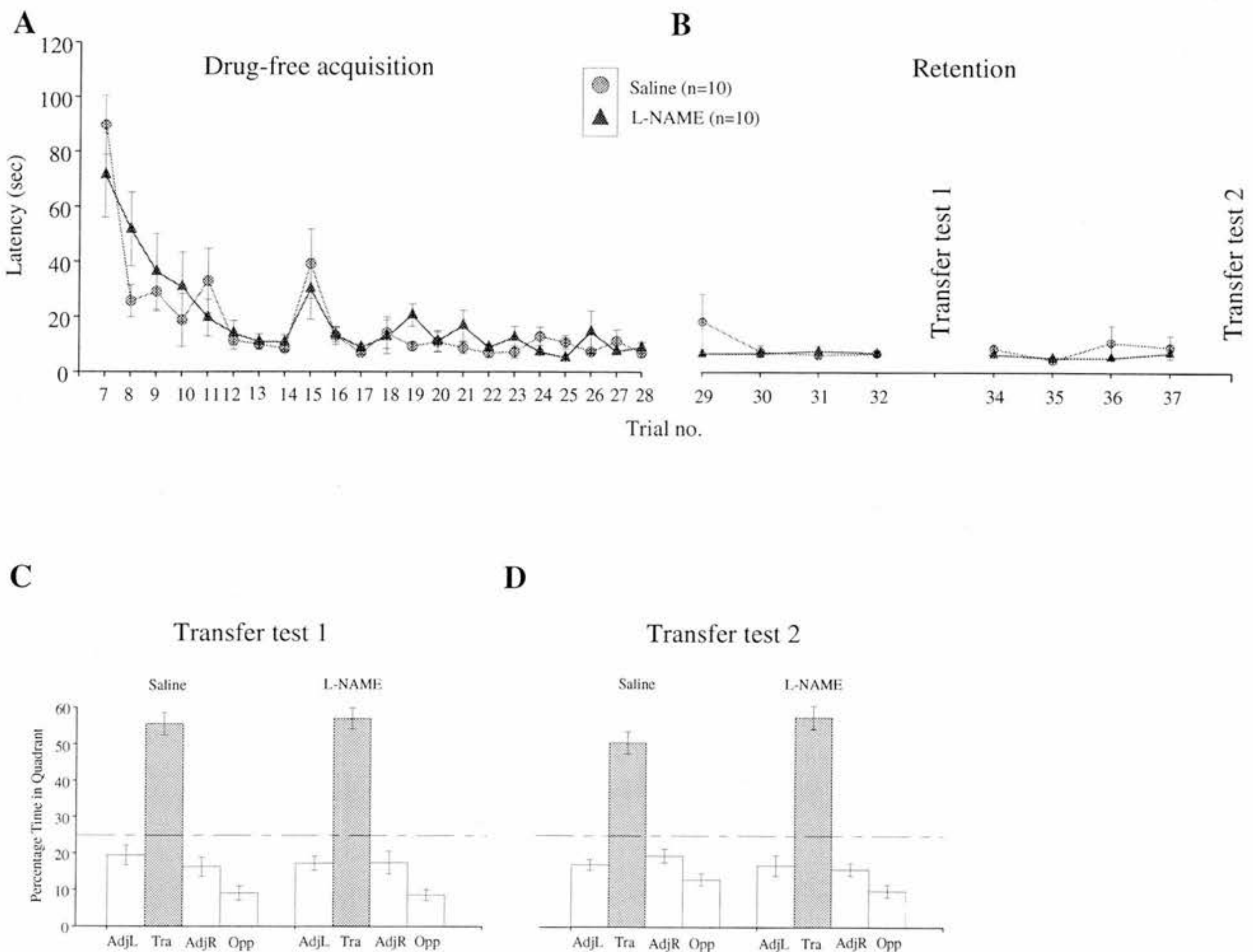
All animals were capable of swimming around the pool until the platform was located and then climbing onto it. There was no visible sign of any sensorimotor disturbance either during swimming or in attempting to climb onto the platform. On the first day of spatial training, the 75 mg/kg L-NAME–treated animals appeared to be swimming faster than those in the other groups. An ANOVA of the mean swim speeds for each animal on the first day of spatial training revealed a significant difference [$F(5,47) = 3.41$; $p < 0.025$] and subsequent Tukey's HSD pairwise comparisons indicated that only the 75 mg/kg L-NAME group (mean = 0.28 ± 0.01 m/sec) differed significantly from the saline group (mean = 0.23 ± 0.01 m/sec).

All animals showed a progressive decline in escape latency with training (Fig. 1*A*). An ANOVA of escape latencies for the 3 d of spatial training revealed an overall effect of group [$F(5,47) = 9.14$; $p < 0.0001$] and trial [$F(17,799) = 28.54$; $p < 0.0001$], and a groups by trials interaction [$F(85,799) = 1.36$; $p < 0.05$]. Further analysis, using Tukey's HSD pairwise comparisons, showed that the 75 mg/kg L-NAME group performed significantly worse than the other groups ($p < 0.01$). Apart from the first trial, for which there was no difference in escape latency between groups ($F < 1$), this deficit was more pronounced over the early trials and, by the end of training, the 75 mg/kg L-NAME–treated rats were escaping the pool as quickly as the others, as reflected in the groups by trials interaction.

The rats' memory of the platform location was assessed in the transfer test (Fig. 1*B,C*). All groups spent more time searching in the quadrant of the pool in which the platform had been located (the "training" quadrant), suggesting that the animals had learned something about the former location of the platform, but the groups differed with respect to the extent of learning [groups by quadrants interaction: $F(10,141) = 2.34$; $p < 0.01$]. A second ANOVA of the percentage time spent in the training quadrant revealed only a significant effect of group [$F(5,47) = 2.96$; $p < 0.05$], and subsequent Tukey's HSD pairwise comparisons showed that the 75 mg/kg L-NAME group was spending significantly less time in the training quadrant than the saline group ($p < 0.05$), and less time than that shown by the 10 mg/kg L-NAME, D-NAME, and the L-NAME + arginine groups considered together ($p < 0.025$). None of these three groups, or the L-arginine alone group, differed significantly from the saline-injected controls.

Immediately after the transfer test, the rats were given the "cue" task. All rats escaped from the pool rapidly (Fig. 1*D*) and there was no significant difference between the groups ($F < 1$).

The result of this first study is in agreement with Chapman et al. (1992) in demonstrating that L-NAME causes an impair-



ure 2. Experiment 2. L-NAME (75 mg/kg) does not affect the retention of previously learned spatial information. *A,* Mean escape latencies ing the initial, drug-free, spatial training. *B,* Mean escape latencies during retention. *C,* The mean percentage time spent in the four quadrants he pool (organized with respect to the training quadrant) during the first transfer test after four retention trials (trial 33). *D,* Transfer test 2 after ıt retention trials (trial 38).

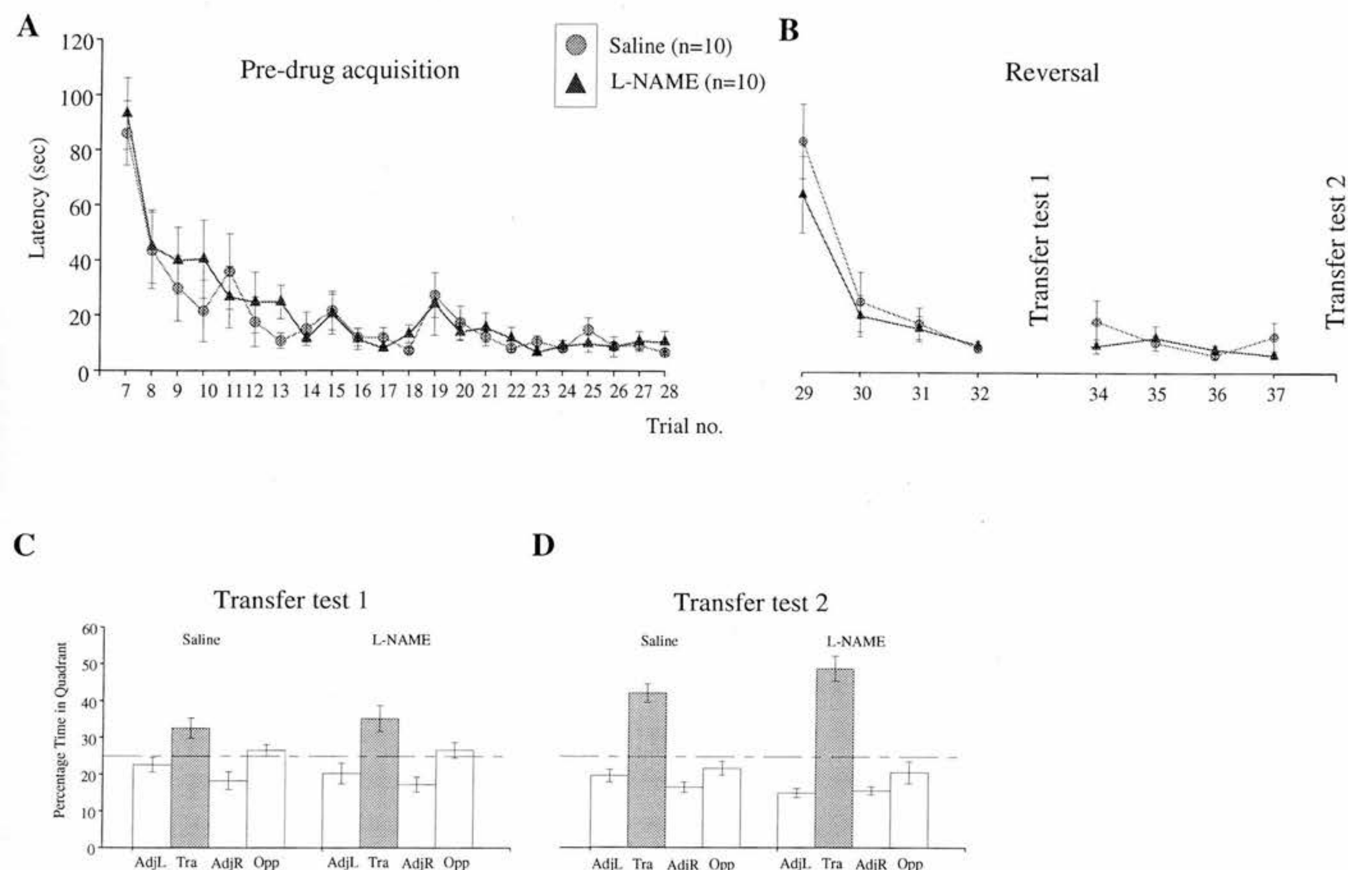
ıed, persistent activation of NO synthase, then the addition .-arginine should reverse the enzyme blockade and result in immediate expression of LTP. The fact that arginine only erses the L-methyl arginine inhibition of LTP if present dur- the tetanic stimulation supports the idea that NO is involved nduction but not long-term expression. Accordingly, if LTP substrate for spatial learning, and if NO is involved in LTP, n one might predict that NO synthase inhibition would pre- t new learning but spare retention, in a manner parallel to behavioral results obtained with D-AP5.

cedure

lowing 1 d of nonspatial pretraining (trials 1–6), rats (n = were extensively trained (in the absence of any drug injec- ı) to find a fixed location, hidden escape platform (3 d of r trials/day, 3 d of two trials/day, and 4 d of one trial/day; ls 7–28). Following a 3 d interval (in order to equate the ning protocol with that used in the D-AP5 experiment of rris et al., 1990), they then received another 8 d of training ı one trial per day to the same platform location. At this ;e, the rats were injected with saline (n = 10) or L-NAME mg/kg; n = 10) each day, 1 hr prior to the start of behavioral ing. Performance was assessed with a first transfer test, 24 hr after trial 32 (and immediately before trial 34), and again 24 hr after trial 37.

Results

During the initial, drug-free, spatial training, all animals learned something about the the location of the hidden platform as indicated by direct swim paths and short escape latencies on the later training trials (Fig. 2*A*). On the first trial after drug administration (trial 29), both groups of animals showed good retention of the platform location and escaped rapidly from the water with no significant difference in terms of escape latency [$F(1,18) = 1.39$; $p = 0.25$]. All rats continued to escape rapidly from the pool during the eight training trials of the drug phase (Fig. 2*B*). An ANOVA revealed no effect of group [$F(1,18) = 1.10$; $p = 0.31$] or trial [$F(7,126) = 1.04$; $p = 0.41$], nor a groups by trials interaction [$F(7,126) = 1.04$; $p = 0.41$]. The two transfer tests (trials 33 and 38; Fig. 2*C,D*) also showed that both groups searched persistently in the training quadrant. An ANOVA of performance in the first transfer test showed a significant effect of quadrant [$F(2,54) = 105.81$; $p < 0.001$] but no groups by quadrants interaction ($F < 1$). Similar results were obtained in the second transfer test [quadrants $F(2,54) = 111.59$; $p < 0.001$; groups by quadrants interaction $F(2,54) = 1.73$; $p > 0.10$].



igure 4. Experiment 4. L-NAME does not impair the acquisition of a novel platform location in a familiar spatial environment (reversal). *A,* ean escape latencies during the initial, drug-free, spatial training. *B,* Mean escape latencies during reversal. L-NAME (75 mg/kg) did not impair e acquisition of a novel platform location in a familiar spatial environment. *C,* The mean percentage time spent in the four quadrants of the ol (organized with respect to the training quadrant) during the first transfer test after four reversal trials (trial 33). *D,* Transfer test 2 after eight versal trials (trial 38).

o groups were indistinguishable on subsequent training sesons. The basis for the longer escape latencies on day 1 is nclear, but a contribution may be that the L-NAME-treated imals showed a greater tendency than saline-treated animals ward failing to escape from the water at all within 60 sec $F(1,19) = 4.05$; $p = 0.06$].

L-NAME did not affect the rate at which criterion levels of erformance were attained in the visual discrimination task. here was, however, a subtle effect of the inhibitor on perforance on the first day of training that resulted in prolonged cape latencies and a tendency to fail to climb onto the stable atform within 60 sec. This apparent "first day" effect of NAME in the watermaze could be of more consequence in a ore rapidly acquired task such as the spatial reference memory sk, and thus may contribute toward the deficit observed in periment 1.

xperiment 4: the effect of L-NAME on acquisition of a ovel platform location in a familiar spatial environment

he results of experiments 1–3 suggest a profile of impairment milar to that reported to occur following chronic intravencular infusion of AP5: an impairment of spatial learning witht effect upon retention or visual discrimination learning (Mors et al., 1986b, 1990). However, the strikingly longer escape tencies of the L-NAME group during day 1 of the visual discrimination task points to a different possibility. Specifically, L-NAME may cause a transient and nonspecific impairment that can affect performance in several different types of learning, rather than a direct effect on the process of spatial learning per se.

A series of experiments was conducted to distinguish these two possibilities. The first of these, run in parallel with experiment 2, began with the same drug-free initial training but, during the drug phase of the experiment, the platform was moved to the opposite quadrant of the pool (a type of "reversal" task). As previously mentioned, D-AP5 has been reported to impair performance in such a reversal task, presumably because new learning is involved (Morris et al., 1990).

Procedure

Separate groups of rats were trained as in experiment 2 ($n = 10$ per group), with the exception that, during the drug phase, the platform was moved to the opposite quadrant of the pool (i.e., rats trained initially to SW had the platform moved to NE).

Results

As in experiment 2, all animals demonstrated a considerable reduction in escape latencies during initial training (Fig. 4*A*). On the first trial during the drug administration phase (trial 29),

ɔre time in the new location and, thus, their escape latencies luced across trials (Fig. 4*B*). An ANOVA revealed a signifiıt improvement across trials [$F(7,126) = 21.02$; $p < 0.0001$], t no effect of group [$F(1,18) = 2.17$; $p = 0.16$] nor any groups trials interaction ($F < 1$). Transfer tests conducted 24 hr after ıls 32 and 37 also showed that L-NAME had no effect on the ןuisition of the new platform location (Fig. 4*C,D*). An ANO- of the first transfer test revealed a significant effect of quadıt [$F(2,54) = 12.60$; $p < 0.001$], but no groups by quadrants eraction ($F < 1$). Similarly, on the second transfer test, there s a significant effect of quadrant [$F(2,54) = 59.93$; $p < 0.001$] again no interaction with the drug treatment [$F(2,54) =$ 3; $p > 0.10$]. Therefore, and somewhat surprisingly, L-NAME l not impair the learning of a novel platform location in a niliar spatial environment.

ɔeriment 5: the effect of L-NAME on spatial learning in ıovel environment by experienced animals previously ined in another watermaze

e lack of an effect of L-NAME on the "reversal" task (ex-ּiment 4) may be explained in two ways. First, the amount new learning required to solve the "reversal" task may be ּy small. During the initial drug-free phase, a relatively deed representation of the environment will already have been med and so, when the platform is moved, the only modifiion required of that representation is to encode the new goal ation. There is no need to "reverse" the learned spatial reonships between extramaze cues. Learning a new goal loion in a familiar environment may necessitate very little, if /, synaptic plasticity. Alternatively, it is possible that the NAME-induced deficit in experiment 1 is, as noted above, ɛ to an action of the drug on some process other than spatial rning which detrimentally affects performance early in train- . To distinguish these two possibilities, a further experiment s conducted in which the initial watermaze training was carɖ out in a different spatial environment from that used during drug phase (Gallagher, 1985; Shapiro and O'Connor, 1992). th this training protocol, the experienced animals then have learn about a wholly novel spatial environment during the ıg phase, in contrast to the "reversal" task in which they have y to learn a new platform location. In this experiment, the ining protocol was otherwise identical to that used in the ɛntion/reversal experiments (experiments 2 and 4).

ɔcedure

llowing 1 d of nonspatial pretraining (trials 1–6), the rats (*n* 24) were given spatial training in watermaze 1 (3 d of four ıls/day, 3 d of two trials/day, and 4 d of one trial/day; trials 28). Following a 3 d interval (in order to equate the training ɔtocol to that used in experiments 2 and 4), a subgroup of ht rats (made up of four animals from each of the saline and NAME groups) was given a transfer test in watermaze 2 (trial in order to assess whether training in watermaze 1 resulted a spatial bias towards any one quadrant in watermaze 2. mediately following this test, these and the remaining rats gan their eight trials of spatial training in watermaze 2 (1 trial/ /), being injected with saline ($n = 12$) or L-NAME (75 mg/ $n = 12$) 1 hr prior to the start of behavioral testing each day. watermaze 1, the platform was located in one of the four ıdrants of the pool. In watermaze 2, the platform was located ɛither the NE or SW quadrant. For every four rats trained to articular platform position in watermaze 2, each of the four platform positions had been used during training in watermaze 1. Assignment of rats to groups was counterbalanced with respect to the platform position in both watermazes 1 and 2 and performance over the last four training trials in watermaze 1 (trials 25–28). Performance in watermaze 2 was assessed with respect to escape latency and using a transfer test, 24 hr after trial 33 (and immediately before trial 35) and again 24 hr after trial 38. Finally, the rats were returned to watermaze 1 and retention of the platform location was assessed with a transfer test in the absence of any drug injection (trial 40).

Results

During spatial training in watermaze 1, both groups of animals learned to escape from the water rapidly. An ANOVA of escape latencies revealed a highly significant improvement across trials [$F(21,462) = 10.42$; $p < 0.0001$; Fig. 5*A*).

The first transfer test in watermaze 2 (trial 29), before the start of spatial training, was analyzed in two ways. First, the data was expressed in terms of the "future" training quadrant (Fig. 5*B*). An ANOVA showed no significant bias to any one quadrant ($F < 1$). Second, the data was reanalyzed in terms of the actual quadrants described in terms of compass directions (Fig. 5*C*); this showed a small bias toward the NW and SW quadrants [$F(2,21) = 4.43$; $p < 0.025$]. This corresponds to a bias toward the door into the watermaze room and the location of the carrying cage.

The rats learned about the new spatial environment extremely rapidly, showing a mean escape latency of less than 20 sec by trial 4. An ANOVA of the escape latencies from the eight training trials in watermaze 2 (Fig. 5*D*) revealed that there was a significant improvement across trials [$F(7,54) = 3.87$; $p < 0.001$] but no overall effect of group [$F < 1$]. There was, however, a just significant groups by trials interaction [$F(7,54) = 2.10$; $p =$ 0.05]. A subsequent analysis of simple main effects revealed group differences on trials 32 and 38 ($p < 0.05$). However, the fact that on trial 32 the saline group was taking, on average, longer to escape, while on trial 38 the opposite was true, suggests that these differences are most likely due to random fluctuations in performance rather than to some substantive underlying cause.

An ANOVA of the transfer test (trial 34; Fig. 5*E*), conducted after four training trials in watermaze 2, revealed a strong bias toward the training quadrant [$F(2,66) = 18.13$; $p < 0.001$]. There was no groups by quadrants interaction ($F < 1$). This result was repeated in the second transfer test [trial 39; Fig. 5*F*; $F(2,66) = 78.34$; $p < 0.001$], with there again being no interaction with drug treatment ($F < 1$).

Finally, when the rats were returned to watermaze 1, both groups still showed a significant and equivalent bias toward the appropriate training quadrant reflecting their memory of the original training environment despite the intervening training (trial 40; Fig. 5*G*). An ANOVA showed a significant effect of quadrant [$F(2,66) = 9.01$; $p < 0.001$] with no groups by quadrants interaction ($F < 1$).

In summary, L-NAME did not impair the acquisition of spatial reference memory in experienced animals that had previously been trained on a watermaze task in a different spatial environment.

Experiment 6: the effect of L-NAME on spatial reference memory (one trial per day)

Examining the pattern of results obtained so far, it is possible that L-NAME only impairs performance early in training in

mals receive only one training trial per day. This lack of ect is, statistically, neither a "ceiling" nor a "floor" effect in t both groups showed a progressive increase in the bias to-rd the training quadrant over the three transfer tests.

scussion

e main findings of this series of experiments were that animals ected intraperitoneally with a dose of L-NAME sufficient to ng about a >90% reduction in hippocampal NO synthase nnerman et al., 1994) showed (1) impaired performance in h a spatial reference memory and a visual discrimination k on early training trials (when these were conducted with ltiple trials per session), and (2) that the same dose of L-NAME s without effect on performance in retention, reversal learn-, the learning of a new spatial environment in watermaze-erienced animals and, even, acquisition of the basic water-ze task in experimentally naive animals trained at one trial day.

es L-NAME impair spatial learning?

ese results are consistent with previously published reports the effects of systemic NO synthase inhibitors on learning he watermaze (Chapman et al., 1992). L-NAME impairs the uisition of a spatial reference memory task with multiple ls per session but does not affect the retention of previously ned spatial information. This impairment is dose related, eoselective, and reversed by coadministration of L-arginine. an attempt to determine the behavioral specificity of this pairment, performance in a visual discrimination task was essed. The rate at which criterion levels of performance were ined was unaffected by L-NAME. This apparent dissociation ween the effects of L-NAME on performance of a spatial task l a visual discrimination task is, in several respects, similar hat obtained with D-AP5 (Morris et al., 1986b), and with ctive hippocampal lesions (Morris et al., 1986a). This sug-ts that L-NAME–treated rats do not exhibit a gross senso-otor impairment and can indeed learn some tasks normally. Closer analysis of the visual discrimination task revealed, vever, that the L-NAME animals took significantly longer to ape from the pool during the first training session. This deficit mpted the suggestion that L-NAME causes a transient but re general disturbance of function extending beyond the do-in of spatial learning. Irrespective of the nature of this dis-pance, it could also account for the apparent impairment of tial learning in experiment 1, bearing in mind that the 18 ls of training took place over only 3 d and the deficit appears be largest over the first 2 d. If L-NAME were, on the other d, actually disrupting the mechanisms underlying spatial ning, then rats should be impaired relative to controls with-regard to intertrial interval or the number of trials per ses-. The results of the platform reversal task (experiment 4) the two watermaze tasks (experiment 5) raises problems for hypothesis and suggests that the initial deficit (experiment ; due to a subtle but less specific effect. The result of the one l/day spatial reference memory task (experiment 6) further s into question the hypothesis that L-NAME is disrupting tial learning. Clearly, we cannot rule out the possibility that ore complete inhibition of NO synthase may result in a real airment of spatial learning. Gene knockouts of brain NO thase may provide an opportunity to realize more complete bition (Huang et al., 1993).

What are the implications for the relationship between LTP and learning?

Given the fact that NO synthase inhibitors block the induction of LTP *in vitro,* our interpretation of the behavioral results would appear to contradict the hypothesis that LTP and certain kinds of learning share a common underlying mechanism. This is not so: in a parallel study (following article, Bannerman et al., 1994), we show that the very same dose and route of administration of L-NAME (causing a >90% inhibition of NO synthase whether given acutely or chronically) does not appear to block the induction of NMDA-dependent LTP in the dentate gyrus *in vivo.* Thus, an accurate description of the two studies is that a compound that does *not* block LTP does *not* impair spatial learning. This result does not contradict the hypothesis of a link between LTP and learning. L-NAME did, however, cause a number of physiological and behavioral changes—a reduction in dentate field potentials, cerebrovascular changes, and a transient behavioral dysfunction affecting both a hippocampal-independent and a hippocampal-dependent task.

What is the nature of the functional disturbance induced by L-NAME?

In attempting to provide an explanation for the observed pattern of results, two distinct but related questions need to be addressed. First, what is it about the various tasks used in this study that results in impaired performance in some, but not other, paradigms? Second, what possible physiological actions of L-NAME may be responsible for the effects on performance? It may then be possible to determine how these two factors interact to produce the observed pattern of results.

Why is impaired performance seen in some, but not other, paradigms? L-NAME impairs performance in tasks involving training with multiple trials and short ITIs. In contrast, L-NAME–treated rats receiving just one trial/day are unimpaired. One possibility is that the physical demands of a task involving multiple trials are much greater and that animals become tired following repeated swims. Drug treatment may accentuate this fatigue and this, in turn, could indirectly interfere with the animals' ability to learn. This account predicts a deficit in both the spatial and the visual discrimination tasks for as long as the animals are taking a considerable time to escape from the water. As performance improves, however, the drug-induced deficit would be expected to disappear—as it did in both experiment 1 and 3. Additionally, there are several potential differences at a psychological level between the multiple- and single-trial paradigms. With one trial/day, the rats find the platform and are then removed to their home cage for the rest of the day—a satisfactory escape. In contrast, with multiple trials, having first found the platform, the rats are soon after put back into the water for further trials. Thus, the platform may be less rewarding with multiple trials offering lesser incentive to escape. Repeated sequential exposure to the aversive elements of the watermaze in the multiple trial paradigm may, quite separately, be more anxiogenic. L-NAME treatment could conceivably interact with the altered psychological state of the animal during multiple-trial paradigms in such a way as to interfere, albeit indirectly, with the ability to learn. In the single-trial paradigms, however, locating the platform results in a complete escape from the water for 24 hr, which could potentially make the task less stressful. Similarly, as animals become more familiar with the procedural demands of the task during training,

APPENDIX D

ıibition of Nitric Oxide Synthase Does Not Prevent the Induction of ɔng-Term Potentiation *in vivo*

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ic oxide (NO), a putative intercellular messenger in the S, may be involved in certain forms of synaptic plasticity l learning. This article reports a series of experiments estigating whether an inhibitor of NO synthase, N_ω-nitro-ginine methyl ester (L-NAME), affects long-term poten-ion (LTP) *in vivo,* as the results of recent *in vitro* exper-nts would predict. L-NAME, given as an acute injection a dose sufficient to inhibit hippocampal NO synthase 0%), had no effect on perforant path–dentate gyrus LTP uced by a strongly suprathreshold tetanus, but appeared mpair LTP induced by a weak near-threshold tetanus that y be more physiologically relevant. However, subsequent dies revealed that chronic L-NAME treatment (>95% in-tion of NO synthase) had no effect upon LTP induction, that acute (but not chronic) treatment resulted in a grad-but significant reduction in nontetanized baseline field entials. The baseline shift appeared to be of a magnitude icient to account for the apparent impairment of weak nus-induced LTP. This possibility was further examined two-hemisphere experiment in which the time course of nges in the field EPSP of the nontetanized pathway served he within-subject control for the tetanized pathway. No airment of LTP induction was observed; indeed, if any-g, there was a trend for greater potentiation with L-NAME. ause NO has also been implicated in the control of va-ilation, the effect of L-NAME on cerebrovascular function also investigated. Peripheral blood pressure was sig-cantly increased by L-NAME at the same dose that af-ted the field EPSP. Local cerebral glucose utilization was hanged, while local cerebral blood flow decreased sig-cantly in various brain regions, including the hippocam-, indicating an uncoupling of cerebral metabolism and od flow. Thus, while NO synthase inhibition does not ap-r to limit the induction of LTP *in vivo,* it does reduce the e of baseline field EPSPs and affect local cerebrovascular ction.

Key words: nitric oxide, long-term potentiation, hippo-npus, rat, cerebrovascular, flow–metabolism coupling]

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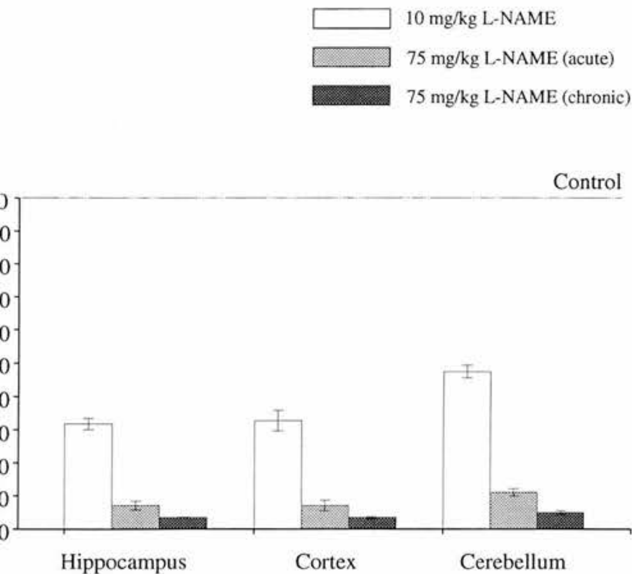
Associative long-term potentiation (LTP) is widely considered to be a model of activity-dependent synaptic plasticity that could be involved in certain forms of learning and memory (Bliss and Lømo, 1973; Morris et al., 1990; Bliss and Collingridge, 1993). The induction of LTP requires the activation of postsynaptic NMDA receptors (Collingridge et al., 1983; Malenka, 1991), and its expression shows a high degree of topological specificity to activated synapses (Kelso et al., 1986; Wigström et al., 1986; Bonhoeffer et al., 1989). However, there is evidence suggesting that the expression of LTP involves an increase in presynaptic transmitter release (Dolphin et al., 1982; Bekkers and Stevens, 1990; Malinow and Tsien, 1990). This implies that there may be an intercellular messenger from postsynaptic to presynaptic neurons.

One candidate intercellular messenger is nitric oxide (NO). NO has the appropriate properties of being freely diffusable and short-lived, as well as being synthesized by a calcium/calmodulin-dependent enzyme, nitric oxide synthase (Garthwaite et al., 1988; Bredt and Snyder, 1992). Evidence supporting the putative role of NO as an intercellular messenger has come from experiments demonstrating that administration of NO synthase inhibitors, or of the extracellular NO scavenger hemoglobin, blocks the induction of hippocampal LTP *in vitro* (Böhme et al., 1991; O'Dell et al., 1991; Schuman and Madison, 1991; Haley et al., 1992). In addition, NO or NO donors (e.g., sodium nitroprusside) can induce synaptic enhancement, either alone (O'Dell et al., 1991; Bon et al., 1992) or when combined with presynaptic activation (Zhuo et al., 1993).

The importance of LTP depends in part on its efficacy as a model of learning and memory. There is evidence suggesting that systemic injection of NO synthase inhibitors affects performance on several different learning tasks (Chapman et al., 1992; Hölscher and Rose, 1992; Böhme et al., 1993). However, it is not known whether inhibitors of NO synthase administered in this way disrupt the establishment of LTP *in vivo.* This is of great importance when considering the possible use of NO synthase inhibitors as a novel means to investigate the relationship between LTP and learning.

Because NO is involved in several distinct signaling pathways in blood vessels (Moncada, 1992), an investigation of the physiological effects of inhibition of NO synthase *in vivo* should also include an examination of its effect upon cerebrovascular function. Accordingly, we measured the effects of L-NAME upon blood pressure, local cerebral glucose utilization (LCGU), and local cerebral blood flow (LCBF).



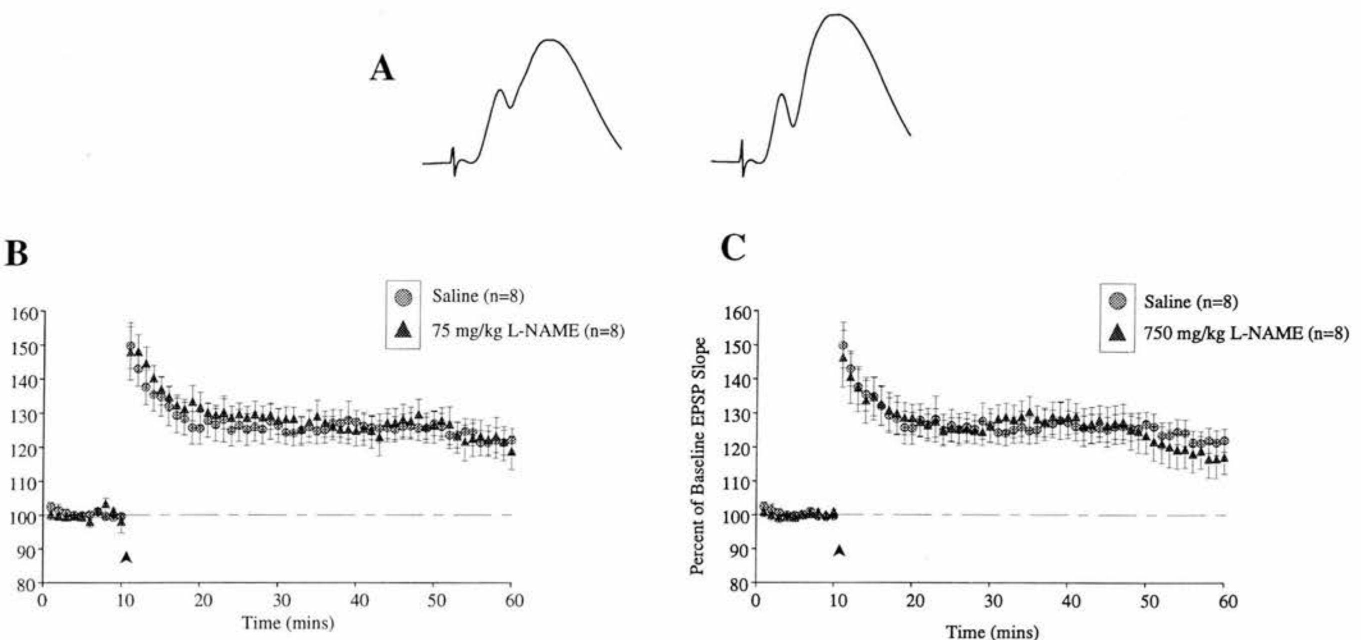
re 1. Experiment 1. L-NAME inhibits NO synthase activity. NO hase activity was determined by measuring the conversion of ^{3}H-ine to ^{3}H-citrulline. L-NAME produced a dose-related impairment O synthase activity in the brain. NOS activity in saline-injected rols was, in hippocampus, 2.09 ± 0.21; cortex, 1.40 ± 0.11; and bellum, 3.11 ± 0.25 dpm × 10^{5}.

er across groups (L-NAME, 75 mg/kg = 2.94 ± 0.29 mV/ c; L-NAME, 750 mg/kg = 3.05 ± 0.48 mV/msec; saline = 5 ± 0.62 mV/msec; $F < 1$). The potentiation elicited by this nus was unaffected by L-NAME (Fig. 2*A,B*). The mean slope he field EPSP evoked by 700 μA test pulses delivered at 0.1 and measured between 45 and 50 min after the tetanus was .4 ± 5.2% for L-NAME animals, and 121.7 ± 3.6% for trols, relative to a baseline averaged over a 5 min period immediately prior to the tetanus. Animals given 750 mg/kg of L-NAME ($n = 8$), 10 times the initial dose, also demonstrated normal LTP (117.1 ± 5.0%; Fig. 2*C*). An ANOVA of the LTP in these three groups showed that they did not differ ($F < 1$).

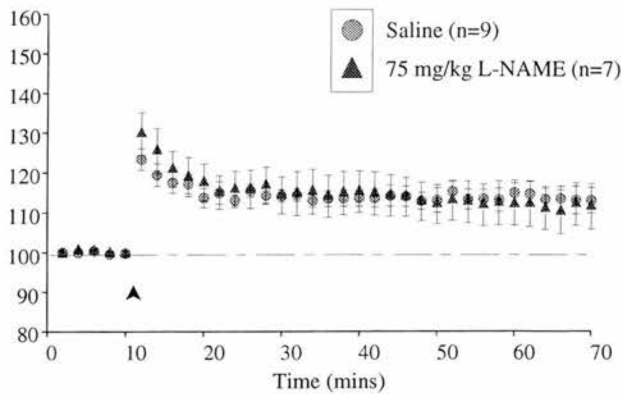
Experiment 3: the effect of L-NAME upon LTP induced by a just suprathreshold tetanus

In view of the inability of L-NAME to affect LTP induced by strongly suprathreshold tetanic stimuli, we altered the tetanic stimulation parameters to a protocol close to the threshold for LTP induction. The number of tetanic trains, pulses per train, train frequency, and pulse intensity were all reduced until only a minimal but measurable amount of LTP was observed in control animals. The experimental protocol was also changed such that test pulse frequency was reduced to 0.05 Hz and test pulse intensity to between 300 and 600 μA (the intensity required to elicit a response that was 50% of the maximum EPSP slope). Accordingly, baseline measurements were taken over a 10 min period immediately prior to the tetanus and the amount of potentiation measured between 50 and 60 min post-tetanus.

The mean absolute slope of the baseline field EPSPs did not differ between the L-NAME (75 mg/kg; 2.86 ± 0.35 mV/msec; $n = 10$) and saline (2.75 ± 0.34 mV/msec; $n = 7$; $F < 1$) groups. L-NAME had no significant effect on the initial potentiation seen 0–4 min after the tetanus ($F < 1$) but, if anything, there was greater potentiation in the L-NAME (121 ± 3%) than in the saline group (116 ± 3%; Fig. 3*A*). The slope of the field EPSP declined in both groups over the course of the next hour. However, this decline was substantially greater in the L-NAME group: (1) the mean slope potentiation 50–60 min post-tetanus shown by the saline group (110.5 ± 4.7%) was significantly greater than that of the L-NAME group [98.0 ± 2.9%; $F(1,15) = 6.26$, $p < 0.025$]; (2) the saline group showed significant potentiation relative to its pretetanus baseline ($t = 2.31$, df = 6, $p < 0.05$) while the L-NAME–treated animals did not ($t <$



re 2. Experiment 2. L-NAME does not affect LTP induced by a strongly suprathreshold tetanus. *A*, Representative field EPSPs recorded from granule cell layer of the dentate gyrus following perforant path stimulation before (*left*) and after (*right*) tetanic stimulation. *B*, L-NAME (75 kg) does not inhibit the induction of LTP induced by a strongly suprathreshold tetanus. Tetanus given at *arrow*. *C*, A 10-fold increase in the of L-NAME (750 mg/kg) was also ineffective.



ıre 4. Experiment 4. Chronic treatment (10–20 d) with L-NAME mg/kg) does not block the induction of LTP induced by a just athreshold tetanus.

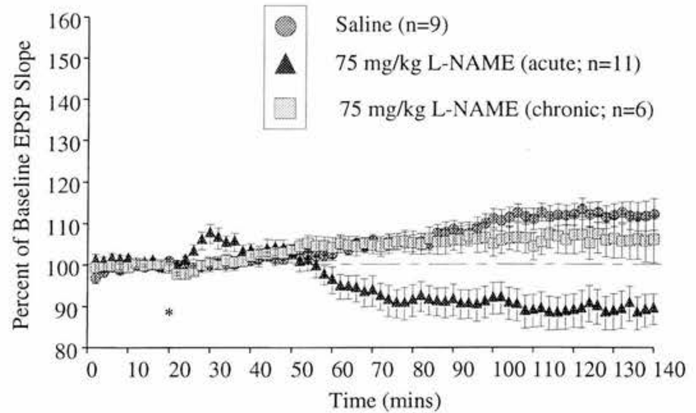


Figure 5. Experiment 5. Acute L-NAME (75 mg/kg) causes changes in baseline field potentials over an extended 2 hr baseline in the absence of a tetanus. Chronic L-NAME (75 mg/kg) treatment produced a profile of effect similar to saline-injected animals. Injection at *asterisk.*

-defined time course, with most of the decline occurring ng the first 60 min after drug injection. Interestingly, the ›nically treated L-NAME group showed neither the short- ı increase immediately after the last injection, nor the grad- decline thereafter (see Fig. 5). In fact, the time course of the e changes in this group did not differ from those of the ıe-treated group [50–60 min, $F < 1$; 110–120 min, $F(1,13)$ .18, $p > 0.10$].

ɔr purposes of comparison with previous LTP experiments his study (e.g., experiments 3 and 4), it is important to sure the change in "baseline" during the *second* hour starting nin postinjection. A further ANOVA was therefore con- .ed on the acutely treated groups only, comparing the mean es 50–60 min postinjection with those 110–120 min postin- on (i.e., equivalent to the analysis used in experiment 3). ; showed that the slope function for the L-NAME–treated declined by 1.7 ± 3.5%, while the saline-treated rats in- sed by 6.3 ± 2.2% [$F(1,18) = 3.46$, $p = 0.08$]. While this ıge is not significant in itself, the absolute difference between ..-NAME- and saline-treated rats amounts to 8.0%, sufficient ccount for the apparent blockade of LTP in experiment 3. he same token, the lack of difference in the baseline between saline- and the chronically treated L-NAME groups, taken ther with the results of experiment 4, is consistent with this ›unt.

eriment 6: the effect of L-NAME upon induction of LTP weak tetanus using a nontetanized hemisphere as a in-subject control pathway

ıe light of the results of experiment 5, it is possible that the ırent blockade of weak tetanus LTP (experiment 3) can be ained by underlying changes in the baselines. However, ex- ments 3 and 5 were conducted using separate animals. Ac- ingly, a two-hemisphere experiment was conducted in which perforant path of one hemisphere received a weak tetanus e the other served as a within-subject control pathway (Bliss Lømo, 1973). Animals were prepared in the usual way pting that stimulating and recording electrodes were im- ted bilaterally. The animals were left for 1 hr prior to the of recording. Potentials were elicited by low-frequency test es to each hemisphere (0.05 Hz, 500 μA), interlaced such the rats received one stimulus every 10 sec. After a 20 min line, they were injected with L-NAME (75 mg/kg; $n = 12$) or saline ($n = 12$) and, 60 min later, a weak tetanus was delivered to one hemisphere only. Test pulses continued for a further 60 min. During the course of this study, two reports that intrace- rebroventricular administration of L-NAME reduced LTP of the dentate population spike were published (Iga et al., 1993; Mizutani et al., 1993). Changes in the amplitude of the popu- lation spike can occur for several reasons other than an alteration in synaptic efficacy (McNaughton et al., 1978). Nevertheless, we decided to examine LTP of the population spike in a subset of the animals participating in this study.

There were no significant differences in the mean absolute slope values of the preinjection baselines of the L-NAME- and saline-treated animals, or between the tetanized and nonteta- nized hemispheres [L-NAME: tetanized = 4.38 ± 0.51 mV/msec; nontet = 3.47 ± 0.38 mV/msec; saline: tet = 3.80 ± 0.35 mV/msec; nontet = 3.47 ± 0.54 mV/msec; groups $F < 1$; tet/nontet $F(1,22) = 3.20$, $p > 0.05$]. All potentials were initially normalized with respect to the preinjection baseline (10 min period immediately prior to injection, i.e., 10–20 min in Fig. 6*A*). The first result of note is that over 2 hr L-NAME had an effect upon field EPSPs comparable to that seen in experiment 5. Figure 6*A* shows the drug-induced change on what served throughout as the nontetanized control pathway; L-NAME caused an immediate increase in slope followed by a gradual decline, while the saline pathway showed a slow but steady increase. There was a 16.1% difference between the L-NAME and saline groups at the 80 min time point on Figure 6*A* (i.e., 60 min after drug injection and the point at which a tetanus was delivered to the experimental pathway). This difference has to be taken into account to assess the amount of LTP in the two groups—a point shown graphically in Figure 6, *B* and *C,* which plot the results for both hemispheres in the L-NAME and saline groups, respectively. These graphs appear to show some poten- tiation in both groups, but it is difficult to assess this quanti- tatively due to the gradually changing baselines. If one looks solely at the experimental pathway and normalizes with respect to the 10 min period prior to the tetanus (70–80 min), the outcome is a result similar to the finding reported in experiment 3: apparently greater potentiation in the saline group (Fig. 6*D*). However, if the nontetanized pathway (also normalized with respect to the 70–80 min time period) is used as a within-subject control at each time point (t) throughout the experiment, [i.e.,

le 1. The effects of L-NAME on cerebrovascular function in the awake rat

	Saline		L-NAME			
			10 mg/kg		75 mg/kg	
ameters	Before	After 1 hr	Before	After 1 hr	Before	After 1 hr
ma glucose (mg/liter)	1.57 ± 0.10	1.53 ± 0.10	1.52 ± 0.20	1.42 ± 0.10	1.55 ± 0.10	1.31 ± 0.09
	7.38 ± 0.01	7.38 ± 0.01	7.35 ± 0.06	7.39 ± 0.04	7.41 ± 0.02	7.41 ± 0.02
)$_2$ (mm Hg)	40.2 ± 1.0	39.0 ± 1.0	39.1 ± 2.0	41.7 ± 2.1	42.4 ± 2.0	38.5 ± 2.9
(mm Hg)	87.2 ± 2.2	88.8 ± 3.3	91.7 ± 3.2	89.8 ± 2.9	89.4 ± 3.0	92.6 ± 2.8
n arterial BP	131 ± 5	139 ± 4	133 ± 2	160 ± 4*†	133 ± 4	173 ± 8*†

e were no significant differences between groups in the physiological status of the animals prior to injection. One hour after the injection of either 10 or 75 mg/kg AME, mean arterial blood pressure was significantly increased above preinjection levels, and was significantly higher than saline-injected controls. No other iological variables were significantly altered. Data are presented as mean ± SEM for $n = 4$ in each group, measured immediately before the intraperitoneal injection ther saline of L-NAME, and again 1 hr later.

nificantly different from preinjection value (Scheffé test).

nificantly different from the appropriate saline-injected control value (Scheffé test).

owing the reports of both Iga et al. (1993) and Mizutani et 1993), we also examined LTP of the population spike in a set of animals participating in this study. To be included, nimal had to have a population spike of >1 mV pretetanus. results (Fig. 6*F*) indicate that 50–60 min post-tetanus as ch population spike LTP was shown by the L-NAME–treated (166.9 ± 16.9%; $n = 9$) as by the saline group (150.2 ± %; $n = 9$). These values did not differ ($F < 1$).

eriment 7: the effect of L-NAME upon cerebrovascular tion in the awake rat

intraperitoneal injection of both doses of L-NAME resulted gnificant increases in mean arterial blood pressure (MAPB), ch was relatively rapid in onset (within 5 min) and was ntained throughout the experimental period (Table 1). Al- ugh the higher dose of L-NAME (75 mg/kg) produced a ntly greater hypertensive response, there was no significant erence in MABP between the two treatment groups. Neither of L-NAME significantly altered any of the other physio- cal variables measured (Table 1).

here were no significant changes in LCGU in any of the pocampal fields measured in this study following either of two doses of L-NAME. Only in the copula pyramis of the bellum was a small but statistically significant decrease in GU observed (−18%) following both doses of L-NAME. In rast, decreases in cerebral blood flow were observed in all pocampal fields and throughout the cerebellum 70 min after injection of L-NAME (10 mg/kg), although in two areas, bellar white matter and the caudal CA1 field of the hip- mpus, decreases were not statistically significant. Increasing dose of L-NAME to 75 mg/kg had no further effect on LCBF, there was no significant difference in response between the doses. Thus, at both doses of L-NAME, and in each of the brain areas analyzed in this study, the ratio of mean LCBF ean LCGU was decreased (Fig. 7). Such decreases in LCBF e absence of any changes in LCGU indicate an uncoupling e fundamental relationship which normally exists between bral blood flow and the metabolic demands of brain tissue.

cussion

main findings of this series of experiments were that (1) aperitoneal injections of L-NAME caused a dose-related in- tion of NO synthase with both a single acute and a series ronic 75 mg/kg injections resulting in >90% inhibition in hippocampus; (2) L-NAME, at this and a 10-fold higher dose, was without effect upon LTP induced by a strong tetanus and recorded in the dentate gyrus *in vivo*; (3) L-NAME caused an apparent inhibition of LTP *in vivo* when induced by a weak tetanus, but subsequent experiments using a within-subject control pathway suggest that this is primarily a consequence of an underlying decrease in the size of the baseline field EPSP; and (4) L-NAME caused a reduction in cerebral blood flow but no change in cerebral glucose utilization.

Has NO synthase been inhibited?

As the main finding of this report is that an inhibitor of NO synthase, L-NAME, does not appear to block the induction of LTP *in vivo* induced by either weak or strong tetanic stimuli, it

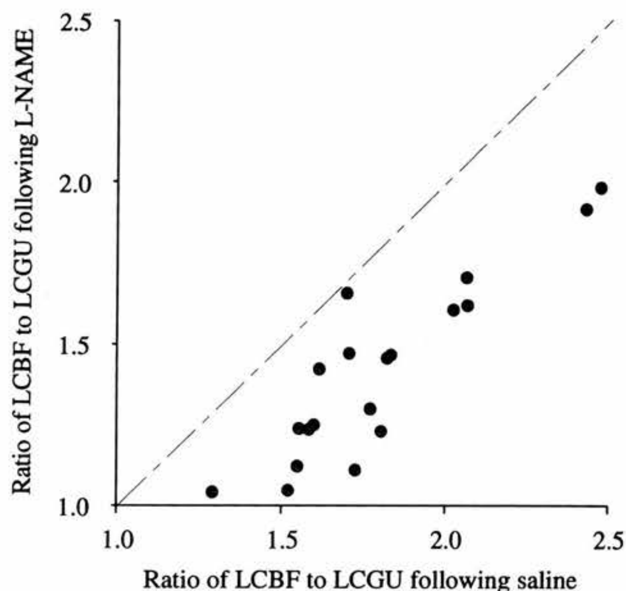


Figure 7. Experiment 7. L-NAME uncouples cerebral blood flow and metabolism. The ratio of local cerebral blood flow to local cerebral glucose metabolism is lower for animals given a single injection of L-NAME (75 mg/kg) than for saline controls. If the flow–metabolism relationship was unchanged by L-NAME, all points (each representing measurements from different subregions of the cerebellum or hippocampus) would fall along the line of identity (illustrated). The shift in the ratios of LCBF to LCGU is indicative of a flow–metabolism uncoupling.

ıal synaptic transmission (e.g., size of the baseline field P).

does L-NAME have little effect in chronically treated ıals?

tment with L-NAME over the course of several days should d ample opportunity for synaptic transmission to stabilize new lower baseline. Further injection of L-NAME would pe expected to produce additional decrements in the slope e field potentials—as we found (experiment 5). Thus, if the rent blockade of weak tetanus LTP seen in experiment 3 really an artifact of a gradually changing baseline, one d not expect such LTP to be impaired in chronically treated ıals. This result is exactly what was obtained (experiment ın alternative possible explanation for the failure to block following chronic administration of L-NAME in terms of loping tolerance seems unlikely in the light of our own and er et al.'s (1991) data, showing that the level of NO synthase pition increases with repeated injections.

does L-NAME fail to block dentate LTP in vivo?

findings, together with those of Barnes et al. (1994), indicate inhibition of NO synthase fails to block dentate LTP *in* There are at least three possible explanations for the ab- of a blockade of LTP with systemic administration of an synthase inhibitor. First, residual NO synthase activity (4– ı our case) may be sufficient to support normal LTP. While are as yet no pharmacological means to ensure complete cade of NO synthase activity, gene knockout experiments ain NO synthase may provide such an opportunity (Huang , 1993). Second, NO may play a threshold modulatory role TP induction such that other messengers (e.g., CO, Stevens Wang, 1993; arachadonic acid, Williams et al., 1989) could ibute to the induction of potentiation under these exper- tal conditions. Third, NO may have no role in the induc- of LTP *in vivo.*

th respect to the last alternative, although several labora- s have reported that NO synthase inhibition blocks the ction of LTP, others have suggested that this effect is de- ent upon experimental conditions (e.g., temperature, age e animal, tetanus parameters; Gribkoff and Lum-Ragan, ; Chetkovich et al., 1993; Haley et al., 1993; Williams et 993). It is not, therefore, altogether unprecedented that the er procedural differences of this study (e.g., *in vivo* vs *in* systemic administration vs bath application) have resulted block of LTP. A separate consideration is the use of the rant path–dentate gyrus synapse rather than the Schaffer eral–CA1 synapse used more frequently in hippocampal . We chose to study synapses in the dentate gyrus because exhibit a relatively high density of NO synthase (Bredt et 990, 1991), and because this is a frequently used and re- method of studying hippocampal LTP *in vivo.* In addition, are no a priori grounds to believe that LTP in perforant dentate gyrus synapses is fundamentally different from of Schaffer collateral–CA1 synapses (e.g., NMDA receptor ndency). Even so, we cannot rule out the possibility that esults may not apply to other hippocampal subfields, and dy of CA1 LTP *in vivo* would be worthwhile.

cent studies by Mizutani et al. (1993) and Iga et al. (1993) t that infusion of L-NAME into the lateral ventricle blocks nduction of LTP of the population spike in the dentate *in vivo.* Our data (experiment 6) does not show any blockade of LTP either in terms of the slope of the field EPSP or of the population spike. There are at least two differences in procedure that may account for these anomolous results. First, the strain and age of the animals may be relevant. Second, the intracerebroventricular route of administration of the drug may result in higher levels of enzyme inhibition in the hippocampus than the >95% inhibition we achieved. In addition, it should be noted that Mizutani et al. (1993) report significantly smaller initial potentiation in animals treated with NO synthase inhibitors, which is in contrast to most other studies measuring EPSPs both *in vitro* and *in vivo,* and raises the possibility that intracerebroventricular administration may have additional physiological effects. Further investigations are underway in our laboratory.

Can L-NAME induce apparently greater LTP?

One intriguing observation of experiment 6 was that the level of long-lasting synaptic potentiation in L-NAME–treated animals appeared to be greater than in control animals when expressed as a ratio of tetanized to nontetanized hemispheres. Although this effect was not significant ($p = 0.07$), there is a clear trend suggesting an L-NAME–induced enhancement of LTP. Because NO can block NMDA receptors (Izumi et al., 1992; Lei et al., 1992; Manzoni et al., 1992), inhibition of NO synthase may result in more NMDA receptor activation during the tetanus and thus elicit greater or more long-lasting potentiation. The magnitude of such an effect may vary as a function of the extent of NMDA receptor activation and thus be determined by the size of the tetanus. Interestingly, we did not observe any trend toward greater LTP in experiment 1, in which a strong tetanus was used. However, the results of experiment 1 are plotted without taking into account any differential changes in the baseline that might have occurred in saline and L-NAME–treated rats (for which we have no data because all animals were tetanized). If changes similar to those measured in experiment 5 occurred in experiment 1, the LTP shown by the L-NAME group is likely to have been greater than that of the saline group. We do not, however, seek to overemphasize this enhanced LTP phenomenon: the effect did not reach statistical significance and is calculated in the presence of gradually changing baselines in both the L-NAME and saline groups. Further study seems warranted.

What are the implications for behavioral studies?

There are several reports in the literature indicating that NO synthase inhibitors impair performance in a variety of learning paradigms including tasks thought to require the integrity of the hippocampus (Chapman et al., 1992; Hölscher and Rose, 1992; Böhme et al., 1993). Although it is by no means certain if, and to what extent, the mechanisms of LTP may also be involved in learning, the lack of an effect of L-NAME on the induction of hippocampal LTP *in vivo* would appear to call into question whether NO synthase inhibition is producing a learning impairment through an effect on LTP. This does not, however, preclude the possibility that NO synthase inhibition is interfering with some other aspect of hippocampal physiology (Hanbauer et al., 1992; Lonart et al., 1992; Wood et al., 1992) which may result in a spatial learning impairment, or changes in brain function elsewhere which may account for apparent impairments of other types of learning. It is unclear to what extent the L-NAME-induced reduction in the size of dentate field EPSPs would affect the normal function of the hippocampus or other

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