CORTICAL NEURONES IN ALZHEIMER'S DISEASE

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ABSTRACT

Two major neurotransmitter systems, cholinergic and glutamatergic, have been studied, mainly in the cortex of patients with Alzheimer's disease. Choline acetyltransferase (ChAT) activity was assayed in 15 areas of the cerebral cortex taken post-mortem from patients with Alzheimer's disease and controls. The number of neurofibrillary tangles was determined in an adjacent tissue sample. ChAT reduction and tangle numbers were maximal in structures of the medial temporal lobe (the uncus, amygdala, hippocampus and parahippocampal gyrus), severe in the neocortex on the lateral surface of the temporal lobe, moderate in the association cortex of the cortex of the parietal and frontal lobes and minimal in the primary somatic and visual sensory areas. These results are interpreted as providing support for the hypothesis that the pathological process in Alzheimer's disease may spread along a sequence of cortico-cortical connections between the main sensory area and the hippocampal formation.

Earlier work in this laboratory suggested that glutamatergic neurones are involved in this process. Furthermore tangles appear to be localized in pyramidal cells which probably use glutamate as their transmitter. Due to a lack of a sutable enzyme marker for glutamatergic cells, this type of cell is extremely difficult to investigate in humans. A drug with possible efficacy in Alzheimer's disease has been examined for effects on glutamatergic neurones (using laboratory animals). In addition a glutamate receptor subtype has been studied in detail, using post-mortem human tissue.

Tetrahydro-9-aminoacridine (tacrine), an alleged drug for the treatment of Alzheimer's disease was examined for effects on glutamatergic neurones in rat brain. The Ca^{2+}-dependent release and Na^{+}-dependent uptake of amino acids in tissue prisms were inhibited by the drug. Extracellular amino acid concentrations (collected by in vivo microdialysis and measured by HPLC with fluorometric detection) were elevated by the drug. However, none of these effects were observed with concentrations thought to be clinically relevant suggesting that the alleged clinical benefit is dependent on the well documented cholinomimetic actions of this drug.

The binding of [^{3}H]-(+)-5-methyl-10 ,11-dihydro-5H-dibenzo [a,d] cyclohepten-5,10 imine maleate ([^{3}H]-MK-801) and [^{3}H]-1-[1-(2-thienyl)cyclohexyl]piperidine ([^{3}H]-TCP) to the N-methyl D-aspartate (NMDA) receptor complex were studied in control post-mortem human brain taken from all cortical areas which was pooled before study. Binding of both ligands was stimulated by glutamate and glycine but the addition of both glutamate and glycine together resulted in an additional effect on [^{3}H]-MK-801 binding only. Saturation analysis revealed approximately twice as many high affinity sites for [^{3}H]-MK-801 than for [^{3}H]-TCP binding. Cortical tissue from the temporal lobe, a severely affected area in Alzheimer's disease, and the frontal lobe, moderately affected, from a number of patients were assayed by radioligand binding for the density of the NMDA receptor complex using [^{3}H]-MK-801 and [^{3}H]-TCP binding . There did not appear to be an alteration in the density of this receptor in Alzheimer's disease in the temporal cortex but there was a decrease in [^{3}H]-MK-801 binding in the frontal cortex.

The modulation of the NMDA receptor complex by glutamate, glycine, zinc and a polyamine, was examined in post-mortem human brain. In control brain the modulation by all four substances was similar to that in rat brain indicating that the NMDA receptor complex is similar in rat and human brain. In Alzheimer's disease tissue, there appeared to be a selective impairment of regulation by glycine and spermidine. These data are discussed in terms of a starting point for rational pharmacotherapy for Alzheimer's disease.

ABBREVIATIONS

ACh	acetylcholine.
AD	Alzheimer's disease.
AMPA	α-amino-3-hydroxy-5-methylisoxazole-4-propionate.
L-AP4	L-2-amino-4-phosphonobutanoic acid.
AP5	2- amino-5-phosphono- pentanoate.
BA	Brodmann area.
ChAT	choline acetyltransferase.
CNS	central nervous system.
CSF	cerebrospinal fluid.
GABA	gamma-amino butyric acid.
GAD	glutamate decarboxylase.
Glu	glutamate.
Gly	glycine
HCA	homocysteic acid.
5-HT	5-hydroxytryptamine.
HPLC	high performance liquid chromatography.
KRP	Krebs Ringer phosphate.
mCPP	m-chlorophenyl piperazine.
MK-801	(+)-5-methyl-10 ,11-dihydro-5H-dibenzo [a,d] cyclohepten-5,10 imine maleate.
nbM	nucleus basalis of Meynert.
NMDA	N-Methyl-D-aspartate.
OPA	ortho-phthaldialdehyde.
PCP	phencyclidine.
PET	positron emission tomography.
PHF	paired helical filament.
PMSF	phenyl methyl sulphonyl fluoride
SLIR	somatostatin-like immunoreactivity.
Tacrine	tetrahydro-9-aminoacridine.
TCA	tricarboxylic acid.
TCP	1-[1-(2-thienyl)cyclohexyl]piperidine.
SKF 10047	N-allylnormetazocine.
Sper	spermidine.

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CHAPTER 1

INTRODUCTION

1.1. Alzheimer's Disease- general overview.

Dementia is the gradual global impairment of higher cognitive function that occurs in 5-10% of the population over 65 years old and more than 20% of those aged over 80 (Katzman, 1976, Plum, 1979, Garland & Cross, 1982). About 40% of the entire population is over 55 years of age, so the syndrome has widespread social and economic implications for patient care.

Causes of progressive dementia make up more than 60 disorders including multi-infarct dementia, Pick's disease, Huntington's chorea, Parkinson's disease, hydrocephalus, brain tumours, trauma (eg. dementia pugilistica) and cerebral infections (eg. meningitis). Dementia may also be secondary to systemic disease eg. dementia in alcoholics (Korsakoff's psychosis), Vitamin B_{12} and folate deficiency, hepatic encephalopathy and renal dialysis. Of all the origins of progressive dementia, Alzheimer's disease is the major cause, irrespective of the age of onset. An estimated 36% of patients of presenile age (65 years or less) suffer from Alzheimer's disease (36%, Marsden & Harrison 1972) and this increases to 50% in senile groups (Tomlinson 1977b). Although clinical symptoms and morphological symptoms are usually more severe in the presenile group (Rossor et al., 1984), the two groups are regarded as synonymous and the term "Alzheimer's disease" will be used to describe dementia of Alzheimer type, irrespective of age of onset. The primary cause of Alzheimer's disease is unknown, but a number of aetiological factors have been proposed (eg aluminium, virus see Katzman,1986, Price, 1986, Pearson & Powell, 1989, Francis & Bowen, 1990).

The characterstic histological changes in the brain now associated with Alzheimer's disease were first described by Alois Alzheimer (1907). The disease follows a common prognosis though the symptoms vary from patient to patient (McKahn et al., 1984, Swash et al., 1985, Neary et al., 1986). In the early stages, memory loss

becomes apparent which is difficult to distinguish from benign senile forgetfulness. As the disease progresses, the patient enters a confused phase with more global impairment of cognitive functioning. Changes in high cortical functions, such as language, perceptuo-spatial relationships and problem solving, become more apparent. In the final phase, the patient becomes aimless and may hallucinate or become restless and agitated. The disease course lasts an average of about 6 years, though 10 years is not uncommon (Sulkava et al., 1983).

1.2. Diagnosis

The clinical diagnosis of Alzheimer's disease is based on the exclusion of other dementing disorders that may be present with similar clinical features. Clinical evaluation involves the taking of case histories and the performing of mental status tests along with physical and neurological examinations.

Perhaps the most widely used criteria for the clinical diagnosis of Alzheimer's disease are those specified by the American Psychiatric Association in their Diagnostic and Statistical Manual of Mental Disorders (DSM-III). The DSM-III diagnosis of dementia requires evidence of both cognitive and functional loss in a patient. Such changes become apparent when a mental status examination is performed. The two most common tests of mental status are the information-concentration-orientation test and the mini-mental state examination (Blessed et al., 1968). The former test is entirely verbal and measures recent and past memory, orientation and concentration. The mini-mental state test is broader and it also tests drawing, writing and language (Katzman et al., 1988).

Other diagnostic tools that may be useful in differentiating Alzheimer's disease from other dementing disorders include the use of blood tests, electroencephalograms, scanning techniques (see section on macroscopic features) and the taking of biopsy specimens (see section on microscopic features).

1.3. Neuropathology

1.3.1 Macroscopic features.

Alzheimer's disease has been well defined morphologically (Corsellis, 1976, Tomlinson, 1977a, 1977b, Tomlinson & Corsellis, 1984). The brain is usually shrunken with the weight often reduced from 1200-1450g to 1000g or less. Cortical atrophy is prominent and, although usually widespread, tends to affect frontal and temporal lobes most noticeably. Atrophy does not always accompany this disease, for occasionally severe and characteristic microscopic degeneration occur without any obvious evidence of tissue shrinkage. When the brain is cut coronally, the appearance of atrophy is enhanced by widening of the sulci, narrowing of the cortical ribbon and moderate to severe dilation of the lateral and third ventricles.

Brain atrophy post-mortem has been estimated by weighing the brain (see Jellinger and Reiderer, 1984, Bowen et al., 1989) comparing brain volume with cranial capacity (Hubbard & Anderson, 1981) and by measuring either cortical thickness (Terry et al., 1981, Mann et al., 1985) or length (Duyckaerts et al., 1985). Most neuropathological assessments highlight the temporal cortex as being most affected. The parietal cortex has often not been studied or it has not been possible to measure the volume of the frontal and parietal cortex separately.

A clinical syndrome of parietal lobe dysfunction is common in Alzheimer's disease, and positron emission tomography (PET) studies with fluorodeoxyglucose has demonstrated a focus of glucose hypometabolism in the association areas of this region. Such a scanning technique is likely to be affected by loss of brain tissue, and as yet there are no means whereby this tissue loss can be assessed in the living brain precisely (Chawluk et al., 1987). This is not the case for post-mortem studies. Atrophy of the temporal lobe has already been objectively assessed by weighing samples, and the 20% reduction in weight agrees with brain volume measurements (Bowen et al., 1979).

1.3.2. Microscopic changes

The definitive diagnosis of Alzheimer's disease depends on confirmation by microscopic investigation of the presence of characteristic changes in the cerebral

cortex. During the subjects' life this can only be achieved by taking cerebral biopsy samples.

The two major microscopic changes which are associated with Alzheimer's disease are neurofibrillary tangles and senile plaques. Less prominent histological abnormalities include granulovacuolar degeneration, Hirano body formation, neuropil degeneration, and vascular amyloid deposition. Plaques and tangles, although present in normal age

ing brain, are limited to cortical areas and the hippocampus in particular and their densities are much greater in Alzheimer's disease (Blessed et al. 1968). The high densities of plaques and tangles in the cerebral cortex and hippocampus are characteristic of Alzheimer's disease pathology and are diagnostic of Alzheimer's disease post-mortem. Their numbers in post-mortem specimens have also been correlated with clinical symptoms (eg Wilcock & Esiri, 1982).

i)Senile Plaques

Although found in small numbers in normal, aged non-demented subjects, large numbers of neuritic plaques in the cerebral cortex have been found in two neuropathological disorders, Alzheimer's disease and trisomy 21. They are spherical 20-200 μm lesions which consist of abnormal neurites that surround an amyloid core. Abnormal mitochondria, lysosomes, straight or helical filaments are found in many (but not all) these dystrophic neurites. The helical filaments resemble those found in tangles. The type of transmitter-releasing neurone associated with plaque formation is not clear and immunocytochemical studies have found associations with many transmitter cell types (Struble et al., 1987, Walker et al. 1988) including cholinergic (Struble et al., 1982, Kitt et al., 1984) peptidergic (Roberts et al., 1985) and GABAergic (Oertel et al., 1983, Walker et al., 1985).

Plaques are found to be widespread in the cerebral cortex and may occupy between one-third and one half of the content of the cortex in silver stained preparation (see Tomlinson & Corsellis, 1984). Plaque counts are greatest in the temporal lobe, parahippocampal gyrus, hippocampus and amygdala (Brun 1983, Rogers & Morrison 1985). In severe cases of Alzheimer's disease, no area of the cortex is spared. In

the subcortex, they are present in nuclei which receive fibres from cortical areas (usually containing tangles), like the hippocampus, claustrum and striatum (Rudelli et al., 1984, Tomlinson & Corsellis, 1984).

ii) Neurofibrillary tangles

The presence of tangles is not specific to Alzheimer's disease as these lesions are also apparent in progressive supranuclear palsy, Pick's disease, dementia pugilistica, Guam Parkinson-dementia complex and Hallervarden-Spatz variants (Dickson et al., 1985, Rasool & Selkoe, 1985, Probst et al., 1983, Eidelberg et al., 1987). It may be inferred that tangle formation is a non-specific response of particular neurones to a variety of cerebral insults. But this does not render the understanding of the pathogenesis of tangle formation unimportant because of their rather selective but abundant presence in Alzheimer's disease brains despite a common process.

Tangles are intracellular structures consisting of abnormal protein aggregations called paired helical filaments (PHFs, Kidd, 1963). These PHFs comprise mainly two 10 nm wide filaments twisted about each other in pairs (Wisniewski et al., 1976). The maximum width of a PHF is 20-24 nm, including the fuzzy, granular outer coat that characterizes PHFs in situ. Some filaments may be straight and unpaired (Yagashita et al., 1981) and coexist with PHF in the same tangle. Both types of filaments are also present in neuritic plaques. The pattern of tangle pathology in the cortex is similar to that of plaques, with temporal and limbic regions being severely affected (Wilcock & Esiri, 1982, Pearson et al., 1985, Mann et al., 1986, Esiri et al., 1989). This distribution of pathology is discussed in section 1.11.3.

1.4. Neurotransmitter abnormalities

Studies of neurotransmitter systems in Alzheimer's disease have mainly involved post-mortem and biopsy tissue and cerebrospinal fluid (CSF) but blood and urine as well as extraneural tissues eg platelets have also been studied. Analyses of such samples have included measurements of neurotransmitter and metabolite concentrations, enzyme activities, receptor densities, the uptake and release of neurotransmitters and most recently measurement of mRNAs coding for receptor proteins (Harrison

et al., 1990, Harrison & Pearson, 1990).

1.4.1. Human studies of neurotransmitter function.

Whereas inbred strains of laboratory animals provide a consistent, homogeneous population for investigation, the human population formsa heterogeneous group. As a result, larger variations are to be expected in any biochemical measurements compared with rat studies. There are many variables associated with postmortem material such as drug treatment, the temperature at which the body was maintained before autopsy, time interval between death and autopsy, cerebral hemisphere selected for analysis, anatomical precision during dissection, the method of sampling, the tissue time interval between autopsy and freezing and the duration of storage. Of particular importance in the study of Alzheimer's disease tissue obtained post-mortem is consideration of the pre-mortem conditions such as coma, respiratory disease and hypoxia. Since terminal bronchopneumonia (Corsellis, 1962) and reduced cerebral blood flow (Ingvar & Gustafson, 1970) usually occur in patients with senile dementia and other manuscripts report a profound effect of coma and bronchopneumonia in control tissue (McGeer & McGeer, 1976, Bowen et al., 1976a, 1977a, Iversen et al., 1978, Palmer et al., 1988, Dodd et al., 1988, Procter et al., 1989, Procter et al., 1990, Harrison et al., 1990, Harrison & Pearson, 1990) consideration of this factor in these studies cannot be overestimated.

Antemortem studies of neurosurgical tissue and CSF circumvent these influences, but inherent variables such as gender and age are unavoidable. The availability of biopsy tissue is limited and therefore cannot be routinely used. Lumbar CSF has been more readily available for studies but it is not certain that neurotransmitters in the lumbar CSF reflect neurotransmitters in the brain.

For radioligand binding studies below, the homogenisation of all layers of the cortical ribbon may present problems with the interpretation of data, as neuronal populations are differentially distributed within different layers of the cortex (see Greenamyre & Young, 1989). A further complication is the freezing necessary for

storage of human material. Tissue fractionation from previously frozen tissue is not equivalent to fresh tissue (Schwarz, 1980, Hardy et al., 1987,). In addition it has been proposed that when pathological conditions are to be studied, minimal tissue fractionation should be employed in order to reduce the possibility that the constituents of fractions from diseased and control tissue may not be equivalent (Procter et al., 1990).

1.4.2 Neocortical transmitter deficits.

The function and integrity of transmitter systems, especially the cholinergic system, have been extensively studied in Alzheimer's disease for several reasons. Any selective change in a neurotransmitter may provide a starting point for unravelling the underlying pathogenesis of the disease. Knowledge of which neurones are affected may allow the development of drugs which alleviate the symptoms of the disease in an analogous fashion to L-DOPA administration in Parkinson's disease.

Although Alzheimer's disease was first described by Alzheimer in 1907, it was not until the mid seventies that a clear loss of cholinergic function was described (see the seminal review of DeKosky and Bass, 1985), based on reduced choline acetyltransferase (ChAT) activity. Since then, the loss of several other neurotransmitters of ascending tracts, as well as of interneurones within the cortex, have been reported. However, the cholinergic deficit remains the most consistent change.

i) Acetylcholine and the cholinergic hypothesis.

An early finding of post-mortem biochemical studies of Alzheimer's disease was a marked reduction in the activity of choline acetyltransferase (ChAT), an enyme enriched within cholinergic varicosities (Tucek, 1967, Fonnum, 1970) and responsible for the synthesis of the neurotransmitter acetylcholine from choline and acetyl-coenzyme A. This reduction (to less than 50% of control values) in the neocortex and hippocampus has been confirmed in all studies in which it has been examined (Bowen et al., 1976, Davies & Maloney, 1976, Perry et al., 1977, White et al.,

1977, Reisine et al., 1978, Davies, 1979, Rossor et al., 1982a). However, ChAT does not limit the rate of acetylcholine synthesis under normal circumstances (Marchbanks, 1977, Tucek, 1978), so the integrity of cholinergic function remained equivocal until access to ante-mortem tissue permitted dynamic measures of cholinergic activity to be assessed. Tissue prisms ("mini-slices") from fresh samples of neocortex from patients with Alzheimer's disease were shown to have a reduced capacity to synthesize acetylcholine (under both resting and stimulated conditions) in both frontal and temporal lobes (Sims et al., 1983a). Measurements of high affinity [^{3}H]-choline uptake were similarly reduced in tissue obtained ante-mortem and post-mortem (Rylett et al., 1983, Sims et al., 1983a). The concordance of these findings strongly suggest loss (in at least a functional sense) of cholinergic varicosities from the cortex and indicate that post-mortem measures of ChAT activity do provide a reliable estimate of the cholinergic deficit in Alzheimer's disease.

Loss of indices of presynaptic cholinergic function is consistent with a number of histological investigations that have reported loss of neurones from the medial forebrain nucleus basalis of Meynert (nbM, Ishino & Otssuki, 1975, Whitehouse et al., 1981, 1982, Perry et al., 1982, Arendt et al., 1983, Tagliavini & Pilleri, 1983, Wilcock et al., 1983, McGeer et al., 1984, Mann et al., 1984a, Saper et al., 1985), a discontinuous group of predominantly large multipolar neurones (Mesulam et al., 1983). Some immunohistochemical studies (using ChAT-specific antibodies) have shown the nbM to be entirely cholinergic. One such study (Pearson et al., 1983) has suggested that nbM neurones do not completely degenerate but shrink. It seems likely, therefore, that cholinergic dysfunction precedes structural loss of these cells as the loss of ChAT activity (90%) in the nbM is almost 3 times greater than the extent of cell loss (Perry et al., 1982).

Plaque formation in the neocortex has been found to relate to both ChAT activity in this area (Perry et al., 1981, Mountjoy et al., 1984) and cell loss from the nbM (Arendt et al., 1985). This is consistent with the hypothesis that cholinergic neurones contribute to the formation of neuritic plaques (Price et al., 1982), but it remains

unclear whether the primary lesion occurs in the nbM (see Saper et al., 1985) or in the cortex (see Pearson et al., 1983). A relationship between cholinergic denervation and cortical damage is further suggested by correlations between ChAT activity and tangle formation (Wilcock et al., 1982, Mountjoy et al., 1984). More recently (Kato et al., 1988) neurofibrillary tangle bearing neurones were observed selectively in the nbM ipsilateral to a large middle cerebral artery infarct on one side of the brain. Considering the widespread projection of small nbM axons to the ipsilateral cerebral cortex this suggests that formation of neurofibrillary tangles can occur as a retrograde reaction in nbM neurones secondary to infarctions

Clinical assessment of the severity of dementia has been shown to to relate to ChAT activity post-mortem (Perry et al., 1978, 1981, Wilcock et al., 1982, Collerton, 1986) and to the capacity of tissue obtained ante-mortem to synthesize acetylcholine (Francis et al., 1985, Neary et al., 1986c).

This finding of an apparently specific subcortical degeneration which accounted for the major neurochemical deficit in the cortex of post-mortem Alzheimer's disease brains soon stimulated a considerable interest in the possibility of specific replacement therapy with cholinomimetic drugs (Marchbanks, 1982, Bartus et al., 1982, Hollander et al., 1986, Thal, 1990). The feasiblity of such an approach received support from studies demonstrating the stability of cortical acetylcholine muscarinic receptors in Alzheimer's disease (White et al., 1977, Perry et al., 1978, Davies & Verth, 1978, Palacios, 1982, Gottfries et al., 1983, Lange & Henke, 1983).

In a study that distinguished between sub-types of muscarinic receptors, Mash et al. (1985) reported a loss of M2 receptors in Alzheimer's disease tissue from the cerebral cortex. This loss of presumed presynaptic receptors occured in the absence of any changes in the level of post-synaptic M1 receptors. Similarly, nicotinic acetylcholine receptors, which are also presumed to be located presynaptically, are reported reduced in density in Alzheimer's disease tissue from the neocortex

(Whitehouse et al., 1986, Candy et al., 1986b, Perry et al., 1987). More recently Harrison et al (1990) report an increase in muscarinic receptor MRNA in Alzheimer's disease temporal cortex measured by in situ hybridization histochemistry.

In Parkinson's disease muscarinic receptor density is reported increased in response to the cholinergic-dependent cognitive deficits (Dubois et al., 1987, Smith et al., 1988). Neuronal cell loss in the nucleus basalis of Meynert has also been reported in Parkinson's disease, particularly those with dementia (Candy et al., 1983). These differential changes in the muscarinic receptor population may well reflect a fundamental difference in the pathogenisis of the cholinergic deficit in the two disease processes. There is evidence in Parkinson's disease that the primary event is degeneration of subcortical cholinergic neurones in the nucleus basalis of Meynert projecting to the cortex, whereas in Alzheimer's disease neuronal loss may reflect a response to primary cortical abnormalities (see Pearson et al., 1983, Procter et al., 1988).

Also of importance regarding the hypothesis that a deficit of the cholinergic system is of major significance in Alzheimer's disease are studies that have demonstrated a role for the cholinergic system in memory and cognitive function (Drachman & Leavitt, 1974, Bartus et al., 1982). Cholinesterase inhibitors such as physostigmine enhance storage and retrieval of long-term memory information in normal human subjects (Davies et al., 1978). Also, dietary choline has been demonstrated to improve retention in abilities in mice (Bartus et al., 1980). Thus, the theory that a cholinergic abnormality may contribute to the development of some clinical symptoms of Alzheimer's disease has come from studies demonstrating correlations between intellectual impairment, the degree of pathological abnormality and loss of cortical ChAT activity (Bowen et al., 1976b, Perry et al., 1978, Wilcock et al., 1982, Bowen et al., 1983, Mountjoy et al., 1984).

The wealth of data demonstrating a cholinergic deficit in Alzheimer's disease has led several groups to suggest that the disease is one of a specific cholinergic dysfunction

(Bartus et al., 1982, Davies, 1983, Terry & Katzmann, 1983, Coyle et al., 1983). However, attempts to ameliorate the symptoms of Alzheimer's disease using drugs directed at the cholinergic system have produced at best only moderate improvement (Marchbanks, 1982, Hollander et al., 1986). Recently, much interest has been shown in the use of the acetycholinesterase inhibitor tetrahydro-9-aminoacridine (tacrine) for the treatment of Alzheimer's disease (Summers et al., 1986, Thal, 1990, also section 1.13).

As described above, the most widespread pathological feature of Alzheimer's disease is the presence of cortical neurofibrillary tangles and neuritic plaques. However, the major part of cortical ChAT activity is found in terminals of neurones arising from subcortical nuclei and not in intrinsic neurones which show the greatest pathology (Johnson et al., 1981). Furthermore, there is evidence that cortical pathology can be manifest without either severe cell loss in the nbM (Perry et al., 1982, Pearson et al., 1983,) or significantly reduced ChAT activity in this region (Henke & Lange, 1983). In addition, the nbM shows regional differences in the extent of degeneration, with those parts which project to the most severly involved cortical areas showing the greatest pathology (Arendt et al., 1985). These findings suggest that the cholinergic deficit is not a primary event in the pathology of Alzheimer's disease, a theory that is supported by studies showing that experimentally induced cortical damage produces a retrograde degeneration of the nucleus basalis in a manner similar to that seen in Alzheimer's disease (Sofroniew & Pearson 1985). In this manuscript the hypothesis that the cholinergic deficit is due to the secondary degeneration of the cholinergic fibres from the basal nucleus is addressed by a dual study of ChAT activity and neurofibrillary tangle density measurement in a number of cortical areas.

ii) Monoamines.

Noradrenaline: The ascending noradrenergic fibre tracts innervating the cortex arise principally from the locus coeruleus. The enzymes responsible for the synthesis of noradrenaline are unstable post-mortem. This may account for the conflicting results on measurements of dopamine-ß -hydroxylase, which was reported reduced (Cross

et al., 1981, Perry et al., 1981) or unchanged in Alzheimer's disease (Davies and Maloney 1976). Neurochemical studies of the cerebral cortex have thus concentrated on the tissue content of monoamines and their metabolites. Most post-mortem studies have shown that noradrenaline is reduced in Alzheimer's disease (Cross et al., 1981, Perry et al., 1981, Arai et al., 1984a, Palmer et al., 1987b) though this is not always the finding (D'Amato et al., 1987). Concentrations of the major metabolite 3-methoxy-4-hydroxyphenyl-glycol have been found to be reduced, unaltered or elevated (Cross et al., 1983, Palmer et al., 1987b). Analysis of biopsy tissue has confirmed reductions in noradrenaline content of temporal lobe (Palmer et al., 1987c). Noradrenaline uptake was also reduced in temporal cortex of Alzheimer's disease subjects, indicating that the noradrenaline deficit is not an artefact at the end stage of the disease, though noradrenaline release was unchanged in frontal cortex (Palmer et al., 1987a). Post-synaptic α and β adrenergic receptors appear to be relatively unaffected in Alzheimer's disease (Bowen et al., 1983, Cross et al., 1984c) although some reports are of a loss of both sub-types in the hippocampus and nbM (Shimohama et al., 1986, 1987). Noradrenergic deficits have not correlated with dementia ratings, but significant correlations have been obtained between the severity of the clinical symptoms and the noradrenergic deficits in the hypothalamus (Adolfsson et al., 1979).

iii) Dopamine.

Dopaminergic innervation of the cerebral cortex arises from the substantia nigra and ventral tegmental area. Evidence for a dysfunction of dopaminergic neurones in Alzheimer's disease is less conclusive than that for involvement of cholinergic and noradrenergic systems. One study has shown tyrosine hydroxylase to be unchanged (Davies & Maloney, 1976). In post-mortem studies, concentrations of dopamine and one of its minor metabolites, dihydroxy-phenylacetic acid have consistently been found to be unchanged (Gottfries et al., 1983, Arai et al., 1984a, Palmer et al., 1987b). Homovanillic acid, the major dopamine metabolite, was found unaltered or increased (Cross et al., 1983, Palmer et al., 1987b). Ante-mortem tissue determinations have confirmed these results. Dopamine release was also unaffected

(Palmer et al., 1987c). Loss of dopamine D_2 receptors from the neostriatum has been reported, possibly reflecting loss of excitatory amino acid nerve terminals from this region (Cross et al. 1984, Rinne et al., 1986).

iv) Serotonin.

The raphe nuclei of the midbrain nuclei give rise to serotonergic fibres innervating the cerebral cortex. Whereas serotonin (5-hydroxytryptamine, 5-HT) concentrations are reported to be reduced in cortex obtained post-mortem from Alzheimer's disease patients, the concentration of its main metabolite, 5-hydroxyindoleacetic acid (5-HIAA), has generally been found to be unaltered (Bowen et al., 1983, Cross et al., 1983, Arai et al., 1984a, Ichimiya et al., 1986). Reductions in 5-HT content were confirmed in ante-mortem studies, which also found reduced 5-HIAA concentrations. 5-HT uptake and K^+-stimulated release of endogenous 5-HT were found reduced in these studies (Palmer et al., 1987a). Another presynaptic marker, imipramine binding, was reported to be reduced in another study (Bowen et al., 1983, D'Amato et al., 1987).

Binding of [^{3}H]-5-HT (to the 5-HT_1 site) has been shown to be significantly reduced in the areas of cortex examined (Cross et al., 1984a, c, Perry et al., 1984, Cross et al., 1986). Lower binding of [^{3}H] ketanserin (to the 5-HT_2 site) has also been described (Cross et al., 1984c, Perry et al., 1984, Reynolds et al., 1984, Procter et al., 1988a). The binding of [^{3}H]-lysergic acid diethylamide (to both 5-HT_1 and 5-HT_2 sites) was reported significantly reduced in all studies, including the only one to make allowance for tissue atrophy by examination of the entire temporal lobe (Bowen et al., 1979, 1983). Further study of 5-HT_{1A} receptors as a marker of pyramidal cells is required (Middlemiss et al., 1986, Davies et al., 1987, Sprouse & Aghajanian, 1988, Crino et al., 1990). There is some evidence from the histopathological measurements to indicate that intrinsic cortical change and serotonergic denervation are related since significant negative correlations were found between tangle counts and 5-HIAA content, in both frontal and temporal cortex of Alzheimer's disease subjects (Palmer et al., 1987c).

v) Peptides. Neuroactive peptides are widely distributed in the brain. Cholecystokinin, vasopressin, vasoactive intestinal peptide, substance P, thyrotropin releasing hormone, corticotrophin releasing factor, luteinising releasing hormone, neurotensin and somatostatin have all been measured in Alzheimer's disease tissue samples, and reviewed by Beal & Martin (1986). Substance P content has been reported reduced (Crystal & Davies 1982) but this is not a consistent finding (Perry et al., 1981). The most consistent finding is a reduction in somatostatin concentration in post-mortem tissue (Ferrier et al., 1983, Beal et al., 1985, Perry & Perry, 1985, Tamminga et al., 1985, Francis et al., 1987). However, ante-mortem data do not confirm this change (Francis et al., 1987, see next section).

vi) Gamma-aminobutyric acid.

Gamma-aminobutyric acid (GABA) is the predominantly inhibitory transmitter used by the majority of small cortical interneurones and it has been estimated that at least 30 % of the total cortical neuronal population is GABAergic (Jones, 1986). Studies of GABAergic neurotransmission in Alzheimer's disease have emphasised the relative stability of this system (Spillane et al., 1977, Perry et al., 1978, Rossor et al., 1982, Lowe et al., 1988). This sparing of cortical GABAergic neurones when compared with the loss of cortical markers for acetylcholine, noradrenalin and serotonin has led a number of workers to propose that Alzheimer's disease is a disorder characterised by a selective loss of ascending processes from the nucleus basalis, locus coeruleus and dorsal raphe nucleus (Rossor, 1981, Benton et al., 1982 Hardy et al., 1987).

Early studies of cortical GABAergic neurones in Alzheimer's disease relied on measurements of the synthetic enzyme glutamic acid decarboxylase (GAD,), the integrity of which is affected by agonal state (Bowen et al., 1976a,b, 1977, Spillane et al., 1977, Perry et al., 1982). Recent studies have therefore utilised measurements of GABA itself, which appears stable over long autopsy delays and some report no influence of agonal state (Spokes et al., 1979) although in the parietal cortex, Lowe

et al. (1988) found that GABA content of tissue from control patients who died following a protracted terminal illness was reduced to 74% of the values for control tissue from subjects who died suddenly. Furthermore, brain GABA concentrations are known to increase rapidly following death (Minard & Mushawar, 1966) and so the use of post-mortem material to measure GABA is questionable. Significant reductions in cortical GABA concentrations in Alzheimer's disease, particularly in the temporal cortex, have been reported by Ellison et al. (1986), Mountjoy et al. (1984), Arai et al. (1984) Sasaki et al. (1986) and Rossor et al. (1982). The reduction of GABA content seen in Alzheimer's disease shows no correlation with the severity of the disease (Mountjoy et al., 1984).

Studies of GABA receptor integrity have shown that levels remain relatively unchanged in the disorder (Bowen et al., 1979, Cross et al., 1984, 1986). Some studies have attempted to distinguish between, and examine for selective changes in, either the GABA-A or GABA-B sub-types of receptor. It has been reported that both receptor sub-types are reduced in the hippocampus (Chu et al., 1987). In the frontal cortex, this group reported a 50-70% loss of GABA-B receptors while GABA-A receptors were not significantly reduced. This preferential loss of GABA-B receptors in the cortex has been suggested to reflect a degeneration of glutamatergic, noradrenergic and serotonergic terminals on which these receptors are believed to exist (Chu et al., 1987).

There is evidence that neuropepides are co-transmitters with some GABAergic neurones (Hendry et al., 1984, Alho et al., 1988). Reductions in the tissue concentrations of somatostatin-like immunoreactivity (SLIR) has been reported in many cortical regions of post-mortem brains from Alzheimer's disease subjects (Davies et al., 1980, Francis et al., 1987, Lowe et al., 1988) whilst other studies report small changes or no loss in Alzheimer's disease tissue (Rossor et al., 1980, Perry et al., 1981). Furthermore, although Francis et al. (1987) report a reduction of SLIR in post-mortem tissue from Alzheimer's disease subjects, this loss is not seen in tissue taken ante-mortem. In a unique group of histologically verified

Alzheimer's disease patients, Francis et al. (1984) reported a small loss of SLIR in lumbar cerebrospinal fluid whilst other groups report a more marked reduction (eg Oram et al., 1981, Wood et al., 1982).

1.5.Glutamate as a neurotransmitter.

Glutamate is ubiquitously distributed throughout the central nervous system and is intimately involved in general metabolic pathways, including energy metabolism, ammonia detoxification and as a precursor for peptides and proteins and transmitter GABA.

Electrophysiological studies demonstrated that glutamate produced a powerful, widespread excitation in a non-stereoselective manner. In view of these original findings (see Watkins & Evans, 1981, Cotman et al., 1981, Fonnum, 1984, McLennan, 1987), glutamate was not thought to act as neurotransmitter. Since this time, numerous studies have demonstrated that glutamate fulfills many of the criteria required for classification as a neurotransmitter-

i) Glutamate is specifically released by physiological stimuli in high enough concentrations to elicit a response

ii) Glutamate demonstrates identity of action with the naturally occuring compound

iii) The action of glutamate is rapidly terminated by specific mechanisms (see Watkins & Evans, 1981, Cotman et al., 1981, Fonnum, 1984).

1.5.1. Synthesis of glutamate .

The synthesis and metabolism of glutamate are compartmentalised in a complex manner (see Fig. 1.1). Glutamate can be produced by transamination of the corresponding keto-acid 2-oxoglutarate by aspartate aminotransferase (Fonnum, 1984, Bradford, 1986). 2-oxoglutarate is a tricarboxylic acid (TCA) cycle intermediate that can be synthesized in the nerve terminal. It can also be transported to the terminal from astroglial cells (Carter et al., 1986). Replenishment of 2-oxoglutarate derived from glial cells can be achieved by carbon dioxide fixation

FIGURE 1.1. SYNTHESIS AND METABOLISM OF GLUTAMATE IN THE CNS

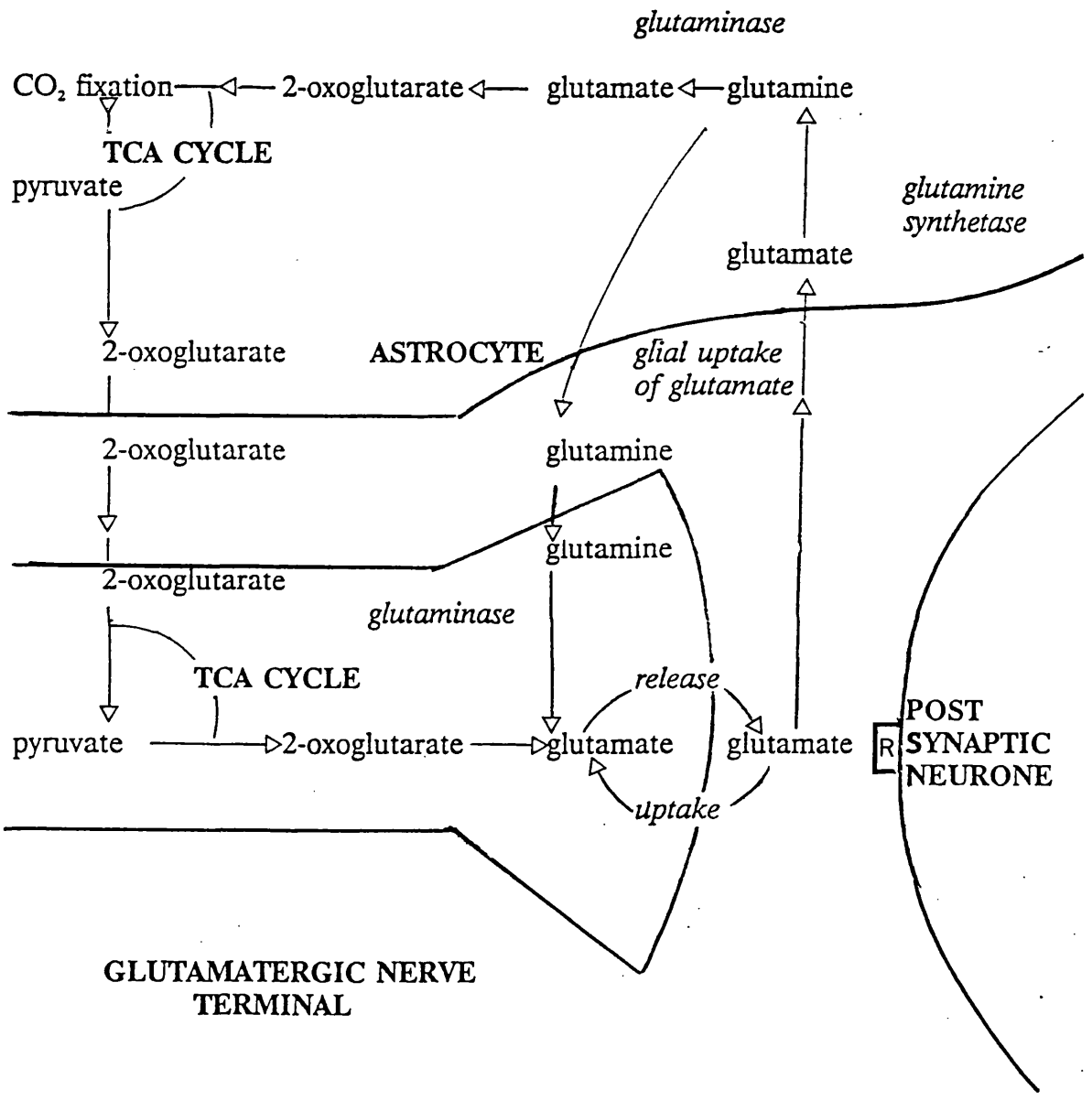


Figure modified from Carter et al. (1986).

mediated by pyruvate carboxylase (Carter et al., 1986, Bradford, 1986).

Glutamate can also be produced from glutamine by the action of phosphate activated glutaminase, a mitochondrial enzyme that is preferentially although not exclusively located to the nerve terminal. The action of this enzyme is strongly inhibited by glutamate and ammonia, the end products of this reaction. This end product inhibition may serve as the major regulatory step for controlling transmitter glutamate synthesis. Glutamate is also produced from ornithine, arginine and proline metabolism. The rate of synthesis from these sources is slow and is unlikely to provide a major contribution to the transmitter pool. (Fonnum, 1984, Bradford, 1986, Nicklas et al., 1987).

1.5.2. Synaptic release.

Synaptosomal cytoplasm contains millimolar concentrations of glutamate (DeBelleroche & Bradford, 1977). The mechanism of release of transmitter glutamate from nerve terminals has been the source of much discussion. DeBelleroche & Bradford (1977) have proposed that glutamate released during neurotransmission comes mainly from a cytoplasmic source. However, recent advances in methodology for studying endogenous glutamate release have provided a new insight into the compartmentalisation and differential Ca^{2+}-dependencies of glutamate release from nerve ending preparations. Using an enzyme-linked fluorimetric assay for measuring the rapid release of endogenous glutamate, Nicholls and colleagues have demonstrated a pool equivalent to 15-20% of total synaptosomal glutamate that can be released in a Ca^{2+}-dependent manner upon depolarisation of the plasma membrane (Nicholls & Sihra, 1986). This pool originates from a non-cytoplasmic source and release has been suggested to represent an exocytotic mechanism (Nicholls & Sihra, 1986, Nicholls et al., 1987, Sanchez-Prieto et al., 1987). Glutamate can also be released by carrier-independent leakage and also by a Ca^{2+}-independent mechanism that may represent a reversal of the uptake process (Kanner & Sharon, 1978, Nicholls & Sihra, 1986, Nicholls et al., 1987,).

1.6. Neuronal pathways.

A specific high affinity uptake mechanism appear to provide the major means of limiting the post-synaptic actions of glutamate (Logan & Snyder, 1972, Bennett et al., 1973). This mechanism has therefore been used as a biochemical marker for glutamatergic nerve terminals and has proven useful in tracing glutamatergic pathways in the brains of experimental animals (see Cotman et al., 1981, Fagg & Foster, 1983, Fonnum, 1984, Cotman et al., 1987).

Surgical and chemical induced denervations, combined with uptake measurements on the target area, have demonstrated uptake to be localised to the terminals of particular anatomically defined pathways (Storm-Mathisen, 1977). Furthermore, quantitative autoradiographic studies of [^{3}H]-glutamate uptake in hippocampal slices have demonstrated a preferential labelling of neuronal rather than glial elements (Storm-Mathisen & Iversen, 1979). However, it should be noted that glial uptake is also prominent. For example, intracisternal injections of [^{14}C]-glutamate rapidly results in a high radiospecific activity of total brain glutamine, owing to glial cell uptake and metabolism (Berl et al., 1962).

Glutamatergic pathways can also be traced by autoradiographic localisation of radiolabelled D-aspartate (a non-metabolisable glutamate analogue) following its uptake by intact nerve terminals and retrograde transport to the parent neuronal perikarya (Streit, 1980). Other techniques for identifying glutamatergic pathways include electrophysiology and the use of immunocytochemistry with staining for glutamate and aspartate-like immunoreactivity (see Otterson & Storm-Mathisen, 1987, Cotman et al., 1987).

Using the techniques outlined above, a number of cell types and neuronal pathways have been identified that utilise glutamate as a neurotransmitter. These include hippocampal granule and CA3 pyramidal cells (Fonnum & Walaas, 1978), and both the perforant path input to the hippocampus and hippocampal output to the subiculum (White et al., 1977, Storm-Mathisen et al., 1983). Other glutamatergic

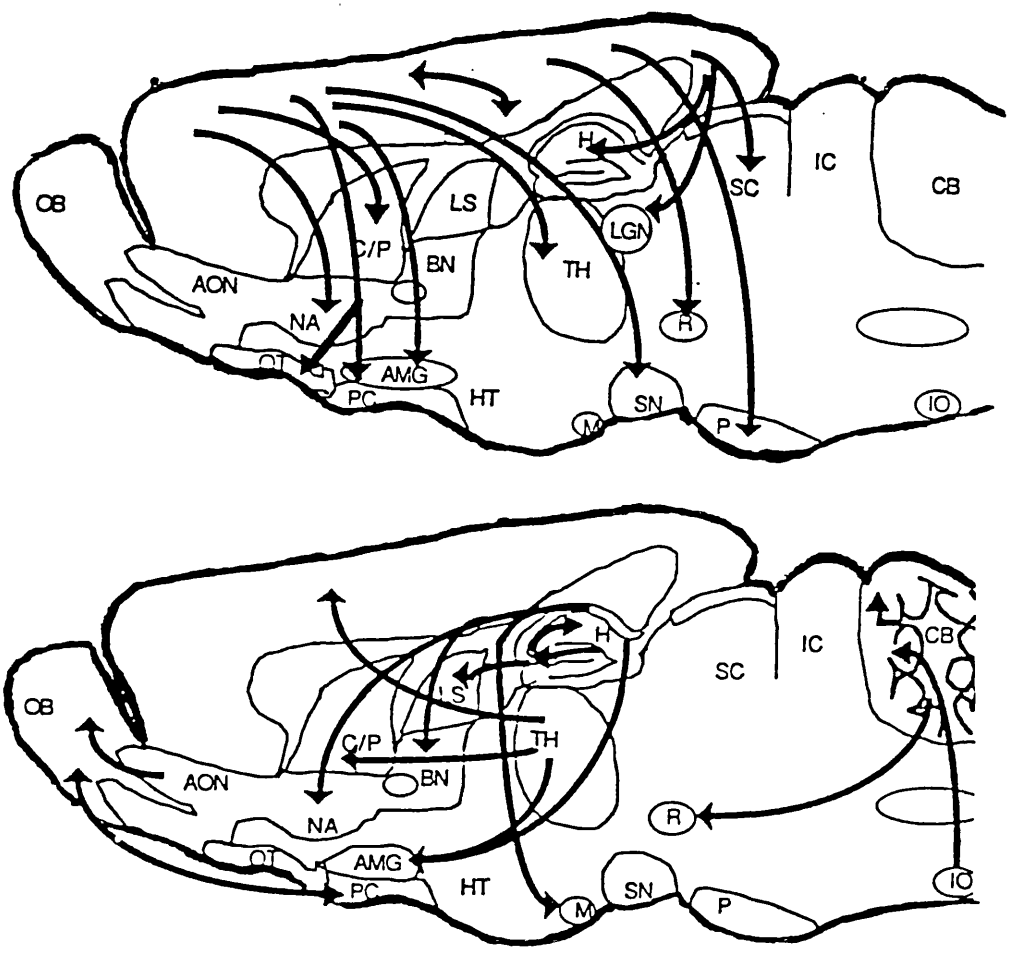
pathways include a number of corticofugal systems such as that to the amygdala (Walker & Fonnum, 1983) and those to the neostriatum and thalamus (Fonnum et al., 1981). Glutamate is also the major neurotransmitter in cerebellar parallel climbing fibres (Wicklund et al., 1982, Rohde et al., 1979). These pathways, together with those subcortical pathways for which there is evidence that glutamate is the neurotransmitter are represented diagramatically in Fig. 1.2.

The evidence outlined above implies that glutamate is the major excitatory amino acid neurotransmitter in the central nervous system. However, it is important to recognise that other acidic amino acids may act as neurotransmitters of excitatory pathways, including aspartate, homocysteic acid and cysteine sulfinic acid (See Do et al., 1986, Palmer et al., 1989). It is also thought that N-acetylaspartylglutamate is either a co-transmitter or a discrete transmitter in this system (Zaczek et al., 1983, Coyle et al., 1990).

1.7.Post-synaptic mechanisms .

The post-synaptic actions of glutamate are mediated by at least four and possibly five receptor sub-types (Fig. 1.3. also see Young & Fagg, 1990). Three of these receptors were defined in the mid to late 1970's by electrophysiological analyses of the actions of large numbers of excitatory amino acid analogues (Watkins & Evans, 1981, McLennan, 1987, Watkins et al., 1987, Young & Fagg, 1990). These were defined as the N-methyl D-aspartate (NMDA), quisqualate and kainate receptor, since these endogenous agonists evoke pharmacologically distinct excitatory responses when applied to neurones in the CNS (Foster & Fagg, 1984, Mayer & Westbrook, 1987, Stone & Burton, 1988, Watkins et al., 1990, Lodge & Johnson, 1990). More recently, the quisqualate receptor has been re-defined as the α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptor because AMPA has greater selectivity than quisqualate. In the early 1980's the existence of a fourth receptor sub-type was proposed on the basis of the potent antagonist properties of L-AP4 at subpopulations of identified brain and spinal cord excitatory synapses (Foster & Fagg, 1984, Cotman & Iversen, 1987, Monaghan et al., 1989). More recently, a

FIGURE 1.2 SUMMARY OF EXCITATORY AMINO ACID USING PATHWAYS IN RAT BRAIN



A = corticofugal pathways. **B** = Allocortical and subcortical pathways. Abbreviations: **OB** = olfactory bulb, **AON** = anterior olfactory nucleus, **NA** = nucleus accumbens, **OT**= olfactory tubercle, **AMG** = amygdala, **PC** = pyriform cortex, **C/P** = caudate putamen, **LS** = lateral septum, **BN** = bed nucleus of the stria terminals, **HT** = hypothalamus, **M** = mamillary bodies, **TH** = thalamus, **H** = hippocampus, **LGN** = lateral geniculate nucleus, **SN** = substantia nigra, **R** = red nucleus, **SC** = superior colliculus, **IC** = inferior colliculus, **P** = pontine nucleus, **CB**, cerebellum and **IO** = inferior olivary complex. Figure taken from Cotman et al. (1987).

fifth subtype which is insensitive to AMPA, but sensitive to quisqualate, ibotenate and trans-1-amino-cyclopentyl-1,2-dicarboxylic acid (trans-ACPD)has been reported. By contrast with the previous receptor sub-types which are linked to ion channels, it is linked to phosphoinositol metabolism and is therefore called the metabotropic receptor (Sladeczek et al., 1989, Schoepp & Johnson, 1989). Table 1.1. descibes the classification of these receptors, their agonists and antagonists.

1.7.1. N-methyl D-aspartate receptors.

The most extensively characterised glutamate receptor is the NMDA sub-type. Progress in the study of this receptor can be largely ascribed to the development of potent, selective, competitive antagonists that have little activity at the other receptor sub-types (see Watkins et al., 1990). Using the potent NMDA antagonists AP5 and AP7 the NMDA receptor complex was demonstrated to be involved in the generation of excitatory synaptic responses in a number of pathways. For example NMDA receptor complexes are involved in the generation of late excitatory post-synaptic potentials (EPSPs) at neocortical pyramidal cells following stimulation of sub-cortical afferent fibres (Thomson, 1986) and in the generation of late EPSPs at hippocampal pyramidal cells following stimulation of Schaffer collateral fibres (Herron et al., 1985).

Evidence from electrophysiological studies has demonstrated that agonists acting at the NMDA receptor complex generate responses via a voltage-dependent conductance mechanism (Mayer & Westbrook, 1984, Ascher & Nowack, 1987). Magnesium appears to enter the channel associated with the NMDA receptor (Fig.1.3) and interact in such a way as to impede the passage of other ions (MacDonald & Nowack, 1990). This voltage sensitivity is dependent upon extracellular magnesium ion concentration (Nowack et al., 1984). At membrane potentials around resting and at normal extracellular magnesium concentrations, NMDA-activated channels are blocked and there is little current flow. However, as the membrane is depolarised (probably via non-NMDA receptors) the channel block by magnesium is removed and the resulting inward ion flux further amplifies

FIGURE 1.3 DIAGRAMMATIC REPRESENTATION OF THREE EXCITATORY AMINO ACID RECEPTOR SUB-TYPES.

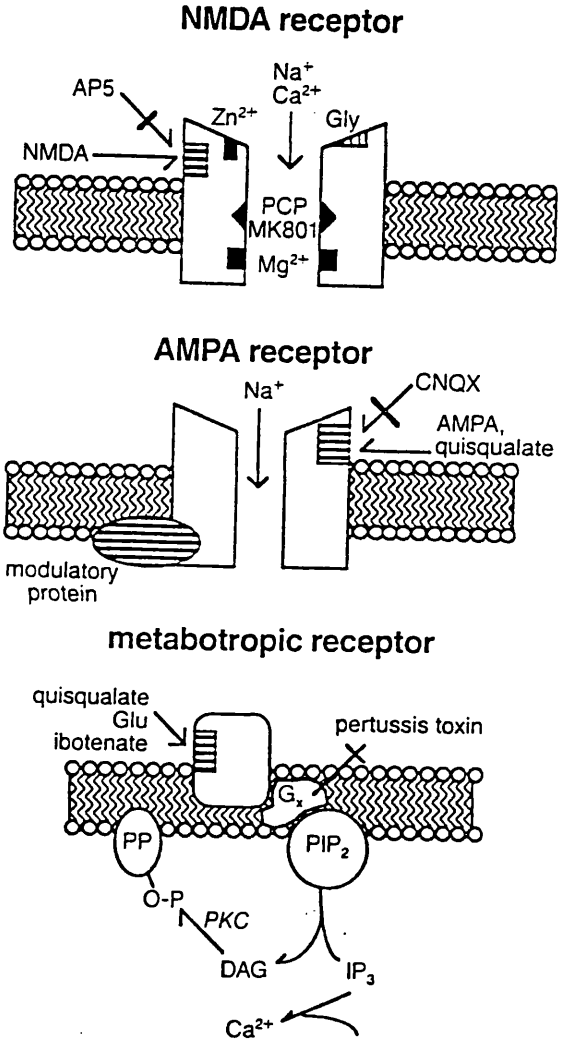


Figure taken from Young & Fagg (1990). The NMDA receptor gates a cation channel that is permeable to Ca^{2+} and Na^{+} and is gated by Mg^{2+} in a voltage - dependent manner. There may be an additional modulatory site for polyamines but its precise location is not known (see section 1.7.3).

The AMPA receptor may also be the kainate receptor. It appears to gate cation conductances that underlie fast depolarizing responses at most excitatory synapses. It appears to be influenced by a modulatory protein.

The metabotropic receptor is linked to phoshoinositol metabolism. At present it has limited pharmacology but appears enriched in the cerebellum.

the depolarization. There are a number of additional modulators of the NMDA receptor complex, these are discussed below.

An issue of considerable interest is the existence of sub-types of the NMDA receptor complex. Autoradiographic studies have suggested that, although NMDA, glycine and channel ligand (eg MK-801 and TCP) binding sites are generally present in a the constant ratio throughout the CNS, clear exceptions are apparent. Particularly striking discrepancies have been observed in the cerebellar granule cell layer, where density of glycine and NMDA binding sites is relatively high but that of channel ligand binding sites is relatively low (Maragos et al., 1988, Monaghan et al., 1989, McDonald et al., 1990). Such variations may reflect regional differences in NMDA receptor sub-types; alternatively each binding site may exist in multiple affinity states which are regulated differently in different regions (Stone & Burton, 1988, Monaghan et al., 1989). Indeed, several groups have postulated the existence of agonist and antagonist preferring forms of the NMDA receptor complex on the basis of disimilar pharmacological profiles of [^{3}H]-glutamate and [^{3}H]-CPP binding. Monaghan et al. (1988) have shown that these sites are differentially distributed and reciprocally regulated by glycine. However, even in the presence of glycine or a glycine antagonist, inhibitors such as AP5 display regional variations in potency, suggesting that two distinct binding sites may exist. One possibilty therefore is that, as in the case of nicotinic cholinoceptors and GABA receptors, multiple genes may exist for NMDA receptor subunits with similar but not identical structures. This has now been demonstrated for both the AMPA (Keinanen et al., 1990) and kainate (Hollmann et al., 1989, Gregor et al., 1989, Wader et al., 1989) receptor subtypes.

Bowery et al., 1988).

1.7.2 Glycine modulation of the NMDA receptor complex..

In addition to the agonists and antagonists for the NMDA receptor complex outlined in Table 1.1, there are a number of compounds that act at the now well characterised glycine modulatory site of the NMDA receptor complex (see Fig. 1.3). The original observation by Johnson & Ascher (1987) that the responses of neuronal membrane patches to NMDA were much larger when the patch was positioned close to the parent cell than when it was separated from the cell by fresh quantities of

TABLE 1.1. CLASSIFICATION OF GLUTAMATE RECEPTOR SUB-TYPES. (modified from Fagg et al., 1986, Watkins et al., 1987, Young & Fagg, 1990, Watkins et al., 1990).

RECEPTOR	AGONISTS in order of selectivity	ANTAGONISTS * denotes non -competitive	EFFECTOR	COMMENTS
NMDA	NMDA trans-2,3-PDA ibotenate L-aspartate L-glutamate quisqualate quinolinate	D-AP5, CPP CGS19755 CGP37849 MK-801* TCP* Ketamine* PCP* SKF 10047*	Na^+/ Ca^{2+}/ K^+	Allosteric modulators. Discussed in section 1.7) Responsible for slow component in repetitive activity generated by non-NMDA receptors. Important in synaptic plasticity (section 1.8)
AMPA	AMPA ATPA 4-AHCP 5-HPCA Br-HIBO quisqualate L-glutamate L-aspartate	NBQX CNQX DNQX	Na^+/ K^+	Parallel disribution to NMDA receptors Involved in fast component of EPSP's.
KAINATE	Kainate L-glutamate domoate acromelic acid 5-bromo-willardine.	CNQX DNQX BBPzDA CBPzDA kynurenate gamma-DGG GAMS	Na^+/ K^+	Not definitely distinct from AMPA receptor. On dorsal root fibres and may be presynaptic.
L-AP4	L-AP4 L-glutamate L-O-phospho -serine	?	?	Inferred from specific depressant effect of L-AP4 on some pathways. May decrease glutamate release and may be presynaptic.
METABOTROPIC	L-glutamate quisqualte ibotenate trans-ACPD	?	IP_3/ DAG	

Abbreviations: **AMPA**,α-amino-3-hydroxy-5-methylisoxazole-4-propionate; **L-AP4**, L-2-amino-4-phosphono butanoic acid;PDA, piperidine dicarboxylate; **AP5**, 2- amino-5-phosphono- pentanoate; **ATPA**, tert-butyl analogue of AMPA; **BBPzDA & CBPzDA** p-bromo and p-chloro derivatives of piperazine dicarboxylic acid; **Br-HIBO**, 4- bromohomoibotenic acid; CPP, 3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonate; **CGP37849**, D-l-2- amino-4-methyl-5- phosphono-3-petanoic acid; **CGS19755**, cis-4-phosphonomethyl-2-piperidine carboxylic acid; **CNQX**, 6-cyano-7-nitroquinoxaline-2,3- dione; **DAG**, diacyl glycerol; **DNQX**, 6,7-dinitroquinoxaline-2,3-dione (NBQX is tricyclic analogue); **GAMMA-DGG**, gamma-D-glutamylglycine; **GAMS**, gamma-D-glutamyl aminomethyl sulphonate; **HPCA**, 3-hydroxy-4,5,6,7-tetrahydroisoxazole [4,5-c] -pyridinecarboxylic acid; **IP3**, inositol triphosphate; **MK-801**, (+)-5-methyl-10 ,11-dihydro-5H-dibenzo [a,d] cyclohepten-5,10- imine maleate; **PCP**, phencyclidine; **TCP**, 1-[1-(2-thienyl)cyclohexyl]piperidine; **SKF 10047**, N-allylnormetazocine; **trans-ACPD**, trans-1-aminocyclopentyl-1,3-dicarboxyalte.

medium was soon ascribed to the release of glycine from the cultured cells. They then demonstrated that co-application of sub-micromolar concentrations of glycine greatly enhanced responses to NMDA. This enhanced response reflects an increase in the frequency of opening of the channel associated with the NMDA receptor complex (see Fig 1.3). Several groups using a wide variety of techniques have since confirmed this finding in rat brain (for example Ransom & Duchenes, 1989, Javitt & Zukin, 1989, Ransom & Stec, 1989, Loo et al., 1987, Reynolds & Miller, 1987, Foster & Wong, 1987, Monahan et al., 1989). The development of quantitative receptor autoradiography for this strychnine insensitive glycine site (Bristow et al., 1986, Cotman et al., 1987) has allowed the distribution of this site to be studied in the CNS. There is a high degree of correlation between the distribution of the NMDA recognition site and the glycine site in most brain regions (Cotman et al., 1987, Maragos et al., 1987). Furthermore, this glycine modulatory site site has been well characterised using [^{3}H]-glycine binding in rat brain with a K_d of between 100-200 nM (Kishimoto et al., 1981, Bristow et al.,1986, McDonald et al., 1990) and a maximum density of 6.2 pmol/mg protein in the stratum radiatum, CA1 (McDonald et al., 1990).

In xenopus oocytes injected with rat brain mRNA, which then express the NMDA receptor complex, conditions relatively free of glycine can be achieved. When glycine is added to the bathing medium the cells respond to the application of NMDA but they fail to respond in the absence of glycine (Kleckner & Dingledine 1988). Therefore, NMDA receptor complex activation has an absolute requirement for low concentrations of glycine.

The study of the NMDA receptor complex in human brain is less thorough, although reports (Jansen et al., 1989a,b, Hubbard et al., 1989, Kornhuber et al., 1989, Procter et al., 1989a, 1990) indicate that the NMDA receptor complex is similar in rat and human cortex. In human brain this receptor has been studied largely by radioligand binding assays. It is well known that in rat brain [^{3}H]-MK-801 and [^{3}H]-TCP which are potent, selective NMDA receptor antagonists bind to

a site associated with the open state of the channel (Foster & Wong, 1987, also see Fig. 1.3). The binding of [^{3}H]-MK-801 and [^{3}H]-TCP have been shown to be sensitive to a number of modulators which appear to change the frequency of channel opening. Therefore, these ligands provide a useful model system with which to study the interactions of drugs and modulators of the NMDA receptor complex. The NMDA recognition site (Kornhuber et al., 1988), and the binding of ligands for example [^{3}H]-MK-801 to the channel appear stable up to 24 h post-mortem (Procter et al., 1990). An extensive characterisation of the NMDA receptor complex in Alzheimer's disease and control tissue using radioligand binding of [^{3}H]-MK-801 and [^{3}H]-TCP binding was a major aim of this study.

Procter and colleagues (1990) have recently characterised the glycine modulatory site in human brain using [^{3}H]-glycine binding and although the binding was variable due to the problems associated with the study of human tissue the K_d value appeared similar in rat and human cortex.

1.7.3. Polyamine modulation of the NMDA receptor complex.

In rat brain an additional modulatory site on the NMDA receptor complex was described by Ransom & Stec (1988). In extensively washed membrane preparations, binding of [^{3}H]-MK-801 was stimulated by spermidine with an EC_{50} of 19.4 μM. There appeared to be an allosteric interaction of spermidine, glutamate and glycine since the EC_{50} was significantly reduced in the presence of glutamate and glycine. Carter et al. (1989) reported that the atypical NMDA receptor complex antagonist, ifenprodil (Carter et al., 1988), which appeared to show considerable neuroprotective properties (see section 1.9) was active at the polyamine site associated with the NMDA receptor complex. This stimulated considerable interest in this site but reports have been conflicting. In rat brain it appears in vitro studies indicate that spermidine shares the same properties as glutamate and glycine except that it has a biphasic action. It appears to stimulate binding of channel ligands at low concentrations but inhibits at high concentrations (Ransom & Stec, 1989, Reynolds & Miller, 1989, Williams et al., 1989, Sacaan & Johnson, 1989, Robinson et al.,

1990). However, reports in vivo are of a negative modulation only (Rao et al., 1990).

The precise location and indeed the existence of a discrete polyamine modulatory site on the NMDA receptor complex has yet to be proven. Original reports of the action of ifenprodil (Carter et al., 1989) suggested that this compound acts competitively at the polyamine site. However, subsequent reports have failed to confirm this (Reynolds & Miller, 1989, Robinson et al., 1990). A number of other polyamines appear to have effects at this receptor, either stimulatory (spermine, ^{1}N-acetyl spermidine, ^{1}N-acetyl spermine, ornithine, S-adenosyl-methionine and putreanine) or inhibitory (putrescine, Robinson et al., 1990, Williams et al., 1989). This structure activity relationship may suggest that the polyamine site is a discrete locus on the NMDA receptor complex. Furthermore, a concentration-dependent increase in the EC_{50} for spermidine was seen in the presence of putrescine, characteristic of a competitive mode of inhibition for this compound. The characteristics of the polyamine modulatory site in control and Alzheimer's disease brain tissue are presented in this thesis.

1.7.4. Zinc modulation of the NMDA receptor complex.

The divalent cation zinc has been shown to block NMDA-induced currents selectively but by contrast with magnesium does so in a relatively voltage-independent manner (Peters et al., 1987, Westbrook & Mayer, 1987). A recent report (Christine & Choi, 1990) suggests a biphasic effect of zinc on NMDA receptor-mediated channel currents in cortical neurones. At concentrations of between 1-10 μM a concentration-dependent reduction in the channel opening frequency was reported which was voltage-insensitive. By contrast, between 10-100μM zinc there was a voltage-dependent blockade of fast channels only. Zinc also reduces the binding of [^{3}H]-MK-801 and [^{3}H]-TCP to the channel associated with the NMDA receptor complex at a site distinct from other inhibitors (Reynolds et al., 1987, Reynolds & Miller, 1988). The physiological significance of the zinc site is unknown but zinc ions which are contained in high concentrations in mossy fibres

are released upon neuronal activity (Assaf & Chung, 1984) and zinc also blocks the excitotoxic action of NMDA on cortical neurones in culture (Koh & Choi, 1988, see section 1.9). The zinc modulatory site has been investigated in human brain (Hubbard et al., 1989) and appears to share the same properties as the site in rat brain. In this thesis results are presented on the effects of zinc on the NMDA receptor complex in control and Alzheimer's disease brain tissue.

1.7.2. The AMPA receptor.

Receptor autoradiographic studies using [^{3}H]-AMPA show that AMPA receptors are localized in the telencephalic regions, with high density in the hippocampus, cortex, lateral septum, striatum and the molecular layer of the cerebellum (Monaghan et al., 1989, Nielsen et al., 1988). This distribution corresponds closely to that of the NMDA receptor and may suggest that these two receptors act together to activate the post-synaptic neurone. Electrophysiological findings that fast synaptic responses are often blocked by non-NMDA receptor antagonists (see Table 1.1), and that NMDA receptor responses are apparent only under certain circumstances support this view (Mayer & Westbrook, 1987, Monaghan et al., 1989). It is possible, therefore, that the AMPA receptor may be responsible for the depolarization necessary for many excitatory synapses in the brain.

1.7.3. The Kainate receptor.

Although this was the first excitatory amino acid binding site to be labelled selectively using radioligands (see Pearce & Bowen, 1984, Foster & Fagg, 1984), the relationship of the [^{3}H]-kainate binding site to the receptor mediating the action of kainate remains unclear. Several investigators have suggested that the physiological effects of kainate are mediated by the AMPA receptor. [^{3}H]-kainate binds to high and low affinity sites in the brain with K_d values in the low nanomolar range. However, low micromolar concentrations are required to elicit an excitatory response. At these micromolar concentrations, kainate has been shown to interact with the AMPA binding site (Mayer & Westbrook, 1984, Monaghan et al., 1989). Similarly, the antagonists CNQX and DNQX (Table 1.1) are some fivefold more

potent as inhibitors of [^{3}H]-AMPA binding than of [^{3}H]-kainate binding but equipotent as antagonists of kainate and AMPA- evoked increases in neuronal firing (Honore et al., 1988). Therefore it seems possible that these agonists are acting via the same receptor to mediate their action. Despite these observations, kainate does appear to mediate some actions independent of the AMPA receptor. In cultured neurones, kainate and quisqualate activate channels with dissimilar conductance and desensitization properties (Westbrook & Mayer, 1984, Mayer & Vyklicky, 1989). Furthermore, spinal-C fibre afferents are depolarized by kainate and other excitatory amino acid analogues with the same order of potency as found for high affinity [^{3}H]-kainate binding sites in isolated brain membranes (Foster & Fagg, 1984, Monaghan et al., 1989). Finally, in autoradiographic experiments, the distribution of high affinity [^{3}H]-kainate binding sites is different to that for AMPA or NMDA (Young & Fagg, 1990) but corresponds well to those brain regions (eg hippocampal area CA3, cortex, lateral septum) that are susceptible to the neurotoxic actions of kainate (see section 1.9).

1.7.4. The metabotropic receptor.

In addition to the channel-linked receptors mediating fast depolarizing responses in the CNS, an excitatory amino acid receptor exists that is coupled to phosphoinositol metabolism (Nicoletti et al., 1986, Sladeczek ett al., 1985, 1988). Binding to this site is distinguished by its high affinity for quisqualate, ibotenate, glutamate and trans-1-amino-cyclopentyl-1,2-dicarboxylic acid but low affinity for AMPA, kainate and NMDA (Cha et al., 1990, also see Table 1.1). At present there is not a specific label for this site and its measurement relies on the inaccurate exclusion of other receptor sub-types. However, these receptors appear present in human brain and appear enriched in the cerebellar molecular layer, lateral septum, striatum and cingulate and entorhinal cortex (Young et al., 1990). The precise function of these binding sites is unclear.

1.8. Excitatory amino acids in learning, memory and plasticity..

Due to their function as excitatory neurotransmitters, excitatory amino acids appear to play important roles in learning and memory, developmental plasticity, neuronal survival and dendritic outgrowth and regression. In the hippocampus, NMDA antagonists can prevent certain aspects of development of long term potentiation (LTP), a model of memory formation (Collingridge & Bliss, 1987, Collingridge et al., 1983, Harris et al., 1984). These agents also impair spatial discrimination learning in rats (Morris et al., 1986). In some cases, normal synaptic plastiscity is dependent on NMDA receptor complex activation (Rauschecker & Hahn, 1987, McCabe & Horn, 1989). Similarly, normal activity-dependent developmental plasticity of the visual system can be disrupted by NMDA antagonists (Kleinschmidt et al., 1986). It has also been suggested that activation of the NMDA receptor may exert a trophic influence during development. During differentiation, activation of the NMDA receptor complex promoted the survival of cultured cerebellar granule cells (Balazs et al., 1988). This effect was stage-dependent and blocked by a competitive NMDA antagonist. Furthermore, at higher, subtoxic doses, excitatory amino acids can cause regression and simplification of the dendritic arbor of the hippocampal pyramidal cells in vitro (Mattson, 1988, Mattson et al., 1988). Excitatory amino acids therefore have biphasic effects, promoting growth and differentiation at low concentrations and inhibiting them at higher concentrations.

1.9. Excitatory amino acids as neurotoxins.

An additional property of excitatory amino acids is their neurotoxicity (Olney et al., 1971, Rothman & Olney, 1986, Rothman & Olney, 1987). The ability of excitatory amino acids to cause neuronal depolarization correlates with their ability to produce axon sparing neurotoxic lesions and has led to the concept of excitotoxicity (Olney et al., 1971). Both the excitatory and the toxic effects of excitatory amino acids can be blocked by receptor antagonists suggesting that both phenomena are mediated by receptors. Excitotoxicity can occur by activation of NMDA, AMPA or kainate receptors. The mechanism of neurotoxicity appears to involve two main processes (Rothman & Olney, 1987). First, depolarization can result in passive chloride

influx, which causes subsequent cation and water entry and may lead to osmotic lysis. Second, depolarization through NMDA receptors also causes calcium and sodium influx (Choi, 1987), which leads to delayed neuronal damage due to mitochondrial dysfunction and protease and lipase activation. Other factors may also contribute to excitotoxicity. For example, under hypoxic/ ischemic conditions, high affinity glutamate uptake is depressed such that glutamate may exert more prolonged effects upon its receptor (Silverstein et al., 1986). In addition, when neurones are unable to maintain their normal membrane potential, the voltage -dependent magnesium gating of the NMDA receptor complex is lifted and the neurones are more susceptible to NMDA receptor complex mediated damage (Novelli et al., 1988). Thus, neurotoxicity appears enhanced by processes that disrupt cellular energy metabolism.

Because of their ubiquitous distribution and neurotoxic potential, it has been proposed that excitatory amino acids may be involved in the pathogenesis of both acute and chronic neurodegenerative disorders (Olney et al., 1971, Engelsen, 1986, Greenamyre, 1986, Rothman & Olney, 1987, Choi, 1990). For example, several lines of evidence suggest that excitotoxicity is involved in hypoxic/ischaemic brain damage (Rothman & Olney, 1987). Hypoxia/ischemia causes a simultaneous large increase in glutamate release (Beneviste et al., 1982) and a marked depression of high-affinity glutamate uptake (Silverstein et al., 1986). Removal of excitatory amino acid innervation to a given region protects against subsequent hypoxic/ischemic damage (Wieloch, 1985). Global hypoxia/ischemia produces preferential damage of the superficial cortical layers, the CA1 region of the hippocampus, striatum and cerebellar Purkinje cells, regions with high densities of excitatory amino acid receptors (Brierly et al., 1984, Greenamyre et al., 1985, Monaghan et al., 1985). Perhaps more importantly, in models of global and focal hypoxia/ ischemia, excitatory amino acid antagonists exert a profound protective effect (Meldrum & Garthwaite, 1990).

There are a number of potential therapeutic targets on the NMDA receptor complex for protection against hypoxia/ischemia (see Fig. 1.3, Choi, 1990). Antagonists acting at the NMDA recognition site or the site for dissociative anaesthetics within the channel have been shown to reduce neuronal injury in a number of different injury paradigms in vivo (Simon et al., 1984, Faden & Simon, 1988, Hayes et al., 1988, McIntosh et al., 1988, Ozyurt et al., 1988, George et al., 1988, Clifford et al., 1989), and in vitro (Goldberg et al., 1988, Monyer et al., 1989, Tecoma et al., 1989). The best characterized class of non-competitive NMDA receptor complex antagonists do not compete for the NMDA recognition site but rather associate with the site for dissociative anaesthetics within the channel (Fig.1.3) and therefore impede cation flow through the channel (Kemp et al., 1987, Lodge et al., 1987). These receptor linked channel blockers include MK-801, TCP, phencyclidine, ketamine and benzomorphan "σ opiates" (Wong et al., 1986) and penetrate the blood-brain barrier well. Another characteristic is use-dependent blockade, increasing levels of agonist produce increasingly rapid onset and recovery of blockade (Karschin et al., 1988). The outstanding concerns specific to these channel ligands are in the area of neurotoxicity. These drugs possess unacceptable phencyclidine-like psychomimetic effects (Koek et al., 1989) such as catalepsy and memory disruption and it seems unlikely that these compounds will be of value in the clinic. Futhermore, a report that low doses of MK-801 induced vacuoles in the posterior cingulate and retrospinal cortices of rats (Olney et al., 1989) questions the use of this type of therapy further.

Competitive NMDA recognition site antagonists such as AP5 (Table 1.1) are generally quite polar and therefore do not cross the blood-brain barrier as well as the non-competitive antagonists. However it is possible that highly potent antagonists such as CPP (Lehmann et al., 1988, Table 1.1) may be effective with penetration of relatively small quantities. Several other competitive NMDA antagonists have been described (Table 1.1). Of special interest is the phosphonoamino acid CGP 37849 which exhibit anticonvulsant activity after oral administration (Fagg et al., 1990).

The use of glycine site antagonists (Fig.1.3.) such as 7-chlorokynurenic acid (Kemp et al., 1988) can reduce neuronal injury in vivo (Tridgett & Foster, 1988) and in vitro (Shalaby et al., 1989, Hartley et al., 1990). However, like NMDA recognition site antagonists, available compounds are polar and do not cross the blood-brain barrier. An alternative approach may be to reduce the availability of endogenous glycine in the vicinity of the receptor, but at present no mechanism for this exists.

More recently, attention has been focussed on the polyamine modulatory site of the NMDA receptor complex as a target for therapeutic intervention. In vivo, the divalent polyamine putrescine is derived from the intracellular decarboxyaltion of ornithine and then converted to spermidine (trivalent) and spermine (tetravalent). Reverse pathways for metabolism also exist. NMDA -evoked transmitter release and Ca^{2+} flux has recently been shown to be blocked by an ornithine decarboxyalse inhibitor, an effect reversed by putrescine (Siddique et al., 1988). Furthermore, it has been reported that the concentration of spermidine in gerbil cortex is approximately 250 nmol/g and that following ischaemia and reperfusion, this concentration remains constant (Paschen et al., 1988). Although it is not known what proportion of the polyamine site is accesible to the NMDA receptor complex it seems likely from in vitro data that the receptors are tonically activated by polyamines (Robinson et al., 1990). If this is the case then antagonists at the polyamine site may be useful for modulating NMDA receptor complex function. The concentration of putrescine is much lower (10 nmol/g) and while there is a three-fold increase in the concentration of putrescine in gerbil cortex following ischemia and reperfusion (Paschen et al., 1988) it is not clear if this would be sufficient to antagonize polyamine potentiation of NMDA receptor complex activity. Ifenprodil and its' derivative SL 82 0715 have been proposed to interact selectively with this polyamine modulatory site but other reports disagree (see section 1.7.3). Both drugs reduce infarct volume in a rat stroke model (Gotti et al., 1988) and appear to be relatively free of adverse effects associated with NMDA receptor complex blockade (Jackson et al., 1988, Tricklebank et al., 1989).

There is evidence suggesting a role for excitatory amino acid in the pathogenesis in several conditions including Huntington's disease (Simon et al., 1984, Rothman & Olney, 1987, Foster et al., 1988), epilepsy (Sloviter, 1983), hypoglycemic brain damage (Wieloch, 1985), and amytrophic lateral sclerosis (Spencer et al., 1987).

1.10. Excitatory amino acid markers in Alzheimer's disease.

1.10.1 Excitatory amino acid concentrations.

Glutamate and aspartate are intimately involved in intermediary metabolism in the central nervous system (Fig.1.1) and the neurotransmitter pool of these amino acids is only a small fraction of the total. However, in regions with dense glutamatergic innervation, the neurotransmitter pool of glutamate and aspartate may be as high as 30-40% of the total (Fonnum, 1984). There have been a number of studies of excitatory amino acid concentrations in Alzheimer's disease and control brains but aspartate has not been found reduced. Some of these studies have shown significant decreases in glutamate concentration in cortex and hippocampus (Arai et al., 1985, Ellison et al., 1986, Sasaki, 1986) but others have shown no change (Perry et al., 1984, Perry et al., 1987). A different approach was used by Hyman and co-workers (1987) who microdissected a discrete region of the hippocampus which was apparently enriched in glutamatergic nerve endings. They reported an 83% reduction in glutamate and this observation supports the earlier finding of Korey et al. (1961) reported in abstract form, that in Alzheimer's disease biopsy tissue "the free amino acid pool demonstrates a decrease in the glutamic acid group". Workers from this laboratory have also examined amino acid concentrations in biopsy samples from Alzheimer's disease patients at this early stage of the disease (Procter et al., 1988); a significant decrease in glutamate concentration in the temporal cortex was found. Autopsy samples obtained within three hours of death were analyzed as well and a much larger decrease in glutamate content was found in Alzheimer's disease. The glutamate content of the Alzheimer's disease biopsy samples correlated with pyramidal neurone density in cortical layer III.(Lowe et al., 1990). Amino acids have also been measured in the cerebrospinal fluid of Alzheimer's disease subjects and controls (see Procter et al., 1989a) but there were no differences in aspartic acid and

glutamate in Alzheimer's disease.

In summary, measurements of excitatory amino acid concentrations have yielded conflicting results. However, when these measurements were made in regions of dense glutamatergic innervation, in biopsy samples or in post-mortem samples obtained within three hours of death, there did appear to be significant decreases in glutamate concentration in Alzheimer's disease.

1.10.2 Excitatory amino acid uptake and release.

More specific biochemical markers for excitatory amino acid containing neurones include Na^+-dependent high affinity uptake and Ca^{2+}-dependent release (Fonnum, 1984, Greenamyre, 1986). High-affinity uptake was measured in a synaptosomal preparation of previously frozen brains from Alzheimer's disease and control subjects (Hardy et al., 1987). Under these conditions there was a 60 % decrease of uptake sites in cortical and hippocampal regions. However, studies in rat brain have indicated that freezing results in a 50-80% decrease in uptake activity compared with fresh tissue, and prolonged freezing results in a further decrement of uptake (Schwarz, 1980, Hardy et al., 1987).

More recently, some laboratories have examined Na^+-dependent D-[^{3}H]aspartic acid binding because it was thought that this would be a more stable marker of the uptake site (Palmer et al., 1986, Cross et al., 1987, Cowburn et al., 1988, Simpson et al., 1988). Unfortunately in homogenates (Danbolt & Storm-Mathisen, 1986) and more recently in autoradiographic assays (Bridges et al., 1988, Greenamyre et al., 1990), Na^+-dependent D-[^{3}H]aspartic acid binding has not been found to have properties consistent with neuronal presynaptic uptake sites. In homogenates (Danbolt & Storm-Mathisen, 1986), thorough washing of membranes in distilled water abolishes subsequent Na^+-dependent D-[^{3}H]aspartic acid binding. This and several other inconsistencies have led authors to conclude that D-[^{3}H]aspartic acid binding does not represent binding to the transport carrier. In an autoradiographic assay (Greenamyre et al., 1990), Na^+-dependent D-[^{3}H]aspartic acid binding has a

regional distribution unlike that expected for a presynaptic neuronal uptake site. Furthermore, lesions of excitatory amino acid pathways do not result in decreased binding in the terminal fields of those pathways, contrary to what would be expected for a marker of the uptake site (Bridges et al., 1988, Greenamyre et al., 1990).

In this laboratory a 40-50% decrease in D-[^{3}H]aspartic acid uptake into tissue prisms containing synaptosomes was observed in Alzheimer's disease using fresh unfrozen tissue obtained shortly after death (Procter et al., 1988). The Ca^{2+}-dependence of release of glutamate and aspartate could not be demonstrated from either the Alzheimer's disease or the control samples.

1.10.3. Excitatory amino acid immunocytochemistry.

Another approach for assessment of excitatory amino acid neurones is by means of excitatory amino acid immunocytochemistry (Storm-Mathisen, 1988). The interpretation of glutamate and aspartate immunocytochemistry is somewhat controversial and may depend, in part, on the particular antibody used. It is also unclear whether antibodies to these compounds are labelling a metabolic or neurotransmitter pool (Ottersen & Bramham, 1988). Despite these uncertanties, it appears that in many regions glutamate and aspartate immunocytochemistry label populations of putative excitatory amino acid containing neurones. This technique has been applied to the study of Alzheimer's disease tissue and it has been shown by two different laborotories that glutamate immunoreactivity is localized in cortical and hippocampal pyramidal neurones, many of which contain neurofibrillary tangles (Kowall et al., 1987, Maragos et al., 1986). In the future, it may be possible to use immunochemistry to perform quantative morphometric studies of excitatory amino acid containing neurones in Alzheimer's disease tissue.

1.10.4. Excitatory amino acid receptors in Alzheimer's disease.

In addition to the alterations described above in presynaptic measures of excitatory amino acid containing neurones there is also some evidence of a receptor

dysfunction although the data are conflicting, in particular for the NMDA receptor (see Table 1.4). Pearce et al. (1984) reported an increase in [^{3}H]-glutamate binding in the caudate nucleus in Alzheimer's disease. This was interpreted as reflecting elevated receptor numbers in response to a loss or dysfunction of descending glutamatergic tracts, a situation analagous to that seen with hemidecortication in rats (Roberts et al., 1982) Greenamyre et al. (1985) described a loss of glutamate binding sites in the neocortex of patients with Alzheimer's disease compared with normal controls and Huntington's disease. When sub-types of excitatory amino acid receptors were examined it appeared that the NMDA receptor complex was affected more severely than the AMPA receptor. A similar loss of the NMDA receptor complex was found in the hippocampus of Alzheimer's disease tissue (Greenamyre et al., 1987, Represa et al., 1988, Penney et al., 1990). However, a number of reports suggested no loss of this receptor in Alzheimer's disease (Geddes et al., 1986, Cowburn et al., 1988a,b, see Table 1.2).

Maragos et al., (1987) found a loss of binding of [^{3}H]-TCP to the channel associated with the NMDA receptor complex in CA1, CA3 and the subiculum of the hippocampus of Alzheimer's disease subjects. A 40% loss of binding of [^{3}H]-TCP to the channel was reported by Monaghan et al. (1987). Interestingly, although this report is of an average 40% loss of binding to the channel in CA1 and no significant loss of NMDA site, they found a correlation coefficient of 0.92 between the two sites in the same brain. Simpson et al. (1988) examined ligand binding ([^{3}H]-TCP) to the channel in homogenates from Alzheimer's disease tissue and found no significant change in the hippocampus the temporal cortex or the caudate nucleus. They did, however find a reduction in the frontal cortex.

The reports of non-NMDA receptor sub-types are also conflicting (see Table 1.2). Some find kainate receptor density unchanged in Alzheimer's disease (Cross et al., 1986a, Cowburn et al., 1988), based on binding performed in cortical membrane homogenates as well as in the caudate nucleus. (Pearce & Bowen 1984). However using autoradiography, Geddes et al. (1986) described an expanded distribution of

binding to the kainate receptor in the molecular layer of the dentate gyrus in Alzheimer's disease hippocampi, but the overall density of binding was unchanged. By contrast, Represa et al. (1988) reported a significant loss of kainate sites in the dentate gyrus and no expansion of the distribution of these sites. Penney et al. (1990) also report a significant loss of kainate binding sites in the hippocampus. In the deep layers of the frontal cortex, Chalmers et al. (1990) report a 70% increase in the density of kainate binding sites. These reports of a change rely on the measurement of density in discrete layers and regions of the cerebral cortex or hippocampus. By contrast two recent reports of [^{3}H]-AMPA binding report no change in this site in Alzheimer's disease tissue from the hippocampus (Penney et al., 1990) or the cortex (Chalmers et al., 1990).

There may be several reasons for the discrepancies in the results of glutamate receptor binding studies in Alzheimer's disease reported by different laboratories. First, differences in assay conditions and buffers used may yield different results and there are few studies where more than one radioligand concentration has been studied. Secondly, binding to homogenates may mask changes in discrete laminae. Thirdly, the severity of the disease may determine the degree of receptor changes measured, this may be further complicated by loss of cortical cells organised in a columnar manner (see Hauw, 1986, Procter et al.,1988). Further studies are necessary to clarify these issues. In the present investigation Scatchard analysis was undertaken using two extensively characterised ligands which bind to the NMDA receptor complex. A loss of glycine stimulation of the NMDA receptor complex in Alzheimer's disease was reported by Procter et al. (1989a, b). Since then other modulators of the NMDA receptor complex have been described in rat brain. In this study the modulation of this receptor by glycine, zinc and spermidine have been investigated in human brain.

In summary, measurement of excitatory amino acid markers including glutamate concentration, synaptosomal uptake and immunocytochemistry provides evidence for a loss of excitatory amino acid releasing neurones. Because the excitatory amino

TABLE 1.2 SUMMARY OF STUDIES ON NMDA RECEPTOR COMPLEX DENSITY IN ALZHEIMER'S DISEASE.

	REFERENCE	ASSAY	BRAIN REGION	OTHER INFORMATION
LOSS OF NMDA RECEPTORS	Greenamyre et al 1985	NMDA sens.[^{3}H]-glu AR	Cortex	layers I & II only
	Greenamyre et al 1987	NMDA sens.[^{3}H]-glu AR	Hippo	loss of "Quis" binding sites
	Maragos et al 1987	[^{3}H]-TCP AR	Hippo	
	Represa et al 1988	NMDA sens.[^{3}H]-glu AR	Hippo	loss of kainate binding sites
	Simpson et al 1988a	[^{3}H]-TCP binding	F cortex	no loss T cortex, caudate, hippo.
	Chalmers et al 1990	NMDA sens.[^{3}H]-glu AR	F cortex	layers I and II only, unrelated to tangles
	Ninomiya et al 1990	[^{3}H]-TCP binding	F cortex	B_{max},AD 237,Control 441 fmol/mg protein
	Penney et al 1990	[^{3}H]-TCP AR & NMDA sens [^{3}H]-glut AR	Hippo	
NO LOSS OF NMDA RECEPTOR	Geddes et al 1986	NMDA sens.[^{3}H]-glu AR	Hippo	no change in kainate binding
	Monaghan et al 1987	NMDA sens.[^{3}H]-glu AR	Hippo	
	Cowburn et al 1988a	NMDA sens.[^{3}H]-glu B	T cortex	
	Cowburn et al 1988b	NMDA sens.[^{3}H]-glu B	Hippo, caudate F & T cortex	Scatchard analysis employed
	Mouradian et al 1988	[^{3}H]-MK-801 binding	Hippo,F,T,O,P cort.	
	Simpson et al 1988	[^{3}H]-TCP binding	T cortex, caudate & hippo.	
	Procter et al 1989	[^{3}H]-MK-801 binding	Cerebellum F,T,P cortex	Effect of glycine regulation

NMDA sens.[^{3}H]-glut AR represents NMDA sensitive [^{3}H]-glutamate autoradiography, NMDA sens.[^{3}H]-glu B refers to studies using homogenate. F, T, O, P are frontal, temporal, occipital and parietal respectively.

acid releasing neurones themselves may receive extensive excitatory amino acid input, it would be expected that degeneration of these cells (pyramidal cells in some areas of the neocortex and CA1 region of the hippocampus) would result in a loss of excitatory amino acid receptors. The results are confusing (see Table 1.4) but some studies have shown a loss of these receptors in particular in the frontal cortex. Of critical importance but still unanswered, is the question of when during the course of the disease these changes in markers of excitatory amino acid transmission occur.

1.11. The relationship of excitatory amino acids to pathology in Alzheimer's disease.

1.11.1 Neurofibrillary tangles.

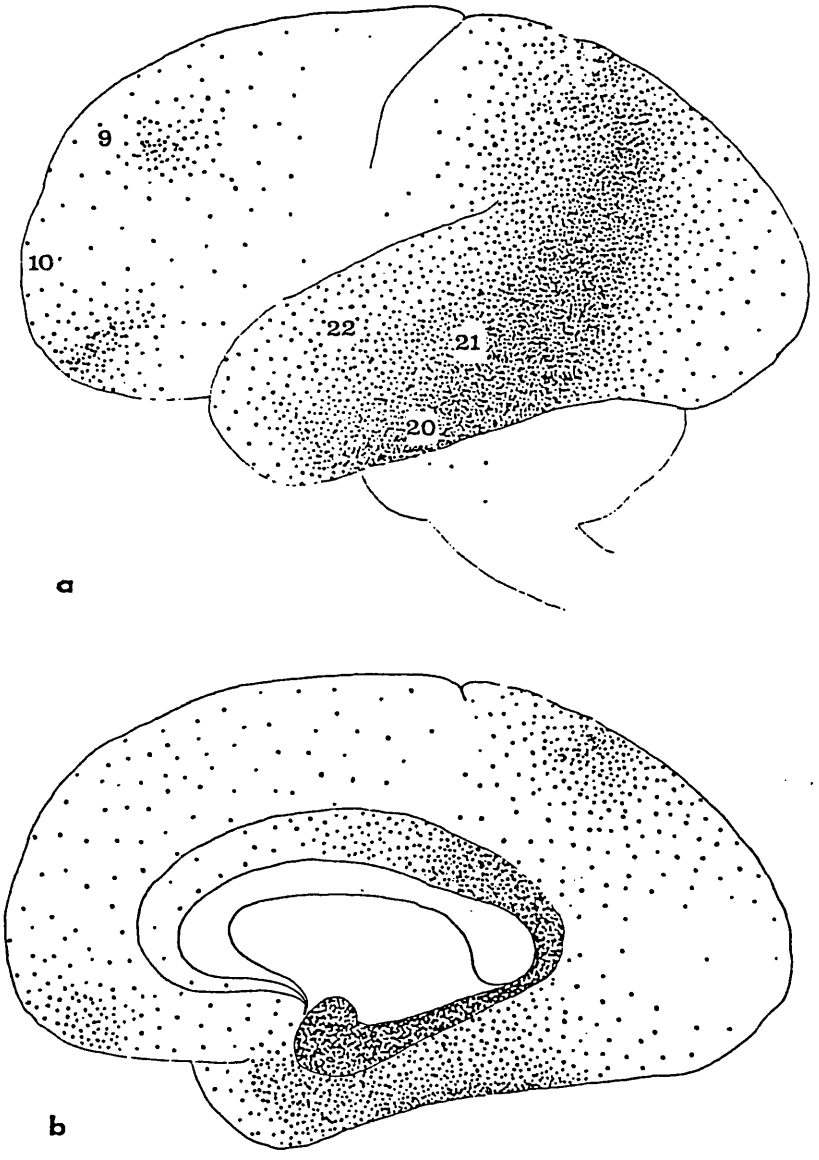
A characteristic neuropathological finding in Alzheimer's disease is the presence of neurofibrillary tangles in the perikarya of many neurones in the cortex, hippocampus and basal forebrain (see section 1.3). Interesting observations have been reported relating excitatory amino acids to neurofibrillary tangles. Results of one study (which has not been replicated) suggested that incubation of cultured human neurones with aspartate and glutamate induced the formation of paired helical filaments similar to those which make up neurofibrillary tangles (DeBoni & Crapper-McLachlan, 1985). Antibodies against the abnormal cytoskeletal elements found in Alzheimer's disease have not been used to determine whether the epitopes on the paired helical filaments described in this culture experiment are similar to those of the paired helical filaments in Alzheimer's disease. On the other hand, immunocytochemical data do suggest that the antigenic composition of neurofibrillary tangles observed in the amyotrophic lateral sclerosis-parkinsonism-dementia of Guam are similar to that of tangles observed in Alzheimer's disease (Shankar et al., 1989). This observation suggests that there is a common pathogenetic mechanism for neurofibrillary tangle formation in these diseases. Because the pathogenesis of amyotrophic lateral sclerosis-parkinsonism-dementia of Guam has been tentatively linked to an excitotoxin acting at the NMDA receptor complex (Spencer et al., 1987), a similar excitotoxic mechanism may play a role in the formation of neurofibrillary tangles in Alzheimer's disease. The mechanism by

which excitatory amino acids induce paired helical filaments is unknown. One might speculate that excitatory amino acids, by opening calcium channels or by activation of the inositol phospholipid cascade, could activate protein kinases and cause abnormal phosphorylation of neurofilament or tau proteins. Such a possibility has been proposed as a mechanism for neurofibrillary degeneration (Iqbal et al., 1986).

1.11.2. Distribution of plaques and tangles.

Systematic examination of the distribution of neurofibrillary tangles and senile plaques suggest that these lesions do not occur randomly, but may have an anatomical basis (Fig.1.4). The "association" cortex of the parieto-temporal and frontal lobes and the cingulate cortex are the most severely affected. The motor cortex and the major sensory areas are little, if at all, affected. The olfactory system appears to be unique in being the only sensory system that is affected and structures associated with it also show pathological change (Pearson & Powell, 1989). On the basis of this distribution of the pathology and of the clinical features there is the possibility that the olfactory system is the initial site of involvement and that the disease process spreads along known cortico-cortical fibre connections which contain excitatory amino acids (Pearson et al., 1985). The parts of the neocortex that are affected are the same as those that have been shown in the monkey to be connected by a sequence of links which starts in the main sensory areas and passes through the "association" cortex of the parieto-temporal and frontal lobes to the hippocampal formation (Jones & Powell, 1970). Each succesive step in this orderly sequence through the parieto-temporal lobe is reciprocally connected both with the preceding step and with the corresponding step in the frontal lobe. In addition, the hippocampal formation and the adjoining areas of the cortex send fibres back to a number of these "association" areas (Kosel et al., 1982, Van Hoesen, 1982). The least affected areas in Alzheimer's disease are at the beginning of this sequence, themost severe at the end and the "association" areas occupy an intermediate position. It is possible that the distribution in the neocortex itself may depend in part upon its connections with the amygdala. The cortico-medial group of the amygdaloid nuclei receive fibres directly from the olfactory bulb and in turn are interconnected

FIGURE 1.4. SCHEMATIC REPRESENTATION OF THE DISTRIBUTION AND SEVERITY OF PATHOLOGICAL CHANGES IN ALZHEIMER'S DISEASE



The numbers indentify cortical areas studied in the present work - a represents the lateral and b the medial surface of the brain. The darker the area the more pronounced degeneration, white areas are spared. Adapted from Brun, 1983.

with the basolateral group (Kretteck & Price, 1978, Ottersen, 1982, Aggleton, 1985). The latter group are connected with widespread areas in the neocortex (Aggleton et al., 1980, Amaral & Price, 1984) and it is significant that in the monkey the most severely affected in Alzheimer's disease and the least connections are with the motor and sensory areas that are least affected by the pathological changes. As well as through the sequence of cortico-cortical fibres between areas of the neocortex, the disease process may spread along these connections between the amygdala and the neocortex.

These hypotheses are extremely difficult to test since it is impossible to take repeated brain samples from patients during the course of the disease. However, if these hypotheses are correct, it is likely that no area of the cortex that is only indirectly linked to the hippocampal formation by cortico-cortical excitatory amino acid containing fibres will be affected in Alzheimer's disease. That is unless intervening areas with relaying neurones more closely situated to the hippocampal formation are also affected. Furthermore, the more closely these areas are situated to the hippocampal formation and to the olfactory structures, and the stronger the connection to the amygdala, the more severe would be their involvement in the disease. Some of these hypotheses have been tested in collaborative studies described in this thesis by measuring neurofibrillary tangle density. If excitatory amino acid releasing cortico-cortical fibres play an important role in the pathology of Alzheimer's disease the excitatory amino acid receptor-mediated mechanisms may play a role in the distribution of pathology and contribute to the degeneration of neurones which are already compromised.

1.11.4. Dendritic changes.

During the course of Alzheimer's disease there is a loss of dendritic elements and a progressive simplification of the dendritic arbor of cortical neurones (Mehraein et al., 1975, Mann et al., 1986). Subtoxic doses of glutamate induce identical regression of dendrites in cultured hippocampal neurones (Mattson, 1988, Mattson et al., 1988). The affected dendrites are often found primarily on pyramidal projection

cells which normally receive extensive synaptic input from other cortical excitatory amino acid containing neurones. A loss of the dendritic arbor would be expected to impair communication between regions of cortex.

In some cases, in addition to the loss of normal dendrites and dendritic spines, there is a proliferation of abnormal, bizarre dendritic structures (Probst et al., 1983, Ihara, 1987,). In these instances, aberrant filopodia are often present. Similar filopodia-like structures are seen transiently after focal application of glutamate in cultured neurones (Smith, 1987). Thus in cultured neurones, excitatory amino acids can reproduce some of the dendritic changes seen in Alzheimer's disease. The relevance to the changes in Alzheimer's disease of this potential for excitatory amino acids to modify dendritic structure is not known.

1.12. The relationship of excitatory amino acid changes to clinical manifestations of Alzheimer's disease.

Patients with Alzheimer's disease become progressively forgetful and develop profound abnormalities of cognition and orientation: signs of cortical disconnection such as apraxias, aphasias and agnosias are also common and it is these symptoms that differentiate Alzheimer's disease from other purely cholinergic deficits and a defect in excitatory amino acid neurotransmission may contribute to this clinical picture. Since excitatory amino acids appear crucial in learning and memory (see section 1.36) and activation of the NMDA receptor complex is required for the development of long-term potentiation the important role of these receptors in cognition cannot be underestimated. Furthermore, cortical and basal forebrain cholinergic deficits alone are probably not sufficient to cause Alzheimer's disease (Kish et al., 1988). It seems likely therefore, that a disruption of excitatory amino acid transmission contributes to the learning and memory deficits in Alzheimer's disease. The clinical signs of cortical disconnection may also result from disruption of glutamatergic pathways.

1.13. Therapeutic modulation of excitatory amino acids in Alzheimer's disease .

Previously, treatment has been aimed at brain cholinergic activity The approach of oral precuror loading using choline (usually in the form of lecithin) was well tolerated but failed to improve the symptoms of Alzheimer's disease (see Gauthier et al., 1990). A direct muscarinic agonist, bethanacol chloride was infused intracerebro-ventricularly using one dose only but there was little improvement (Harbaugh et al., 1984). There have been a large number of reports suggesting that this type of therapy is unsuccesful in Alzheimer's disease. More recently, an inhibitor of acetylcholinesterase activity, tetrahydroaminoacridine (tacrine) in oral doses of up to 200 mg a day was reported to improve patients (Summers et al., 1986, see Thal et al., 1990). Tacrine is a potent acetylcholinesterase inhibitor with an IC_{50} of 0.1-0.2 μM (Pearce & Potter, 1988). At a concentration above 1 μM it also acts as a muscarinic antagonist (M1 and M2, Pearce & Potter, 1988). Thus if tacrine were present in brain at concentrations in the low micromolar range, the blockade of muscarinic receptors would be expected to offset the anticholinesterase effects of tacrine. At slightly higher concentrations, tacrine antagonises the NMDA receptor complex (Albin et al., 1988). It has recently been found in mice that a single dose of tacrine which produces a therapeutic plasma level, could result in at least a ten-fold concentration of tacrine in the brain, with peak tacrine concentrations of 2.4 μM (Liston et al., 1988). Conceivably, on a regular dosing schedule, steady-state concentrations of tacrine in brain could be even higher than after a single dose used in this study. At this reported concentration tacrine could be expected to have significant cholinergic antagonist effects and would also act at the NMDA receptor complex. This raises the possibilty that any beneficial effect of tacrine on the cholinergic system may be offset by actions at the NMDA receptor complex. A recent report (Hodges et al., 1990) reports that treatment with low doses of tacrine substantially improved radial maze performance in rats. However, acetylcholine content was not changed and inhibition of brain acetylcholinesterase activity was only marginally increased by this treatment regime. The authors conclude that the action of tacrine is probably not as an acetylcholinestrase inhibitor. There are a number of additional effects of tacrine that could account for the reported improvement in

patients. Tacrine inhibits "high affinity" uptake of choline (Drukarch et al., 1987, Tachiki et al., 1988, Sherman & Messamore, 1988, Robinson et al., 1989)and in vitro findings indicate that, in the low micromolar range, tacrine modulates monoaminergic transmission (Drukarch et al., 1987, Robinson et al., 1989). The effects of this compound on excitatory amino acid release and uptake have not been examined previously and results are reported in this thesis.

It has been suggested that clinical trials examining the effect of glutamate in this disorder are warranted (Deutsch & Morihisa, 1988). While augmentation of excitatory amino acid transmission with either glutamate may seem attractive, this approach is potentially quite dangerous because of excitotoxic properties of excitatory amino acids. Under normal circumstances the acidic amino acid transport system by which glutamate is translocated across the blood brain barrier is saturated with ambient concentrations of glutamate (Pardridge, 1977). Furthermore, the concentration of glutamate in the brain is approximately 5-10 mM. Thus it seems unlikely that manipulation of plasma concentrations would significantly alter brain levels unless extremely high doses were used (Toth & Lajtha, 1981). In Alzheimer's disease there may be a disruption of the blood-brain barrier (Wisniewski & Koslowski, 1982) and glutamate may enter the brain more freely. Because of loss of neuronal glutamate uptake sites, higher levels of glutamate would more likely be toxic.

1.14 Neuronal loss

Loss of nerve cells throughout most areas of cortex has long been the impression of neuropathological examination (Corsellis, 1976, Tomlinson, 1977). However, quantitative measurements of cell numbers in the cerebral cortex is complicated by the 13-18% reduction in brain volume (Hubbard & Anderson, 1981; Miller et al., 1980). Early studies suggested that nerve cell numbers were unaltered (Tomlinson & Henderson, 1976, Terry et al., 1977, Terry, 1979) whereas more recent observations, using an image analyzer that makes allowance for atrophy by counting cells in columns of cerebral cortex rather than in individual fields, indicate that large

(presumably pyramidal) neurones are depleted by 23-46% (Terry et al., 1981, Mountjoy et al., 1983, Hubbard and Anderson, 1985). This work has been confirmed and extended by Mann & colleagues who counted the number of pyramidal neurones in cortical layers III and V and made allowance for atrophy by taking measurements of cortical thickness using an ocular micrometer (see Mann et al., 1985), rather than an image analyzer (see above). The mean cortical atrophy was estimated to be about 30%, which is approximately twice the value obtained by the quantitative morphometric studies of Hubbard and Anderson (1981) and Miller et al. (1980). Reduced numbers of pyramidal cells were found in the neocortex of Alzheimer tissue obtained both post-mortem (66-75% loss; Mann et al., 1985) and ante-mortem (56-60%loss; Neary et al., 1986). It is possible that shrinkage could account for at least part of the reduction of large cells as neuronal atrophy, with 20-40% shrinkage of nuclei, has been shown to affect scattered neurones in the cerebral cortex (Mann et al., 1981a). However, automated image analysis of cells of various size does indicate that loss of large cells is prominent (Terry et al., 1981, Mountjoy et al., 1983, Hubbard & Anderson, 1985).

The precise elements of cortical circuitry comprising these pathological changes have remained obscure, but pyramidal cells are probably intimately involved in the formation of plaques and tangles. The abundance of these neurones makes it likely that they make a substantial contribution to the degenerating neurites that partially constitute plaques. Moreover, the neurofibrillary tangles accumulate in the cytoplasm of neurones which generally resemble pyramidal cells in size, shape and dendritic morphology. Just as loss of pyramidal cells in the cortex are localized to layers 3 and 5, so tangles are found predominantly in pyramidal neurones in these layers (Braak & Braak, 1985, Pearson et al., 1985, Lewis et al., 1987). When examined quantitatively, the tangles are not distributed within a cortical area in a single random pattern; rather they exhibit patchy clustering which resembles the patchy distribution of the cells of origin of cortical "association" fibres (Pearson et al., 1985). Histological changes are also reported post-mortem in the hippocampus and amygdala which are components of the limbic system. Both exhibit severe cell

loss and presence of plaques and tangles. A third component of the limbic system is the olfactory pathway which is unique amongst the sensory systems in being severely affected in Alzheimer's disease (Hooper and Vogel, 1976, Herzog & Kemper, 1980, Ball, 1977, Ball et al., 1985, Reyes et al., 1987, Najlerahim & Bowen, 1988). Such findings led Pearson & Powell (1989) to suggest that Alzheimer's disease may be caused by entry of a viral agent/toxin via the olfactory system. Recently Talamo and coworkers (1989) supported this hypothesis when they reported that nasal epithelium taken from Alzheimer's disease patients showed pathological changes and abnormal reactions with phosphorylated neurofilaments.

Mann and colleagues (1988) determined plaque and tangle densities in the cortex of brains which were obtained at autopsy from Alzheimer's disease patients who had previously undergone diagnostic craniotomy. Senile plaque density did not consistently change from biopsy to death; neurofibrillary tangle density either did not change or it decreased from biopsy to death. By contrast, the density of pyramidal cells was found to be significantly reduced in post-mortem specimens when compared to those obtained ante-mortem, a similar result has been obtained with synapse counts (DeKosky & Scheff, 1990). Correlations with the severity of cognitive impairment were also found (see also Neary et al., 1986) so these findings suggest that the pathological change giving rise to clinical symptoms during the course of the disease may be loss of the pyramidal neurones in the cortex. Loss of the these neurones may account for much of the cerebral atrophy seen in Alzheimer's disease (see above).

Neuronal loss also appears to be associated with a number of discrete subcortical nuclei which gives rise to corticopetal fibres which innervate the cerebral cortex. These affected nuclei are associated with distinct neurotransmitter systems and includes the nbM (Whitehouse et al., 1981, 1982, Wilcock et al., 1983, Saper et al., 1985), the locus coeruleus (Bondareff et al., 1982, Iversen et al., 1983, Mann et al., 1980, 1983, 1986, Zweig et al., 1988) and the dorsal raphe (Ishii, 1966, Curcio & Kemper, 1984, Yamamoto & Hirano, 1985, Zweig et al., 1988) giving rise to

cholinergic, noradrenergic and serotonergic neurones respectively. A number of the above studies indicate that this neuronal loss may be associated with neurofibrillary tangle formation.

1.15 Aims of the Present Study

Studies reviewed above provide histological evidence for a loss of cortical pyramidal neurones in Alzheimer's disease. Despite the evidence that L-glutamic acid is the major transmitter of these cells in experimental animals, these neurones are extremely difficult to study in human brain due to lack of suitable markers. This thesis addresses glutamate- releasing neurones using three different methodological approaches.

1. The hypothesis that the disease process in Alzheimer's disease spreads along a sequence of cortico-cortical connections (probably glutamate-releasing) was tested. Choline acetyltransferase activity and neurofibrillary tangle density were measured in adjacent samples from various areas of the brain from Alzheimer's disease and control subjects. These areas were selected as examples of the cortex known to have a defined proximity in terms of their fibre connections, with the entorhinal cortex, based on comparison with equivalent areas of the monkey brain.

2. In a second series of experiments, the integrity of the NMDA receptor complex, the best characterised glutamate receptor sub-type, was determined in Alzheimer's disease compared with controls. This study was performed in view of the conflicting disease-related data and also because of the probable role of the receptor complex in learning and memory and in the phenomenon of excitotoxic neuronal cell death. To facilitate this study, a comparison was made in human brain of two radioligands ([^{3}H]-MK-801 and [^{3}H]-TCP) that appear to label the channel associated with the NMDA receptor complex. The modulation of the NMDA receptor complex by glutamate, glycine, polyamines and zinc has also been studied using [^{3}H]-MK-801 binding in human brain. This was also considered important in terms of future treatment strategies for at least the symptoms of Alzheimer's disease.

3. Finally, tetrahydro-9-aminoacridine (tacrine) , an alleged drug for the treatment of Alzheimer's disease was examined for effects on glutamatergic neurones in rat brain. In this study extracellular amino acids, including aspartate and glutamate, were collected by in vivo microdialysis and concentrations determined by high performance liquid chromatography. The Ca^{2+}-dependent, K^{+}-evoked release of amino acids and also the Na^{+}-dependent uptake of [^{3}H]-D-aspartate were measured in the neocortex in vitro. In addition the effect of tacrine on [^{3}H]-MK-801 binding was assessed.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

L-forms of amino acids, spermidine HCl, acetyl Co A and $ZnCl_2$ were purchased from Sigma Chemical Co. (Poole, UK). D-[2,3,^{3}H]-aspartate (22 Ci/mmol) were purchased from Amersham Chemical Co. (Aylesbury, Buckinghamshire, UK). [^{3}H]-(+)-5-methyl-10,11-dihydro 5H-dibenzo[a,d]cyclohepten-5,10-imine maleate (MK-801, 17.8- 24.8 Ci/mmol) and [^{3}H]-N-[1-(2-thienyl)cyclohexyl]piperidine (TCP, 48.9 Ci/mmol) and 1-[^{3}H]-acetyl CoA (1.6 Ci/mmol) were purchased from New England Nuclear, Stevenage. HPLC grade methanol was obtained from Rathburn Chemicals (Walkerburn, UK). Emulsifier safe was purchased from Packard (Pangbourne, UK). AnalR grade sodium dihydrogen phosphate (NaH_2PO_4), disodium hydrogen phosphate (Na_2HPO_4), sodium tetraborate, absolute ethanol, bovine serum albumin (fraction V) and 2-mercaptoethanol were all purchased from BDH Ltd (Poole, UK). Tetrahydroaminoacridine (tacrine) was supplied by Aldrich Chemical Co.(Gillingham, UK). 2-amino-5-phosphonopentanoate (AP5) and 7-chlorokynurenic acid were obtained from Cambridge Research Biochemicals Ltd, Cambridge. MK-801 and ifenprodil were kind donations from Merck Sharp and Dohme, Harlow and Synthelabo, Paris respectively. Phencyclidine (PCP), N-allylnormetazocine (SKF 10 047) and TCP were kind gifts from Astra Neuroscience Research Unit, London. All other chemicals were of the highest grade of purity available.

2.2. Human brain samples.

2.2.1. Conventional autopsy samples.

Control postmortem brains were obtained from the Radcliffe Infirmary, Oxford (Dr. M.M. Esiri), St. Pancras Public Mortuary, London (Prof. J.M. Cameron) and Guy's Hospital, London (Drs. S. Cordner and K.A.L. Lee). Brains from patients with a clinical diagnosis of Alzheimer's disease were obtained from the Radcliffe Infirmary (Prof. G.K. Wilcock and Dr. M.M. Esiri) and Runwell Hospital, Wickford (Prof.

J.A.N. Corsellis and staff). Clinical diagnosis was confirmed by histological examination at the Radcliffe Infirmary and Runwell Hospital. The brains were dissected sagitally, one hemisphere was used for histological examination and the other was frozen at -70°C until required for neurochemical studies (carried out after histological diagnosis, to eliminate infectious conditions that may masquerade as Alzheimer's disease). Subjects with Alzheimer's disease had a clinical diagnosis together with senile plaque formation and neurofibrillary degeneration consistent with a diagnosis of Alzheimer's disease (Bowen et al., 1976a, Wilcock and Esiri, 1982). There was no selection of cases by disease severity, gender, drug treatment or agonal status. Left and right hemispheres were randomly allocated for each type of analysis.

2.2.2. Neurosurgical samples.

Samples of grey matter and underlying white matter of temporal and frontal lobes were obtained by neurosurgery as previously described (Bowen et al., 1982a, Neary et al., 1986a, Sims et al., 1983a). Control samples were from patients undergoing neurosurgical treatment for tumours (craniopharyngioma, glioma, meningioma, pituitary tumour, epidermoid cyst) or aneurysms, for which removal of apparently normal tissue was a necessary part of the surgical procedure. These samples were obtained from the National Hospital for Nervous Diseases, London (Prof. L. Symon, Dr. D.N. Grant, Mr. R.D. Hayward, Mr. D.G.T. Thomas). Less than 5 min after removal from the brain, samples were placed in ice-cold KRP-A (see section 2.6.1). After 20-40 min. in buffer, the tissue was dissected at 4°C. The outer 2 mm (approximately) of specimens was discarded if diathermy was used and grey matter (containing all cortical layers) was dissected free of meninges and white matter. It was then minced with a scalpel to obtain a homogeneous sample. Representative aliquots were maintained at -196°C prior to neurochemical assay. All specimens were obtained under procedures approved by the relevant Hospital Ethical Committees in both London and Manchester.

2.2.3.Brodmann Area samples with neurofibrillary tangle estimation.

A further group of samples were obtained (Dr M.M. Esiri, Oxford) post-mortem from 15 selected areas of the brain from demented and non-demented subjects. Approximately 1 cm^3 was removed and immediately frozen at -70°C. Before assay, the sample was thawed at 0-4°C and the grey matter including all cortical layers dissected free of meninges and white matter. Grey matter only was mixed with a scalpel, placed in an eppendorf tube and frozen at -70°C until measurement of choline acetyl transferase (ChAT) activity. An adjacent sample was formalin-fixed and paraffin sections of these areas stained with Cross' modification of the Palmgren stain (see Cross, 1982) for neurofibrillary tangles (NFT, kindly carried out by Dr M.M. Esiri, Oxford).

2.3. Estimation of choline acetyltransferase activity .

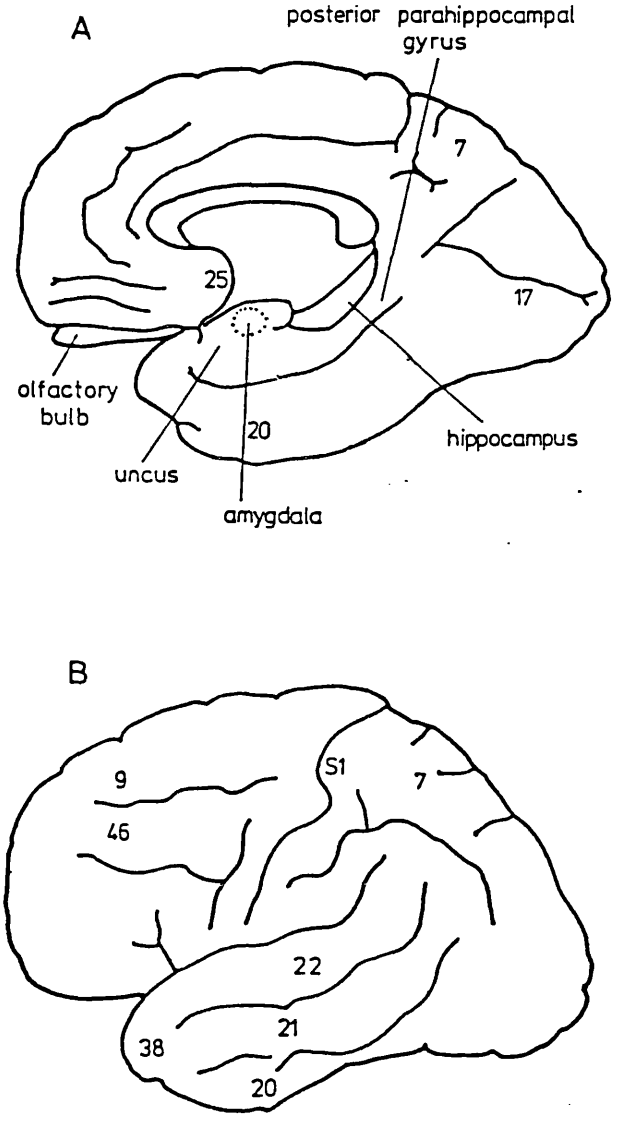
2.3.1.Tissue homogenisation.

Previously dissected tissue (section 2.2) from a number of Brodmann areas (BA) from control and Alzheimer's disease subjects (details of patient age, sex and delay to post-mortem given in section 3.1) was allowed to thaw at 4°C. The areas examined were the following: uncus, amygdala, hippocampus, TH/TF (the posterior part of the parahippocampal gyrus and BA 20, 21, 22, 38, 7, 25, 46, 13, 9, 3/1/2 (SI) and 17, see Fig. 2.1). Approximately 100 mg tissue was homogenised in 5 vols of 10 mM sodium phosphate buffer (pH 7.4) using a manually driven glass-teflon homogenizer assembly (Tri-R Stir, 10 up and down passes). The homogenate was transferred into an eppendorf tube and 2 x 50 μl aliquots removed and frozen at -20°C for later protein estimation (see section 2.8). One μl of a 10% Triton solution (final concentration 1%) was added to the homogenate and this mixture was stored for 1h at 4°C before estimation of ChAT activity.

2.3.2. Estimation of ChAT activity.

ChAT activity was estimated using an adaptation of the method of Fonnum, (1975). Microtubes were placed on ice, 2 μl of homogenate from each sample was placed in each tube, 6 tubes per sample. Two tubes were placed immediately in a boiling

FIGURE 2.1. SCHEMATIC REPRESENTATION SHOWING THE AREAS WHERE ChAT ACTIVITY AND NEUROFIBRILLARY TANGLES WERE MEASURED.



Areas have been indicated on outlines of the medial (A) and lateral (B) surfaces of the cerebral hemisphere. the numbers indicated are Brodmann areas and S1 is the primary somatic sensory area (BA1/2/3). Area 13 on the orbital surface of the hemisphere is not shown.

water bath for 10 min (blanks) and were then included in the assay. Five μl of a mixture of choline chloride (final concentration 10 mM), sodium chloride (300 mM), serine sulphate (0.1 mM), sodium phospahte buffer (700 mM), EDTA (10 mM), acetyl CoA (75 mM) and 25 μl [^{3}H]-acetyl CoA was added to the microtube containing the homogenate and incubated at 37°C for 10 min. The reaction was terminated by the rapid addition of 5 ml ice-cold 10 mM phosphate buffer via a syringe to the microtube placed in a glass scintillation vial. Two ml Kalignost (0.5 gm tetraphenylboron dissolved in 100 ml acetonitrile) was added to the scintillation vial and the mixture shaken vigorously. Finally, 10 ml scintillant (1 litre toluene, 200 mg 1,4-Di-2-(5-phenyloxazolyl)-benzene and 4 g 2,5-diphenyloxazole) was added. Radioactivity was immediately estimated by liquid scintillation spectrometry.

2.4. Radioligand binding experiments for measurement of the NMDA receptor complex.

2.4.1. Tissue samples.

Human tissue was from a sub-group of a previously described series (Procter et al., 1989). Details of subject age, delay to post-mortem, sex, pre-mortem status, laterality are given in the appropriate results section. Slices of frozen tissue were thawed to -20°C and the brain area required was removed. The tissue was subsequently thawed at 0-4°C when the cortical grey matter including all cortical layers was separated from the underlying white matter and the meninges. Approximately equal amounts of the caudate nucleus and the putamen were also dissected and pooled in equal amounts, hereafter called the striatum. Rats (male Lister-hooded, 300-500 g) were killed by cervical dislocation and their brains removed. Cortical grey matter was dissected exactly as described above.

2.4.2. Membrane preparation.

i)Crude membrane preparation.

Membranes were prepared by a modification of the method of Procter et al., (1989a). Grey matter from human or rat brain was homogenised in 10 vol (weight/vol) ice-cold 50 mM Tris-HCl buffer (pH 7.4) with a glass teflon Potter-

Elvejhem homogenizer and centrifuged at 18 000 g for 25 min to obtain the total particulate fraction, material previously shown to contain cell debris, nuclear fraction and crude mitochondrial fraction of previously unfrozen brain (Bowen et al., 1977). The pellet was resuspended in 75 vol 50 mM Tris-HCl buffer (pH 7.4) using an Ultra-Turrax homogenizer and centrifuged for 25 min at 30 000 g. This washing was repeated once with 5 mM Tris-HCl buffer (pH 7.4) and then 3 times with distilled de-ionized water. The resultant pellet was stored at -70°C for at least 18 h and not more than 1 week. On the day of the assay membrane pellets were thawed at room temperature, resuspended in 75 vol 5 mM Tris-HCl buffer (pH 7.4) and centrifuged at 30 000 g for 25 min. This was repeated 3 times before a final resuspension in 75 vol 5 mM Tris-HCl buffer (pH 7.4).

ii)Purified membrane preparation.

Cortical grey matter was homogenized in 10 vol 20 mM imidazole-HCl (pH 7.2) containing 320 mM sucrose as described above. This homogenate was centrifuged at 15 000g for 15 min to obtain the total particulate fraction, material previously shown to contain cell debris, nuclear fraction and crude mitochondrial fraction of a previously unfrozen brain (Bowen et al., 1977). The pellet was resuspended in 4 ml 320 mM sucrose using an Ultra-Turrax homogenizer, was layered onto 18 ml 800 mM sucrose and centrifuged with a swing out head at 50 000 g for 40 min. This sucrose gradient yielded two subfractions, one with the buoyant density of myelin, at the interface between 320 mM and 800 mM sucrose, and all the material that penetrated the 800 mM sucrose from which was derived the purified membranes (White, 1978). The purified membrane pellet was resuspended in 320 mM sucrose (25 ml) and centrifuged at 20 000 g for 15 min. The pellet was resuspended in 75 vol 50 mM Tris-HCl buffer (pH 7.4) and the washing procedure was continued exactly as described above.

2.4.3. Binding assays.

Assays were performed according to the method of Wong et al. (1986). Incubations were in triplicate in a total volume of 1ml 5 mM Tris-HCl buffer (pH 7.4) except for

purified membranes when 0.5 ml was used and contained 200- 400 μg protein, [^{3}H]-MK-801 or [^{3}H]-TCP (5 nM except for saturation analysis) and various concentrations of glutamate, glycine and test compounds where appropriate. Non-specific binding of both [^{3}H]-MK-801 and [^{3}H]-TCP was determined with either 100 μM (Chapter 4.1) or 10 μM MK-801 (Chapter 4.2.) and was typically less than 10 % of the total [^{3}H]-MK-801 and 20 % of the total [^{3}H]-TCP binding. Membranes were incubated at 25°C for 45 min (non-equilibrium conditions) 2 h or 4 h (equilibrium conditions). Incubations were terminated by rapid filtration over Whatman GF/B filters (previously maintained in 0.1% polyethyleneimine for 1 h) using either a Millipore filter manifold (chapter 5) or a Skatron cell harvester (Chapters 4 and 6). Radioactivity on the filters was determined by liquid scintillation spectrometry. Total membrane protein content was estimated as described in section 2.8.

2.5. In vivo microdialysis.

2.5.1 Anaesthetized animals.

Male Porton-Wistar rats were maintained on a 12 h light-dark cycle with access to food and water. Animals (body weight 180-280 g) were anaesthetized by intraperitoneal (IP) injection of chloral hydrate (400 mg/kg) and placed in a stereotaxic frame. After retracting the overlying skin and temporal muscles from the bone, a hole was drilled above the prefrontal cortex and the microdialysis probe (membrane 0.5 mm outer diameter x 2 mm, Carnegie Medicin, Stockholm, Sweden) was slowly lowered into position (co-ordinates AP 3.5 mm, ML 2.5 mm from Bregma, DV 3.0 mm, Paxinos & Watson, 1982). The tissue was perfused (2 μl/min) with a freshly prepared, prefiltered (0.2 μM filter) Ringer solution (composition in mM: NaCl, 141; Na_2HPO_4, 10; KCl, 5; $CaCl_2$, 1.3; $MgCl_2$, 1.3; pH 7.4). After 1 h of perfusion, serial 10 min fractions were collected on iced water and frozen for later amino acid determination. Tacrine was applied either through the probe (in the perfusion media) or systemically (IP injection).

2.5.2. Conscious animals.

Another group of animals (body weight 130-230 g) were anaesthetized with pentobarbitone (60 mg/kg, IP). The probe was secured in position (same coordinates as above) with two stainless steel anchor screws and cyanoacrylic cement. The day after implantation, the conscious rat was placed in a plexiglass cage (25 x 25 x 30 cm) and the probe perfused with Ringer solution. Serial samples were collected in 10 min fractions following a 60 min equilibration period. Either tacrine (freshly prepared in Ringer solution) or vehicle was injected intraperitoneally at the start of the seventh fraction.

Animals were killed with pentobarbitone and decapitated at the end of each experiment. The position of the probe was marked with ink and the brains were removed and fixed in 10% (vol/vol) formaldehyde. The position of the probe was then assessed by visual examination of coronal sections.

2.5.3.Estimation of recovery of amino acids through the dialysis membrane.

The tip of the probe was perfused in 1.5 ml of Ringer solution containing 1-5 nmol/ml of amino acid standards and perfused (2 μl/min). After a 60 min equilibration period, the mean perfusate concentration of 20 μl fractions was expressed relative to the concentration of the solution outside the probe. Recoveries were 11 $\pm$ 1% for aspartate and 13 $\pm$ 3% for glutamate (mean +_ SD, n = 3). Recoveries of other amino acids ranged from 6 to 16%, except for GABA which was 34%. All extracellular concentrations are expressed without correction for in vitro recovery.

2.6.In vitro uptake and release of amino acids.

2.6.1.Tissue Preparation.

Male Porton-Wistar rats (body weight 300 - 400 g) were used. Following decapitation, the brain was removed and the neocortex dissected free of other structures and white matter. Tissue (about 300 mg for uptake and 600 mg for release) was chopped with a McIlwain tissue chopper (Mickle Ltd, Gomshall, Surrey) at 0.3 mm intervals in two directions separated by 45°, as described (Procter et al.,

1988). The resulting tissue prisms were added to 10 ml of ice-cold Krebs-Ringer Phosphate buffer (KRP-A; composition in mM: NaCl, 141; glucose, 10; Na_2HPO_4, 10; KCl, 5; $CaCl_2$, 1.3; $MgSO_4$, 1.3; pH 7.4) oxygenated with 100% oxygen (as were all subsequent buffers), dispersed with a wide-bore pipette tip and allowed to settle. Supernatant (6 ml) was removed and the suspension transferred to a flask. The volume was adjusted either to 20 ml (uptake) or 40 ml (release) with KRP-A and, following application of 100% oxygen (45 s), the suspension of tissue prisms was preincubated at 37°C for 30 min with constant shaking. The suspension was then placed on ice and the tissue prisms allowed to settle. For uptake experiments, 16 ml of the supernatant was removed and replaced with an equal volume of KRP-A. For release experiments, 32 ml of supernatant was removed and replaced with an equal volume of KRP-B (low sodium buffer, composition in mM: choline chloride, 141; glucose, 10; Na_2HPO_4, 10; KCl, 5; $CaCl_2$, 1.3; $MgSO_4$, 1.3; pH 7.4). Washing was repeated and 75% of the supernatant fraction was removed before the suspension was decanted into a glass vial. The flask was rinsed with appropriate KRP to give a final volume of either 8 ml (uptake) or 12 ml (release) containing the tissue prisms. The suspensions were gently stirred magnetically and aliquots were removed for assay of D-[3H]-aspartic acid uptake and release of amino acids.

2.6.2. Specific uptake of D-[^{3}H]aspartic acid.

Specific uptake of D-[2,3,^{3}H] aspartic acid, 22 Ci/mmol) was determined as previously described (Procter et al., 1988). Aliquots (940 μl) of KRP-A containing varying concentrations of tacrine (100 μm - 1.5 mM) were added to incubation tubes (2 ml capped polypropylene tubes, Walter Sarstedt, UK) containing 100 μl of the suspension of prisms (0.1 - 0.5 mg of protein). The tubes were preincubated for 15 min either at 30°C with constant shaking or on ice (2°C). Then, 40 μl of D-[^{3}H]aspartic acid was added to give a final D-aspartic acid concentration of 300 nM (0.6 μCi/tube). Following a 4 min incubation period, the tubes were briefly placed on ice prior to centrifugation at 4°C (15,000 g for 1 min; Buckard Koolspin). The prisms were washed twice by centrifugation with 1.5 ml of ice-cold KRP-A. After discarding the final supernatant fraction, 1 ml of 5% (weight/vol)

trichloroacetic acid was added, and the suspension was sonicated (ultrasonic cleaning bath) for 3 min before centrifugation. The radioactivity of the supernatant fraction was determined by liquid scintillation counting and the pellet was used to determine protein content as described in section 2.8. Incubations were performed in quadruplicate and the results are expressed relative to the protein content of the pellet. Specific uptake was determined by subtracting uptake at 2 °C from that occurring at 30°C.

2.6.3. Ca^{2+}-dependent, K^+-evoked release of amino acids.

Aliquots (0.5 ml) of the suspension of prisms (approximately 1 mg protein) were placed in incubation tubes. Tubes were centrifuged (Eppendorf Microfuge) at 12,000 g for 2 s, the supernatant buffer was removed and replaced with 0.7 ml of either KRP-B or KRP-C buffer (zero Ca^{2+}, low sodium, composition in mM: choline chloride, 141; glucose, 10; Na_2HPO_4, 10; KCl, 5; ethyleneglycol-bis-(beta-aminoethylether) N,N'-tetra acetic acid, 2; $CaCl_2$, 1.3; $MgSO_4$, 1.3; pH 7.4). Each sample was assayed in quadruplicate $\pm$ Ca^{2+} in the presence of between 0.05 mM and 5 mM tacrine. After preincubation with tacrine for 15 min at 37°C with constant shaking, 14 μl of 2.25 M KCl was added to each tube to give a final concentration of 50 mM K^+. Prisms were incubated for a further 2 min and then 0.7 ml of ice-cold KRP-C was added. Tubes were centrifuged (12 000 g, for 2 min) and 1 ml of the supernatant fraction was maintained at -70°C until measurement of amino acid concentrations by HPLC. The pellet was used for protein determination as described in section 2.8.

2.7.High Performance Liquid Chromatography of amino acids.

2.7.1.Apparatus.

The HPLC system (Fig. 2.2) consisted of a solvent delivery system (Model 300/O2; Applied Chromatography Systems, Macclesfield, Cheshire, UK) linked to a gradient controller (Trilab 2000, Trivector Scientific, Sandy, Bedfordshire, UK). This was connected to a fluorometric detector with a 370 nm excitation filter and 418-700 nm emission filter (Fluoromonitor 3, LDC, Stone, UK). Injection of samples into this

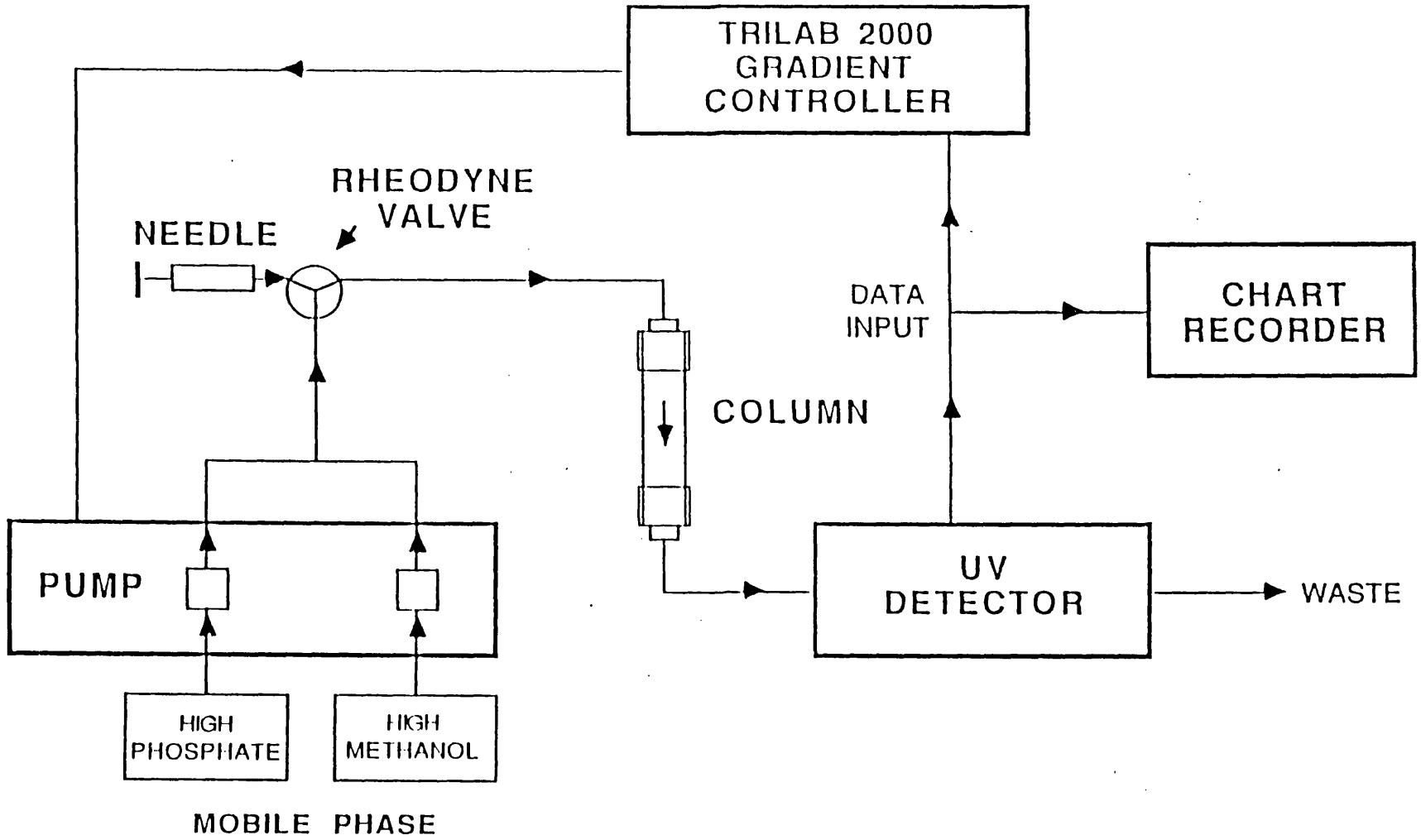


FIGURE 2.2 DIAGRAMMATIC REPRESENTATION OF HPLC APPARATUS.

system was done using an injection valve with a 50 μl loop (Model 7125, Rheodyne, Cutati, CA, USA).

2.7.2.Preparation of Phosphate Buffer .

70.2 g of sodium dihydrogen orthophosphate ($NaH2PO_4.2H2O$) and 17.9 g of disodium hydrogen orthophosphate ($Na_2HPO_4.12H_2O$) were dissolved in 5 l distilled, deionized water and the pH adjusted to 5.7 or 4.5 with 0.1 M phosphoric acid. An equal volume of distilled, deionized water was added to give a stock 0.05 M buffer and the resultant phosphate buffer was stored in Winchester bottles.

2.7.3.Preparation of mobile phase.

The eluant was a mixture of the phosphate buffer and methanol. For gradient elution, the mobile phase consisted of a low and high methanol buffer. Buffer A consisted of phosphate buffer pH 5.7: methanol, 80:20 (600 : 150 ml respectively) and buffer B consisted of phosphate buffer pH 4.5: methanol, 20:80 (75 : 300 ml respectively). The mobile phases were filtered using a filter pump assembly (Millipore (UK) Ltd/ Waters Chromatography Division, Harrow, Middlesex) and gassed for 5 min with helium prior to running through the column. Fresh buffer was made up for each day of analysis.

2.7.4.Preparation of the OPA/ 2-mercaptoethanol derivatizing solution.

81 mg of OPA was dissolved in 15 ml absolute ethanol. 15 ml of 0.1 M borate buffer (3.81 g di-sodium tetraborate [Borax; $Na_2B_4O_7.10H_2O$] dissolved with heating in 100 ml water) was added, followed by 60 μl of 2-mercaptoethanol. The mixture was kept overnight before use and away from light during its usage. Fresh solutions were made every week. Each sample for HPLC analysis was run in duplicate with an internal standard present except for amino acid release samples.

2.7.5.Derivatizing procedure.

To prepare amino acid standards or samples for injection onto the column, a 20 μl aliquot was taken and 80 μl OPA solution added. After thorough mixing (Vortex

mixer), the injection loop was washed with distilled, deionized water. After 2 min, the sample or standard was injected onto the column.

2.7.6.Preparation of amino acid standards.

A stock solution (1 mg/ml) for each standard amino acid (aspartate, homocysteic acid, glutamate, asparagine, serine, glutamine, glycine, taurine, alanine, GABA, was prepared in 0.1M HCl and stored at -70°C. These were diluted appropriately to make a mixed standard.

2.7.7. Gradient assisted chromatography.

Two gradients were developed to elute amino acids in biological samples.
Each gradient consisted of varying numbers of segments. The duration and degree of change of buffer composition was computed as shown in Table 2.1. The slope value refers to the rate of buffer change. A slope of one denotes a linear rise over a given time period. A positive slope begins with an initial slow rise, which is steep at the end of the segment whereas a negative slope results in the opposite, with a fast initial rise which then slows down. For positive and negative slopes 2 denotes the most steep curve and 5 the least steep. The slope value does not alter the composition of buffer at the end of the segment, but alters the rate at which that composition is reached. Amino acid peaks were quantified by measuring peak area (Tri-lab Vector microcomputer). The concentration of amino acids was calculated from amino acid standards run daily. Standard curves run for each one showed that the fluorescence response was linear over a wide range of amino acid concentrations.

2.8. Protein assay.

The assay was adapted from the method of Lowry et al. (1951). Tissue pellets from in vitro uptake and release experiments were incubated in 1 ml 1 M NaOH solution in a water bath at 37°C with constant shaking for 60-90 min until the pellet was dissolved. Tissue homogenates were incubated for 60 min at 37°C in NaOH to give a final concentration of 1 M in 1 ml. Aliquots of protein (approximately 100 μg, in triplicate) were placed into glass tubes and diluted with an equal volume of distilled

water. The volume was adjusted to 1 ml with 0.5 M NaOH. A standard curve was constructed using 25- 250 μg bovine serum albumin, fraction V (dissolved in 0.5 M NaOH to give 1 mg/ml). Standards, protein samples and blanks were treated simultaneously with 5 ml of a solution containing sodium carbonate (2% w/v), copper sulphate (0.01% weight/vol) and sodium potassium tartrate (0.02% weight/vol). Colour development was initiated by addition of 0.5 ml Folin-Ciocalteu's reagent (diluted 1:2 vol/vol with distilled water). The tubes were vortexed and allowed to stand at room temperature for 45 min before reading the optical density of each tube at 660 nm using a Gilford Spectrophotometer (Model 300 N).

2.9. Statistics and data analysis.

For comparisons between groups with similar population variance (Fisher F-test), parametric statistics were used; single comparisons were by the Students t-test and multiple comparisons by one way analysis of variance (ANOVA) followed by the least significant difference test.

For variables where the population variance of groups were significantly different, non-parametric tests were used. Kruskal-Wallis ANOVA was used for multiple comparisons and the Mann-Whitney U test for single comparisons and for groups with a significant ANOVA result (null hypothesis rejected at $p < 0.01$). Relationships between variables were assessed by Spearman's Rank Correlation Coefficient (Rs). For paired samples data were analysed by the paired t-test or the Wilcoxon's signed rank test. All probabilities refer to two-tailed tests and were considered significant when $p < 0.05$.

Radioligand binding experiments were analysed using the EBDA LIGAND programme of MacPherson (1985). Details of these analyses are given where appropriate.

TABLE 2.1. GRADIENTS USED TO ELUTE AMINO ACIDS IN RAT DIALYSATE AND RAT NEOCORTEX

Gradient 1. MEASUREMENT OF A NUMBER OF AMINO ACIDS IN RAT CORTICAL DIALYSATE.

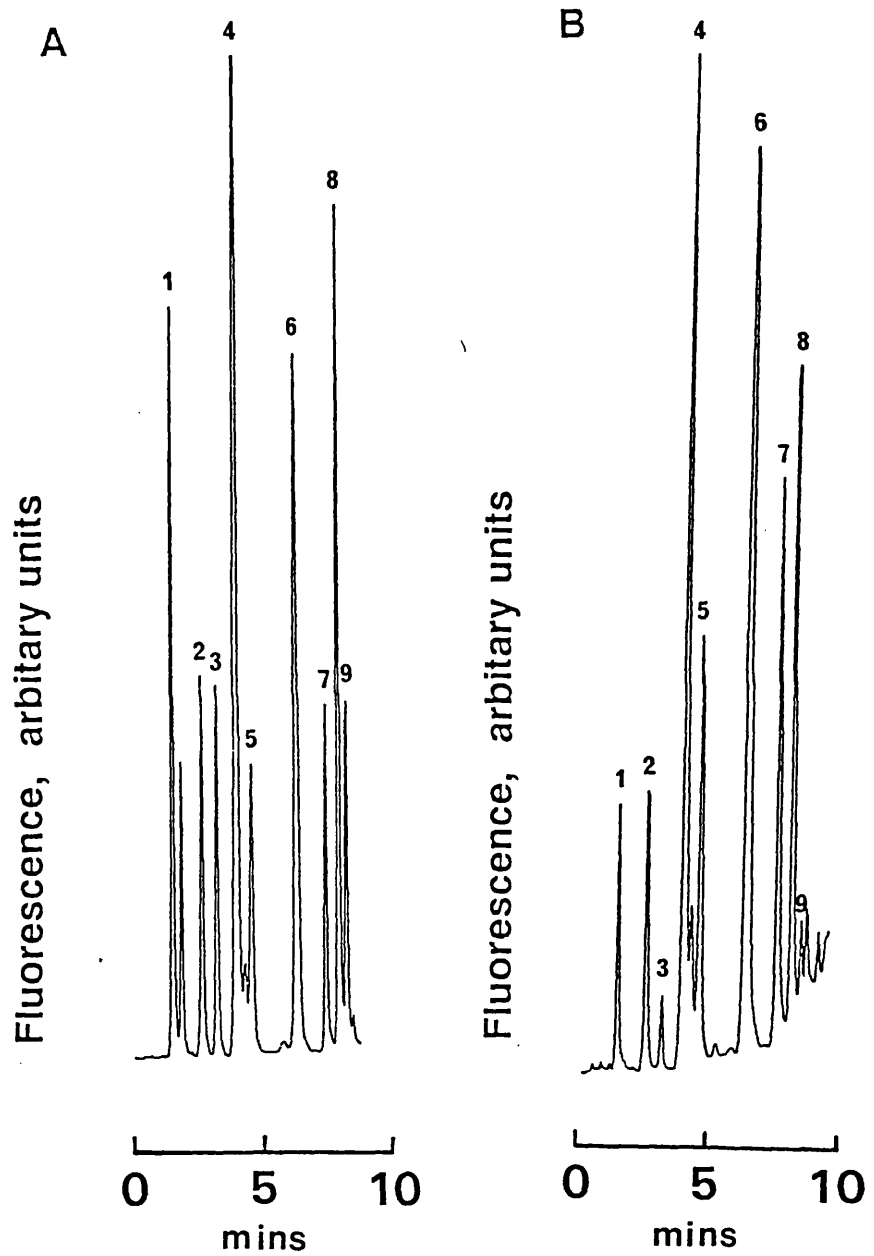
Gradient segment	Time (mins.)	Buffer %A	%B	Slope
0	0-0	95	5	0
1	0-5	80	20	1
2	5-8	50	50	2
3	8-11	45	55	1
4	11-12	30	70	1
5	12-15	95	5	1

Gradient 2. MEASUREMENT OF Ca^{2+}-DEPENDENT, K^{+}-EVOKED RELEASE OF ASPARTATE, GLUTAMATE AND GABA FROM RAT CORTEX

Gradient segment	Time (mins.)	Buffer %A	%B	Slope
0	0-0	85	15	0
1	0-8	53	47	-4
2	8-14	48	52	2
3	14-16	15	85	1
4	16-19	85	15	1

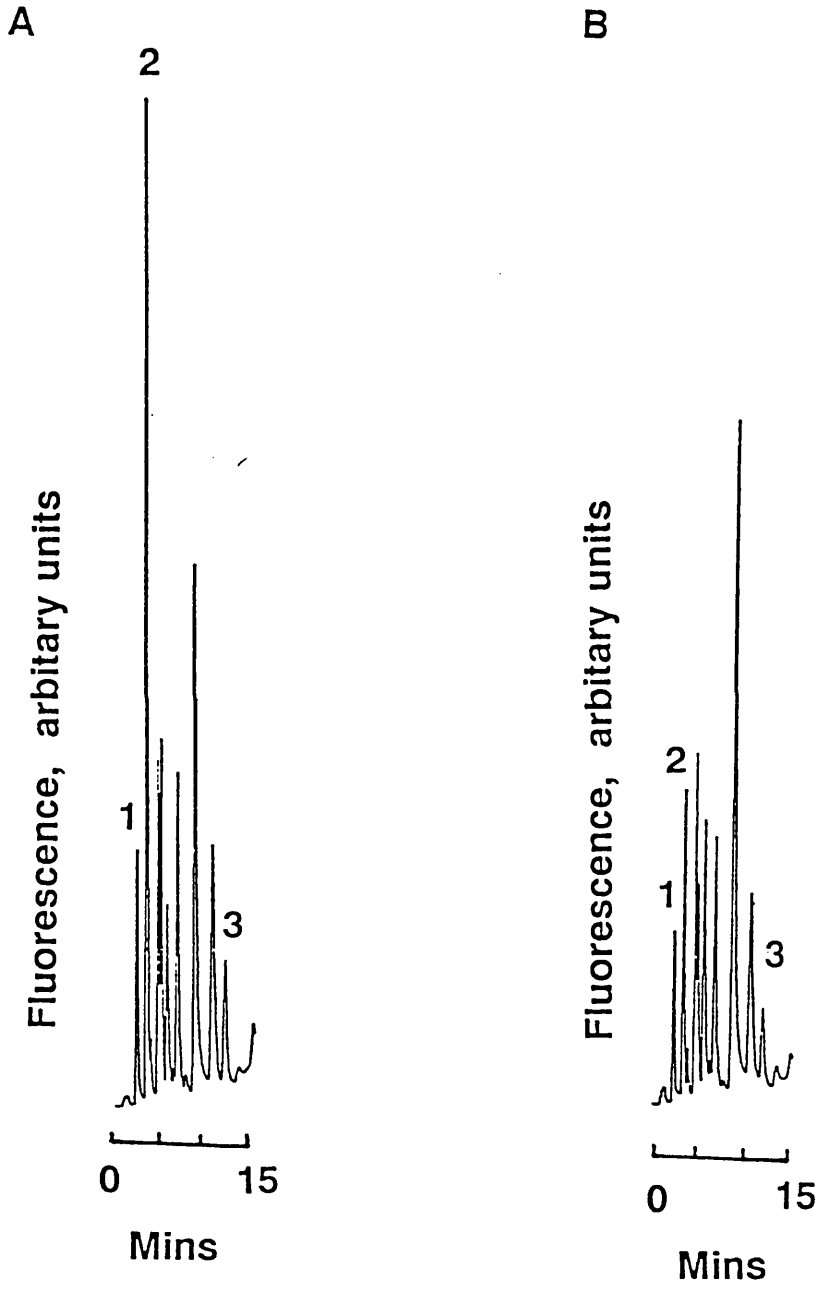
Representative chromatograms are given in Fig.2.3 and 2.4.

FIGURE 2.3. REPRESENTATIVE CHROMATOGRAM FOR DETERMINATION OF A NUMBER OF AMINO ACIDS IN RAT CORTICAL DIALYSATE.



Chromatogram was obtained using gradient 1, Table 2.1. Retention times are given below the x-axis in minutes. A: authentic standard mixture; B: rat cortical dialysate. 1 = aspartate, 2 = glutamate, 3 = asparagine, 4 = glutamine, 5 = serine, 6 = glycine, 7 = taurine, 8 = alanine, 9 = GABA. The unidentified peak eluting after aspartate in the standard is homocysteic acid which was not detected in the dialysate.

FIGURE 2.4. REPRESENTATIVE CHROMATOGRAM FOR DETERMINATION OF Ca^{2+}-DEPENDENT, K^+-EVOKED RELEASE OF ASPARTATE, GLUTAMATE AND GABA FROM RAT NEOCORTEX.



Chromatogram was obtained using gradient 2, Table 2.1. Retention times are given below the x-axis in minutes. A: 1 = aspartate, 2 = glutamate, 3 = GABA in medium containing 50 mM K^+, 1.3 mM Ca^{2+} and 1.3 mM Mg^{2+}.
B: aspartate, glutamate and GABA in medium containing 50 mM K^+, and 2.6 mM Mg^{2+} and 2 mM EGTA (zero Ca^{2+}).

CHAPTER 3

CHOLINE ACETYLTRANSFERASE ACTIVITY AND NEUROFIBRILLARY TANGLE DISTRIBUTION IN ALZHEIMER'S DISEASE

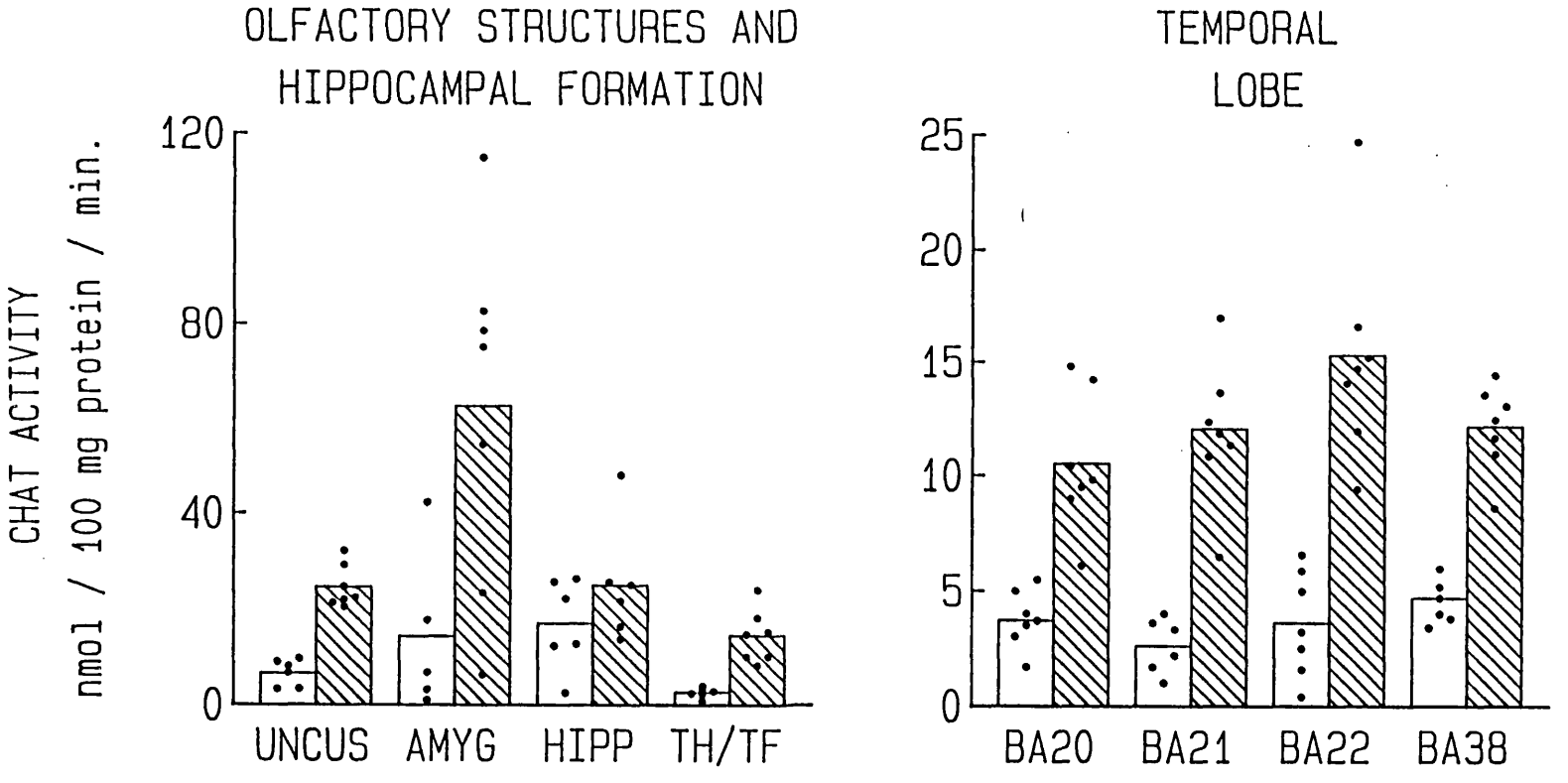
3.1.RESULTS

In a number of areas of the cortex, the olfactory sructures and the hippocampal formation from a series of control tissue (n = 7, see section 2.2.3) ChAT activity was not related to age (range 70 - 79 y), post-mortem delay (range 48 - 96 h) or sex (4 males, 3 females) in any of the areas studied (data not shown). The assays of ChAT activity in Alzheimer's disease tissue (n = 7, age range 58 - 90 y, delay to post-mortem 12 - 120 h, 3 males, 4 females studied) showed a significant decrease in most of the 15 areas studied compared with control tissue ($p < 0.05$, Mann-Whitney U-test; Figs. 3.1 and 3.2). There were three exceptions, BA25 of the cerebral cortex, the hippocampus and the amygdala where the data was subject to unexplained variability (Fig. 3.1). The loss of ChAT activity was most severe in the olfactory structures and the hippocampal formation, the mean reduction in activity compared with controls for this group of structures (the uncus, amygdala, hippocampus and TH/TF, the posterior part of the parahippocampal gyrus) being 73 $\pm$ 3% (Fig. 3.3). The loss of ChAT activity was also severe in the temporal lobe (BA20, 21, 22 and 38), the mean reduction in these areas being 69 $\pm$ 5% (Fig. 3.3). However, the loss was only moderate in the parietal and frontal lobes (45 $\pm$ 9%, BA7, 46, 9 and 25) and also in the sensory cortex (48%, BA17 and S1, Fig. 3.3).

Although there was some degree of variability in the absolute numbers of neurofibrillary tangles between each case, there are findings that are consistent throughout this group of brains. Firstly, the reduction in ChAT activity relates well to the number of neurofibrillary tangles in each group of structures (Fig. 3.3). The mean number of tangles (1941 $\pm$ 345/ mm^3, Table 3.1) was maximal in the parts of the brain related to the olfactory structures and hippocampal formation, severe in

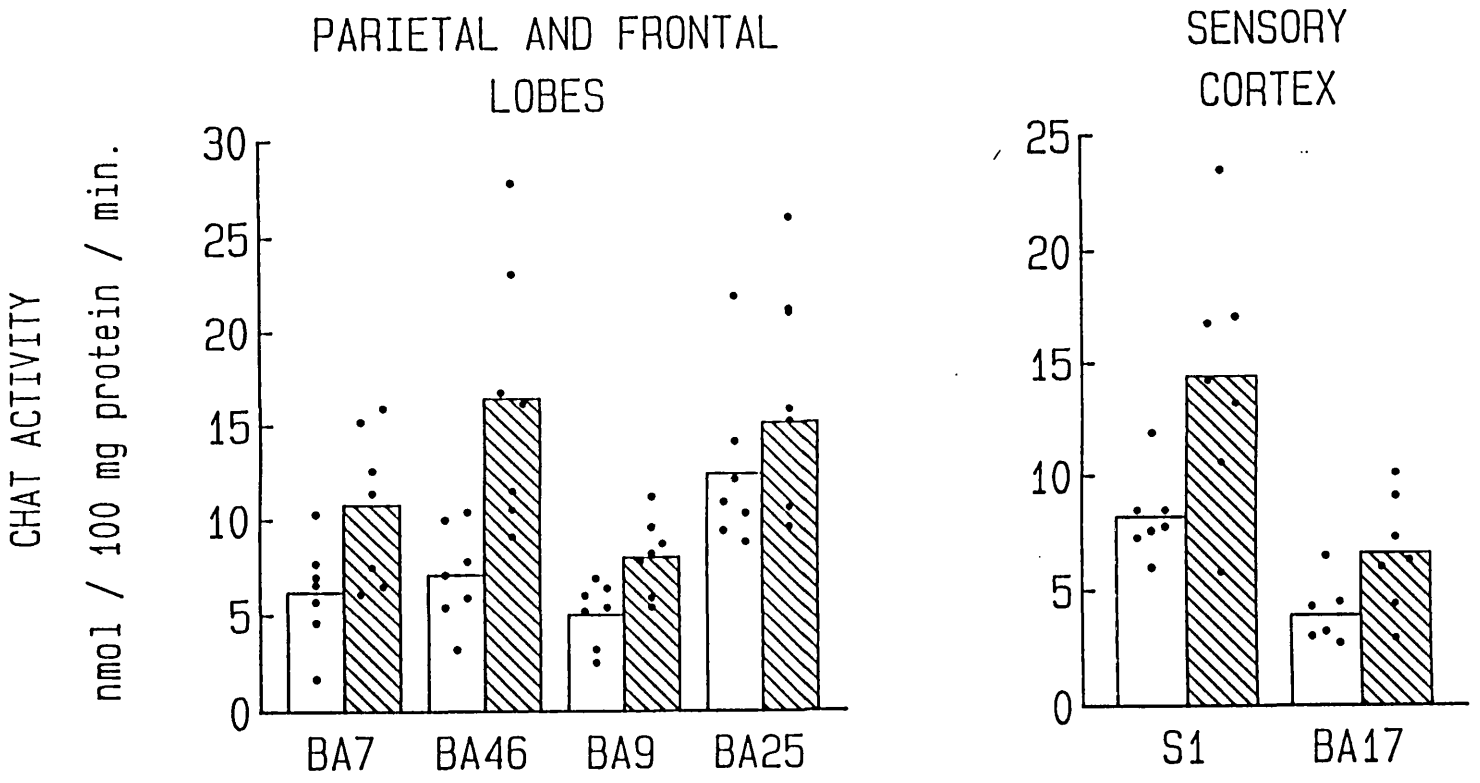
the temporal lobe (1356 $\pm$ 213/ mm^3), moderate in the parietal and frontal lobes (385 $\pm$ 44/ mm^3) and low in the sensory cortex (28/ mm^3).

FIGURE 3.1. CHOLINE ACETYLTRANSFERASE ACTIVITY MEASURED IN THE OLFACTORY STRUCTURES AND THE TEMPORAL LOBE IN POST-MORTEM ALZHEIMER'S DISEASE AND CONTROL TISSUE.



Histobars are the mean value for each group and the points identify values for the individual subjects.
Open bars are Alzheimer's disease tissue, hatched bars are control. In Alzheimer's disease, ChAT activity showed a significant reduction ($p < 0.05$, Mann-Whitney U-test) in most areas studies except the amygdala and hippocampus where the values were subject to unexplained variability.
Abbreviations: BA = Brodmann Area; Amyg = Amygdala; Hipp = Hippocampus; TH/TF = posterior part of parahippocampal gyrus.

FIGURE 3.2. CHOLINE ACETYLTRANSFERASE ACTIVITY MEASURED IN A NUMBER OF AREAS IN THE PARIETAL AND FRONTAL LOBES AND THE SENSORY CORTEX IN POST-MORTEM ALZHEIMER'S DISEASE AND CONTROL TISSUE.



Histobars are the mean value for each group and the points identify values for the individual subjects.
Open bars are Alzheimer's disease tissue, hatched bars are control. In Alzheimer's disease, ChAT activity showed a significant reduction ($p < 0.05$, Mann-Whitney U-test) in most areas studies except BA25 where the values were subject to unexplained variability.
Abbreviations: BA = Brodmann Area; S1 = BA1/2/3.

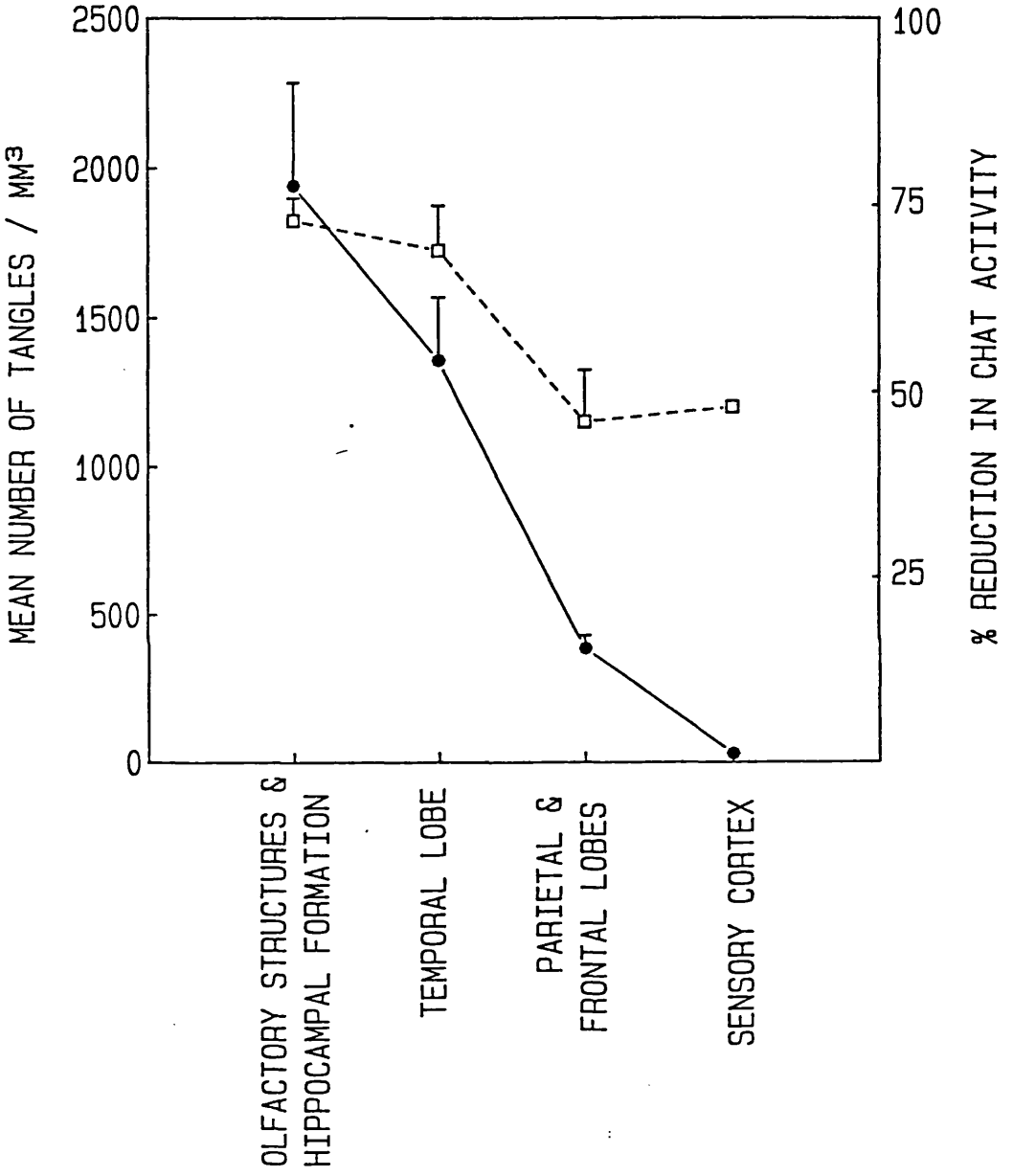
TABLE 3.1. NUMBER OF NEUROFIBRILLARY TANGLES/mm³ IN THE OLFACTORY AND HIPPOCAMPAL FORMATION AND THE TEMPORAL, FRONTAL AND PARIETAL LOBES IN POST-MORTEM ALZHEIMER'S DISEASE TISSUE.

	OLFACTORY STRUCTURES AND HIPPOCAMPAL FORMATION				TEMPORAL LOBE			
	UNCUS	AMYG	HIPPO	TH/TF	BA20	BA21	BA22	BA38
MEAN TANGLE NUMBER/mm³ ± S.E.M.	2565±497	1526±408	2492±528	1180±306	1926±496	1180±330	926 ± 392	1391 ± 250
NUMBER OF CASES.	6	5	6	7	7	7	7	7
MEAN VALUE FOR GROUP OF STRUCTURES±S.E.M.	**1941 ± 345**				**1356 ± 213**			

	PARIETAL AND FRONTAL LOBES				SENSORY CORTEX	
	BA7	BA46	BA9	BA25	S1	BA17
MEAN TANGLE NUMBER/mm³ ± S.E.M.	443±147	256±87	437±170	404±86	41±22	13±9
NUMBER OF CASES	7	7	7	7	7	7
MEAN VALUE FOR GROUP OF STRUCTURES ± S.E.M	**385 ± 44**				**27**	

The mean number of tangles/mm³ in the four major groups of structures is given in bold typeface.

FIGURE 3.3. RELATIONSHIP BETWEEN THE MEAN TANGLE COUNTS AND THE PERCENTAGE DECREASE IN ChAT ACTIVITY IN THE FOUR GROUPS OF AREAS STUDIED.



Closed circles represent number of tangles, open squares are percent reduction in ChAT activity.
Values are mean ± SEM. Data are derived from Figs 3.1, 3.2 and Table 3.1.

3.2 DISCUSSION

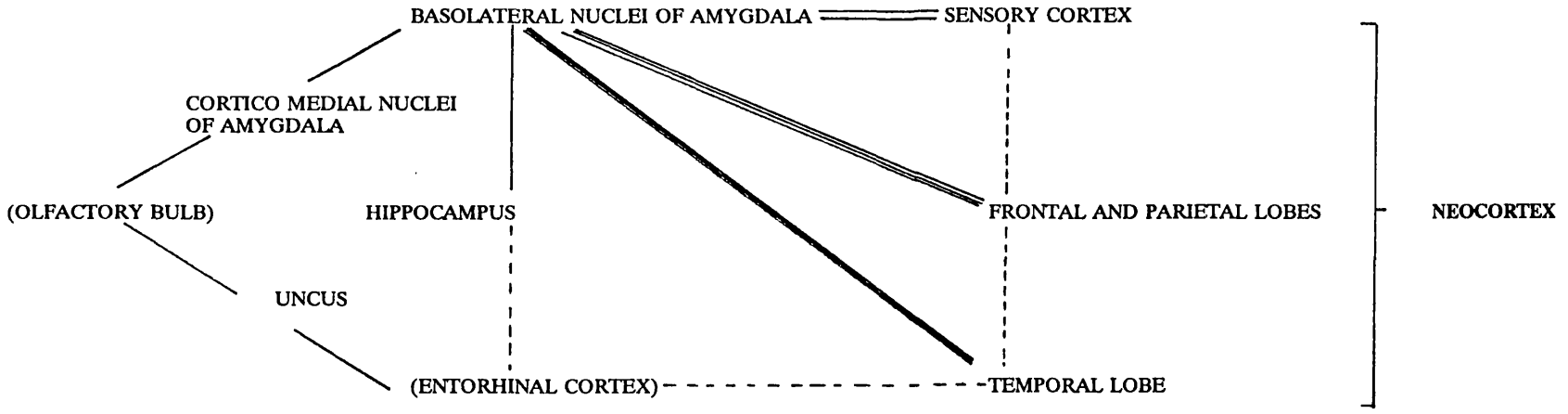
The results of the present quantitative study agree with previous reports on the distribution of the pathological changes in the cerebral hemisphere in Alzheimer's disease whereby the basal and medial temporal lobes are severely affected whilst the frontal and occipital lobes and sensory and motor areas are relatively spared (Brun, 1983, also see Pearson & Powell, 1989).

The purpose of the dual study of ChAT activity and tangle number was to determine the relationship , in any area, between the structural and biochemical changes. A correlation would be expected if the changes in ChAT activity were due to the secondary degeneration of fibres from the basal nucleus. The ChAT value of the areas which have been studied agree quite well with the pathological change indicated by the number of tangles. In the sensory areas the reduction in ChAT activity is clearly shown whereas the tangles are negligible, but it is known that senile plaques are present in this area and therefore the ChAT activity may represent earlier changes than the tangle formation.

The olfactory structures and hippocampal formation show the greatest number of tangles and the most severe reduction in ChAT activity. This may indicate the initial involvement of the olfactory system in Alzheimer's disease (Pearson & Powell, 1989) and indeed it has been shown that nasal epithelium tissue taken at autopsy shows unique pathological changes in morphology, distribution and immunoreactivity of neuronal structures in patients with Alzheimer's disease (Talamo et al., 1989).

It has been suggested (Pearson et al., 1985) that the disease process in Alzheimer's disease may spread in either an anterograde or retrograde direction, along a sequence of cortico-cortical connections or through the fibre connections between the neocortex and the amygdala or by both these sets of connections (Fig.3.4). The severe involvement of the hippocampus is probably due to the direct connections between it and the amygdala (Fig.3.4; Rosene & Van Hoesen, 1977, Kretteck &

FIGURE 3.4. A HIGHLY SCHEMATIC AND SIMPLIFIED DIAGRAM OF THE CONNECTIONS BETWEEN THE AREAS THAT HAVE BEEN STUDIED HERE.



Most of these connections are reciprocal. The connections between the structures in the medial part of the temporal lobe are shown as single lines, those between the amygdala and neocortex as double lines, the density indicated by the degree of shading. The sequence of cortico-cortical connections between the areas of neocortex are broken lines.
Structures in parenthesis were not included in this study. Figure taken from Esiri et al., 1990.

Price, 1977, Aggleton, 1985). The areas on the lateral surface of the temporal lobe that have been studied (BA20, 21, 22 and 38) here have both direct and reciprocal connections with the basolateral nuclei of the amygdala (Kosel et al., 1982) and they also also form the final steps between the three major sensory systems and the hippocampal formation (Jones and Powell, 1970). The severe reduction in ChAT activity and the high number of tangles in all of these areas may therefore be considered to support the suggestion that the disease process spreads from the structures in the medial temporal lobe (amygdala, uncus and hippocampal formation) to the neocortex along these connections. This is supported further by a recent study by Hubbard and colleagues (1990) who reported early tangle formation in the hippocampus and substantia innominata while the neocortex in these subjects was relatively free of tangles.

The parietal lobe (BA7) and the lateral surface of the frontal lobe (BA40 and BA9) are only moderately affected, and this may reflect their intermediate position on the sequence of cortico-cortical connections (Fig.3.4). It would have been expected from it's later position on the sequence of connections that BA25 on the medial surface would have contained a greater number of tangles and show a greater reduction in ChAT activity. Indeed, the reduction in ChAT did not reach significance in this area.

The minimal involvement of the primary visual and the somatic sensory areas agrees with earlier accounts of the distribution of pathology in this disease (Pearson & Powell, 1989) and with hypotheses that the disease may spread in a retrograde way along the sequence of cortico-cortical connections that begins in the sensory areas and ends in the hippocampal formation.

The number of tangles and the severity of ChAT activity reduction in the different areas of cortex that have been studied here parallel remarkably closely the density of the connections between any individual area and the amygdala (Aggleton et al.,

1980, Amaral & Price, 1984). Dense connections have been described in the monkey, between the amygdala and the lateral surface of the temporal lobe which has the maximum number of tangles, whereas there are sensory areas with few such amygdala-cortical connections and minimal number of tangles and ChAT reduction (see Esiri et al., 1990). It is possible therefore that the disease process in Alzheimer's disease may pass along the reciprocal connections between the amygdala and the cortex instead of, or as well as, along the sequence of cortico-cortical connections. It is obvious that the areas of cortex with the greatest density of connections with the amygdala and the highest number of tangles and severe ChAT reduction are also those which are situated late on the series of links between the sensory areas and the hippocampus.

The correspondence between the reduction in ChAT activity, the number of tangles and the degree of pathological change is particularly clear in the areas on the lateral surface of the temporal lobe. Brodmann areas 20, 21, 22 and 38 all have dense reciprocal connections with the amygdala (Aggleton et al., 1980, Amaral & Price, 1984, Herzog and Van Hoesen, 1976, Turner et al., 1980) and they also have the highest number of tangles and greatest reduction in ChAT in the whole of the neocortex. The number of tangles and reduction in ChAT in BA7 of the parietal lobe is substantially less than in the temporal lobe and this agrees with the quite localised origin and termination of the connection with the amygdala to the part of this area in the depth of the intraparietal sulcus in the monkey (Aggleton et al., 1980, Amaral & Price, 1984). In the frontal lobe the modest number of tangles and ChAT reduction in BA9 and BA46 on the lateral surface agrees with the smaller number of fibres from the amygdala to these areas, but the severity of ChAT reduction and the number of tangles in BA25 on the medial surface is less than the predicted values predicted on the basis of the relatively dense projections to it from the amygdala (Aggleton et al., 1980, Amaral & Price, 1984). The consistently severe involvement of the amygdala in all brains and the suggestion that the disease spreads thence to the neocortex agree with two other findings. Marked ChAT

deficits have been found in the amygdala in cases of Alzheimer's disease with pathological change but with no significant decrease in ChAT activity in either the neocortex and the hippocampus (Palmer et al., 1986) and there is early and severe involvement of the amygdala in Down's Syndrome (Mann & Esiri, 1989).

Just as the reciprocal connections between the amygdala and the neocortex may be one factor responsible for the distribution of the pathology and ChAT loss in the neocortex so also may be the connections of the amygdala with other subcortical structures. Thus, the presence of neurofibrillary tangles in such sites as the basal nucleus and the hypothalamus and the raphe nucleus of the brain stem may be attributed to their connections with the amygdala. It would be interesting to note whether these areas have reduced ChAT activity.

This study has clearly indicated that the temporal lobe is severely affected in Alzheimer's disease whilst the frontal lobe is relatively unaffected. For the remainder of this thesis both these areas are compared in Alzheimer's disease and control tissue. In this way, since many of the measures are expressed relative to unit mass, problems associated with with the loss of cells organized in a columnar manner in Alzheimer's disease (see Procter et al., 1988) in the temporal cortex can in part be overcome using tissue from the frontal cortex where the pathology is less obvious.

CHAPTER 4

THE NMDA RECEPTOR COMPLEX IN HUMAN BRAIN.

4.1 COMPARISON OF [^{3}H]-MK-801 AND [^{3}H]-TCP BINDING IN HUMAN BRAIN.

RESULTS

All results in this section apply to human tissue unless otherwise stated. Membrane preparations were either from a number of cortical regions (predominantly the parietal and occipital lobes) which were pooled before assay or an equal amount of the caudate and putamen which was pooled before assay, hereafter called striatum. The crude membrane preparation (chosen in order to reduce the possibility that the constituents of fractions from diseased and control tissue may not be equivalent) was derived from the "total particulate fraction", (cell debris, nuclear fraction and the crude mitochondrial fraction) hereafter referred to as "crude membrane preparation" (Bowen et al., 1977, see section 2.4.2).

4.1.1. Stimulation of [^{3}H]-MK-801 and [^{3}H]-TCP binding by glutamate and glycine.

Well washed membranes from 4 control brains (age range 57 - 83 y, delay to post-mortem range 4 - 24 h, 3 right and 1 left hemisphere, 2 male, 2 female and no subject had died following a protracted terminal illness) were assayed (see section 2.4.3; incubated at 25°C for 2 h). In the absence of exogenous amino acids (basal conditions) the specific binding of [^{3}H]-MK-801 and [^{3}H]-TCP was 140 $\pm$ 20 and 108 $\pm$ 7 fmol/mg protein respectively (mean $\pm$ SEM)). In the same experiment, when either glutamate or glycine was added to the incubation medium a dose-dependent increase in the binding of both ligands was observed (Fig. 4.1). When glutamate and glycine were added together in the incubation medium, an additional effect was seen on [^{3}H]-MK-801 binding but not on [^{3}H]-TCP binding (Fig. 4.2). Thus in the presence of both glutamate (30 μM) and glycine (30 μM), [^{3}H]-MK-801 binding was increased to 725 $\pm$ 72 fmol/mg protein (fivefold above basal values,

above) whereas [^{3}H]-TCP binding was only increased to 333 $\pm$ 60 fmol/mg protein (threefold above basal, Fig. 4.2).

4.1.2. Effects of glutamate and glycine on the association rates of [^{3}H]-MK-801 and [^{3}H]-TCP .

Under basal conditions, [^{3}H]-TCP binding was consistent with a slowly associating component (Fig.4.3). The addition of glutamate (100 μM) or glycine (1000 μM) to the incubation medium increased the rate of association of [^{3}H]-TCP. The addition of both glutamate and glycine together did not increase the rate of association of this ligand above that seen with either amino acid alone. In a similar manner, under basal conditions, the binding of [^{3}H]-MK-801 was slow (Fig. 4.3). In the presence of 100 μM glutamate or 1000 μM glycine the association rate was increased. However, in contrast with [^{3}H]-TCP binding, in the presence of both these amino acids the association rate of [^{3}H]-MK-801 was increased above that seen with glutamate or glycine alone.

4.1.3. The effect of spermidine on [^{3}H]-MK-801 and [^{3}H]-TCP binding.

The effect of spermidine was examined on [^{3}H]-MK-801 and [^{3}H]-TCP binding in the absence or the presence of glutamate and glycine. In this section the effects of 30 μM spermidine only are examined, more detailed experiments are described in Chapter 5. After 2 h incubation at 25 °C in the presence of 30 μM spermidine [^{3}H]-MK-801 was increased to 343 $\pm$ 27 fmol / mg protein (mean $\pm$ SEM, n =3) compared with 113 $\pm$ 15 fmol/ mg protein for basal binding. In this experiment this was comparable to the stimulation seen with glutamate and glycine (100 and 1000 μM respectively, Fig. 4.4). The addition of glutamate, glycine and spermidine together was additive, binding was 523 $\pm$ 50 fmol/ mg protein compared with 424 $\pm$ 19 fmol/ mg protein in the presence of glutamate and glycine alone (Fig. 4.4). By contrast, [^{3}H]-TCP binding was inhibited by spermidine to 96 $\pm$ 19 fmol / mg protein compared with 149 $\pm$ 46 fmol / mg protein for basal binding. This was also the case in the presence of glutamate, glycine and spermidine, binding was 122 $\pm$ 16

fmol / mg protein.

4.1.4. Saturation analysis of [^{3}H]-MK-801 and [^{3}H]-TCP binding .

In human cortex, saturation binding experiments were performed in the presence of 100 μM glutamate and 1000 μM glycine (maximally stimulating conditions). Membranes were incubated for 2 h, by which time both ligands had reached equilibrium (Fig 4.3). The data from three separate brains were pooled, and the Scatchard plot of these data was clearly non-linear (Fig. 4.5). Using an iterative curve fitting programme (EBDA LIGAND, MacPherson, 1985), a two- site binding model gave a significantly ($p < 0.05$, F-test) better fit to these data than a one site model. The binding parameters were determined as K_{d1} = 5.8 $\pm$ 0.2 nM, K_{d2} = 16 $\pm$ 2 μM and B_{max1} = 1500 $\pm$ 300 fmol/ mg protein, B_{max2} = 138 $\pm$ 52 pmol/ mg protein (mean $\pm$ SEM).

Saturation analysis of [^{3}H]-TCP binding was more variable between subjects and although the low affinity site seen with [^{3}H]-MK-801 was visible on the Scatchard plot the data from three separate brains could only be individually fitted to a single site binding model after exclusion of these points, with a K_d = 16.8 $\pm$ 1.0 nM and B_{max} = 660 $\pm$ 170 fmol/ mg protein (mean $\pm$ SEM). The number of [^{3}H]-TCP binding sites was therefore significantly less than the number of high affinity [^{3}H]-MK-801 binding sites in the same preparation ($p< 0.01$, Student's t-test Fig. 4.5).

In rat cortex the data were linear for both ligands indicating a single site interaction for [^{3}H]-MK-801 and [^{3}H]-TCP binding to the NMDA receptor complex under the maximally stimulating conditions used for human cortex (100 μM glutamate, 1000 μM glycine, Fig 4.6). There were however significantly more [^{3}H]-MK-801 binding sites (3582 $\pm$ 209 fmol/mg protein) than [^{3}H]-TCP (2089 $\pm$ 205 fmol/mg protein, $p < 0.01$, Student's t-test).

4.1.5. Dissociation of [^{3}H]-MK-801 and [^{3}H]-TCP.

It seemed possible that the difference in the apparent number of high affinity sites labelled by [^{3}H]-MK-801 and [^{3}H]-TCP was due to a difference in the rates of dissociation, in such a way that some [^{3}H]-TCP binding was lost during filtration. A number of experiments were therefore performed using centrifugation to separate bound and free ligand. However, in crude human brain membrane preparations, using this procedure non- specific binding was high and it was impossible to consistently detect a high affinity component for either [^{3}H]-MK-801 or [^{3}H]-TCP (data not shown). When studied using a filtration assay, the dissociation kinetics of both [^{3}H]-MK-801 and [^{3}H]-TCP were complex (Fig. 4.7). It did not appear, however, that there was loss of [^{3}H]-TCP binding during filtration under these conditions. The possibility that a proportion of [^{3}H]-TCP binding is lost extremely rapidly (< 30 secs) cannot be excluded.

4.1.6. Pharmacological specificity of [^{3}H]-MK-801 and [^{3}H]-TCP binding.

A number of compounds active at the cationic channel of the NMDA receptor inhibited both [^{3}H]-MK-801 and [^{3}H]-TCP binding to human cortical membranes in the presence of maximally stimulating concentrations of glutamate and glycine. The rank order of potency of these inhibitors was identical for both [^{3}H]-MK-801 and [^{3}H]-TCP binding namely: MK-801 > TCP > PCP > SKF 10047 (Table 4.1).

AP5 is a well-known competitive inhibitor of the glutamate recognition site (Watkins & Olverman, 1987) as is 7-chlorokynurenic acid of the glycine site (Kemp et al., 1988). When tested in the presence of 100 μM glutamate and 1000 μM glycine, both compounds were inactive. They were therefore tested against [^{3}H]-MK-801 and [^{3}H]-TCP binding using only 1 μM glutamate and 1 μM glycine. AP5 inhibited [^{3}H]-MK-801 and [^{3}H]-TCP binding with comparable potency. By contrast, 7-chlorokynurenic acid inhibited [^{3}H]-MK-801 binding more potently than [^{3}H]-TCP binding (Table 4.1). Similarly, $ZnCl_2$ was more potent an inhibitor of [^{3}H]-MK-

801 than [^{3}H]-TCP binding. All these ligands exhibited Hill coefficients significantly less than unity. Strychnine and remoxipride active at the "sigma-opiate" binding site were inactive against both [^{3}H]-MK-801 and [^{3}H]-TCP binding at concentrations up to 100 μM. Detailed curves were produced for the inhibition of both ligands by MK-801 and TCP and all inhibition curves could be best described by a two-site inhibition model (Fig. 4.8). The ratio of high and low affinity sites differed between [^{3}H]-MK-801 and [^{3}H]-TCP binding binding (Table 4.2). However, for each ligand both TCP and MK-801 displaced a consistent ratio of high and low affinity sites. The main difference between the inhibition curves was that MK-801 displaced the lower affinity [^{3}H]-MK-801 site with an apparent IC_{50} of 294 nM whereas the apparent IC_{50} of MK-801 for the equivalent site labelled by [^{3}H]-TCP was 3175 nM (Table 4.2).

4.1.7. [^{3}H]-MK-801 and [^{3}H]-TCP binding in striatum.

Saturation experiments were performed in the striatum exactly as described for cortical membranes (Fig. 4.9). Again, non- linear regression of the saturation data indicated that [^{3}H]-MK-801 bound to two sites (Table 4.3), the calculated B_{max} for the high affinity site was 1403 $\pm$ 394 fmol/mg protein. For [^{3}H]-TCP binding the calculated B_{max} was 1292 $\pm$ 305 fmol/mg protein. The two values were not significantly different, thus in the striatum [^{3}H]-MK-801 and [^{3}H]-TCP bound to the same number of sites.

The effects of glutamate and glycine on [^{3}H]-MK-801 and [^{3}H]-TCP binding in striatum are shown in Fig. 4.10. Both glutamate and glycine stimulated ligand binding when added alone. However, the combination of glutamate and glycine did not increase the binding of [^{3}H]-MK-801 or [^{3}H]-TCP above that observed in the presence of either amino acid alone.

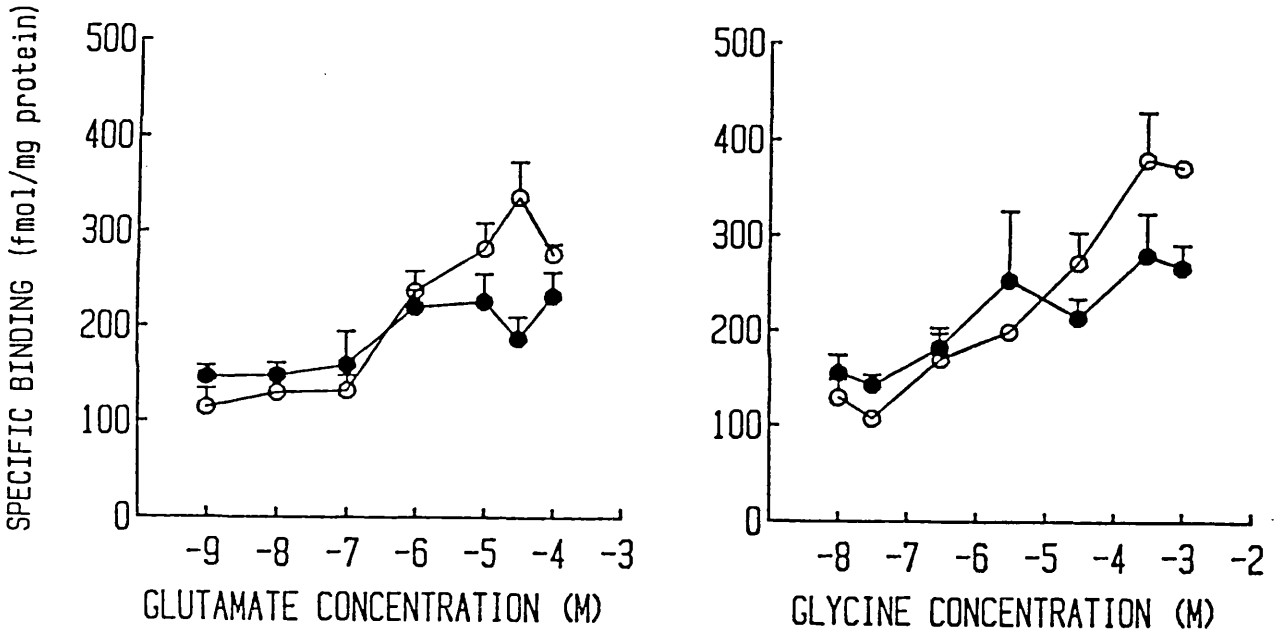
4.1.8. Effect of freezing and thawing cortical tissue on [^{3}H]-MK-801 and [^{3}H]-TCP binding

Frozen cortical tissue (four brains) was thawed, dissected free of meninges and white matter, mixed thoroughly, placed in eppendorf tubes and refrozen at -70°C. This second freezing stage and subsequent thawing did not effect the binding of a number of concentrations of [^{3}H]-MK-801 or [^{3}H]-TCP (Fig. 4.11).

4.1.9. The effect of a number of protease inhibitors on [^{3}H]-MK-801 and [^{3}H]-TCP binding.

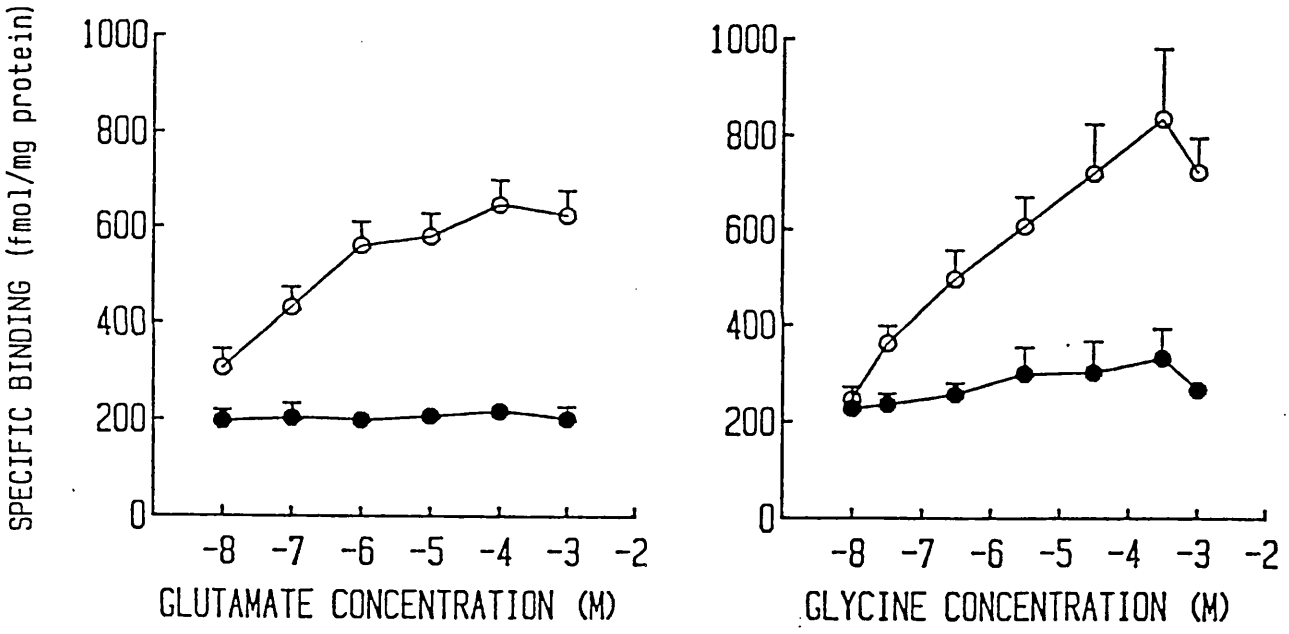
Three protease inhibitors were added to the incubation medium (Bacitracin and phenyl methyl sulphonyl fluoride (PMSF, 10 μg/ml) and aprotinin 3400 units/ml. In the absence of protease inhibitors after a 4h incubation at 25°C in the presence of maximally stimulating glutamate and glycine concentrations, binding of [^{3}H]-MK-801 and [^{3}H]-TCP was 209 and 106 fmol/mg protein respectively for control tissue (n = 2). In the presence of protease inhibitors the binding was 186 and 108 fmol/mg protein respectively. In Alzheimer's disease tissue in the absence of protease inhibitors the binding of [^{3}H]-MK-801 and [^{3}H]-TCP was 217 and 108 fmol/mg protein respectively. In the presence of protease inhibitors the binding was 180 and 108 fmol/mg protein respectively (n = 2, Fig. 4.12).

FIGURE 4.1. STIMULATION OF [^{3}H]-MK-801 AND [^{3}H]-TCP BINDING TO HUMAN CORTICAL MEMBRANES BY GLUTAMATE AND GLYCINE.



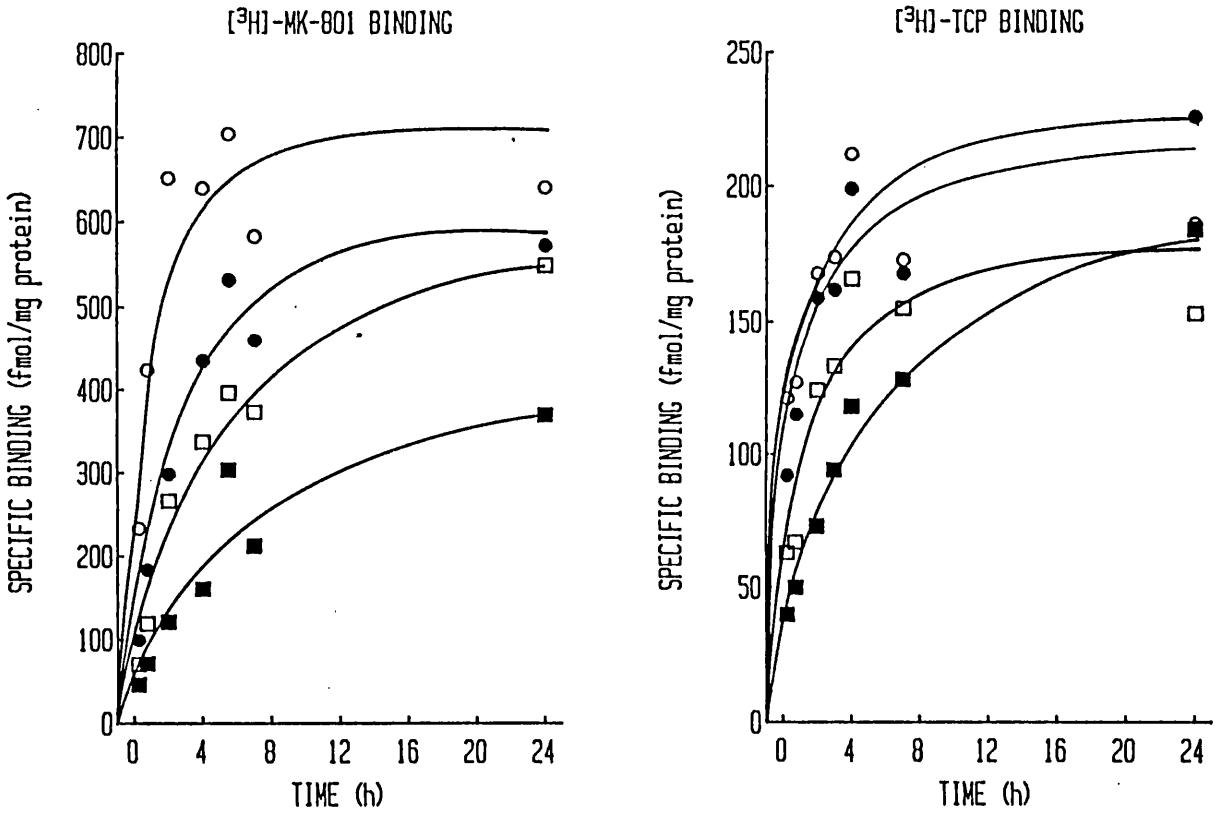
Experiments were performed with membranes incubated for 2 h at 25°C in Tris-HCl buffer using a final concentration of 5 nM [^{3}H]-ligand with increasing concentrations of glutamate or glycine.
Open circles represent [^{3}H]-MK-801 binding, closed circles represent [^{3}H]-TCP binding.
Values are mean + SEM, n = 3.

FIGURE 4.2. STIMULATION OF [^{3}H]-MK-801 AND [^{3}H]-TCP BINDING TO HUMAN CORTICAL MEMBRANES BY GLUTAMATE AND GLYCINE TOGETHER.



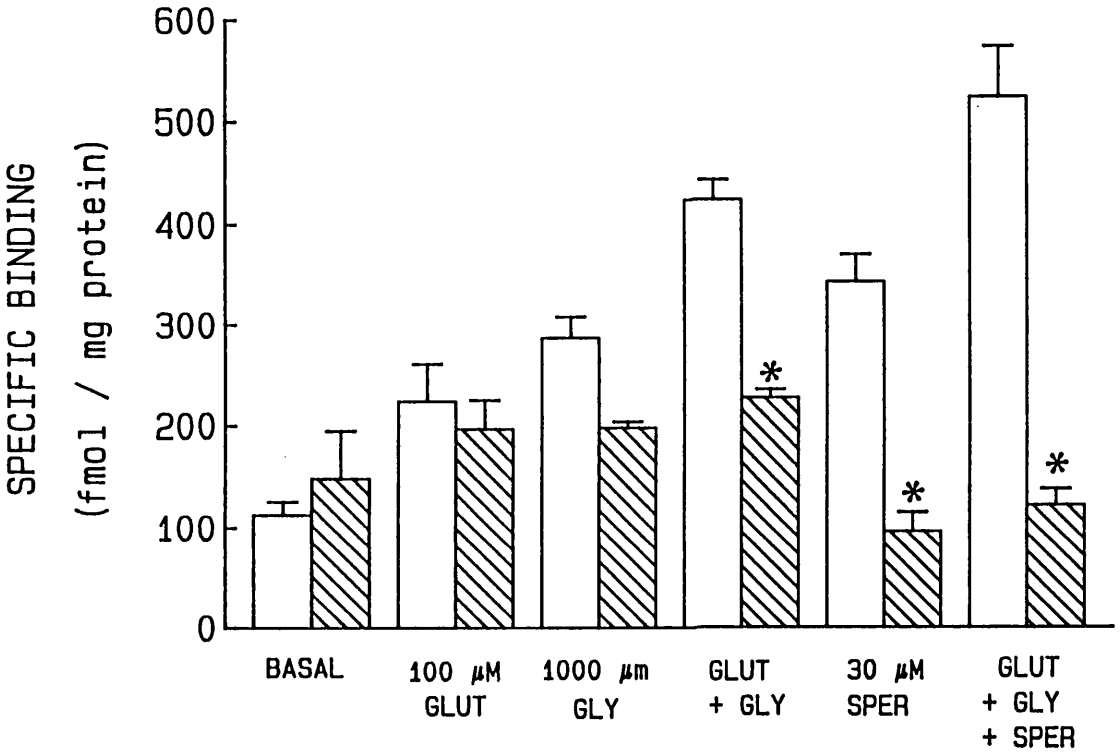
Experiments were performed with membranes incubated for 2 h at 25°C in Tris-HCl buffer using a final concentration of 5 nM [^{3}H]-ligand with increasing concentrations of glutamate in the presence of 30 μM glycine or increasing concentrations of glycine in the presence of 30 μM glutamate.
Open circles represent [^{3}H]-MK-801 binding, closed circles represent [^{3}H]-TCP binding.
Values are mean + SEM, n = 3.

FIGURE 4.3. THE EFFECT OF GLUTAMATE AND GLYCINE ON THE ASSOCIATION OF [^{3}H]-MK-801 AND [^{3}H]-TCP HUMAN CORTICAL MEMBRANES.



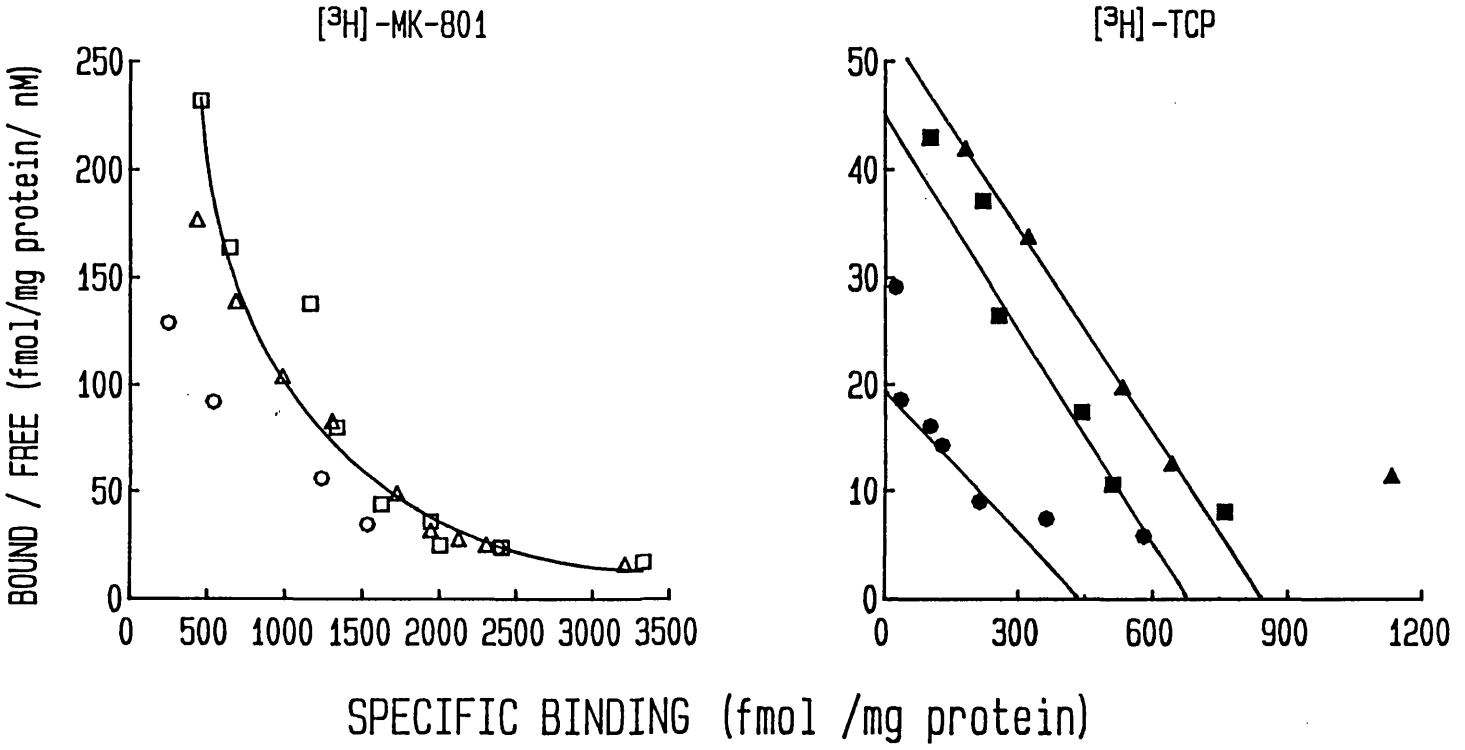
Experiments were performed with membranes incubated in Tris-HCl buffer using a final concentration of 5 nM [^{3}H]-ligand without addition to the incubation medium (■), in the presence of 100 μM glutamate (□), 1000 μM glycine (●) or 100 μM glutamate and 1000 μM glycine (○).
Values are mean, n = 3-5. For clarity error bars are omitted; the SEM was always less than 15% of the mean value.
Logarithmic transformation of the binding data did not result in a linear plot (data not shown).

FIGURE 4.4. THE EFFECT OF GLUTAMATE, GLYCINE AND SPERMIDINE ON [^{3}H]-MK-801 AND [^{3}H]-TCP BINDING TO HUMAN CORTICAL MEMBRANES.



Experiments were performed with membranes incubated for 2 h at 25 °C in Tris-HCl buffer using a final concentration of 5 nM [^{3}H]-ligand in the presence of 100 μM glutamate, 1000 μM glycine, 30 μM spermidine or a combination of these compounds as indicated.
Open bars [^{3}H]-MK-801, hatched bars represent [^{3}H]-TCP binding.
Values are mean $\pm$ SEM, n = 3.
* indicates significantly different to [^{3}H]-MK-801 binding ($p < 0.05$, Student's t-test). .

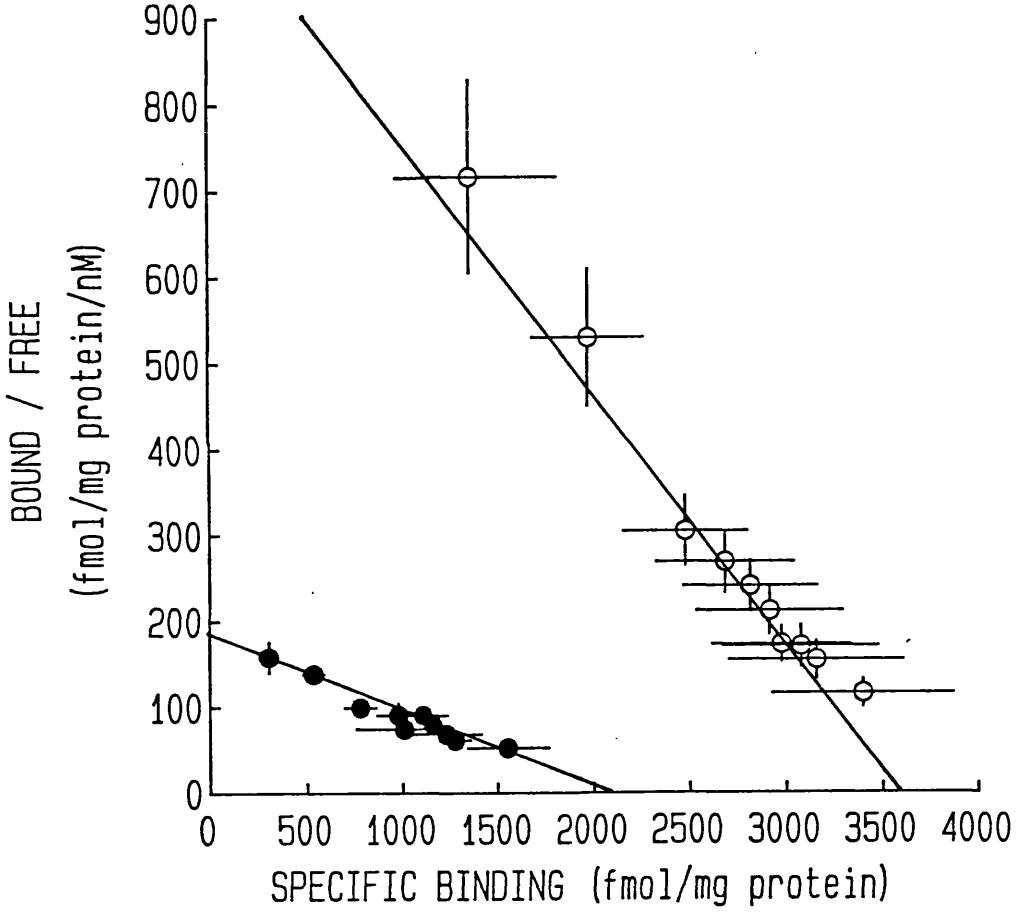
FIGURE 4.5. SATURATION ANALYSIS OF [^{3}H]-MK-801 AND [^{3}H]-TCP BINDING TO HUMAN CORTICAL MEMBRANES.



Experiments were performed with membranes incubated in Tris-HCl buffer using a final concentration of between 1-170nM [^{3}H]-ligand in the presence of 100 μM glutamate and 1000 μM glycine. The different symbols represent binding to 3 separate brains. For [^{3}H]-MK-801 binding the data was pooled for analysis by non-linear regression. For [^{3}H]-TCP binding the data was more variable and each sample was analysed separately.

Binding of [^{3}H]-MK-801 was clearly non-linear and a two-site model gave a significantly better fit than a one site model ($p < 0.05$, F-test). Binding parameters of the high affinity site are given in Table 4.3, parameters for both sites are given in the text.

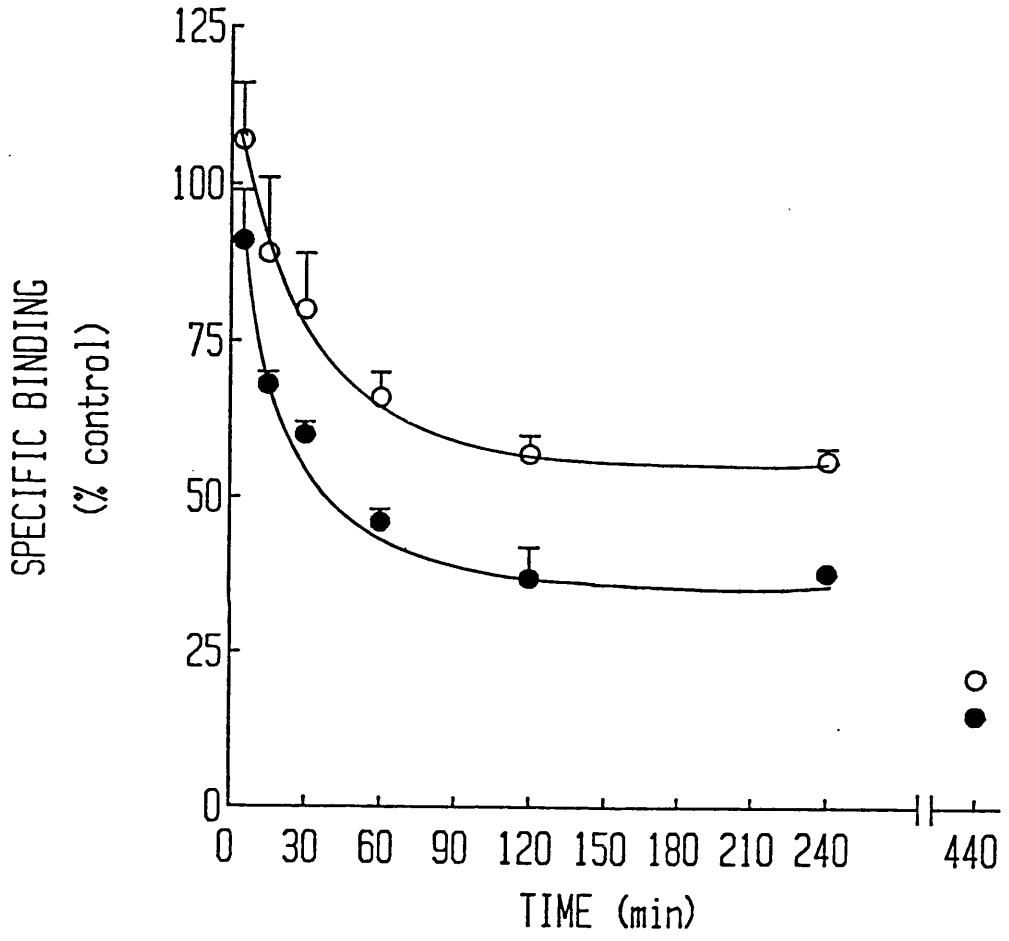
FIGURE 4.6 SATURATION ANALYSIS OF [^{3}H]-MK-801 AND [^{3}H]-TCP BINDING TO RAT CORTICAL MEMBRANES.



Experiments were performed with membranes incubated for 2 h at 25°C in Tris-HCl buffer using a final concentration of between 1-30 nM [^{3}H]-ligand in the presence of 100 μM glutamate and 1000 μM glycine. Non-specific binding was defined with 10 μM MK-801 and was typically less than 20% of the total binding. Using non-linear regression a one-site model gave the best fit for both these ligands. Binding parameters (mean $\pm$ SEM, n = 3) for [^{3}H]-MK-801 were B_{max}= 3582 $\pm$ 209 fmol/mg protein, K_d= 3.2 $\pm$ 0.2 nM, for [^{3}H]-TCP were B_{max}= 2089 $\pm$ 205 fmol/mg protein, K_d= 11.9 $\pm$ 0.6 nM

Open circles represent [^{3}H]-MK-801 binding, closed circles represent [^{3}H]-TCP binding.

FIGURE 4.7. TIME COURSE FOR DISSOCIATION OF[^{3}H]-MK-801 AND [^{3}H]-TCP BINDING IN HUMAN CORTICAL MEMBRANES.



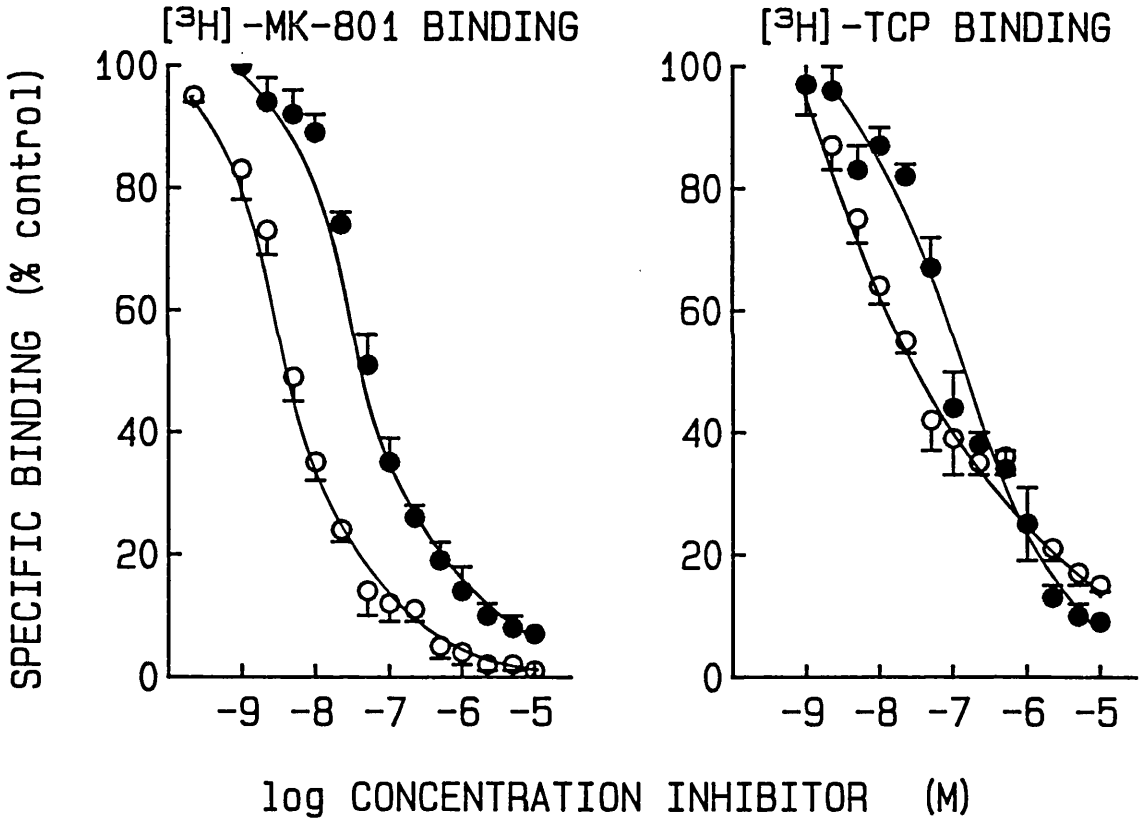
Experiments were performed with membranes incubated in Tris-HCl buffer using a final concentration of 5 nM [^{3}H]-ligand in the presence of 100 μM glutamate and 1000 μM glycine. Dissociation was achieved using 100 μM MK-801.
Open circles represent [^{3}H]-MK-801 binding, closed circles represent [^{3}H]-TCP binding.
Values are mean $\pm$ SEM, n = 3.
Logarithmic transformation of the binding data did not result in a linear plot (data not shown)

TABLE 4.1. PHARMACOLOGY OF [^{3}H]-MK-801 AND [^{3}H]-TCP BINDING TO HUMAN CORTICAL MEMBRANES.

Compound	[^{3}H]-MK-801		[^{3}H]-TCP	
	pIC_{50}	nH	pIC_{50}	nH
MK-801	8.30 ± O.24	0.59	8.29 ± 0.37	0.31
TCP	8.06 ± 0.23	0.49	7.7 3 ± 0.13	0.47
PCP	7.24 ± 0.02	0.78	7.04 ± 0.02	0.80
SKF 10047	6.63 ± 0.06	0.71	6.47 ± 0.04	0.50
$ZnCl_2$	4.68 ± 0.08*	0.54	3.37 ± 0.27	0.52
7-chlorokynurenic acid	4.51 ± 0.05		¥ <4.00	
AP5	4.23 ± 0.06		4.14 ± 0.08	

The activity of a number of ligands was determined with membranes incubated for 2 h at 25 °C in Tris HCl buffer using a final concentration of 5 nM [^{3}H]-ligand in the presence of 100 μM glutamate and 1000 μM glycine, except 7-chlorokynurenic acid and AP5 where 1 μM glutamate and glycine was used. Values are mean ± SEM from 3 separate determinations.
* significantly different to [^{3}H]-TCP value ($p < 0.05$, Student's t-test)
¥ = 40% inhibition at 100 μM .

FIGURE 4.8. DISPLACEMENT OF [^{3}H]-MK-801 AND [^{3}H]-TCP BINDING TO HUMAN CORTICAL MEMBRANES BY MK-801 AND TCP.



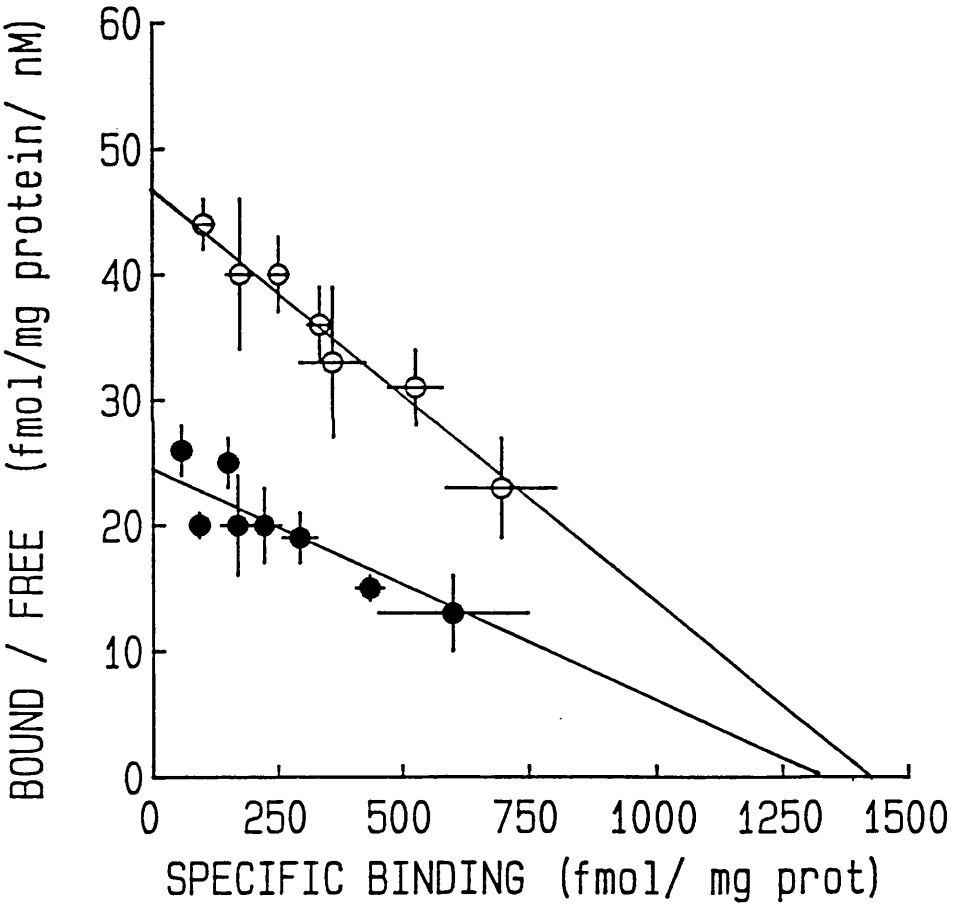
Experiments were performed with membranes incubated in Tris-HCl buffer using a final concentration of 5 nM [^{3}H]-ligand in the presence of 100 μM glutamate and 1000 μM glycine.
Open circles represent MK-801 inhibition, filled circles represent TCP inhibition.
Values are mean $\pm$ SEM, n = 3.
All displacement curves could be fitted to a two-site model, details of this analysis are given in Table 4.2.

TABLE 4.2. KINETIC ANALYSIS OF [^{3}H]-MK-801 AND [^{3}H]-TCP BINDING DISPLACEMENT CURVES IN HUMAN CORTICAL MEMBRANES.

Ligand	DISPLACING AGENT: TCP		DISPLACING AGENT: MK-801	
[^{3}H]-MK-801	K_1 = 17.5 ± 2.2	89 ± 3.5 %	K_1 = 3.2 ± 0.8	89 ± 3.7 %
	K_2 = 2350 ± 1360	11 ± 1 %	K_2 = 294 ± 101	11 ± 3 %
[^{3}H]-TCP	K_1 = 37 ± 19	69 ± 9 %	K_1 = 4.9 ± 2.7	66 ± 36 %
	K_2 = 1724 ± 505	31 ± 7 %	K_2 = 3175 ± 1000	34 ± 6 %

Displacement curves shown in Fig. 4.8 were analysed using non-linear regression. In all cases a two-component model gave a significantly better fit to the data than a one- component model ($p < 0.05$, F-test). Experiments were performed with cortical membranes incubated in Tris HCl buffer using a final concentration of 5 nM [^{3}H]-ligand in the presence of 100 μM glutamate and 1000 μM glycine.
Values are mean ± SEM from 3 separate determinations. Values shown are the apparent IC_{50} values (K_1 and K_2, nM) and the proportions of high and low affinity sites (as percentages).

FIGURE 4.9 SATURATION ANALYSIS OF [^{3}H]-MK-801 AND [^{3}H]-TCP BINDING TO HUMAN STRIATAL MEMBRANES.



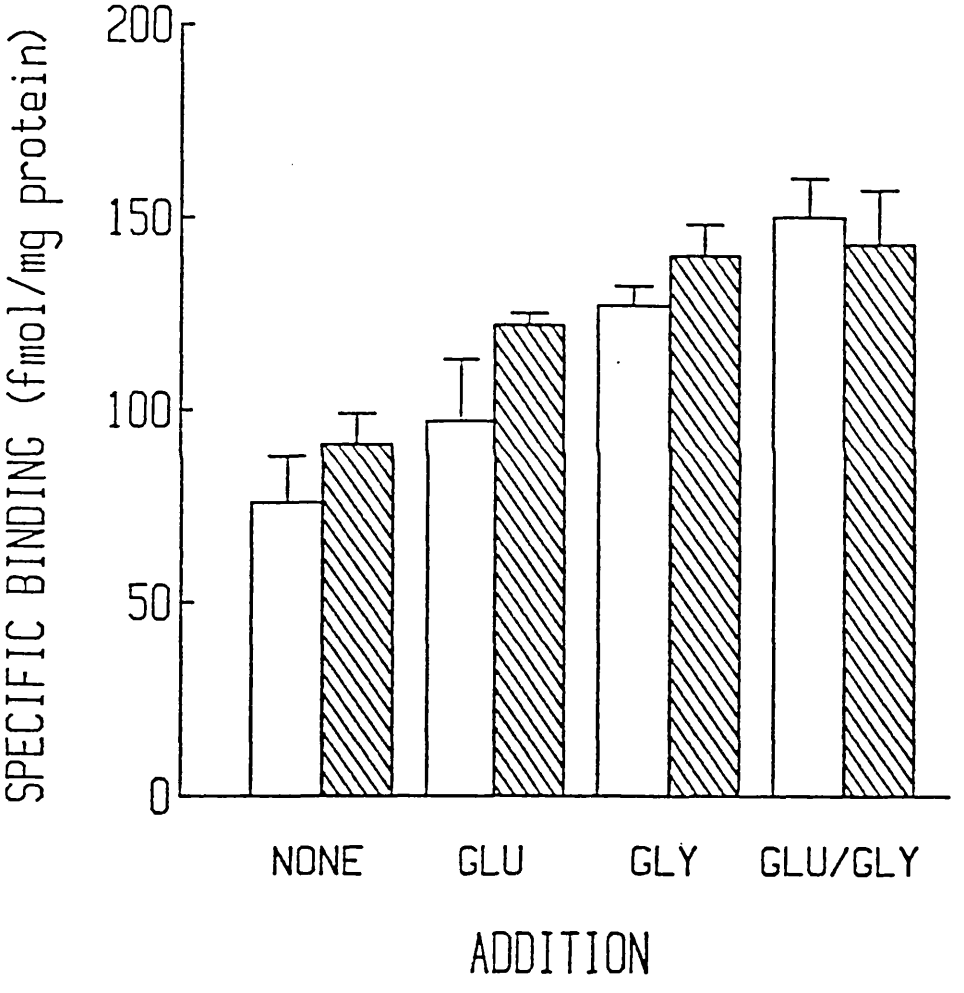
Experiments were performed with membranes incubated for 2 h at 25 °C in Tris-HCl buffer using a final concentration of between 1-50 nM [^{3}H]-ligand in the presence of 100 μM glutamate and 1000 μM glycine.
Open circles represent [^{3}H]-MK-801 binding, closed circles represent [^{3}H]-TCP binding.
Values are mean $\pm$ SEM, n = 3.
Binding parameters are given in Table 4.3.

TABLE 4.3. BINDING PARAMETERS DERIVED FROM SATURATION ANALYSIS OF [^{3}H]-MK-801 AND [^{3}H]-TCP BINDING IN HUMAN CORTICAL AND STRIATAL MEMBRANES.

Brain region	[^{3}H]-MK-801		[^{3}H]-TCP	
Cortex	K_d	5.8 ± 0.2	K_d	16.8 ± 1.0
	B_{max}	1500 ± 300*	B_{max}	660 ± 170
Striatum	K_d	29.8 ± 11.0	K_d	50.4 ± 11.7
	B_{max}	1403 ± 394	B_{max}	1292 ± 305

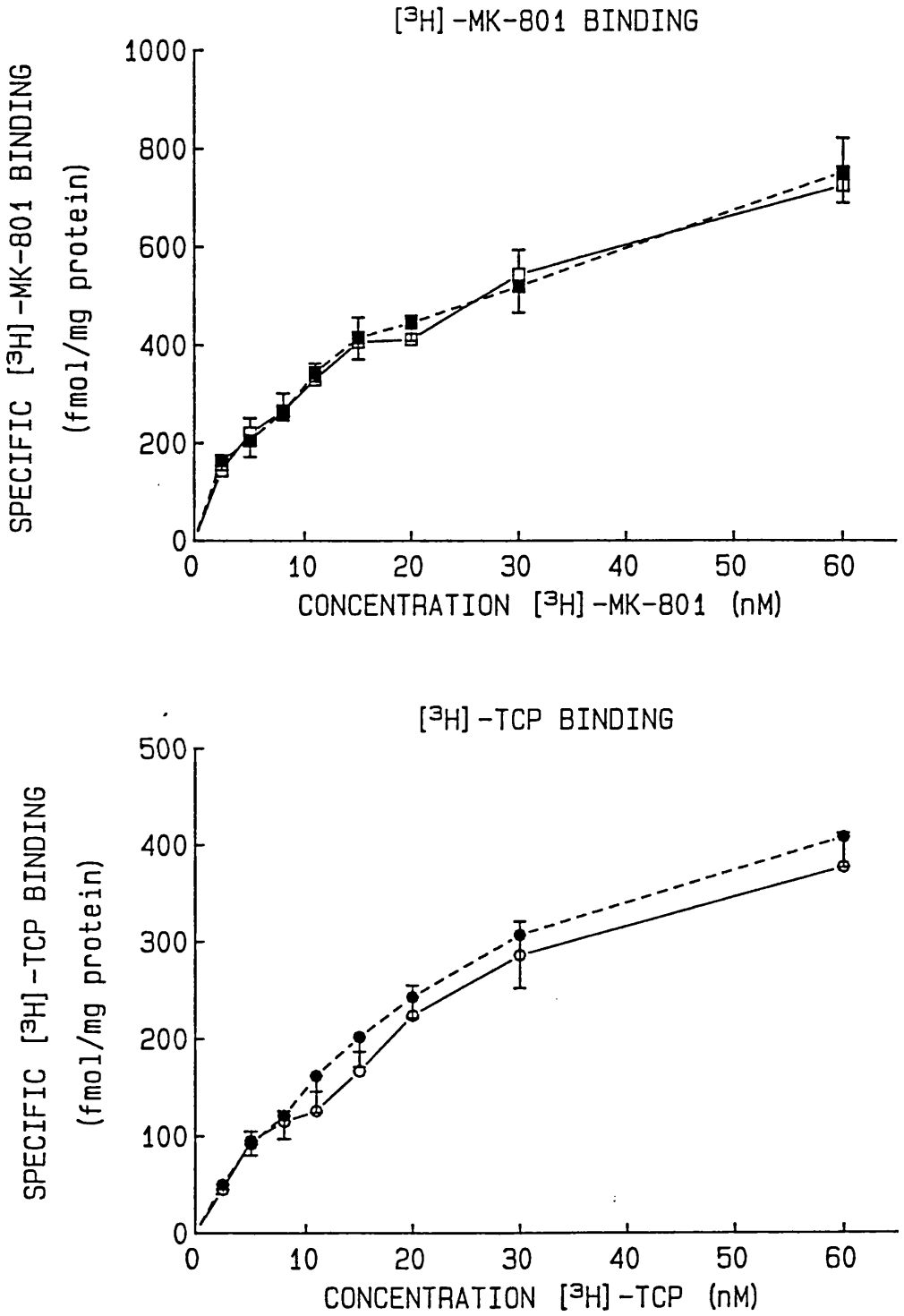
Saturation curves in Figs. 4.5 and 4.9 were analysed using non-linear regression of the high affinity component only.
Experiments were performed with membranes incubated for 2 h at 25 °C in Tris HCl buffer using between 1- 50 nM [^{3}H]-ligand in the presence of 100 μM glutamate and 1000 μM glycine.
Values are mean ± SEM from 3 separate determinations. K_d values shown are nM and B_{max} values are fmol/ mg protein.
* significantly different to [^{3}H]-TCP binding ($p < 0.05$, Student's t-test).

FIGURE 4.10. EFFECTS OF GLUTAMATE AND GLYCINE ON [^{3}H]-MK-801 AND [^{3}H]-TCP BINDING IN HUMAN STRIATAL MEMBRANES.



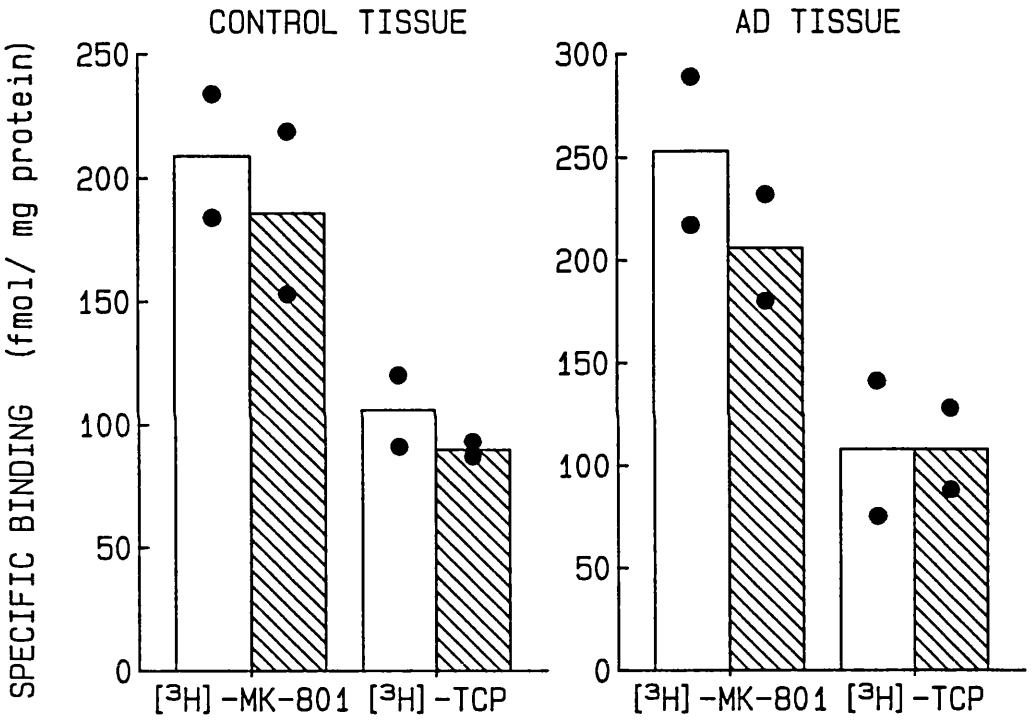
Experiments were performed with membranes incubated for 2 h at 25 °C in Tris-HCl buffer using a final concentration of 5 nM [^{3}H]-ligand in the presence of 100 μM glutamate, 1000 μM glycine or glutamate and glycine together.
Open bars represent [^{3}H]-MK-801 binding, hatched bars represent [^{3}H]-TCP binding.
There were no differences between [^{3}H]-MK-801 and [^{3}H]-TCP binding (Student's t-test).
Values are mean $\pm$ SEM, n = 3.

FIGURE 4.11. THE EFFECT OF FREEZING AND THAWING TISSUE ON [^{3}H]-MK-801 AND [^{3}H]-TCP BINDING TO HUMAN CORTICAL MEMBRANES.



Experiments were performed with membranes incubated at 25 °c in Tris-HCl buffer using a final concentration of between 2-60 nM [^{3}H]-ligand in the presence of 100 μM glutamate and 1000 μM glycine.
Open symbols represent tissue frozen once, filled symbols represent tissue frozen, thawed and refrozen.
Values are mean $\pm$ SEM, n=4.

FIGURE 4.12. THE EFFECT OF THREE PROTEASE INHIBITORS ON [^{3}H]-MK-801 AND [^{3}H]-TCP BINDING TO ALZHEIMER'S DISEASE AND CONTROL TISSUE FROM CORTEX.



Experiments were performed with membranes incubated for 4 h at 25 °C in Tris-HCl buffer using a final concentration of 5 nM [^{3}H]-ligand in the presence of 100 μM glutamate and 1000 μM glycine.
Open bars represent incubation in the absence of protease inhibitor, hatched bars represent incubations in the presence of protease inhibitors (a mixture of bacitracin, PMSF (both 10 μg/ ml) and aprotinin (3400 units/ ml)).
Bars are the mean value from 2 brains, points are individual values. .

4.1.10 DISCUSSION

The results of the present study demonstrate that both [^{3}H]-MK-801 and [^{3}H]-TCP bind to the NMDA receptor complex in human cerebral cortical membranes. The binding of both ligands was stimulated by glutamate and glycine in an analogous fashion to that observed in rat brain (section 1.7.2). In the presence of glutamate and glycine , [^{3}H]-MK-801 and [^{3}H]-TCP binding were inhibited by the competitive NMDA receptor antagonist AP5, and also by 7-chlorokynurenic acid, an antagonist of NMDA receptors which acts primarily at the glycine modulatory site. The inhibition by AP5 and 7-chlorokynurenic acid was not observed at high concentrations of glutamate and glycine which is consistent with competitive inhibition at these sites.

Compounds known to act at the channel of the NMDA receptor complex were also effective inhibitors of both [^{3}H]-MK-801 and [^{3}H]-TCP binding. The order of potency of these compounds was similar to that reported in rat brain and was identical for both ligands. Strychnine, an antagonist of spinal glycine receptors, and remoxipride, which has high affinity for "sigma opiate sites" were inactive with both [^{3}H]-MK-801 and [^{3}H]-TCP binding.

Although the binding of [^{3}H]-MK-801 and [^{3}H]-TCP to cortical membranes was compatible with labelling of the NMDA receptor complex, the binding of both ligands was complex and not compatible with a single non-interacting site model. The binding of [^{3}H]-MK-801 and [^{3}H]-TCP was stimulated by glutamate and glycine, apparently by an allosteric interaction. In the absence of glutamate and glycine, ligand binding to membranes was slow and the association rate was markedly increased by the presence of glutamate and glycine. The finding that these amino acids altered the association rate of these ligands makes it difficult to interpret data from experiments not employing maximal stimulating conditions. Therefore, the saturation and displacement studies were performed in the presence of high concentrations of glutamate and glycine and were incubated for 2 h when equilibrium

had been reached. However, under these conditions the binding of [^{3}H]-MK-801 and [^{3}H]-TCP was complex, as displacement of both ligands by NMDA receptor channel blocking drugs (MK-801, TCP, phencyclidine, SKF10047) displayed low Hill coefficients. Moreover, in the case of [^{3}H]-MK-801, saturation analysis revealed the presence of apparent high and low affinity sites. A low affinity site could not be demonstrated for [^{3}H]-TCP binding, although the data was clearly non- linear. This may be due to variable non-specific binding at high ligand concentrations therefore a pooled analysis could not be undertaken for [^{3}H]-TCP binding. Therefore, the data for [^{3}H]-TCP binding was analysed separately for each brain and there were not sufficient data points to statistically demonstrate a low affinity site. It is unlikely that the shallow inhibition curves observed in displacement experiments are due to [^{3}H]-MK-801 binding to this low affinity site as it can be calculated from the relative binding parameters (Fig. 4.5) that at a concentration of 5 nM only 5% of [^{3}H]-MK-801 binding would be to the low affinity site.

Whilst [^{3}H]-MK-801 and [^{3}H]-TCP binding share many common properties, there are clear differences. Firstly, in cortex, the pattern of stimulation of [^{3}H]-MK-801 binding is different from that of [^{3}H]-TCP. Thus, [^{3}H]-MK-801 binding in the presence of both glutamate and glycine was much greater than in the presence of either amino acid alone, while this combination did not significantly increase [^{3}H]-TCP binding to a greater extent than glycine alone. This has not been reported in rat brain where glycine appears to further stimulate the glutamate- enhanced [^{3}H]-TCP binding (Bonhaus & MacNamara, 1988, Benavides et al., 1988, Johnson et al., 1988, Stirling et al., 1989). These studies however, employed much shorter incubation times than those used here and since glycine increases the association rate of this ligand it is difficult to compare these data. It seems unlikely, however that there is a species difference for the effect of glycine on this receptor, this difference more likely reflects the different experimental conditions employed, including use of membrane preparations that were not equivalent between species. Furthermore, the inhibition on [^{3}H]-TCP binding by spermidine has not been reported in rat brain,

in fact spermidine has been reported to increase [^{3}H]-TCP binding in rat brain (Carter et al., 1989). The dose response- response curve is markedly bell- shaped in this report and it is possible that after 2 h incubation employed here the descending part of the curve is studied. In Chapter 5 the effects of spermidine on the NMDA receptor complex in control brain are studied. In human brain the spermidine stimulation of [^{3}H]-MK-801 binding is also bell- shaped, supporting this idea. It was thought that the difference between [^{3}H]-MK-801 and [^{3}H]-TCP binding could reflect protease digestion of membrane proteins which in some way reduced the effect of glycine on [^{3}H]-TCP binding, particularly since crude homogenate from human brain was incubated for 2 h at 25 °C. A number of protease inhibitors (bacitracin, a peptidase inhibitor, PMSF, a serine cleaving protease inhibitor and aprotinin which inhibits trypsin) were included in the incubation medium. They did not increase [^{3}H]-MK-801 or [^{3}H]-TCP binding and it therefore seems unlikely that proteases are active in these well washed membrane preparations.

The second, and most obvious difference between [^{3}H]-MK-801 and [^{3}H]-TCP binding was the maximum number of high-affinity binding sites for each ligand. There were more than twice as many high affinity binding sites for [^{3}H]-MK-801 than for [^{3}H]-TCP in the cortex. The validity of this result is perhaps strengthened by the observation that both ligands had the same number of high-affinity binding sites in the striatum. Furthermore, in rat brain the difference was apparent indicating that this result is not attributable to post-mortem artifacts and epiphenomena affecting [^{3}H]-TCP binding more than [^{3}H]-MK-801 binding. Despite the different number of binding sites for the two ligands, unlabelled TCP displaced [^{3}H]-MK-801 and [^{3}H]-TCP binding with equal potency, and the same was observed with unlabelled MK-801. On this basis it is difficult to understand why [^{3}H]-MK-801 labels more sites than [^{3}H]-TCP. However, detailed analysis of MK-801 and TCP displacement curves demonstrated that whilst MK-801 has equal affinity for the high affinity component of either [^{3}H]-MK-801 or [^{3}H]-TCP binding, it was considerably more potent at inhibiting the second component of [^{3}H]-MK-801 binding than [^{3}H]-TCP binding.

The calculated IC_{50} of MK-801 at this second site was 294 nM. In saturation studies with [^{3}H]-MK-801 two sites were observed with apparent K_d values of 5.8 nM and 16 μM. It is possible therefore that an additional site with intermediate affinity for MK-801 is present, although the saturation experiments performed here do not permit a reliable estimate of this site to be made. Indeed, this extra site has been reported in human brain (Kornhuber et al., 1989). It is clear that [^{3}H]-TCP does not label this site, as the second component of [^{3}H]-TCP binding was inhibited by MK-801 with a much lower potency.

The non-competitive NMDA antagonists and the competitive antagonist, AP5, did not distinguish between [^{3}H]-MK-801 and [^{3}H]-TCP binding in human cortex. However, both Zn^{2+} and 7-chlorokynurenic acid were more potent inhibitors of [^{3}H]-MK-801 than [^{3}H]-TCP binding and as these compounds probably act at sites distinct from the primary agonist site, these findings suggest that there are differences in the allosteric modulation of [^{3}H]-MK-801 and [^{3}H]-TCP binding. Glutamate and glycine act synergistically to increase the association rate of [^{3}H]-MK-801 binding. However, they were not additive in their effects on [^{3}H]-TCP binding, again suggesting differences in the allosteric modulation of [^{3}H]-MK-801 and [^{3}H]-TCP binding. One interpretation of these differences is that subclasses of NMDA receptors exist, which are differentially modulated by glutamate and glycine (see section 1.7). Alternatively, substates of a single NMDA receptor may exist which differ in their interactions with MK-801 and TCP. The present data do not distinguish between these two possibilities.

Although there is heterogeneity of NMDA receptor binding in cortex, this does not occur in the human striatum where the combination of glycine and glutamate did not synergistically enhance [^{3}H]-MK-801 or [^{3}H]-TCP binding and both ligands bound to an equal number of high affinity sites.

The present data demonstrate differences between the binding of [^{3}H]-MK-801 and [^{3}H]-TCP to the NMDA receptor of human brain, possibly the result of either heterogeneity or differences in the interaction of the ligands with a single receptor. This may be an important observation in the design of novel antagonists of the NMDA receptor which do not have the undesireable effects of some of the presently available compounds (Tricklebank et al., 1989). Furthermore, it may be possible that the pattern of [^{3}H]-MK-801 or [^{3}H]-TCP binding is selectively altered in Alzheimer's disease, a condition associated with loss of glutamatergic neurotransmission (Pearce et al., 1984, Procter et al., 1988, also see Greenamyre & Young, 1989). This is discussed in detail the next section.

4.2 COMPARISON OF [^{3}H]-MK-801 AND [^{3}H]-TCP BINDING IN ALZHEIMER'S DISEASE.

RESULTS

All results in this section apply to human tissue unless otherwise stated. Membrane preparations were either from the temporal cortex (BA20/21) or the frontal cortex (BA9/10). The membrane preparations used were derived from either the "total particulate fraction" as used in section 4.1 hereafter referred to as "crude membrane preparation" or a fraction low in material with the buoyant density of myelin hereafter called "purified membrane preparation" (Bowen et al., 1977, see section 2.4.2).

4.2.1 Comparison of [^{3}H]-MK-801 and [^{3}H]-TCP binding in crude membrane preparation from the frontal cortex.

In superior frontal cortex (BA9/10) the maximum number of high affinity [^{3}H]-MK-801 binding sites was greater than [^{3}H]-TCP binding sites ($p < 0.01$, paired t-test, Fig. 4.13, Table, 4.5) in both control (1222 $\pm$ 161 compared with 897 $\pm$ 114 fmol/mg protein, mean $\pm$ SEM, n = 5, and Alzheimer's disease tissue (1163 $\pm$ 157 compared with 827 $\pm$ 119 fmol/mg protein, n = 5). [^{3}H]-MK-801 also appeared to have a greater affinity for this binding site, the K_d value for [^{3}H]-MK-801 was significantly lower than for [^{3}H]-TCP binding in both control (5.7 $\pm$ 1.0 compared with 15.1 $\pm$ 2.2 nM) and Alzheimer's disease tissue (4.9 $\pm$ 1.0 and 10.6 $\pm$ 1.7 nM; $p < 0.01$, paired t-test, Table 4.5). For control subjects, K_d and B_{max} values for [^{3}H]-MK-801 or [^{3}H]-TCP were not related to age, delay to post-mortem time, sex, hemisphere studied and the way the patient died (see Table 4.4). There were no alterations in either the maximum number of binding sites or in K_d values for either ligand when control tissue was compared with Alzheimer's disease tissue (Table 4.5).

4.2.2 Comparison of [^{3}H]-MK-801 and [^{3}H]-TCP binding in a crude membrane preparation from the temporal cortex.

In temporal cortex (BA 20/21), like the frontal cortex, the maximum number of high affinity [^{3}H]-MK-801 binding sites was significantly higher than [^{3}H]-TCP binding sites ($p < 0.01$, paired t-test, Fig 4.14, Table 4.6) in both control (1243 $\pm$ 77 compared with 989 $\pm$ 27 fmol/ mg protein, n = 6) and Alzheimer's disease tissue (1190 $\pm$ 41 compared with 837 $\pm$ 71 fmol/ mg protein, n = 5.). The K_d value for [^{3}H]-MK-801 was also lower than for [^{3}H]-TCP binding in control tissue (7.0 $\pm$ 1.6 and 16.8 $\pm$ 3.3 nM) and Alzheimer's disease tissue (8.4 $\pm$ 3.8 and 14.5 $\pm$ 3.1 nM). In control tissue, K_d or B_{max} values for [^{3}H]-MK-801 or [^{3}H]-TCP were not related (data not shown) to age, delay to post-mortem time, hemisphere studied and the way the patient died (see Table 4.4).

4.2.3 Comparison of[^{3}H]-MK-801 and [^{3}H]-TCP binding in a purified membrane preparation from the frontal cortex.

To attempt to reduce the variability of binding observed with crude membrane preparations from human brain (see Tables 4.5 & 4.6, particularly K_d values) a sub-fraction of the crude membrane preparation was prepared excluding a fraction of high buoyant density that was enriched in lipids (White et al., 1978, Procter et al., 1990, see methods section 2.4.2). For control tissue the maximum number of binding sites for[^{3}H]-MK-801 was greater than for [^{3}H]-TCP (3317 $\pm$ 173 compared with 2554 $\pm$ 162 fmol / mg protein, n = 8, $p < 0.01$, paired t-test) but not for Alzheimer's disease tissue (2773 $\pm$ 180 compared with 2347 $\pm$ 256 fmol/mg protein, n = 8, Table 4.8) where the number of [^{3}H]-MK-801 sites was reduced ($p < 0.05$, Student's t-test) compared with control. The K_d value for [^{3}H]-MK-801 was lower than for [^{3}H]-TCP binding in control (12.7 $\pm$ 2.3 and 26.8 $\pm$ 3.1 nM) and Alzheimer's disease tissue (13.3 $\pm$ 2.5 and 30.9 $\pm$ 5.7 nM, $p < 0.01$, paired t-test, Table 4.8). However, the K_d values were somewhat higher than in the crude membrane preparation (Table 4.11). The K_d or B_{max} values for [^{3}H]-MK-801 or [^{3}H]-TCP were not related (data not shown) to age, delay to post-mortem time,

hemisphere studied and the way the patient died but there was an apparent effect of sex on the K_d value for [^{3}H]-TCP binding which was lower ($p < 0.05$, Student's t-test) in tissue from males (31.8 $\pm$ 3.4 nM) than females (18.0 $\pm$ 2.0 nM, see Table 4.7).

4.2.4 Comparison of[^{3}H]-MK-801 and [^{3}H]-TCP binding in a purified membrane preparation from the temporal cortex.

The purified membrane preparation described in the previous section was also prepared for the temporal cortex. The maximum number of [^{3}H]-MK-801 binding sites compared with [^{3}H]-TCP binding sites were obviously different (4503 $\pm$ 423 and 2686 $\pm$ 301 fmol/mg protein, n = 6 in control and 4321 $\pm$ 451 and 2943 $\pm$ 377 fmol/mg protein, n = 6 in Alzheimer's disease tissue respectively, Fig 4.16, Table 4.9). In this purified membrane preparation the K_d values for [^{3}H]-MK-801 were lower than for [^{3}H]-TCP binding in control (6.3 $\pm$ 0.8 and 18.2 $\pm$ 2.0 nM) and Alzheimer's disease tissue (9.3 $\pm$ 1.0 and 23.5 $\pm$ 2.7 nM, $p < 0.01$ paired t-test, Table 4.9) and there was a significant reduction in the K_d value for [^{3}H]-MK-801 binding in Alzheimer's disease compared with control subjects ($p < 0.05$, Student's t-test, Table 4.9). However, there were no other differences between control and Alzheimer's disease tissue.

4.2.5 Comparison of membrane protein content in crude and purified membrane preparations from Alzheimer's disease and control tissue in the temporal cortex.

Table 4.10 shows that the mean protein content of the purified membrane preparations were to approximately half the value of the crude membrane preparations ($p < 0.01$, Mann-Whitney U-test). There were no differences in membrane protein content between Alzheimer's disease and control tissue in the temporal or the frontal cortex for either the crude or the purified membrane preparations.

TABLE 4.4 **DEMOGRAPHY OF SUBJECTS STUDIED USING CRUDE MEMBRANE PREPARATION**

	AGE (y)	POST-MORTEM DELAY (h)	HEMISPHERE STUDIED	STORAGE TIME AT -70°C (y)	SEX	AGONAL STATE
FRONTAL CORTEX						
CONTROL TISSUE n = 5	75 - 94 (81±3)	12 - 48 (30±6)	3R, 2L	3 - 8 (5±1)	3F, 2M	3P, 2S
AD TISSUE n = 5	74 - 86 (82±2)	14 - 48 (31±6)	3R, 2L	1 - 8 (4±2)	2F, 1M	3P, 2S
TEMPORAL CORTEX						
CONTROL TISSUE n = 6	65 - 81 (73±2)	3 - 37 (19±6)	3R, 2L	2 - 3 (3±O.2)	2F, 2M	2P, 4S
AD TISSUE n = 5	60 - 81 (70±4)	5 - 38 (18±6)	1R, 2L	1 - 2 (2±0.2)	3F, 2M	4P, 1S

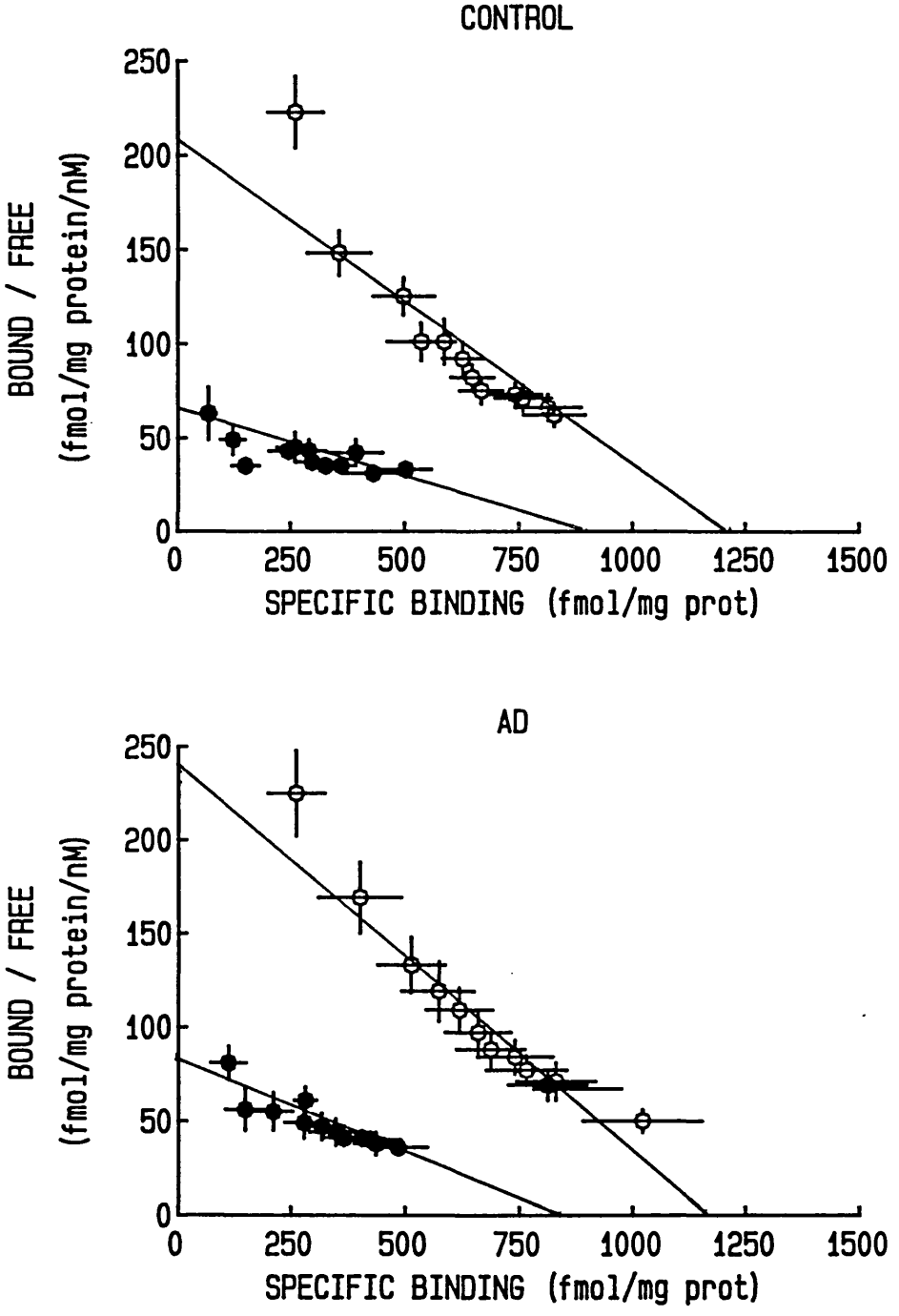
Mean ± SEM values given in parenthesis.
Abbreviations: y = years, h = hours, R = right, L = left, F = female, M = male.
P = protracted death eg bronchopneumonia, S = sudden death eg cardiac arrest.

TABLE 4.5. BINDING PARAMETERS DERIVED FROM SATURATION ANALYSIS OF [^{3}H]-MK-801 AND [^{3}H]-TCP BINDING TO A CRUDE MEMBRANE PREPARATION OF ALZHEIMER'S DISEASE AND CONTROL TISSUE FROM SUPERIOR FRONTAL CORTEX.

	[^{3}H]-MK-801		[^{3}H]-TCP	
CONTROL TISSUE n = 5	K_d	5.7 ± 1.0	K_d	15.1 ± 2.2
	B_{max}	1222 ± 161	B_{max}	897 ± 114
AD TISSUE n = 5	K_d	4.9 ± 1.0	K_d	10.6 ± 1.7
	B_{max}	1163 ± 157	B_{max}	827 ± 119

The saturation curves in Fig. 4.13 were analysed using non-linear regression, for both ligands a one - site model gave the best fit.
Experiments were performed with a crude membrane preparation incubated for 4 h at 25 °C in Tris HCl buffer using a final concentration of between 1-40 nM [^{3}H]-ligand in the presence of 100 μM glutamate and 1000 μM glycine.
Values are mean ± SEM. K_d values shown are nM and B_{max} values are fmol/ mg protein.
All values for [^{3}H]-TCP binding are significantly lower than for [^{3}H]-MK-801 ($p < 0.01$, paired t-test)
There were no significant differences between control and AD tissue. Membrane protein content was similar for control and AD tissue (27.5 ± 3.3 and 27.5 ± 3.9 mg/g wet weight respectively).

FIGURE 4.13. SATURATION ANALYSIS OF [^{3}H]-MK-801 AND [^{3}H]-TCP BINDING TO A CRUDE MEMBRANE PREPARATION OF ALZHEIMER'S DISEASE AND CONTROL TISSUE FROM SUPERIOR FRONTAL CORTEX.



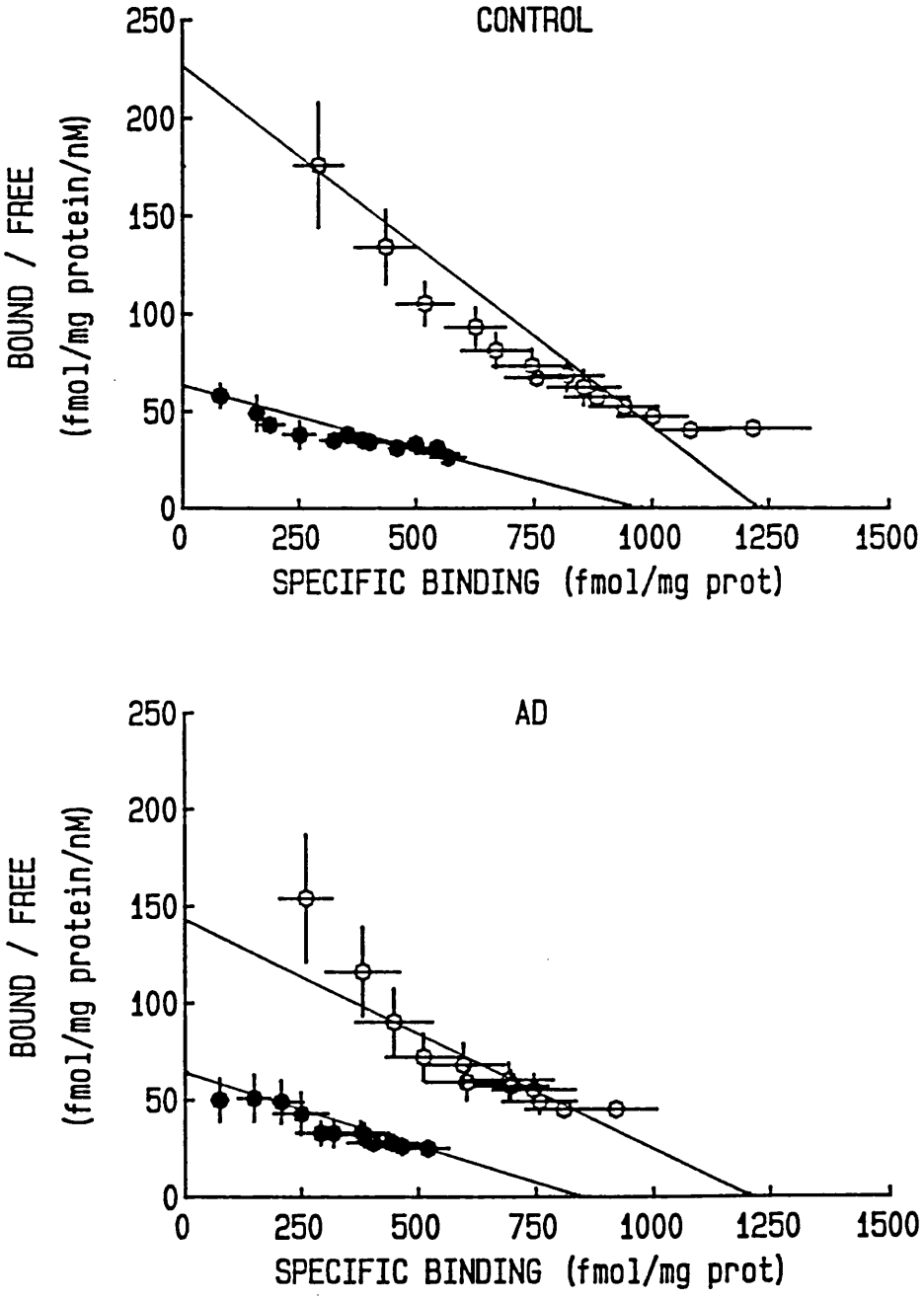
Experiments were performed exactly as described in Table 4.5.
Open circles represent [^{3}H]-MK-801 binding, closed circles represent [^{3}H]-TCP binding.
Values are mean $\pm$ SEM. K_d, B_{max} and n values are given in Table 4.5.
prot identifies protein.

TABLE 4.6. BINDING PARAMETERS DERIVED FROM SATURATION ANALYSIS OF [^{3}H]-MK-801 AND [^{3}H]-TCP BINDING TO A CRUDE MEMBRANE PREPARATION OF ALZHEIMER'S DISEASE AND CONTROL TISSUE FROM TEMPORAL CORTEX.

	[^{3}H]-MK-801		[^{3}H]-TCP	
CONTROL TISSUE n = 6	K_d	7.0 ± 1.6	K_d	16.8 ± 3.3
	B_{max}	1243 ± 77	B_{max}	989 ± 27
AD TISSUE n = 5	K_d	8.4 ± 3.8	K_d	14.5 ± 3.1
	B_{max}	1190 ± 41	B_{max}	837 ± 71

The saturation curves in Fig. 4.14 were analysed using non-linear regression, for both ligands a one - site model gave the best fit.
Experiments were performed with a crude membrane preparation incubated for 4 h at 25 °C in Tris HCl buffer using a final concentration of between 1-30 nM [^{3}H]-ligand in the presence of 100 μM glutamate and 1000 μM glycine.
Values are mean ± SEM. K_d values shown are nM and B_{max} values are fmol/ mg protein.
All values for [^{3}H]-TCP binding are significantly lower than for [^{3}H]-MK-801 ($p < 0.01$, paired t-test)
There were no significant differences between AD and control tissue. Membrane protein content was similar for AD and control tissue (Table 4.10).

FIGURE 4.14. SATURATION ANALYSIS OF [^{3}H]-MK-801 AND [^{3}H]-TCP BINDING TO A CRUDE MEMBRANE PREPARATION OF ALZHEIMER'S DISEASE AND CONTROL TISSUE FROM TEMPORAL CORTEX.



Experiments were performed exactly as described in Table 4.6.
Open circles represent [^{3}H]-MK-801 binding, closed circles represent [^{3}H]-TCP binding.
Values are mean $\pm$ SEM. K_d, B_{max} and n values are given in Table 4.6.
prot identifies protein.

TABLE 4.7 DEMOGRAPHY OF SUBJECTS STUDIED USING A PURIFIED MEMBRANE PREPARATION.

	AGE (y)	POST-MORTEM DELAY (h)	HEMISPHERE STUDIED	STORAGE TIME AT -70°C (y)	SEX	AGONAL STATE
FRONTAL CORTEX						
CONTROL TISSUE n = 8	65 - 94 (79±3)	3 - 48 (26±6)	4R, 1L	2 - 10 (5±1)	3F, 3M	6P, 2S
AD TISSUE n = 8	60 - 93 (80±3)	5 - 48 (24±5)	4R, 1L	1 - 9 (5±1)	2F, 1M	6P, 2S
TEMPORAL CORTEX						
CONTROL TISSUE n = 6	65 - 81 (73±2)	3 - 37 (19±6)	3R, 2L	2 - 3 (3±1)	2F, 2M	2P, 4S
AD TISSUE n = 6	60 - 81 (72±4)	5 - 38 (20±6)	2R, 1L	1 - 2 (1±0.4)	3F, 2M	4P, 2S

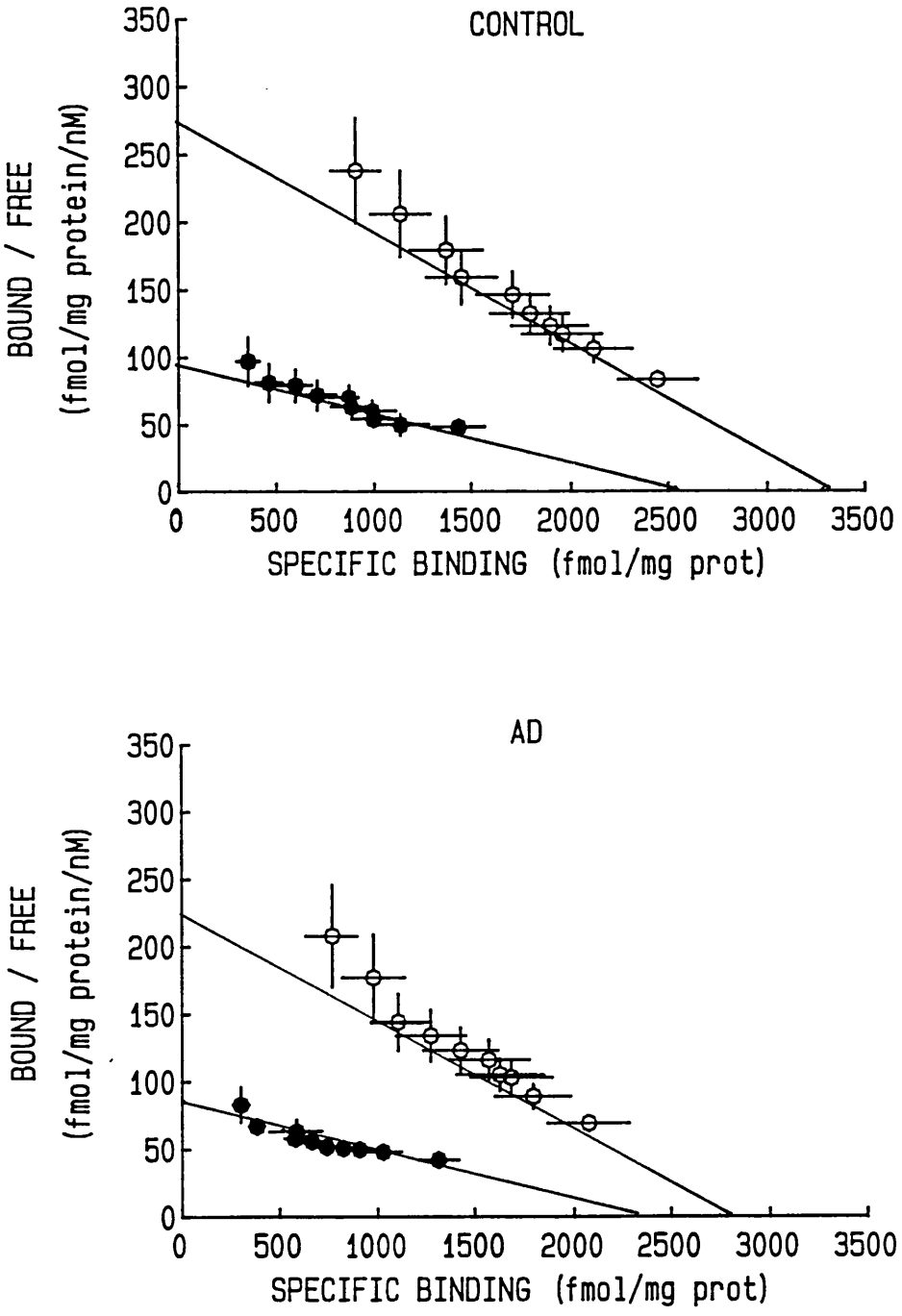
Mean ± SEM values given in parenthesis.
Abbreviations: y = years, h = hours, R = right, L = left, F = female, M = male.
P = protracted death eg bronchopneumonia, S = sudden death eg cardiac arrest.

TABLE 4.8. BINDING PARAMETERS DERIVED FROM SATURATION ANALYSIS OF [^{3}H]-MK-801 AND [^{3}H]-TCP BINDING IN A PURIFIED MEMBRANE PREPARATION OF ALZHEIMER'S DISEASE AND CONTROL TISSUE FROM SUPERIOR FRONTAL CORTEX .

	[^{3}H]-MK-801		[^{3}H]-TCP	
CONTROL TISSUE n = 8	K_d	12.7 ± 2.3	K_d	26.8 ± 3.1
	B_{max}	3317 ± 173	B_{max}	2554 ± 162
AD TISSUE n = 8	K_d	13.3 ± 2.5	K_d	30.9 ± 5.7
	B_{max}	2773 ± 180*	B_{max}	2347 ± 256

The saturation curves in Fig. 4.15 were analysed using non-linear regression, for both ligands a one - site model gave the best fit.
Experiments were performed with membranes incubated for 4 h at 25 °C in Tris HCl buffer using a final concentration of between 1-30 nM [^{3}H]-ligand in the presence of 100 μM glutamate and 1000 μM glycine.
Values are mean ± SEM. K_d values shown are nM and B_{max} values are fmol/ mg protein.
All values for [^{3}H]-TCP binding are significantly lower than for [^{3}H]-MK-801 in control tissue ($p < 0.01$, paired t-test). In AD tissue, the B_{max} for [^{3}H]-MK-801 was reduced and was similar to that for [^{3}H]-TCP binding.
* significantly different to control ($p < 0.05$, Student's t-test).
Membrane protein content was similar for control and AD tissue (16.8 ± 1.3 and 19.3 ± 1.6 mg/g wet weight respectively).

FIGURE 4.15. SATURATION ANALYSIS OF [^{3}H]-MK-801 AND [^{3}H]-TCP BINDING TO A PURIFIED MEMBRANE PREPARATION OF ALZHEIMER'S DISEASE AND CONTROL TISSUE FROM SUPERIOR FRONTAL CORTEX.



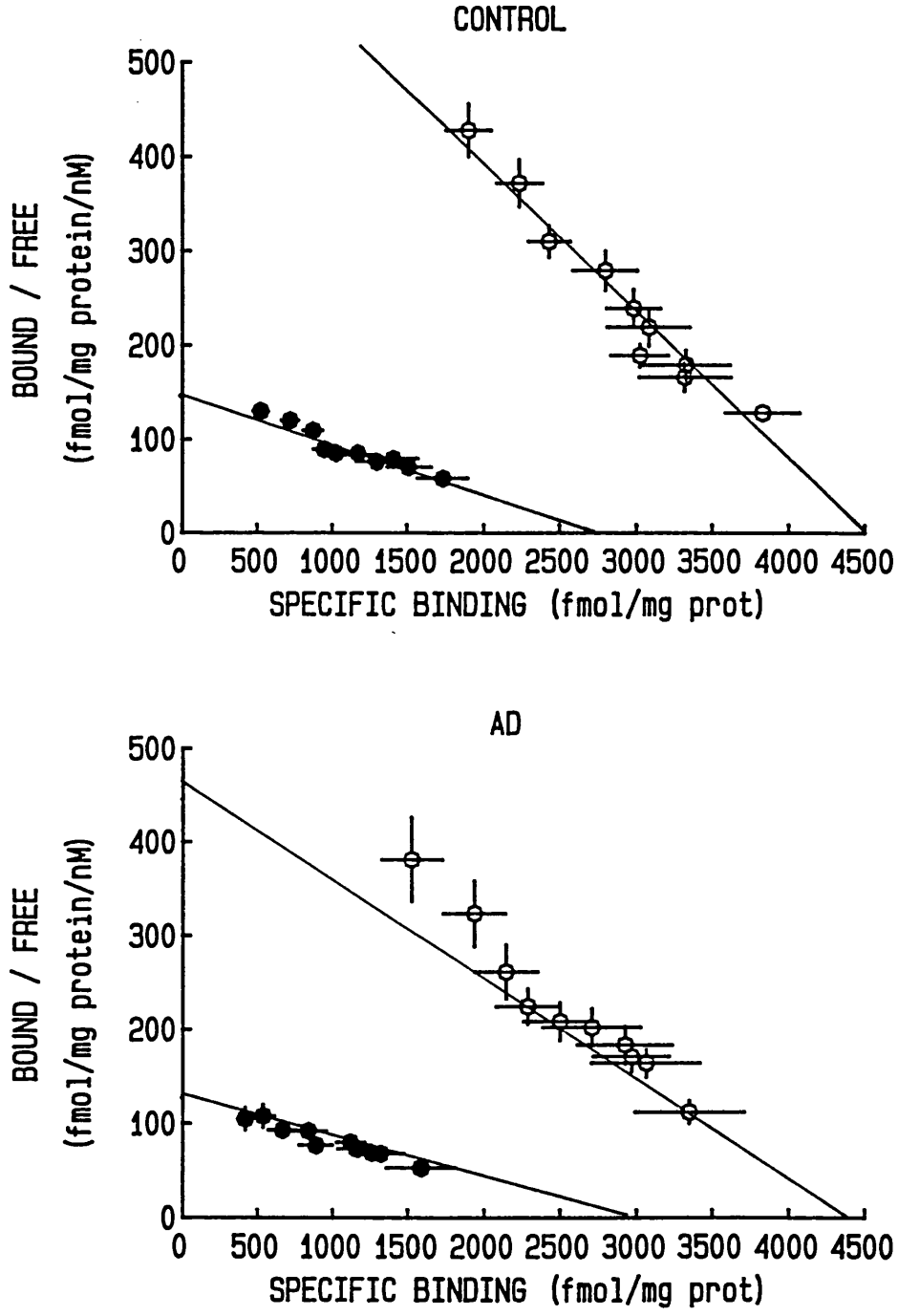
Experiments were performed exactly as described in Table 4.8.
Open circles represent [^{3}H]-MK-801 binding, closed circles represent [^{3}H]-TCP binding.
Values are mean $\pm$ SEM. K_d, B_{max} and n values are given in Table 4.8.
prot identifies protein.

TABLE 4.9. BINDING PARAMETERS DERIVED FROM SATURATION ANALYSIS OF [^{3}H]-MK-801 AND [^{3}H]-TCP BINDING IN A PURIFIED MEMBRANE PREPARATION OF ALZHEIMER'S DISEASE AND CONTROL TISSUE FROM TEMPORAL CORTEX.

	[^{3}H]-MK-801		[^{3}H]-TCP	
CONTROL TISSUE n = 6	K_d	6.3 ± 0.8	K_d	18.2 ± 2.0
	B_{max}	4503 ± 423	B_{max}	2686 ± 301
AD TISSUE n = 6	K_d	9.3 ± 1.0*	K_d	23.5 ± 2.7
	B_{max}	4321 ± 451	B_{max}	2943 ± 377

The saturation curves in Fig. 4.16 were analysed using non-linear regression, for both ligands a one - site model gave the best fit.
Experiments were performed with membranes incubated for 4 h at 25 °C in Tris HCl buffer using a final concentration of between 1-30 nM [^{3}H]-ligand in the presence of 100 μM glutamate and 1000 μM glycine.
Values are mean ± SEM. K_d values shown are nM and B_{max} values are fmol/ mg protein.
All values for [^{3}H]-TCP binding are significantly lower than for [^{3}H]-MK-801 ($p < 0.01$, paired t-test).
* significantly different to control ($p < 0.05$, Student's t-test).
Membrane protein content was similar for AD and control tissue (Table 4.10).

FIGURE 4.16. SATURATION ANALYSIS OF [^{3}H]-MK-801 AND [^{3}H]-TCP BINDING TO A PURIFIED MEMBRANE PREPARATION OF ALZHEIMER'S DISEASE AND CONTROL TISSUE FROM TEMPORAL CORTEX.



Experiments were performed exactly as described in Table 4.9.
Open circles represent [^{3}H]-MK-801 binding, closed circles represent [^{3}H]-TCP binding.
Values are mean $\pm$ SEM. K_d, B_{max} and n values are given in Table 4.9.
prot identifies protein.

TABLE 4.10. MEMBRANE PROTEIN CONTENT IN CRUDE AND A PURIFIED MEMBRANE PREPARATION FROM TEMPORAL CORTEX OF ALZHEIMER'S DISEASE AND CONTROL TISSUE.

	Crude membrane protein content (mg/g wet weight)	Purified membrane protein content (mg/g wet weight)
CONTROL TISSUE n = 6	23.8 ± 2.6*	12.0 ± 0.8
ALZHEIMER'S DISEASE TISSUE n = 5	30.2 ± 4.3*	13.2 ± 1.5

Protein content was estimated exactly as described in the text (materials and methods, section 2.8) using bovine serum albumin as standards.

Values are mean ± SEM.

* indicates significantly different to purified membrane preparation ($p < 0.01$, Mann-Whitney U-test). There was no difference between control and Alzheimer's disease membrane protein content.

TABLE 4.11 SUMMARY OF BINDING PARAMETERS DERIVED FROM SATURATION ANALYSIS OF [^{3}H]-MK-801 AND [^{3}H]-TCP BINDING.

	CRUDE MEMBRANE PREPARATION				PURIFIED MEMBRANE PREPARATION			
	B_{max}		K_d		B_{max}		K_d	
	MK801	TCP	MK801	TCP	MK801	TCP	MK801	TCP
FRONTAL CORTEX								
CONTROL	1222±161	897±119	5.7±1.0	15.1±2.2	3317±173	2554±162	12.7±2.3	26.8±3.1
AD	1163±157	827±119	4.9±1.0	10.6±1.7	2773±180*	2347±256	18.2±3.0	30.9±5.7
TEMPORAL CORTEX								
CONTROL	1243±77	989±27	7.0±1.6	16.8±3.3	4503±423	2686±301	6.3±0.8	18.2±2.0
AD	1190±41	837±71	8.4±3.8	14.5±3.1	4321±451	2943±377	9.3±1.0*	23.5±2.7

All values for [^{3}H]-MK-801 are significantly different to [^{3}H]-TCP ($p < 0.01$, paired t-test) except for AD tissue from the frontal cortex where the subfraction was used.
* significantly different to control tissue ($p < 0.05$, Student's t-test).
For comparison, in the previous section where tissue was taken from all cortical areas B_{max} value for [^{3}H]-MK-801 was 1500 ± 300, for[^{3}H]-TCP binding was 660 ± 170 fmol/mg protein.
n values are given previously, K_d values are nM, B_{max} values are fmol/mg protein.

4.2.6 DISCUSSION

In both the superior frontal and the temporal cortex from Alzheimer's disease and control tissue studied here, [^{3}H]-MK-801 bound to more sites than [^{3}H]-TCP, with one exception, the purified membrane preparation from the frontal cortex. It was with this preparation in this cortical area that in Alzheimer's disease a significant reduction in [^{3}H]-MK-801 binding occurred. This was the only disease-related reduction in receptor number detected, as summarized (Table 4.11).

The difference between the number of sites for [^{3}H]-MK-801 and [^{3}H]-TCP binding was in general less obvious than described in the previous section, as summarized (see legend, Table 4.11). The material studied previously was enriched in tissue from the parietal and occipital lobes and this difference may reflect regional variation in binding characteristics. The nature of this variation needs to be determined in further experiments.

Although there did not appear to be any effect of age, post-mortem delay, agonal state, or laterality on the binding parameters reported here, the number of samples used was small and it cannot be excluded that these factors may influence the data (see Bowen et al., 1976a, 1977, Perry et al., 1982, Palmer et al., 1988 Procter et al., 1989a, 1990, see introduction 1.4.1) and may account for the small difference seen in the number of binding sites in this tissue. The material used in the previous section, where the difference between ligands was greatest was from young patients (mean age 70 $\pm$ 5) with short delay to post-mortem (12 $\pm$ 5 h) and none of these patients died after a protracted terminal illness. By contrast, the samples studied in this section were older, had longer delays to post-mortem and included subjects who had died after a prolonged terminal illness, particularly for the frontal cortex samples where the difference between [^{3}H]-MK-801 and [^{3}H]-TCP binding was much smaller (demographic features: purified membrane preparation; mean age 79 $\pm$ 3 y, mean post-mortem delay 26 $\pm$ 6 h, 6 subjects died after prolonged terminal illness; crude membrane preparation; mean age 81 $\pm$ 3 y, mean post-mortem delay 30 $\pm$ 6 h, 4

subjects died after prolonged terminal illness, see Table 4.4 and 4.7). In addition, Kornhuber et al. (1988) reported an effect of storage time on NMDA-sensitive sites in the human brain. In this study, tissue from the temporal cortex was stored for less than 3 years whereas tissue from the frontal cortex was stored for up to 10 years (see Tables 4.4 and 4.7). These observations also illustrate some of the difficulties that may be encountered when a group of diseased brains need to be compared with control samples, in particular with respect to matching groups for agonal state (ie very few Alzheimer's disease subjects are in the category "sudden death", and it is uncertain whether the pathophysiology of death after protraced illness is equivalent in controls and Alzheimer's disease subjects).

The maximum number of binding sites was increased in the purified membrane preparation compared with crude sample preparations. This is not entirely accounted for by a reduction in membrane protein content since the loss of proteins in the temporal cortex is only 2 fold (Table 4.10) whereas the increase in binding sites was 2.9 fold for [^{3}H]-TCP and 3.8 fold for [^{3}H]-MK-801 binding. It appears therefore that the fractionation procedure results in the loss of some proteins that do not bind either [^{3}H]-MK-801 or [^{3}H]-TCP. It was also apparent that this fractionation procedure improved the assays considerably, particularly with respect to [^{3}H]-TCP binding which was very variable in the crude membrane preparation. Similar effects of tissue purification were reported for [^{3}H]-glycine binding in human brain (Procter et al., 1990). It seems unlikely from these data that the reduction in [^{3}H]-MK-801 binding seen in purified frontal cortex membranes in Alzheimer's disease would have been observed in a crude membrane preparation because the variability seems somewhat excessive and the difference modest.

The K_d values were also higher in the purified membrane preparation compared with the crude preparation in the frontal cortex (see Table 4.11). It is possible that in this purified membrane preparation that equilibration has not been reached despite the addition of very high glutamate (100 μM) and glycine (1000 μM)

concentrations and incubation for 4h. Therefore, the apparent binding affinity is possibly lower than the true K_d at equilibrium conditions. There was a significant reduction in the affinity (increased K_d value) of [^{3}H]-MK-801 in Alzheimer's disease in the temporal cortex using the purified membrane preparation. This was the only disease related change in the affinity of either ligand. Procter et al. (1989a) report a similar effect (using different assay conditions) and it is possible that the apparent change detected in affinity reflects a difference in the equilibration of [^{3}H]-MK-801 in Alzheimer's disease and control tissue. This requires further investigation.

In this study there was no evidence of changes in the number of binding sites for either [^{3}H]-MK-801 or [^{3}H]-TCP, with the exception of [^{3}H]-MK-801 in the purified membrane preparation from the frontal cortex. This result seems to be in agreement with Simpson et al. (1988), Chalmers et al. (1990) and Ninomiya et al. (1990), although Scatchard analysis was not employed (but see Ninomiya et al., 1990) and either [^{3}H]-TCP binding or NMDA-sensitive [^{3}H]-glutamate autoradiography was used. The lack of change in the temporal cortex is in agreement with Cowburn et al. (1998a), Simpson et al. (1988) and Procter et al. (1989a). The pathology of Alzheimer's disease tends to be more prominent in the temporal cortex (Chapter 3) and it is possible that in this region there is no difference in the binding values expressed per milligram of protein because of marked loss of structures (predominantly pyramidal neurones) organized in a columnar manner (see Hauw et al., 1986, Duyckaerts et al., 1985, 1986).

Previous reports of the density of the NMDA receptor complex in Alzheimer's disease have not been consistent. The discrepancy between the findings may reflect the severity of the disease. In this study there was no deliberate selection of cases according to severity. However, in the frontal cortex where the difference between Alzheimer's disease and control tissue was seen, the tissue was taken from slightly older subjects (79 $\pm$ 3 y) than for temporal cortex (73 $\pm$ 2 y). It has been known for some time however that changes are more pronounced in younger Alzheimer

subjects, a finding that indicates that the frailer, older cases die at an earlier stage of pathogenesis of the disease (Bowen et al., 1979).

The discrepancy between reports may also reflect the different methodological approaches used to measure the NMDA receptor complex (see Table 1.4). Since amino acids alter the rate of association of the channel ligands used this makes it difficult to interpret some of the previous studies as maximally stimulating conditions were not used. In this study high concentrations of glutamate and glycine with 4 h incubation conditions were used to ensure that the maximum nimber of sites was assayed.

No alteration was detected in the number of binding sites for these ligands in Alzheimer's disease in the temporal cortex. Since there appears to be a reduction here in apparent affinity of [^{3}H]-MK-801 in Alzheimer's disease, the possibility that the modulation of the receptor-linked ion channel is impaired in Alzheimer's disease was investigated. This is described in the next section.

CHAPTER 5

MODULATION OF THE NMDA RECEPTOR COMPLEX IN CONTROL AND ALZHEIMER'S DISEASE TISSUE

All results in this section were obtained with the crude membrane preparation as used in section 4.1.

RESULTS

5.1 Effect of immediate preterminal state on [^{3}H]-MK-801 binding in control tissue from frontal and temporal cortex.

The effects of glutamate, glycine and zinc on the binding of [^{3}H]-MK-801 under non-equilibrium conditions were compared in control and Alzheimer's disease tissue matched for age, post-mortem delay and storage time at -70 °C (Table 5.1). Measurements were made in the presence of either glutamate (30 μM, "glutamate-stimulated") or glycine (3 μM) plus glutamate (30 μM; "total binding"). Another value "glycine-stimulated binding" has been obtained by subtracting "glutamate-stimulated binding" from "total binding". Initially, the effect of various factors which may influence binding data other than those for which the Alzheimer's disease and control samples were matched, were assessed by dividing control subjects into appropriate subgroups. None of the measures were related to either sex or the hemisphere studied (right or left). However, the immediate preterminal state of the patients influenced the present data since for temporal cortex "glycine-stimulated' binding in control subjects that died of prolonged terminal illness was lower (100 $\pm$ 12 fmol/ mg protein, n = 7) than for subjects who died suddenly (156 $\pm$ 5 fmol/mg protein, n = 5; $p < 0.05$, Mann-Whitney U-test, Table 5.2). "Total binding" also showed this effect (Table 5.2). Furthermore, in the presence of glycine, the zinc IC_{50} value was higher ($p < 0.02$, Mann-Whitney U-test) in subjects who had died following a prolonged terminal illness (47 $\pm$ 11 μM, n=7) than in those who had died suddenly (22 $\pm$ 3 μM, n = 5; Table 5.2). For this reason values for this series of brains from the temporal cortex do not include subjects who died suddenly. However, the frontal cortex of these subjects did not show this effect (Table 5.2).

Few Alzheimer's disease subjects were free of drug medication, but mean binding values of drug-free (n =3) and drug treated subjects (n = 8) were similar. For example in the frontal cortex "total binding" (fmol/ mg protein) values of drug- free subjects (treated in parenthesis) were 240 $\pm$ 52 (239 $\pm$ 28).

5.2 Zinc inhibition of [^{3}H]-MK-801 binding to control tissue from human cortex.

In control tissue taken post- mortem from the parietal cortex, zinc potently inhibited [^{3}H]-MK-801 binding with an IC_{50} value of 30 $\pm$ 6 μM (mean $\pm$ SEM, n=3). In ante- mortem tissue from the frontal cortex the IC_{50} value was similar to that in post-mortem tissue, 23 $\pm$ 3 μM, n=3. By contrast, magnesium had no inhibitory properties at or below 100 μM (Fig. 5.1).

5.3. Zinc inhibition of [^{3}H]-MK-801 binding in Alzheimer's disease tissue from frontal and temporal cortex.

IC_{50} values for zinc were estimated using a number of concentrations between 1 and 100 μM in Alzheimer's disease and control tissue. Zinc potently and dose-dependently inhibited the binding of [^{3}H]-MK-801 in tissue from the frontal and the temporal cortex. There was no difference in the potency of zinc between Alzheimer's disease and control tissue (Fig. 5.2).

5.4 Effect of glutamate and glycine on [^{3}H]-MK-801 binding in control and Alzheimer's disease tissue from frontal and temporal cortex

In both areas of the cerebral cortex examined, binding in the presence of 30 μM glutamate was similar in Alzheimer's disease and control tissue (Fig. 5.3). In the presence of 30 μM glutamate and 3 μM glycine there was some reduction in [^{3}H]-MK-801 binding in Alzheimer's disease compared with control. However, "glycine-stimulated" binding showed the most obvious reduction in both areas in Alzheimer's disease tissue (p = 0.05, Mann-Whitney U-test, Fig 5.3).

5.5 Effect of glutamate and glycine on the association of [^{3}H]-MK-801 in Alzheimer's disease and control tissue.

Control and Alzheimer's disease tissue in the same experiments using frontal cortex from sub- groups of the subjects described above ; demography of groups, values are mean ± SEM for 4 controls (4 with Alzheimer's disease in parenthesis) were age 85 ± 8 (88 ± 3)y, delay to post-mortem 24 ± 1 (24 ± 3)h, storage time at -70 °C 7 ± 1 (6 ± 1)y, 4 right (2 right) hemispheres, 2 females (3 females) and 2 (3) subjects died after protracted terminal illness. The association of [^{3}H]-MK-801 was slow compared with control tissue (Fig. 5.4) using sub- maximal stimulating concentrations of glutamate (30 μM) and glycine (3 μM). This is consistent with the reduction seen in "glycine-stimulated" binding for similar samples (Figure 5.3). However, in the presence of supramaximal concentrations of glutamate (100 μM) and glycine (1000 μM see section 4.1), there was no difference in the association rate of [^{3}H]-MK-801 between Alzheimer's disease and control tissue. This was also the case for [^{3}H]-TCP binding (Fig. 5.5). The demography of groups for 4 controls (4 Alzheimer's disease subjects in parenthesis) was age 85 ± 3 (86 ± 1)y, delay to post-mortem 30 ± 6 (28 ± 7)h, storage time at -70 °C 6 ± 1 (5 ± 1)y, 3 (3) right hemispheres, 1 (1) female, all subjects died after protracted terminal illness.

5.6 Stimulation by spermidine of [^{3}H]-MK-801 binding to control tissue from frontal cortex.

The binding of [^{3}H]-MK-801 was enhanced after 45 min incubation with spermidine at 25 °C. This enhancement was dose- dependent reaching a maximum value of 319 ± 71 fmol/mg protein (n = 4, Fig 5.6) with 200 μM spermidine compared with 56 ± 5 fmol/mg protein in the absence of spermidine; the EC_{50} for spermidine was 89 ± 22 μM. The addition of 1 μM glutamate with 1 μM glycine significantly reduced the EC_{50} to 5.5 ± 0.7 μM ($p = 0.01$, Mann-Whitney U-test, n =4). The addition of supramaximal concentrations of glutamate (100 μM) and glycine (1000 μM, see section 4.1) did not significantly reduce the EC_{50} further (4.9 ± 0.4 μM). The concentration-response curves for spermidine enhancement of [^{3}H]-MK-801 binding were bell- shaped in the presence or absence of amino acids. At concentrations of

spermidine above 300 μM, a heavy precipitate was noticed in the incubation tubes which may account for the apparent inhibition seen at high spermidine concentrations. The demography of the 4 subjects used was age 83 $\pm$ 1 y, delay to post-mortem 33 $\pm$ 1 h, storage time at -70 °C 6 $\pm$ 1 y, 3 right hemispheres, 3 females, 2 subjects died after protracted terminal illness.

5.7 The effect of spermidine on the association of [^{3}H]-MK-801 in control tissue from the frontal cortex.

The association of [^{3}H]-MK-801 to cortical membranes (subjects as in section 5.6) is shown in Fig. 5.7. In the presence of 30 μM spermidine the rate of binding was markedly increased and reached equilibrium after 2 h compared with 6 h in the absence of spermidine.

5.8 The effect of ifenprodil, 7-chlorokynurenic acid and AP5 on [^{3}H]-MK-801 binding in control tissue from the frontal cortex.

In the presence of 300 μM spermidine, [^{3}H]-MK-801 was inhibited by AP5, 7-chlorokynurenic acid and ifenprodil (Table 5.3, subjects as in section 5.6). The rank order of potency for inhibition was AP5 > 7-chlorokynurenic acid > ifenprodil. Specific binding of [^{3}H]-MK-801 was totally abolished by all three inhibitors at a concentration of 1000 μM. Only ifenprodil had a Hill coefficient significantly different from unity (Table 5.3). By contrast AP5 and 7-chlorokynurenic acid had steep inhibition curves. The inhibition of spermidine-stimulated [^{3}H]-MK-801 binding was examined (subjects as in section 5.6) further by studying spermidine concentration-response curves in the presence of increasing concentrations of these three inhibitors (Fig. 5.8). With increasing concentrations of each inhibitor, the maximal binding induced by spermidine was reduced.

5.9. Stimulation of [^{3}H]-MK-801 binding by spermidine in Alzheimer's disease tissue from the frontal cortex.

In the absence of exogenous amino acids basal binding of [^{3}H]-MK-801 was similar for Alzheimer's disease and control tissue (142 $\pm$ 22 and 135 $\pm$ 14 fmol/mg protein respectively, n = 4, Fig 5.9). Spermidine stimulated [^{3}H]-MK-801 binding to control tissue from the frontal cortex in a concentration -dependent manner to a maximum of 370 $\pm$ 18 fmol/mg protein at 300 μM. However, there was no stimulation of [^{3}H]-MK-801 binding by spermidine in Alzheimer's disease tissue, at 300 μM binding was 133 $\pm$ 36 fmol/mg protein (Fig. 5.9). In the presence of 1 μM glutamate and 1 μM glycine basal binding was significantly increased for control tissue to 334 $\pm$ 36 fmol/mg protein. By contrast [^{3}H]-MK-801 binding was not increased in Alzheimer's disease tissue (167 $\pm$ 21 fmol/mg protein) in the presence of glutamate and glycine. Furthermore, although further enhancement of [^{3}H]-MK-801 binding was seen with spermidine in control tissue (maximum value 469 $\pm$ 21 fmol/mg protein with 300 μM spermidine) no further enhancement was seen in Alzheimer's disease tissue (253 $\pm$ 36 fmol/mg protein). The demography of the subjects used, 4 controls (4 with Alzheimer's disease in parenthesis) was age 82 $\pm$ 3 (84 $\pm$ 1)y, delay to post-mortem 24 $\pm$ 0 (29 $\pm$ 1)h, storage time at -70 °C 7 $\pm$ 2 (7 $\pm$ 1)y, 2,(1) females, 3 (3) right hemispheres, 2 (3) subjects died after protracted terminal illness.

TABLE 5.1 DEMOGRAPHY OF SUBJECTS STUDIED.

	AGE (y)	POST-MORTEM DELAY (h)	HEMISPHERE STUDIED	STORAGE TIME AT -70°C (y)	SEX	AGONAL STATE
FRONTAL CORTEX						
CONTROL TISSUE. n = 12	67 - 94 (84±2)	6 - 48 (27±4)	10R, 2L	4 - 9 (7±1)	5F, 7M	6P, 6S
AD TISSUE n = 12	62 - 92 (82±2)	14 - 48 (25±9)	7R, 5L	3 - 8 (5±1)	8F, 4M	11P, 1S
TEMPORAL CORTEX						
CONTROL TISSUE n = 12	67 - 94 (84±2)	6 - 48 (27±4)	10R, 2L	4 - 9 (6±1)	6F, 6M	7P, 5S
AD TISSUE n = 12	62 - 92 (82±2)	14 - 48 (25±9)	7R, 5L	3 - 8 (5±1)	8F, 4M	11P, 1S

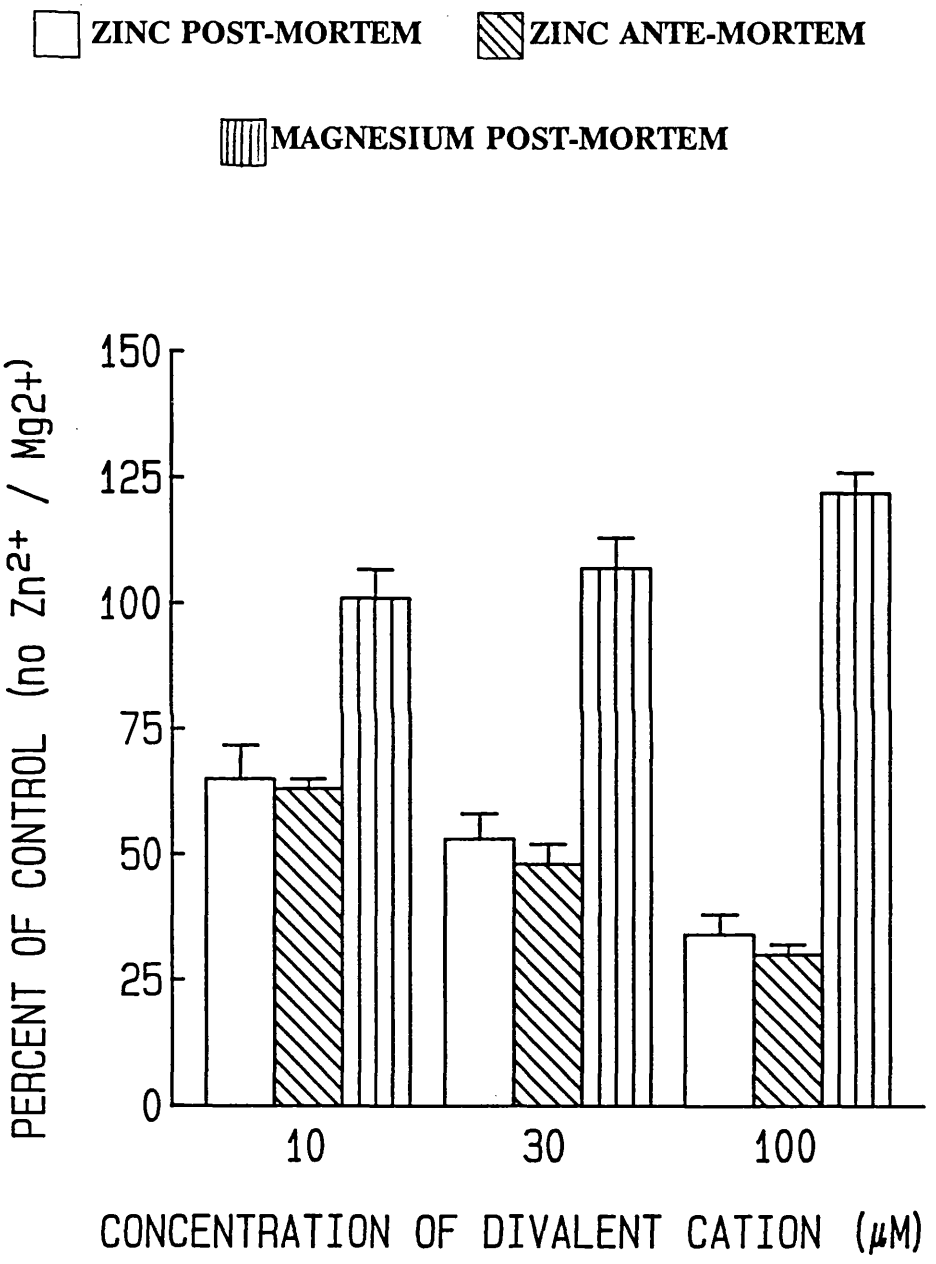
Mean ± SEM values given in parenthesis.
Abbreviations: y = years, h = hours, R = right, L = left, F = female, M = male.
P = protracted death eg bronchopneumonia, S = sudden death eg cardiac arrest,

TABLE 5.2. THE EFFECT OF IMMEDIATE PRETERMINAL STATUS ON THE STIMULATION OF [^{3}H]-MK-801 BINDING BY GLUTAMATE AND GLYCINE IN CONTROL TISSUE FROM FRONTAL AND TEMPORAL CORTEX.

	SPECIFIC BINDING (fmol/ mg protein)		ZINC IC_{50} VALUE (μm)	
	SUDDEN DEATH	PROTRACTED DEATH	SUDDEN DEATH	PROTRACTED DEATH
FRONTAL CORTEX				
Glutamate stimulated	152 ± 23 (6)	192 ± 20 (6)	47 ± 7	64 ± 9
Total binding	271 ± 29 (6)	325 ± 28 (6)	26 ± 6	31 ± 6
Glycine stimulated	119 ± 12 (6)	132 ± 12 (6)		
TEMPORAL CORTEX				
Glutamate stimulated	208 ± 21 (5)	181 ± 13 (7)	53 ± 5	77 ± 14
Total binding	364 ± 29 (5)	281 ± 23 (7)*	22 ± 3	47 ± 13*
Glycine stimulated	156 ± 15 (5)	103 ± 14 (7)*		

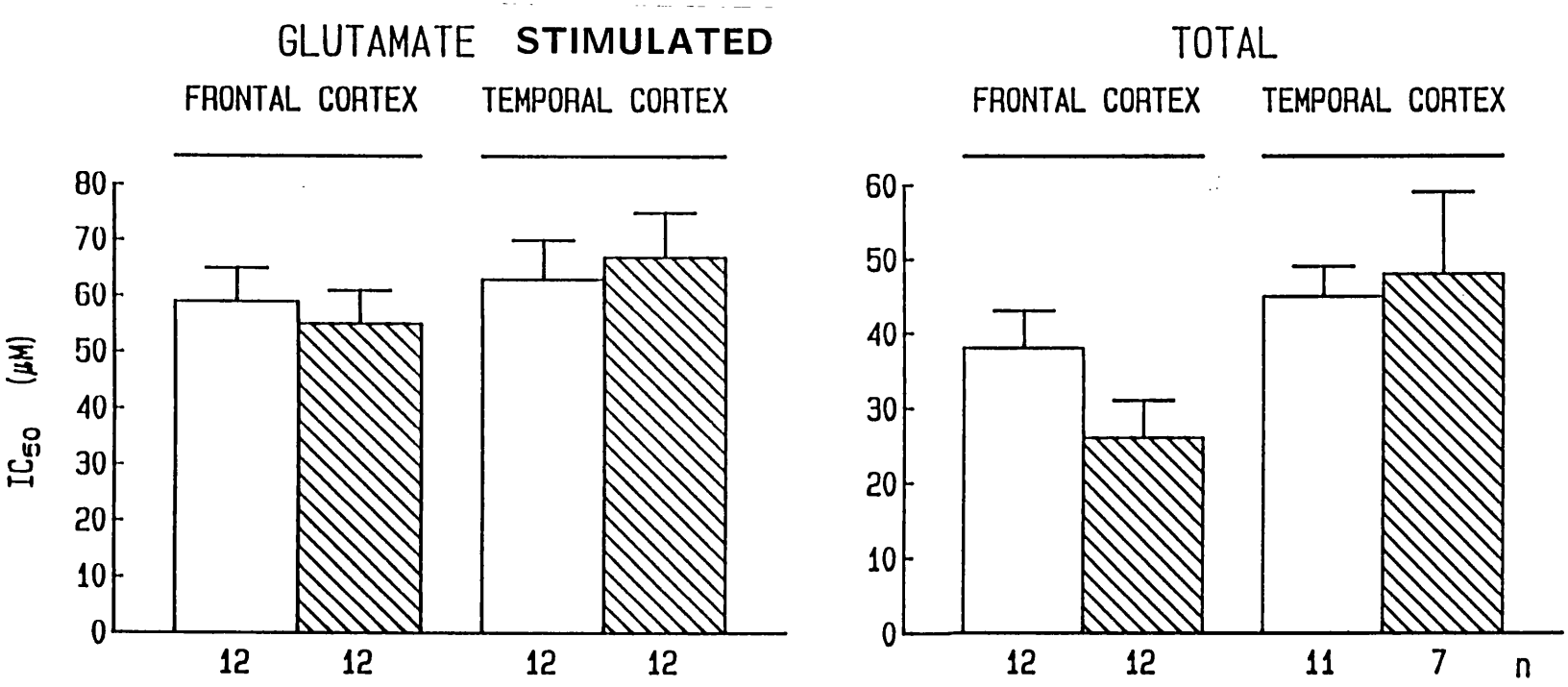
Experiments were performed with membranes incubated for 45 min at 25 °C in Tris-Hcl buffer using a final concentration of 5 nM [^{3}H]-MK-801. Assays were in the presence of either 30 μM glutamate ("glutamate stimulated", B) or 30 μM glutamate and 3 μM glycine ("Total binding", A). "Glycine stimulated" binding was calculated as A - B (data for individual subjects).
Values are mean ± SEM with the number of subjects examined in parenthesis
* significantly different to sudden death ($p < 0.05$, Student's t-test).

FIGURE 5.1. THE EFFECT OF ZINC AND MAGNESIUM ON [^{3}H]-MK-801 BINDING IN CONTROL TISSUE.



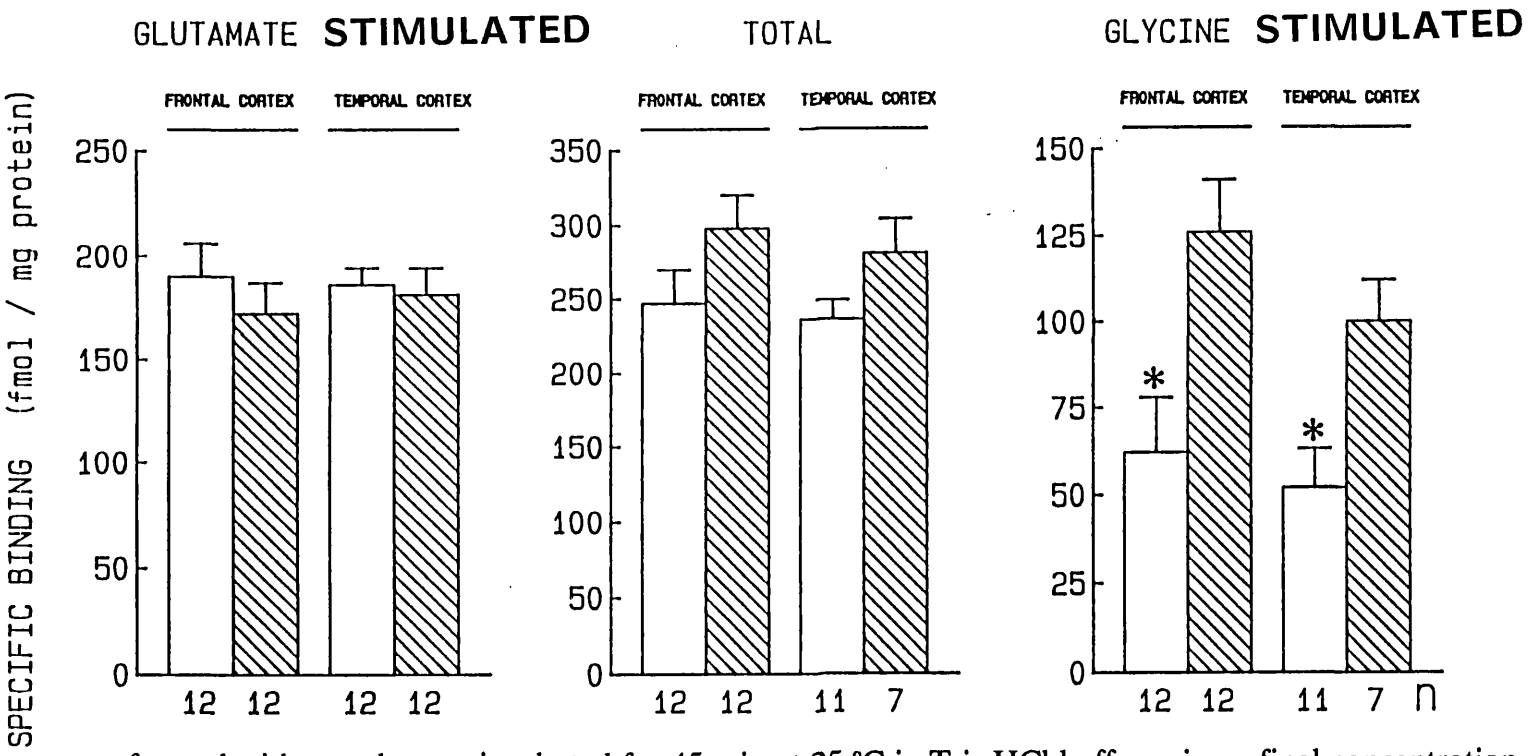
Experiments were performed with membranes incubated at 25 °c in Tris-HCl buffer using a final concentration of 5 nM [^{3}H]-MK-801 in the presence of 30 μM glutamate and 3 μM glycine.
Tissue was obtained from either the parietal cortex (post-mortem) or the frontal cortex (ante-mortem).
Values are mean $\pm$ SEM, n = 3.
All values in the presence of zinc are significantly different from control (Kruskal-Wallis ANOVA and Mann-Whitney U-test)

FIGURE 5.2. THE EFFECT OF ZINC ON [^{3}H]-MK-801 BINDING IN ALZHEIMER'S DISEASE AND CONTROL TISSUE FROM FRONTAL AND TEMPORAL CORTEX



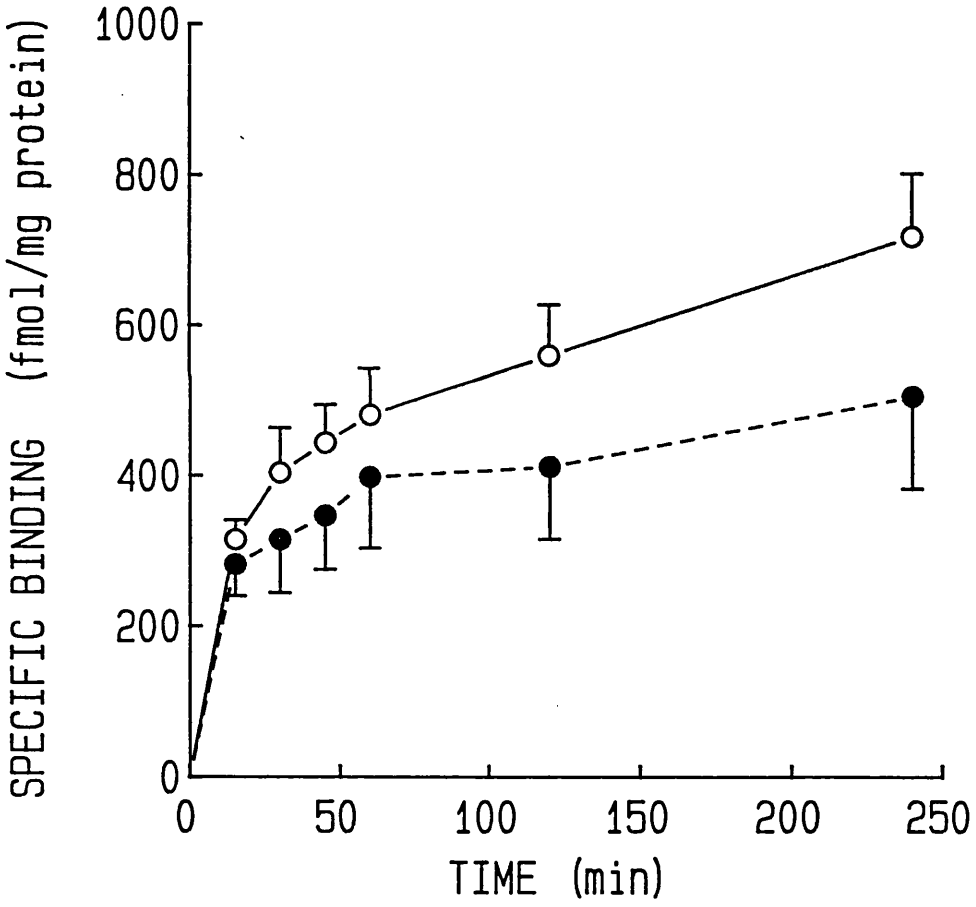
Experiments were performed with membranes incubated for 45 min at 25 °C in Tris-HCl buffer using a final concentration of 5 nM [^{3}H]-MK-801. Assays were in the presence of either 30 μM glutamate ("glutamate stimulated") or 30 μM glutamate and 3 μM glycine ("Total binding"). Values are mean ± SEM with the number of subjects examined beneath the X axis. Where immediate pre-mortem status complicated comparisons (groups where n < 12: see text) only data for subjects with protracted illness is presented.
There were no significant differences between Alzheimer's disaese and control tissue.
Open bars are Alzheimer's disease tissue, hatched bars are control.

FIGURE 5.3. THE EFFECT OF GLUTAMATE AND GLYCINE ON [^{3}H]-MK-801 BINDING IN ALZHEIMER'S DISEASE AND CONTROL TISSUE FROM FRONTAL AND TEMPORAL CORTEX



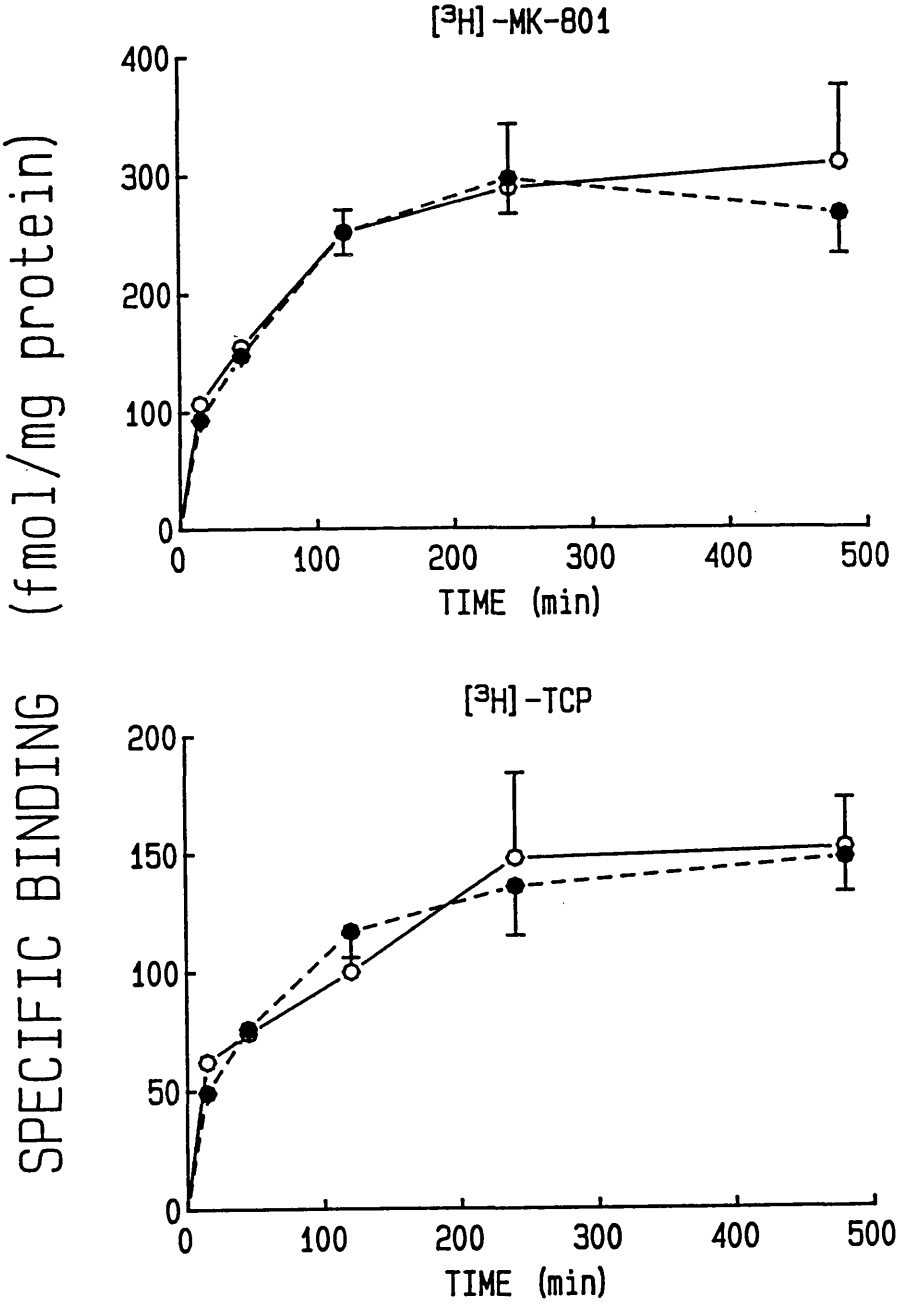
Experiments were performed with membranes incubated for 45 min at 25 °C in Tris-HCl buffer using a final concentration of 5 nM [^{3}H]-MK-801. Assays were in the presence of either 30 μM glutamate ("glutamate-stimulated binding was calculated as A - B (data for individual subjects).
Values are mean $\pm$ SEM with the number of subjects examined beneath the X axis. Where immediate pre-mortem status complicated comparisons (groups where n < 12: see text) only data for subjects with protracted illness is presented.
* significantly different to control (p = 0.05, Mann-Whitney U-test).
Open bars are Alzheimer's disease tissue, hatched bars are control.

FIGURE 5.4. THE ASSOCIATION OF [^{3}H]-MK-801 IN ALZHEIMER'S DISEASE AND CONTROL TISSUE IN THE PRESENCE OF 30 μM GLUTAMATE AND 3 μM GLYCINE



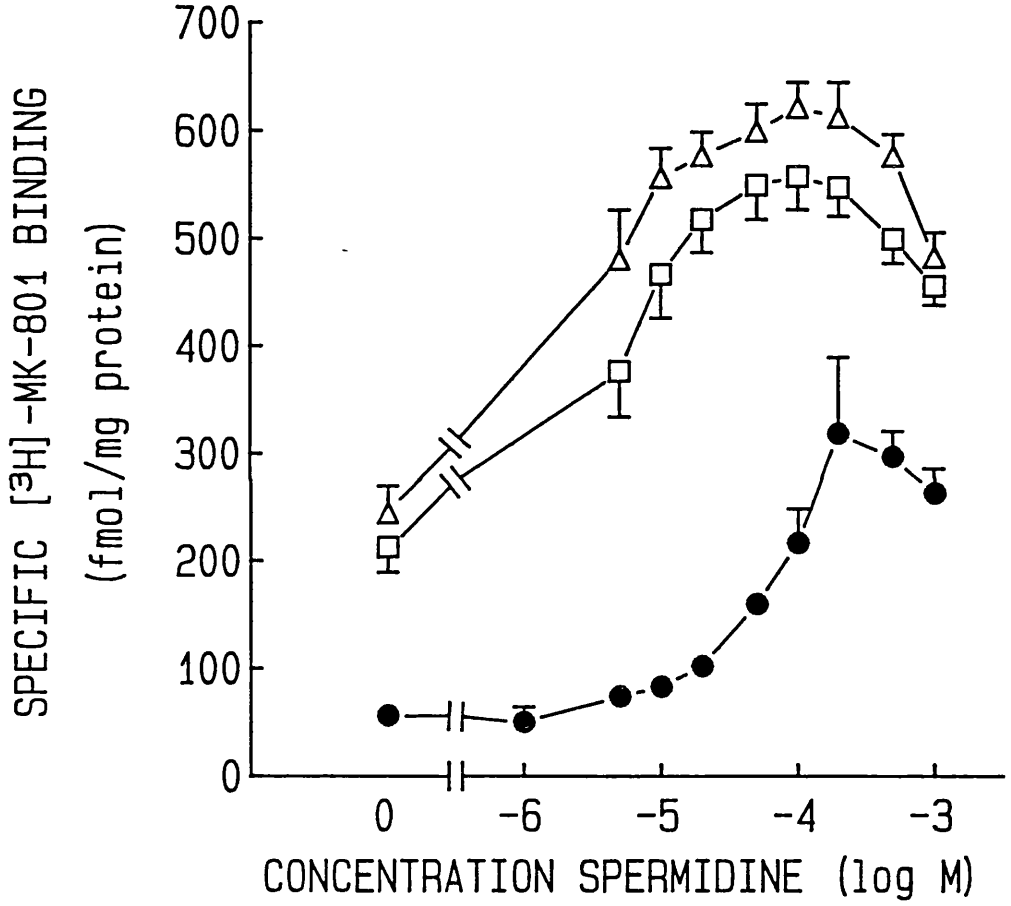
Experiments were performed with membranes from the frontal cortex incubated at 25 °c in Tris-HCl buffer using a final concentration of 5 nM [^{3}H]-MK-801 in the presence of 30 μM glutamate and 3 μM glycine.
Open circles represent control tissue, closed circles represent AD tissue.
Values are mean $\pm$ SEM, n = 4.
There was no significant difference between Alzheimer's disease and control tissue.

FIGURE 5.5. THE ASSOCIATION OF [^{3}H]-MK-801 AND [^{3}H]-TCP IN ALZHEIMER'S DISEASE AND CONTROL TISSUE IN THE PRESENCE OF 100 μM GLUTAMATE AND 1000 μM GLYCINE



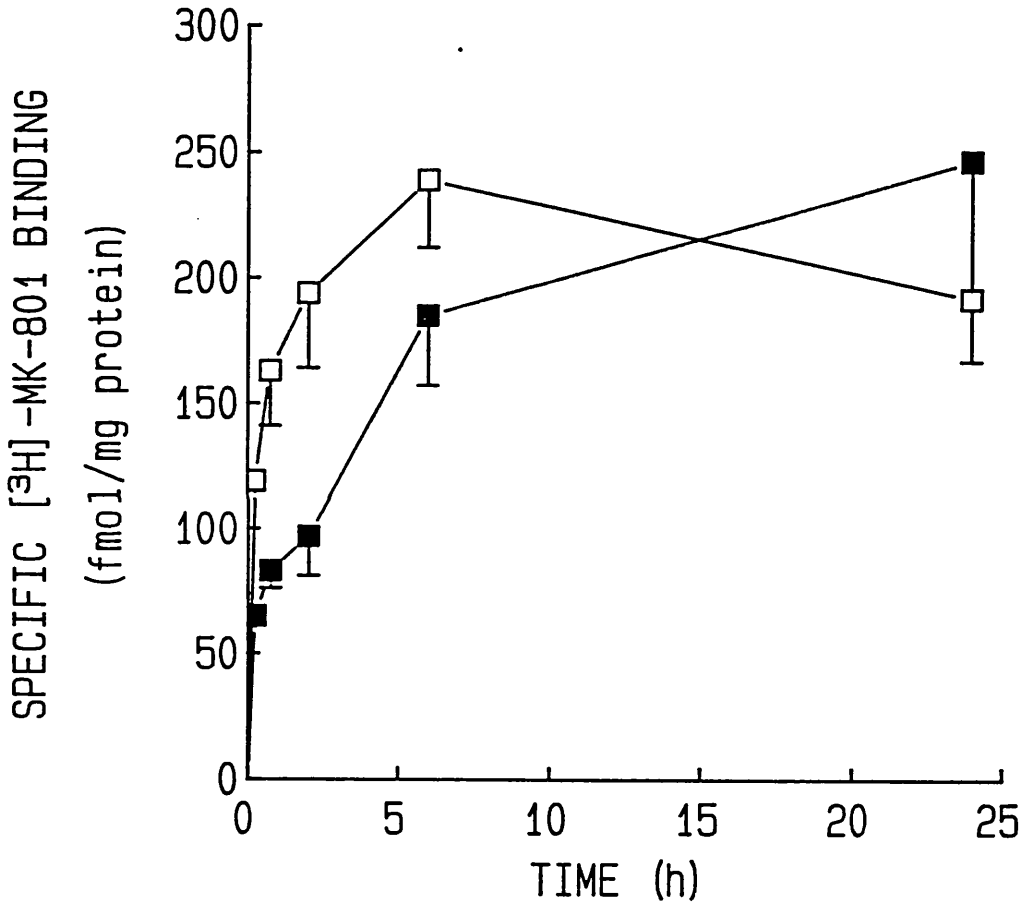
Experiments were performed with membranes from the frontal cortex incubated at 25 °C in Tris-HCl buffer using a final concentration of 5 nM [^{3}H]-ligand in the presence of 100 μM glutamate and 1000 μM glycine. Open circles represent control tissue, closed circles represent AD tissue. Values are mean $\pm$ SEM, n = 4.

FIGURE 5.6. THE EFFECT OF SPERMIDINE ON [^{3}H]-MK-801 BINDING IN CONTROL TISSUE FROM THE FRONTAL CORTEX.



Experiments were performed with membranes from the frontal cortex incubated at 25 °c in Tris-HCl buffer using a final concentration of 5 nM [^{3}H]-MK-801 in the absence (●) or the presence of either 1 μM glutamate and 1 μM glycine (□) or 100 μM glutamate and 1000 μM glycine (△).
Values are mean $\pm$ SEM, n = 4.

FIGURE 5.7. THE EFFECT OF SPERMIDINE ON THE ASSOCIATION OF [^{3}H]-MK-801 IN CONTROL TISSUE FROM THE FRONTAL CORTEX.



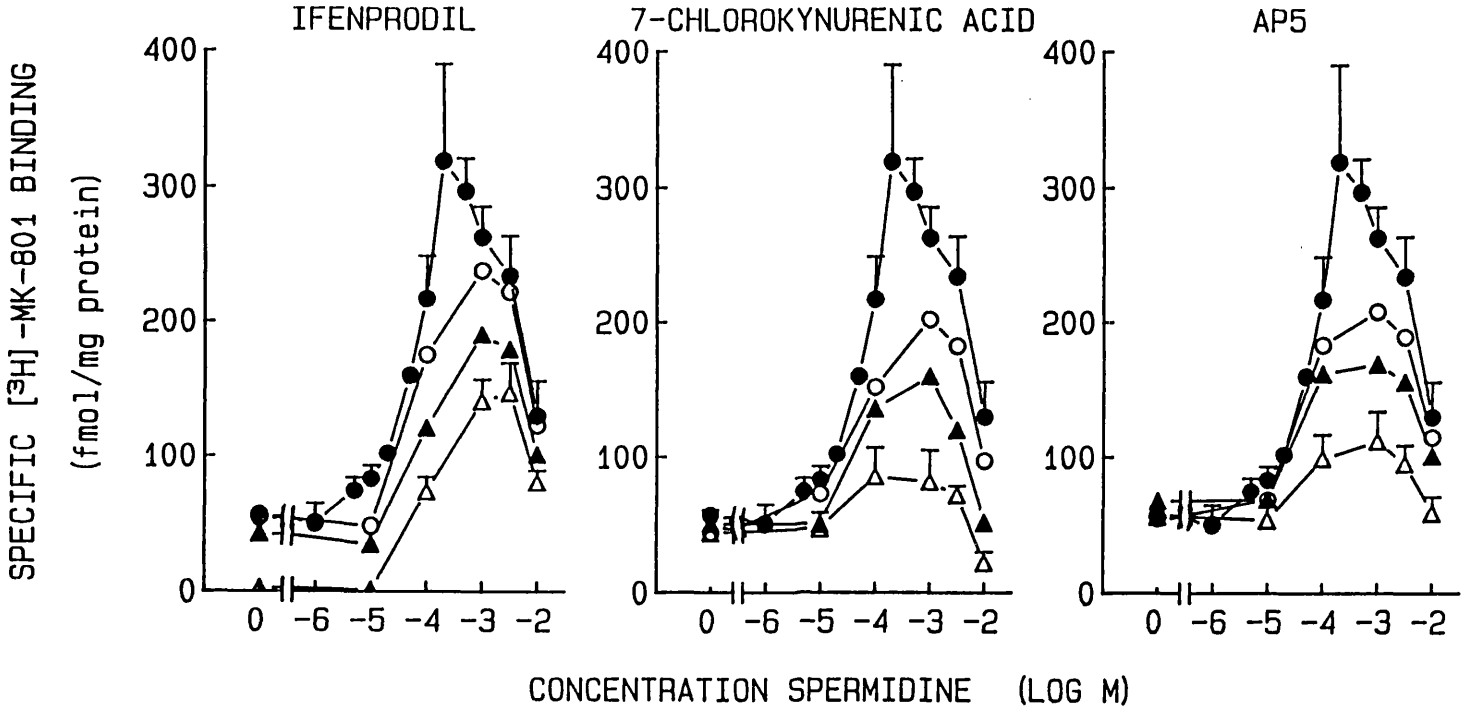
Experiments were performed with membranes from the frontal cortex incubated at 25 °C in Tris-HCl buffer using a final concentration of 5 nM [^{3}H]-MK-801 in the absence (■) or the presence of 30 μM spermidine (□).
Values are mean ± SEM, n = 4.

TABLE 5.3. IC_{50} VALUES FOR IFENPRODIL, 7-CHLOROKYNURENIC ACID AND AP5 INHIBITION OF SPERMIDINE-STIMULATED [^{3}H]-MK-801 BINDING IN HUMAN FRONTAL CORTEX.

DRUG	MEAN IC_{50} VALUE (μM)	HILL COEFFICIENT
IFENPRODIL	91 $\pm$ 28	0.78 $\pm$ 0.05*
7-CHLOROKYNURENIC ACID	24 $\pm$ 19	0.86 $\pm$ 0.06
AP5	0.5 $\pm$ 0.3	1.0 $\pm$ 0.06

Experiments were performed with membranes incubated for 45 min at 25 °C in Tris-HCl buffer using a final concentration of 5 nM [^{3}H]-MK-801 in the presence of 300 μM spermidine and 6 concentrations of drug (0.01 - 1000 μM).
IC_{50} values and Hill coefficients were estimated using linear regression analysis of Hill plots.
Values are mean $\pm$ SEM , n= 4.
* significantly different to unity ($p < 0.05$).

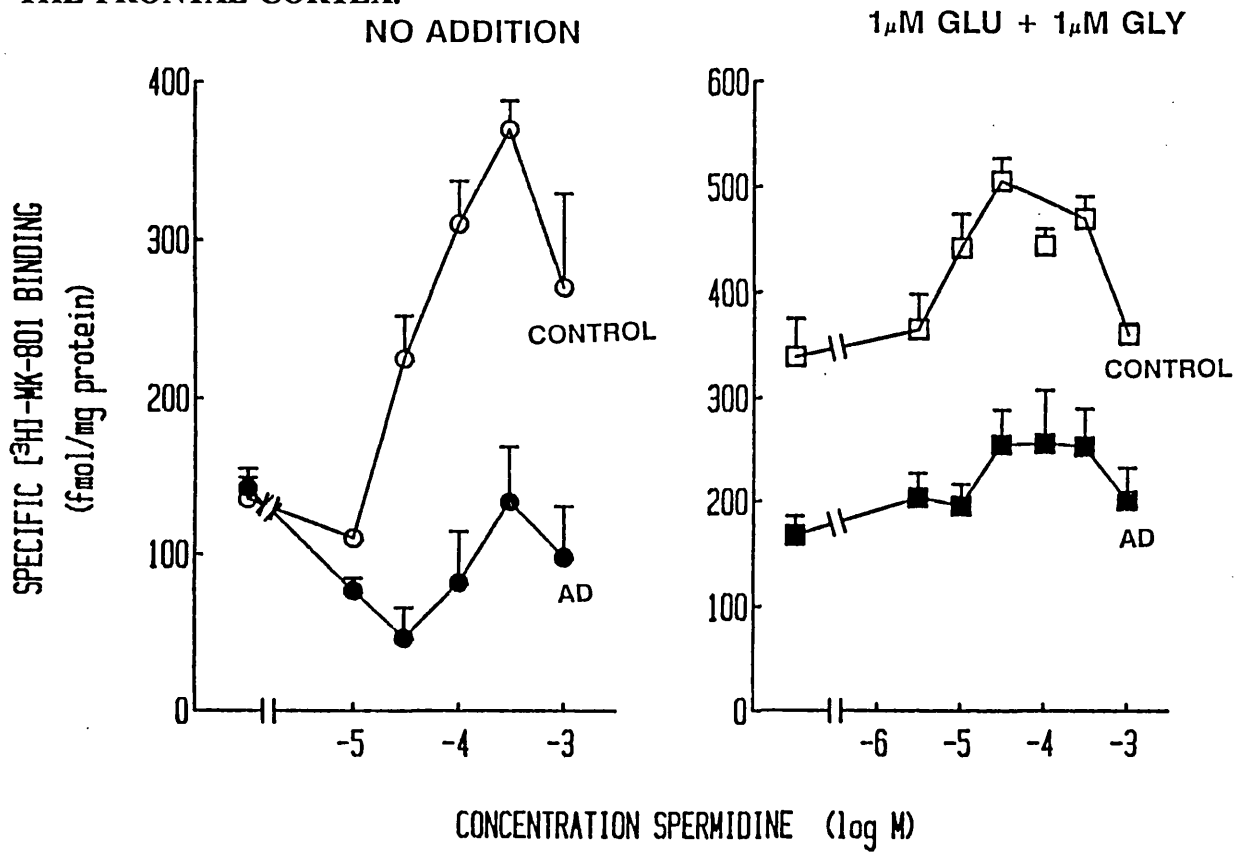
FIGURE 5.8. THE EFFECT OF A NUMBER OF INHIBITORS ON SPERMIDINE -STIMULATED [^{3}H]-MK-801 BINDING IN HUMAN FRONTAL CORTEX.



Experiments were performed with membranes incubated for 45 min at 25 °C in Tris-HCl buffer using a final concentration of 5 nM [^{3}H]-MK-801. Concentration -response curves to spermidine were determined in the absence (●) and in the presence of increasing concentrations of ifenprodil, 1 μM (o), 10 μM (▲) and 100 μM (△); 7-chlorokynurenic acid, 0.3 μM (o), 1 μM (▲) and 3 μM (△); AP5, 0.1 μM (o), 0.3 μM (▲) and 1 μM (△).

Values are mean ± SEM, n = 4. For clarity, error bars were omitted from the lowest two concentrations of inhibitor but were always less than 15% of the mean value. With increasing concentrations of each drug the maximal binding induced by spermidine was reduced ($p < 0.001$, Student's t-test); this is consistent with a non-competitive mode of action for these 3 inhibitors.

FIGURE 5.9. THE EFFECT OF SPERMIDINE ON [^{3}H]-MK-801 BINDING IN ALZHEIMER'S DISEASE AND CONTROL TISSUE FROM THE FRONTAL CORTEX.



Experiments were performed with membranes incubated for 45 min at 25 °C in Tris-HCl buffer using a final concentration of 5 nM [^{3}H]-MK-801 in the absence or the presence of 1 μM glutamate and 1 μM glycine.
Open symbols represent control tissue, closed symbols represent Alzheimer's disease tissue.
Values are mean $\pm$ SEM, n = 4.

5.9 DISCUSSION

Post- mortem human brain is often used for neurochemical research and it is widely known that measures may be influenced by many factors such as patient age, sex, drug treatment, preterminal status and post- mortem delay (see Bowen et al., 1977, and section 1.4.1). In this study there were no effects of age, sex, drug treatment or post- mortem delay. However, in the temporal cortex there seemed to be an effect of preterminal status on the glycine regulation of [^{3}H]-MK-801 binding. Subjects who had died following a prolonged terminal coma had lower [^{3}H]-MK-801 binding in the presence of glycine compared with subjects who had died suddenly. This factor is known to affect other neurochemical measures including glutamate decarboxylase, (Bowen et al., 1976a, Bowen et al., 1977, Spokes, 1979, Perry et al., 1982, Harrison et al., 1990), GABA content (Lowe et al., 1988) and detection of messenger RNAs (Harrison et al., 1990), all of which are reduced following prolonged terminal coma. Furthermore, Procter et al., 1989a reported a similar effect of prolonged terminal coma on glycine regulation of [^{3}H]-MK-801 binding in the frontal cortex. For this reason, in the temporal cortex, in order to separate disease related changes from peri- mortem artifacts, only values for subjects who died following a prolonged terminal coma are presented. Where only one cortical region is studied, tissue was taken from the frontal cortex only, where no significant effect of agonal state was observed in this study.

In the previous chapter the effects of glutamate and glycine on the association of [^{3}H]-MK-801 were described. From these data it was apparent that at least 2 h incubation at 25°C was needed for [^{3}H]-MK-801 to equilibrate even under conditions of high glutamate and glycine stimulation (see section 4.1). Under these maximally stimulating conditions of amino acids no difference in the number of [^{3}H]-MK-801 or [^{3}H]-TCP binding sites between Alzheimer's disease and control tissue was observed with this preparation. Here the modulation of the NMDA receptor complex by glutamate, glycine, spermidine and zinc in both control and Alzheimer's disease tissue has been investigated. Since the modulation of [^{3}H]-MK-801 binding was under study, non-equilibrium conditions were chosen, usually 45 min incubation

at 25°C in the presence of 30 μM glutamate and 3 μM glycine. Furthermore, since [^{3}H]-TCP binding was variable and did not appear to be affected in Alzheimer's disease (see Chapter 4), [^{3}H]-MK-801 was the ligand of choice for this study.

Zinc inhibition of [^{3}H]-MK-801 binding has been reported in rat brain (Reynolds & Miller, 1988) with an IC_{50} value of 6 $\pm$ 1 μM. In this study, in both post- mortem tissue and ante- mortem tissue zinc inhibited [^{3}H]-MK-801 binding with comparable potency (Fig.5.1). However, this was lower than in the rat. Further experiments are needed to investigate whether this is due to the prior freezing of the material, a precaution necessary for work with diseased human tissue (section 2.2.1). Zinc inhibition of [^{3}H]-MK-801 in human brain provides further evidence that the NMDA receptor complex is similar in rat and human cortex. There was no reduction in zinc regulation of [^{3}H]-MK-801 binding in Alzheimer's disease (Fig.5.2).

Spermidine enhancement of [^{3}H]-MK-801 binding was originally reported by Ransom & Stec (1988). This study demonstrates that a similar regulation also occurs in human brain (Fig.5.6). Like zinc, spermidine was markedly less potent in human tissue than in rat brain; EC_{50} = 89 $\pm$ 22 μM compared with 19.4 $\pm$ 4.6 μM reported by Ransom & Stec (1988).

The presence of the precipitate at high spermidine concentrations makes it difficult to interpret the biphasic effect of this compound. It is possible however that spermidine has stimulatory properties at low concentrations whereas it inhibits the binding of [^{3}H]-MK-801 at higher concentrations. In addition, <u>in vitro</u> and <u>in vivo</u> data is contradictory. All reports <u>in vitro</u> (Ransom & Stec, 1988, Carter et al., 1989, Reynolds & Miller, 1989, Williams et al., 1989, Robinson et al., 1990) report a stimulation of the NMDA receptor complex with spermidine whereas a report <u>in vivo</u> is of a negative modulation (Rao et al., 1990). Ifenprodil reversed the spermidine stimulation of [^{3}H]-MK-801 binding in human brain (Fig.5.8). This observed spermidine antagonism is in agreement with Carter et al., (1989) who demonstrated that ifenprodil blocked the enhancement of [^{3}H]-TCP binding to rat

forebrain membranes. At concentrations of ifenprodil below 10 μM these workers report a rightward parallel shift of the dose response curve for spermidine, leading them to suggest that ifenprodil is a competitive antagonist at the polyamine site. However, in the present study ifenprodil did not cause a shift in the ascending part of the concentration response curves but did reduce the maximal binding, consistent with a non-competitive mode of action. Furthermore, the potency of ifenprodil was markedly less than AP5 and 7-chlorokynurenic acid in this system (Table 5.3). Reynolds & Miller (1989) also suggest that ifenprodil is not a competitive antagonist of spermidine- stimulated [^{3}H]-MK-801 binding. The pharmacology of the polyamine site has now been studied in a number of systems but its precise location and mechanism of action at the NMDA receptor complex remains to be established. It is unclear whether further indirect measures of polyamine function (eg [^{3}H]-MK-801 binding) are sensitive enough to elucidate these details.

The excitotoxic hypothesis of cell death (Olney, 1970) received considerable impetus with the realization that Ca^{2+} entry through the NMDA receptor linked channel is a major contributing factor (see section 1.77, also Meldrum & Garthwaite, 1990). Attention has recently been focussed on the anti-ischemic potential of ifenprodil and other compounds which appear to be atypical antagonists of the NMDA receptor complex (eg SL 82.0715 see Carter et al., 1989). If these compounds were acting selectively at a polyamine site associated with the NMDA receptor complex, a different target for the protection from excitotoxic cell death which may underlie some of the pathology associated with Alzheimer's disease may be found.

In the presence of glutamate and glycine the EC_{50} for spermidine was markedly reduced (Fig. 5.6). This suggests that the cooperative modulation of [^{3}H]-MK-801 binding by glutamate, glycine and polyamines observed in rat tissue (Ransom & Stec, 1988) also occurs in human brain. In the presence of maximally stimulating concentrations of glutamate and glycine (100 μM and 1000 μM respectively, see section 4.1), the stimulation of [^{3}H]-MK-801 binding was still observed. This may suggest that spermidine acts at a separate site sensitive only to polyamines and not

at the glutamate or the glycine site. Indeed, this was proposed by Ransom & Stec in rat tissue since they report additional effects of spermidine above that found for concentrations of glutamate and glycine that produce maximal binding. However, an additional effect of spermidine above that seen for glutamate and glycine was not observed by Robinson et al. (1990) using rat brain, this may reflect the different assay conditions used. NMDA receptors expressed in xenopus oocytes may provide ideal conditions for the study of modulators of the NMDA receptor linked channel because endogenous levels of amino acids and polyamines are low. In this system, the effect of spermine was additive above that seen with saturating concentrations of glutamate and glycine (McGurk et al., 1990). Furthermore, spermine was inactive without the addition of low concentrations of glutamate and glycine. Agonist activation of the glutamate site has an absolute requirement for glycine (Kleckner & Dingledine, 1988) and these data suggest an absolute requirement for glutamate or glycine also exists for polyamine activation.

The enhancement of [^{3}H]-MK-801 binding in human cortical membranes clearly reflects an increase in the association rate of the ligand (Fig. 5.7). This effect mimics the action of glutamate and glycine and has also been reported in rat brain by Robinson et al. (1990). Ransom & Stec (1988) have proposed that glutamate and glycine regulate binding by controlling access of ligands to a transiently accesible or "guarded" site located inside the receptor-coupled ion channel. The present data also suggest that a polyamine may also control the access of [^{3}H]-MK-801 to this site.

The effects of glutamate and glycine on [^{3}H]-MK-801 in control tissue were described in detail in section 4.1. Here the effects of glutamate and glycine on [^{3}H]-MK-801 binding in Alzheimer's disease tissue are described and compared with control tissue reported. In this study a severely affected area (temporal cortex, see Chapter 3) and a less affected area (frontal cortex) have been studied. In both these areas, Alzheimer's disease and control tissue was equally sensitive to glutamate (Fig. 5.3), consistent with Cowburn et al. (1988a) who used [^{3}H]-glutamate binding. This was

not found for the effect of glycine, as "glycine-dependent" binding of [^{3}H]-MK-801 was reduced in both frontal (BA 10) and temporal (BA 21/22) cortex (Figure 5.3). The results for frontal cortex agree with Procter et al. (1989a) and the observation in the temporal cortex extends these findings. Previously, BA20 (Procter et al., 1989a) was assayed where the pathology of Alzheimer's disease tends to be more prominent than in BA 21/22 (see Chapter 3, also Fig.1.4). It is possible that different results for these areas of the temporal lobe are because loss of structures organised in a columnar manner are less pronounced than in BA20 (see Procter et al., 1988).

The loss of glycine regulation seen in Alzheimer's disease makes it difficult to interpret the similar reduction in spermidine regulation in the frontal cortex. Since this and other studies (Ransom & Stec, 1988, Robinson et al., 1990) have reported an allosteric interaction between glycine and spermidine it seems likely that an alteration in one "site" will effect the other. Although the membrane preparation involves extensive washing, there is probably still glutamate and glycine present in the tissue (approximately 0.1 nmol/mg protein, see Stirling et al., 1989). Therefore, in this system it is impossible to investigate the action of a modulator at one site only. In the absence of exogenous amino acids and spermidine (basal conditions) there was no difference in [^{3}H]-MK-801 binding in Alzheimer's disease and control tissue. However, in the presence of a number of concentrations of spermidine there was a marked difference in the binding of [^{3}H]-MK-801 in Alzheimer's disease and control tissue. This may suggest that the effect of spermidine is independent of the effect of glycine in Alzheimer's disease tissue. In the presence of glycine, the reduction of spermidine regulation of [^{3}H]-MK-801 binding in Alzheimer's disease is more marked, probably due to an additive effect of the two. The reduction of glycine and spermidine regulation of [^{3}H]-MK-801 binding in Alzheimer's disease may provide a starting point for rational pharmacotherapy. This is discussed more fully in the following sections.

CHAPTER 6

DRUGS AND ALZHEIMER'S DISEASE

RESULTS

6.1 The influence of tacrine on amino acid release in vivo.

The influence of ip administration of 10 mg/kg tacrine on extracellular concentrations of aspartate, asparagine, glutamine, serine, GABA (Fig.6.1), glycine and taurine (data not shown) was assessed over 2 h in perfusates collected by in vivo dialysis from the frontal cortex of conscious animals. The only significant change was in the concentration of glutamine, which was collected 20 min after application of drug. Animals apparently responded to the drug since hypersalivation, hyperlacrimation and tremor always occurred in drug-treated but not in vehicle-treated animals. No convulsions were observed.

Tacrine was also added to the perfusion solution to study its effect on the extracellular concentrations in anaesthetized animals. Drug (1 and 5 mM) was perfused for 40 min, but only the highest concentration had significant effects. Perfusate concentrations of asparagine, GABA, serine (Fig. 6.2) glycine and taurine (data not shown) were unaffected, whereas the concentration of aspartate, glutamate and asparagine were increased (Fig.6.2). Aspartate and glutamate concentrations increased by 168 and 370% respectively (values are the highest amino acid concentrations after treatment expressed as a percentage of the mean concentration before treatment). Glutamine concentration appeared to show even greater changes (787%) but was subject to unexplained variability. This latter change was apparently sensitive to tetrodotoxin (Fig.6.2), whereas the changes in glutamate and aspartate concentration were insensitive to this treatment. All changes were apparent in the first 10 min fraction obtained during drug administration and were maintained during the entire period of application and persisted after the drug was withdrawn (Fig.6.2).

6.2 The influence of tacrine on amino acid release in vitro.

Figure 6.3 shows the effect of tacrine on the K^+ -evoked release of amino acids in the presence and absence of 1.3 mM Ca^{2+}. Studies with cortical tissue prisms showed that the release of glutamate after 4 min incubation was 178 $\pm$ 11 % (n=3) of that after 2 min (Najlerahim et al., 1990). Tacrine at low concentrations (0.05 and 0.1 mM, data for 0.05 mM not shown) did not significantly alter the amount of amino acid released compared with control (no tacrine), in incubations with and without Ca^{2+}. At the higher concentrations of tacrine in the absence of Ca^{2+}, more amino acids were released than control. In the presence of Ca^{2+}, release was inhibited by 1 mM tacrine. The Ca^{2+}-dependent release was calculated for each incubation, based on the numerical difference between the release in the presence and absence of Ca^{2+} (Fig 6.4). Except for 0.05 mM, all the doses of tacrine significantly reduced the Ca^{2+}-dependent release of aspartate and glutamate. Significant effects on GABA were also seen at concentrations of 1 and 5 mM tacrine. The effect of tacrine was specific for aspartate, glutamate and GABA as no effect of this drug was seen on non-transmitter amino acids (data not shown). The inhibition of amino acids release by tacrine was independent of the duration of exposure to the drug (t= 15, 30, 45 and 60 min, data not shown).

6.3 The influence of tacrine on D-[^{3}H]aspartic acid uptake.

Specific uptake of D-[^{3}H]aspartic acid into preparations of neocortical tissue containing synaptosomes was linear for 6 min, displayed saturable kinetics and was dependent on intact structure (sensitive to osmotic lysis) and intracellular energy (inhibited by an uncoupler of oxidative phosphorylation, data not shown). After 15 min pre-incubation, tacrine (50- 1500 μM) inhibited high affinity uptake of D-[^{3}H]aspartic acid in a dose-dependent manner, with a mean ($\pm$ SEM) IC_{50} value of 614 $\pm$ 20 μM (Fig. 6.5). Pre-incubation with tacrine for longer (see above) gave similar results (data not shown).

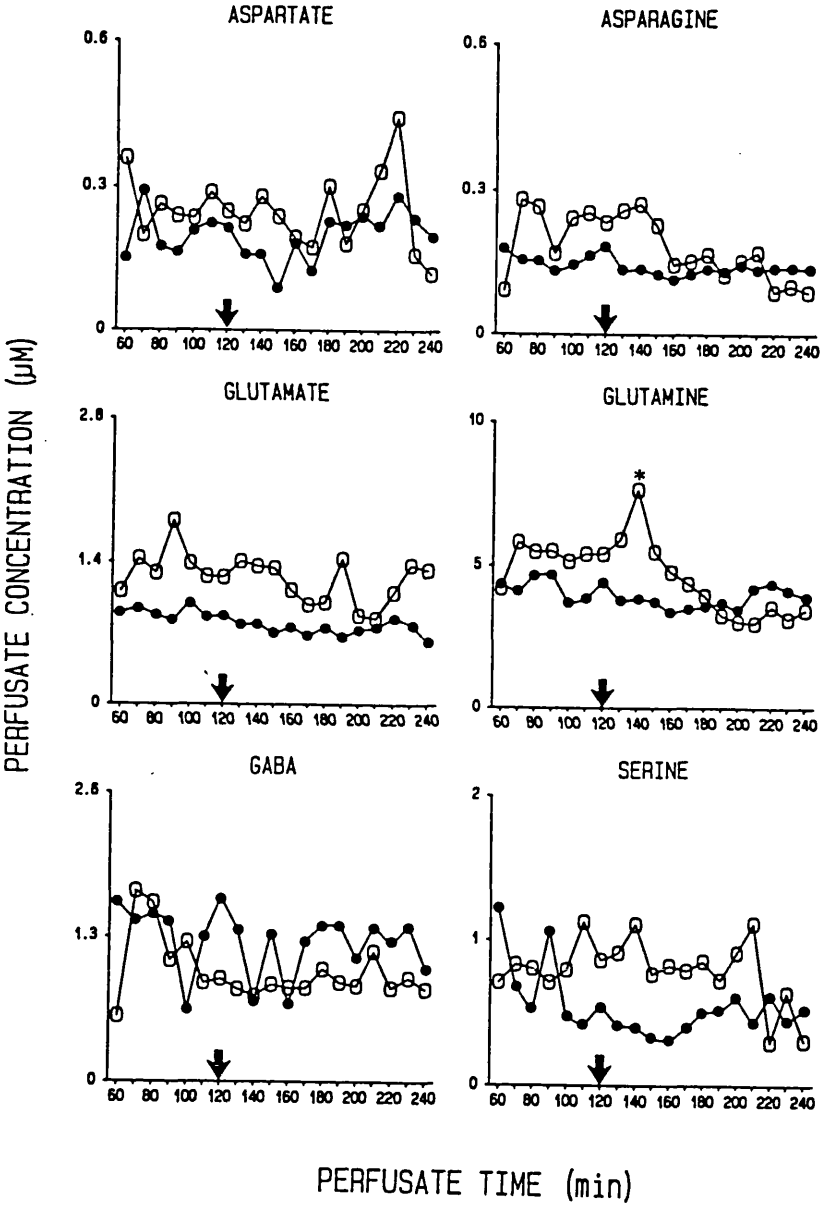
6.4 The influence of tacrine on [^{3}H]-MK-801 binding in rat and human cortex.

In the presence of 30 μM glutamate and 3 μM glycine, [^{3}H]-MK-801 binding was inhibited by high concentrations of tacrine (Fig. 6.6) in both rat and human control tissue from the frontal cortex (age range 71-87 y, delay to post-mortem 14-24 h, storage time at -70 °C 7-10 y, 2 males and 2 females, all right hemispheres,and all of sudden death). By contrast with zinc inhibition, tacrine was approximately equipotent in rat (IC_{50} 66 $\pm$ 11 μM) and human tissue (46 $\pm$ 2 μM).

6.5 The influence of m-chlorophenylpiperazine on [^{3}H]-MK-801 binding in human cortex.

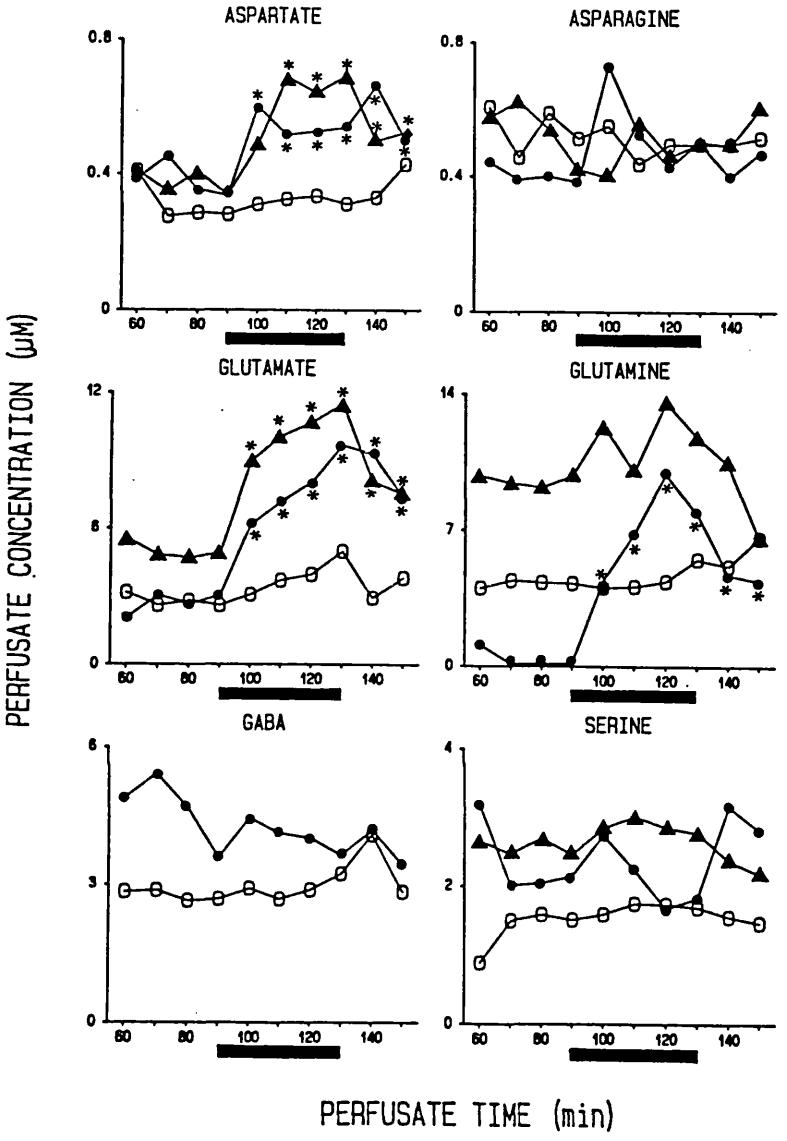
In the presence of 30 μM glutamate and 3 μM glycine, [^{3}H]-MK-801 binding was inhibited by high concentrations of m-chlorophenylpiperazine (mCPP, IC_{50} = 97 $\pm$ 0.6 μM, Fig. 6.7) in human control tissue from the frontal cortex (age range 67-86 y, delay to post-mortem 6-48 h, storage time at -70°C 6-11 y, 2 males and 2 females, 3 right and 1 left hemisphere, all died suddenly. After 45 min incubation at 25 °C mean binding was 382 $\pm$ 34 fmol/ mg protein, n = 4. By contrast, in the presence of 90 μM spermidine, mCPP did not inhibit binding over a concentration range of 1 -300 μM.

FIGURE 6.1. THE EFFECT OF INTRAPERITONEAL ADMINISTRATION OF TACRINE ON THE RELEASE OF AMINO ACIDS IN RAT CORTEX.



Amino acid outflow was measured by in vivo dialysis with HPLC in conscious, freely moving rats. Each 10 min fraction represents the mean of 3-5 independent experiments. The arrow indicates the point of tacrine (10 mg/kg, O) or vehicle (●) administration. Representative SD values: t = 110 min, with vehicle (tacrine in parenthesis) for aspartate, glutamate and glutamine are 0.07 (0.07), 0.65 (0.75), 1.8 (3.8), respectively; and t = 140 min, 0.03 (0.1), 0.75 (0.66) and 2.4 (5.5), respectively. * significantly different to vehicle treated animals ($p < 0.05$, Mann- Whitney U-test)

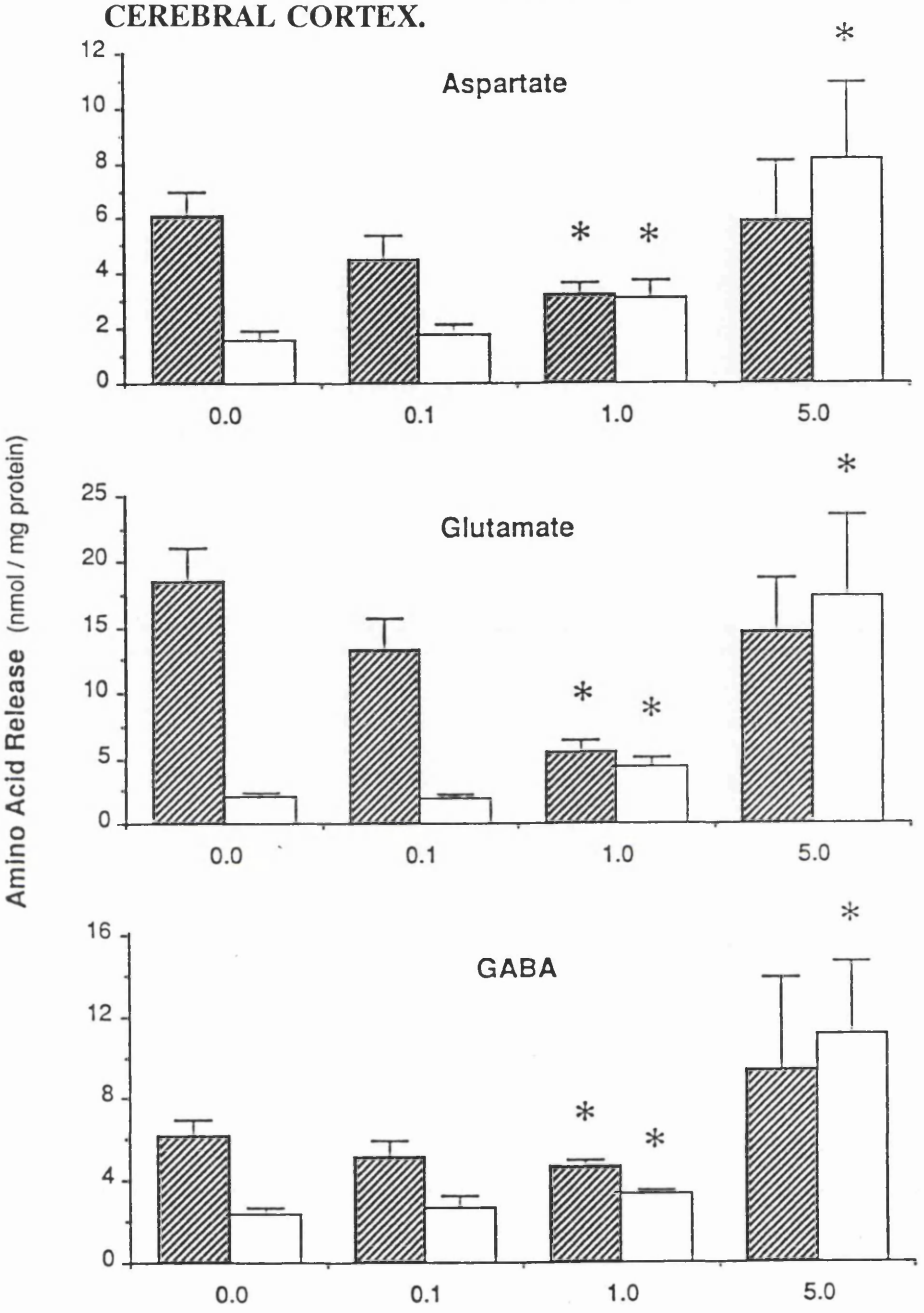
FIGURE 6.2. THE EFFECT OF INTRACORTICAL ADMINISTRATION OF TACRINE ON THE RELEASE OF AMINO ACIDS IN RAT CORTEX.



Amino acid outflow was measured by in vivo dialysis (with HPLC and fluorimetric detection) in anaesthetized rats. Each 10 min fraction represents the mean of 5 independent experiments. The horizontal bar indicates the period of tacrine administration through the dialysis probe (o 1 mM, • 5 mM, ▲5 mM plus 160 pmol tetrodotoxin). GABA values for experiments performed in the presence of tetrodotoxin are not presented because of peak coelution. Representative SD values: t = 80 min, with 1 mM tacrine (5 mM tacrine in parenthesis) for aspartate and glutamate are 0.17 (0.29), 2.2 (1.9); respectively; and t = 120 min, 0.12 (0.22) and 1.6 (0.5) respectively.

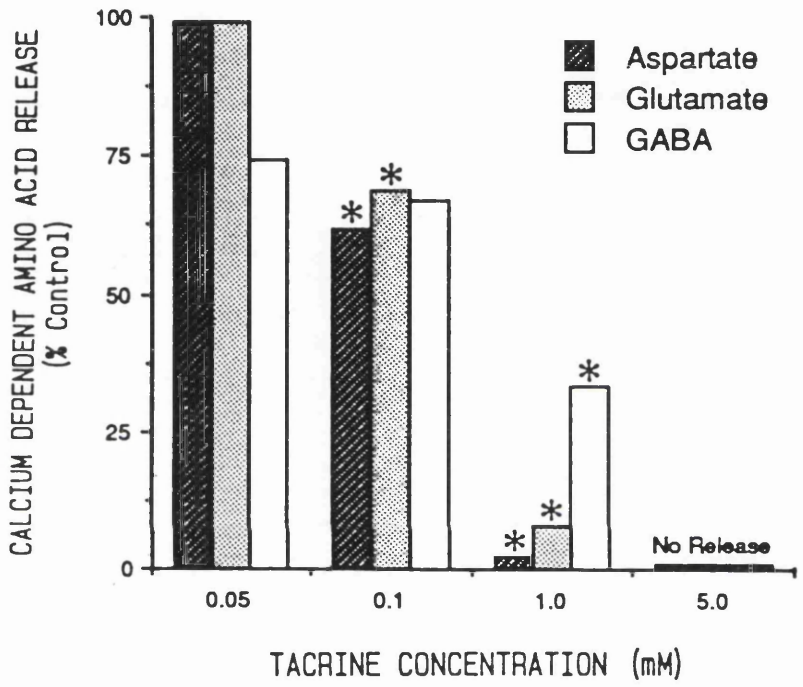
* significantly different to pretreatment values ($p < 0.05$, Wilcoxon's paired-rank test).

FIGURE 6.3. THE EFFECT OF TACRINE ON K^+- EVOKED RELEASE OF TRANSMITTER AMINO ACIDS FROM TISSUE PRISMS OF RAT CEREBRAL CORTEX.



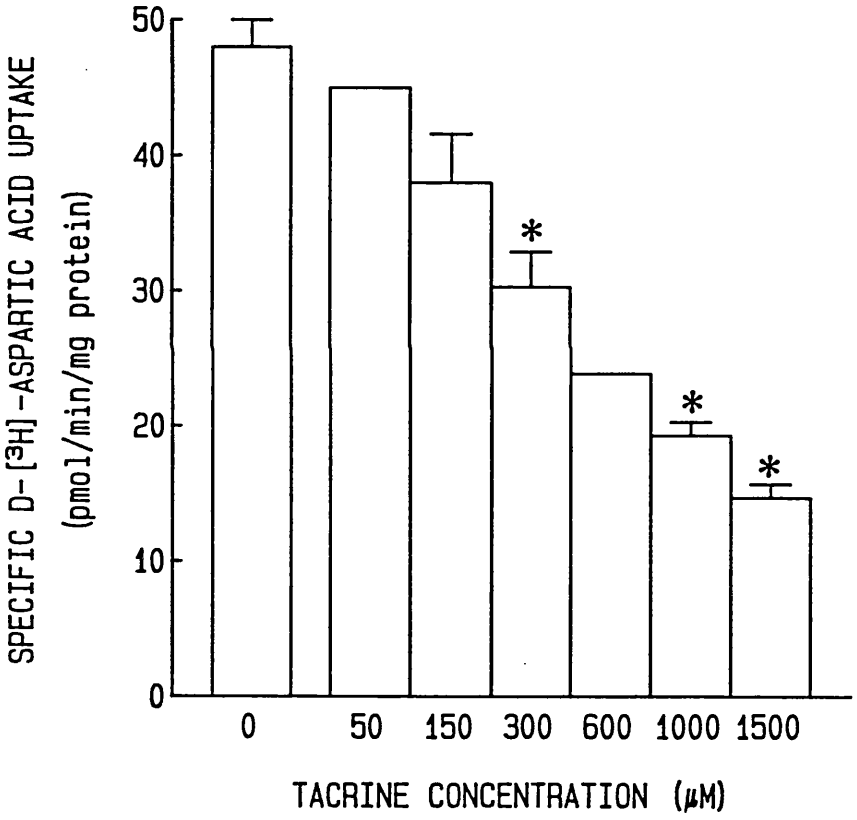
Hatched bars represent incubations in the presence of 1.3 mM Ca^{2+}, open bars in the absence of Ca^{2+} and in the presence of 2 mM EGTA.
Incubations with 0.05 mM tacrine in the presence or absence of Ca^{2+} were not significantly different from control values (data not shown).
Values are mean $\pm$ SD of 3 independent experiments.
* significantly different compared with release in the absence of tacrine ($p < 0.05$, Student's t-test)

FIGURE 6.4. THE EFFECT OF TACRINE ON THE Ca^{2+}-DEPENDENT, K^+ - EVOKED RELEASE OF TRANSMITTER AMINO ACIDS FROM TISSUE PRISMS OF RAT CEREBRAL CORTEX.



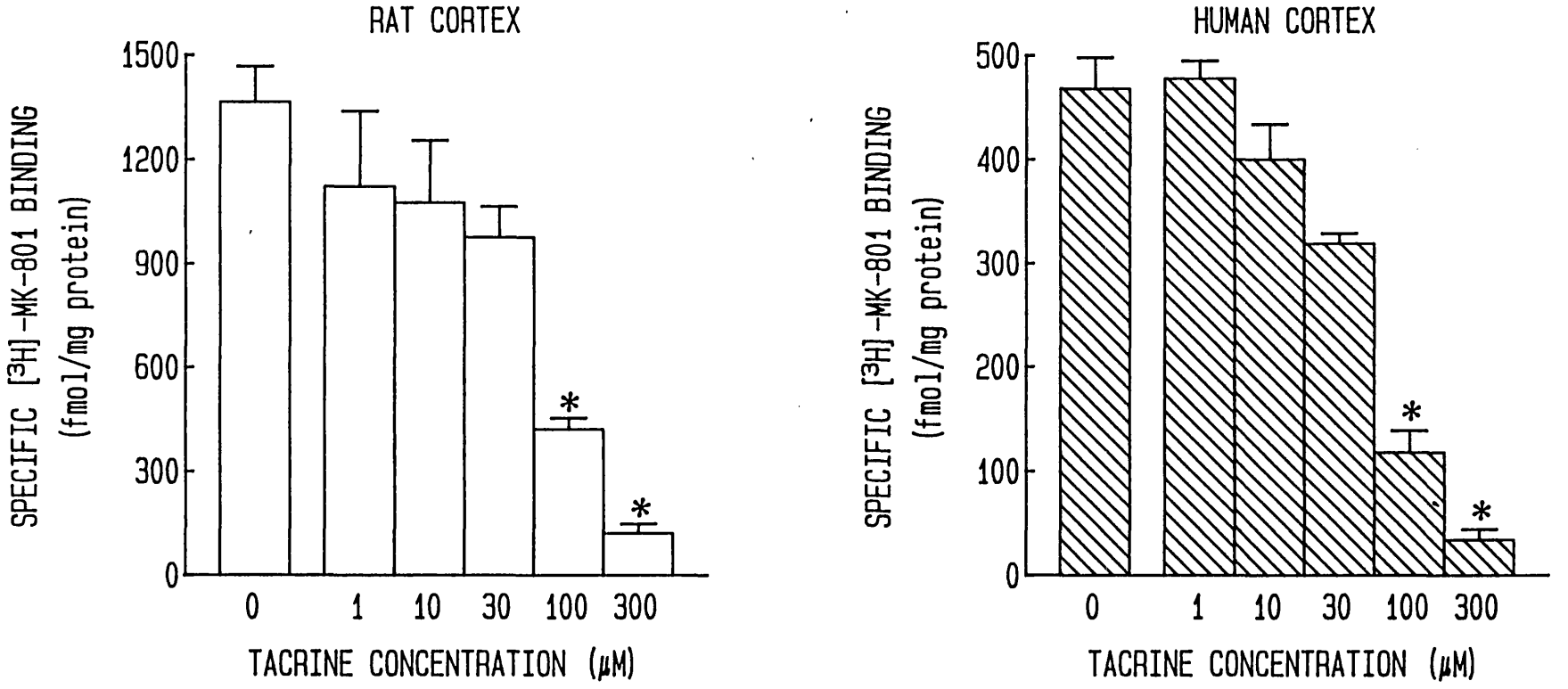
Ca^{2+}-dependent release value for each amino acid was calculated (numerical difference between release in the presence and absence of Ca^{2+}) for control incubations (no tacrine) and various concentrations of drug.
Absolute values for these data are given in Fig.6.3.
* Significantly different compared with release in the absence of tacrine ($p < 0.05$, Student's t-test).

FIGURE 6.5. THE EFFECT OF TACRINE ON THE UPTAKE OF D-[^{3}H]-ASPARTIC ACID INTO TISSUE PRISMS OF RAT CEREBRAL CORTEX.



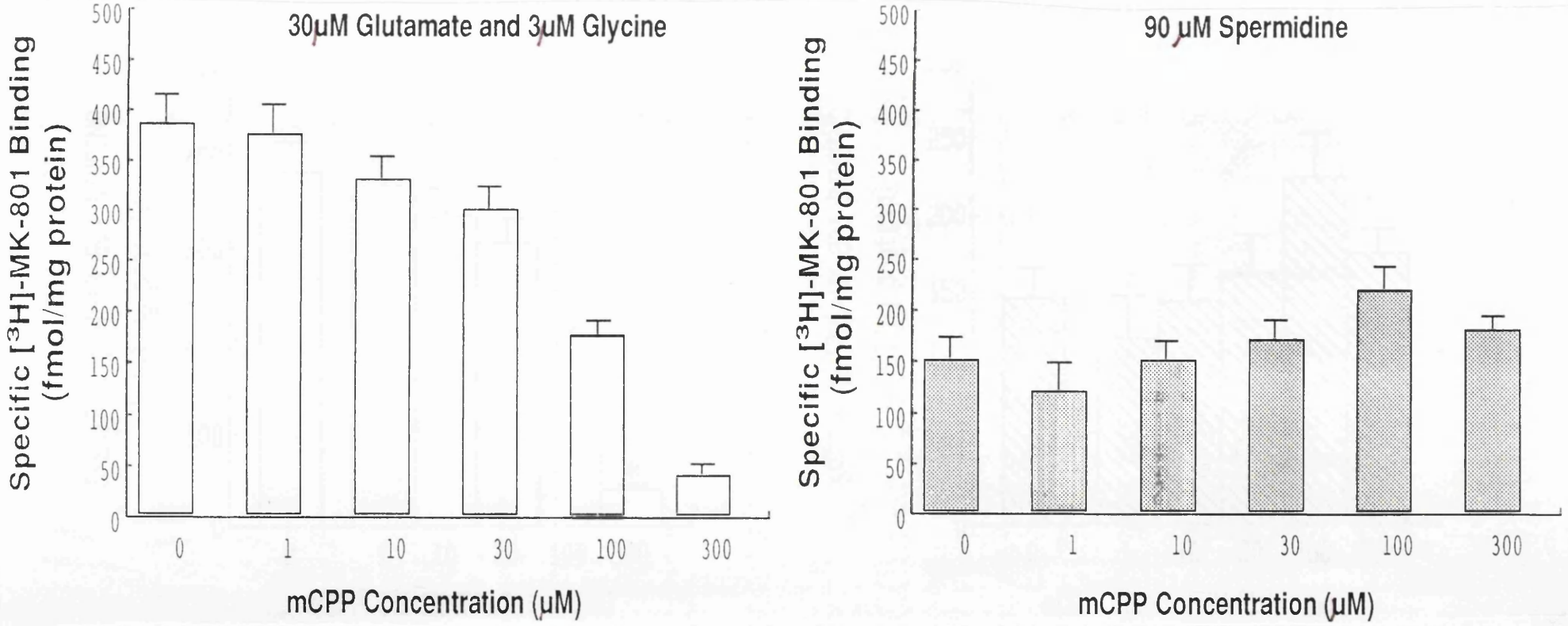
Specific uptake was calculated as the numerical difference between uptake at 30 °C and 2 °C. IC_{50} values were estimated using linear regression analysis of Hill plots. IC_{50} = 614 ± 20 μM.
Values are mean ± SEM of 3 independent experiments.
* significantly different compared with uptake in the absence of tacrine ($p < 0.01$, Student's t-test.

FIGURE 6.6. THE EFFECT OF TACRINE ON [^{3}H]-MK-801 BINDING IN RAT AND HUMAN CORTEX



Experiments were performed with membranes incubated for 45 min at 25 °C in Tris-HCl buffer using a final concentration of 5 nM [^{3}H]-MK-801. Assays were in the presence of 30 μM glutamate and 3 μM glycine.
IC_{50} values were estimated using linear regression analysis of Hill plots, in rat brain = 66 $\pm$ 11 μM, in human brain = 46 $\pm$ 2 μM.
Values are mean $\pm$ SEM (n = 4).
* significantly different compared with binding in the absence of tacrine ($p < 0.01$, Student's t-test).

FIGURE 6.7. THE EFFECT OF mCPP ON [^{3}H]-MK-801 BINDING IN HUMAN CORTEX IN THE PRESENCE OF GLUTAMATE AND GLYCINE OR SPERMIDINE.



Experiments were performed with membranes incubated for 45 min at 25 °C in Tris-HCl buffer using a final concentration of 5 nM [^{3}H]-MK-801. Assays were in the presence of either 30 μM glutamate and 3 μM glycine (open bars) or 90 μM spermidine (hatched bars). IC_{50} values were estimated using linear regression analysis of Hill plots, in the presence of glutamate and glycine was 97 $\pm$ 0.6 μM. Values are mean $\pm$ SEM (n = 4).

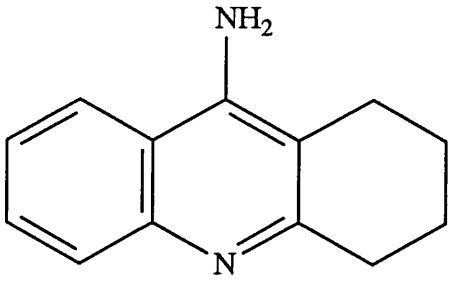
* significantly different compared with binding in the absence of tacrine ($p < 0.01$, Student's t-test).

6.6 DISCUSSION

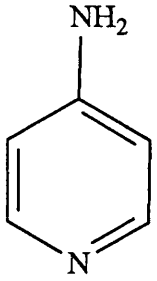
At the onset of this study, clinical efficacy in Alzheimer's disease has been claimed for tacrine (Summers et al., 1986, but see Gauthier et al., 1990, Chatelier & Lacomblez, 1990). It seems unlikely from this study that this efficacy can be attributed to actions on amino acid containing neurones. Application of the highest dose of tacrine to the frontal cortex through the microdialysis probe caused a selective increase in the concentration of aspartate, glutamate and glutamine (Fig. 6.2). Tetrodotoxin, an inhibitor of voltage-dependent sodium channels abolished only the increase in glutamine, suggesting that the changes in the concentrations of aspartate and glutamate were independent of neuronal conductance. Indeed, only the highest concentrations of tacrine increased in vitro Ca^{2+}-independent release of aspartate and glutamate (Fig 6.3). By contrast, Ca^{2+}-dependent release of glutamate and aspartate was inhibited by tacrine. Inhibition was also observed for endogenous GABA, as previously shown for release of radiolabelled GABA (de Belleroche and Gardiner, 1988). However, at high concentrations of tacrine D-[^{3}H]-aspartic acid uptake was reduced and it seems likely therefore that the net effect of tacrine, in vitro at high concentrations, was to increase the extracellular concentration of amino acids. This is consistent with in vivo data when tacrine was perfused through the dialysis probe. The dialysis data, however, was variable, particularly for GABA, glutamate and glutamine and these data suggest that the system used for this study was possibly not sensitive enough to detect small changes in amino acid outflow.

Because of structural similarities (see Fig.6.8), Summers et al., (1986) suggested that tacrine may act by a mechanism similar to that of 4-aminopyridine, a substance shown to increase spontaneous release of transmitter without affecting K^+ -evoked release (Tapia and Sitges, 1982; Barnes et al., 1987). Different modes of action are suggested by the present finding that tacrine inhibits the K^+ -evoked release of amino acids and GABA. This is supported by electrophysiological data which indicates that spontaneous synaptic activity is suppressed by tacrine and promoted by 4-aminopyridine (Rogawski, 1987; Stevens and Cotman, 1987). It is difficult,

FIGURE 6.8. STRUCTURAL SIMILARITY BETWEEN TACRINE AND 4-AMINOPYRIDINE.



Tacrine



4-Aminopyridine

however to suggest a mode of action for tacrine from these data. Since effects are seen at high concentrations and are seen on Ca^{2+}-independent release of amino acids it seems likely that the effects are non-specific and can be attributed to the numerous effects on other neurotransmitter systems (see section 1.13).

A large systemic application of tacrine did not influence the amino acid outflow from the frontal cortex (Fig. 6.1). The drug appears to accumulate in the brain following such ip administration to a concentration of about 10 μM (Liston et al., 1988). It is difficult to relate the concentration of the drug in the intracortical perfusion medium to the concentration achieved in the extracellular fluid, but the present in vitro data do suggest that the lowest effective concentration of tacrine is an order of magnitude higher than can be achieved after ip administration. This effective concentration (100 μM) is much higher than the serum concentration reported to be associated with clinical benefit (30-300 nM, Summers et al., 1986) and is similar to the concentrations shown to attenuate glutamate receptor-mediated neurotoxicity (100 μM - 3 mM, Davenport et al., 1988). In agreement with the action in rat brain (Albin et al., 1988), high concentrations of tacrine inhibit [^{3}H]-MK-801 binding to human brain. It seems unlikely therefore, that tacrine will either potentiate excitotoxicity or impair learning and memory by interaction with glutamate receptors. Furthermore, it seems likely that if tacrine is efficacious in Alzheimer's disease patients (see Thal et al., 1990) it may be through its activity on brain cholinergic systems.

A drug (mCPP; developed as a 5-HT_1 receptor agonist) which appeared to exacerbate some clinical symptoms of Alzheimer's disease to a greater extent than in age matched controls (Lawlor et al., 1989), was also examined for effects on [^{3}H]-MK-801 binding in human brain . However, like tacrine, effects were observed at only high concentrations of mCPP and it seems more likely therefore that the effect is attributable to other mechanisms. Studies with rats (Maura & Raiteri, 1986) have shown that in the hippocampus 5-HT has an inhibitory effect on cholinergic

transmission that is mediated through the 5-HT_1 receptor subtype. It seems possible therefore that the effect of mCPP in this study is to inhibits release of acetylcholine in the hippocampus, disrupting memory. Since the hippocampus is most degenerate in Alzheimer's disease it seems likely that Alzheimer's disease patients would be more sensitive to this disruption than control subjects.

Clinical Implications

The release and uptake data for tacrine indicate that any clinically relevant concentration of tacrine is unlikely to potentiate harmful (neurotoxic and memory impairing) effects. Such systemic doses, however, seems to influence the brain concentrations of other transmitters affected in Alzheimer's disease (eg 5-HT, Palmer et al., 1988, Cross, 1990) and inhibit "high affinity" uptake of choline (Drukarch et al., 1987, Tachiki et al., 1988, Sherman & Messamore, 1988, Robinson et al., 1989). In vitro findings indicate that, in the low micrmolar range, tacrine modulates monoaminergic transmission (Drukarch et al., 1987, Robinson et al., 1989), with similar tissue preparations and drug exposure time to those used here, and inhibits acetylcholinesterase activity (Summers et al., 1986, Drukarch et al., 1987). However, tacrine also antagonizes muscarinic M1 and M2 receptors at concentrations above 1 μM (Pearce & Potter, 1988). Thus if tacrine were present in the brain at low micromolar concentrations the action on muscarinic receptors may offset any effects inhibition of acetycholinesterase. A single dose of tacrine in mice that produces therapeutic plasma levels also results in a ten-fold increase in the concentration of tacrine in the brain (about 3 μM ; Liston et al., 1988). It is not impossible that on a regular dosing schedule, steady-state concentrations of tacrine in the brain could be even higher so the possibility cannot be excluded of an action on the NMDA receptor complex.

In summary tacrine, which is an inhibitor of cholinesterase, is remarkable of its type in that it has been claimed to produce clinical improvement in some patients (see Thal et al 1990). It has been considered for some time that its efficacy may be due

to actions on other, non-cholinergic, systems (e.g. 5-HT) as well as by prolonging the life of released acetylcholine, hence enabling lower concentrations of acetylcholine than normal to stimulate acetylcholine receptors. However, tacrine does not seem to affect excitatory amino acid transmission, at least at clinically relevant doses.

CHAPTER 7

GENERAL DISCUSSION

A striking feature of Alzheimer's disease appears to be the selective pattern of brain degeneration. The quantitative study of tangles density and ChAT reduction in Chapter 3 support the hypothesis that the pathological process in Alzheimer's disease may spread along a sequence of cortico-cortical connections between the main sensory areas and the hippocampal formation. One major goal of research will be to firmly establish the relationship between extracellular amyloid deposits and tangle formation, (see Selkoe, 1990), occuring primarily in pyramidal cells. At present it remains to be proven that amyloid deposition is other than a harmless by product of the pathological mechanism and there is little evidence (Yankner et al., 1989) to the contrary.

The accumulation of tangles in the pyramidal cells, together with loss of these neurones, seem to be important features of the pathology of Alzheimer's disease (see Introduction, section 1.8). These pyramidal cells appear to use glutamate or a related excitatory amino acid as their transmitter (Fonnum, 1984, Bowen & Francis, 1990, Bowen, 1990, Lowe et al., 1990). However, glutamate dysfunction has been difficult to demonstrate due to the lack of a suitable enzyme marker for such neurones. A number of groups have now reported evidence that suggests a loss of cerebral cortical and hippocampal glutamatergic terminals in Alzheimer's disease (Palmer et al., 1986, Cross et al., 1987, Cowburn et al., 1988a,b, Bowen, 1990, Lowe et al., 1990). Studies of glutamate receptor integrity in Alzheimer's disease, including those in this thesis, have concentrated on the NMDA receptor subtype. This was not well characterized in human brain, however this thesis has illustrated that the NMDA receptor complex appears to be similar in rat and human cortex (Chapter 5).

Some investigators have reported that in the cerebral cortex and hippocampus the number of NMDA receptors are reduced in Alzheimer's disease, whereas others have found no loss (see Table 1.4). This discrepancy between the findings of different groups may reflect the severity of the disease process. Alzheimer's disease is a slowly progressive disorder but this feature is rarely acknowledged in the interpretation of post-mortem biochemical studies. The study of biopsy tissue, removed surgically for diagnostic purposes, may help to determine early changes. Preliminary results with such samples (Procter et al., 1989a) suggest that the receptor complex is affected within at least 2 years of the emergence of clinical symptoms. Biopsy samples are so rare (the laboratory has received only 39 specimens from 7 UK centres in the last 12 years, Bowen, 1990) that other approaches are needed to address the issue of disease severity. The assay of ChAT activity in the cortical samples described in Chapter 3, where tangle density is known, is one example. In the future it may be possible to use positron emission tomography to follow changes in receptors throughout the course of the disease (see Fade et al., 1990). Such studies are dependent upon the design and development of suitable ligands, a topic of intensive current research (see Hansen & Krogsgaard-Larsen, 1990).

The discrepancy between results may also reflect different methodological approaches used. Receptor binding with well washed membrane preparations allows precise kinetic and pharmacological measurements without complications of regional tritium quenching (Geary & Wooten, 1983) and residual endogenous compounds (eg glycine, see Greenamyre & Young, 1989) but may mask changes in discrete anatomical laminae which can be studied with receptor autoradiography. Thus in future work, both approaches should be used to study the binding characteristics and the distribution of [^{3}H]-MK-801 and [^{3}H]-TCP and other ligands in human brain.

There are few studies where more than one concentration of radioligand was used to estimate the number of binding sites (Table 1.4). For this reason the present

study employed Scatchard analysis using two different ligands which, after extensive investigation appeared to have different properties but seem to bind to the NMDA receptor complex. [^{3}H]-MK-801 binding in the presence of high concentrations of glutamate and glycine was much greater than in the presence of either amino acid alone. However, this combination did not increase [^{3}H]-TCP binding above that seen in the presence of glutamate or glycine alone. The second and most obvious difference in the binding characteristics of these ligands was that there were more high affinity binding sites for [^{3}H]-MK-801 than for [^{3}H]-TCP in the cortex but not in the striatum. There are two possible explanations for the difference; either the receptor is heterogenous or these ligands interact differently with different states of the same receptor (section 4.1).

A modest reduction in the number of NMDA receptor complexes based on [^{3}H]-MK-801 binding alone, was observed in Alzheimer's disease tissue from the frontal cortex but not temporal cortex (section 4.2). There are two possible explanations for the difference. The pathology of Alzheimer's disease tends to be more prominent in the temporal lobe and it is possible that in this region no difference is detected because of greater loss of structures organized in a columnar manner (see Procter et al., 1988a,b). Alternatively, because all the patients in the study had been hospitalized prior to death, it is possible that inadvertant selection of subjects with prominent behavioural disorders had occurred (Palmer et al., 1988). This latter explanation seems unlikely because in a previous study of [^{3}H]-MK-801 binding in Alzheimer's disease (using different experimental conditions than those used here) a reduction in [^{3}H]-MK-801 binding was seen in those subjects without prominent behavioural disorders (Procter et al., 1989a).

It is now well established that at least three non-NMDA glutamate receptors exist in the mammalian CNS (see Lodge & Collingridge, 1990, also see section 1.7). There is little information about the density and characteristics of these in human brain. In Alzheimer's disease, the kainate receptor is reported increased (Geddes et

al., 1986, Chalmers et al., 1990), decreased (Represa et al., 1988, Penney et al., 1990) or unchanged (Pearce & Bowen, 1984, Cross et al., 1986a, Cowburn et al., 1988a). The involvement of the AMPA and the metabotropic receptor in Alzheimer's disease is clearly understudied (section 1.7) Further research is required as these non-NMDA receptors probably mediate a large proportion of the fast synaptic neurotransmission in the CNS (section 1.7) and also help mediate glutamate neurotoxicity (section 1.9, also see Greenamyre and Young, 1989, Choi, 1990).

The prospect of obtaining meaningful data from diseased post-mortem brain may be questioned as parameters under scrutiny may be influenced by factors such as patient age, sex, drug history, immediate pre-mortem status (sudden death or protracted coma) and post-mortem delay (see Introduction, section 1.4, also Palmer et al., 1988). Interpretation of post-mortem data therefore requires fastidious consideration of these factors in order to separate changes that are due to the brain disease from those that occur as a result of epiphenomena. In this study, evidence was obtained that the glycine regulation of the NMDA receptor complex may be affected by protracted illness just prior to death. It is possible that the ischemia/ hypoxia thought to be associated with prolonged terminal illness is more severe in Alzheimer's disease patients and therefore this loss of glycine regulation may be a reflection of this.

Since the discovery of the presynaptic cholinergic deficit in Alzheimer's disease (see section 1.4.1) attempts at symptomatic treatment at first concentrated on reversing this deficit with choline or lecithin but with little success. In 1986, Summers and collegues reported that oral doses of the cholinesterase inhibitor, tacrine, alleviated symptoms of patients at various stages of Alzheimer's disease. In addition to the cholinestease inhibition properties of tacrine, this drug has effects on a number of neurotransmitters including the glutamatergic system (see section 1.13). However, the in vitro and in vivo data presented here suggest that tacrine is not acting in patients via a glutamatergic mechanism (Chapter 6) Therefore other drugs need to be

designed and developed.

Since the present study found only a modest loss of NMDA receptors and other investigators find evidence of a marked reduction of glutamate-releasing nerve terminals it may be beneficial to enhance glutamatergic transmission in Alzheimer's disease. Manipulation of the glycine site could be an appropriate way to enhance the activity of the remaining pyramidal neurones. The validity of this approach is strengthened by the fact that the NMDA receptor complex appears to be involved in neural pathways underlying memory and behaviour (McCabe & Horn, 1989).

Since excessive activation of the NMDA receptor complex can induce excitotoxic cell death (see Meldrum, 1990 and section 1.46), compounds acting at this site that exhibit only partial agonist characterisics may be the most suitable. The antituberculous drug, D-cycloserine, active in rat learning and memory tasks (Monahan et al., 1989, Herberg & Rose, 1990) and with high affinity for the glycine site associated with the NMDA receptor complex (Hood et al., 1989) but with partial agonist properties is one example and should provide a valuable tool to investigate cognitive disorders such as Alzheimer's disease (Watson et al., 1990). Success would depend on many factors including the magnitude of neuronal loss , the concentration of endogenous glycine near the glutamatergic synapse and the functional state of the NMDA receptor in cortex affected by Alzheimer's disease. If the reduction in the glycine regulation of the NMDA receptor complex (Chapter 5) is a genuine change due directly to brain pathology, drugs such as D-cycloserine may also help to overcome this receptor alteration.

A recent study (DeKosky & Scheff, 1990) has confirmed that there are significant correlations between loss of pyramidal neurones and clinical ratings of dementia determined for patients examined at cerebral biopsy (Neary et al., 1986). This lays further emphasis on the importance of glutamatergic neurotransmission in Alzheimer's disease. Thus, the disappointing results of cholinomimetic drug

treatment of Alzheimer's disease patients so far should not be a reason for despair. Careful post-mortem investigations in the future using normal and diseased tissue will provide additional information in terms of neurochemical anatomy and function. One obvious approach is to concentrate on the excitatory amino acid system, particularly non-NMDA glutamatergic receptor subtypes. It is possible that this target will provide the symptomatic treatment we all search for.

In conclusion, until the cause of the disease has been identified, the devastating nature of this disorder should encourage research into the treatment of symptoms as even modest benefits, if clearly shown and sustained, would be helpful.

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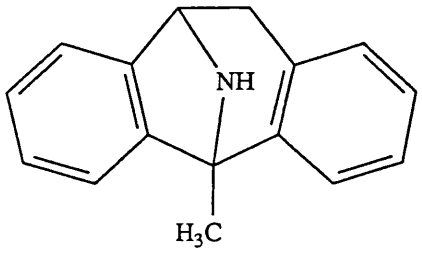
APPENDIX 1A. STRUCTURES OF COMPOUNDS STUDIED IN THIS THESIS



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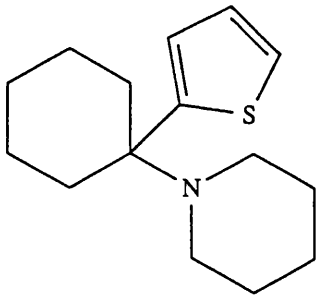

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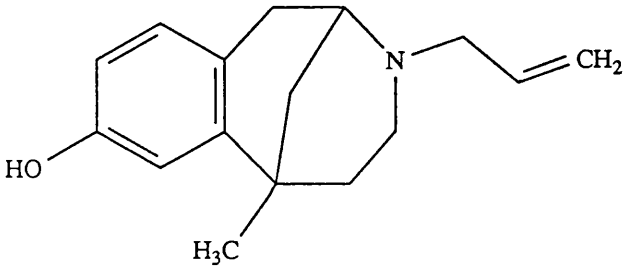

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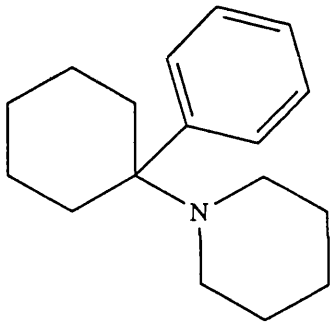

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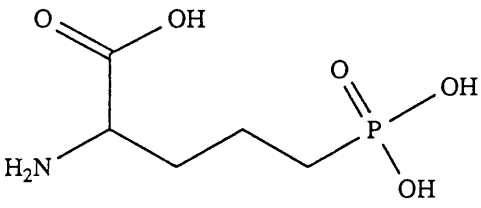
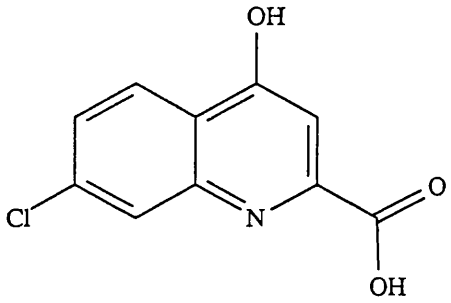
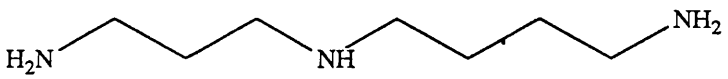


AP-5



7-Chlorokynurenate

APPENDIX 1B. STRUCTURES OF COMPOUNDS STUDIED IN THIS THESIS



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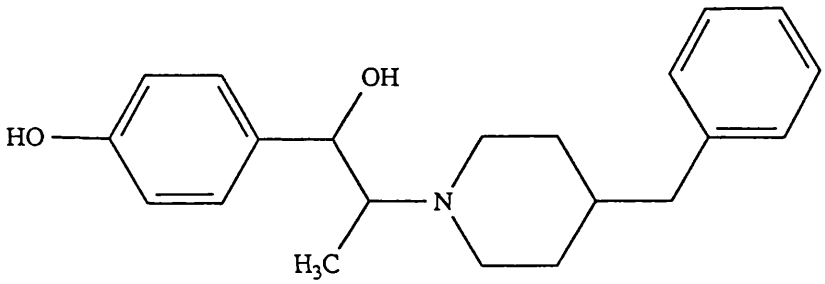

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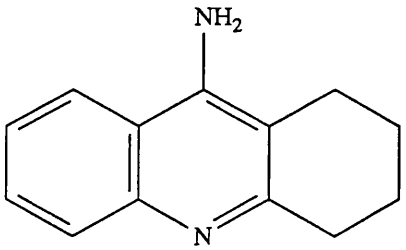
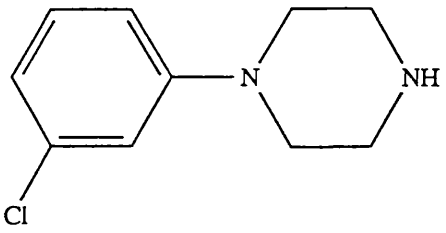


Tacrine



m-CPP

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