ALTERATIONS OF CHOLINERGIC AND SEROTONERGIC NEUROCHEMISTRY IN ALZHEIMER'S DISEASE: CORRELATIONS WITH COGNITVE AND BEHAVIORAL SYMPTOMS

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TABLE OF CONTENTS

PAGE

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

[γ-^{32}P]ATP	Adenosine-5'-[^{32}P] triphosphate
5-HIAA	5-hydroxyindoleacetic acid
5-HT	Serotonin
5-HTT	5-HT transporter or 5-HT reuptake sites
5-HTTLPR	5-HTT linked polymorphic region
Aβ	β-amyloid
ACh	Acetylcholine
AChE	Acetylcholinesterase
AChEI	Acetylcholinesterase inhibitor
AD	Alzheimer's disease
ANOVA	Analysis of variance
ApoE	Apolipoprotein E
APOE	Apolipoprotein E gene
APP	Amyloid precursor proteins
BA11	Brodmann area 11; Orbitofrontal gyrus
BA21/22	Brodmann area 21/22; Superior and midtemporal gyrus
BACE	β-site APP-cleaving-enzyme
B_{max}	Binding density, in fmol/mg protein
BPSD	Behavioral and psychological symptoms of dementia
CERAD	Consortium to Establish a Registry for Alzheimer's Disease
ChAT	Choline acetyltransferase
CI	Confidence interval
CPM	Counts per minute
DAG	Diacylglycerol
DPM	Disintegrations per minute
GDP	Guanosine diphosphate
GPCR	G-protein-coupled receptors
GppNHp	Guanylyl-imidodiphosphate
GTP	Guanosine triphosphate
HDL	High density lipoproteins
IP_3	Inositol-1,4,5-triphosphate

K_D	Binding affinity, in nM
K_i	High affinity binding constant in the absence of G-protein
K_{iG}	High affinity binding constant in the presence of G-protein
LTP	Long term potentiation
MAO	Monoamine oxidase
MAPs	Microtubule-associated proteins
MCI	Minimal cognitive impairment
MMSE	Mini-Mental State Examination
nAChR	Nicotinic receptor
NBM	Nucleus basalis of Meynert
NFTs	Neurofibrillary tangles
NMDA	*N*-methyl-D-aspartate
NPI	Neuropsychiatric Inventory
NR1	*N*-methyl-D-aspartate receptor subunit 1
NR2A	*N*-methyl-D-aspartate receptor subunit 2A
NSB	Non-specific binding
PBE	Present Behavioural Examination
PET	Positron emission tomography
PHFs	Paired helical filaments
PIP_2	Phosphatidylinositol-4,5-bisphosphate
PKC	Protein kinase C
PLC	Phospholipase C
PMI	Postmortem interval
PZ	Pirenzepine
RACK	Receptor for activated C-kinase
S.E.M.	Standard error, mean
SP	Senile plaques
SSRI	Selective serotonin reuptake inhibitor
τ	Tau proteins
TB	Total binding
VLDL	Very low density lipoproteins

SUMMARY

Alzheimer's Disease (AD) is a neurodegenerative disease characterized clinically by progressive cognitive decline and frequently present with behavioral and neuropsychiatric symptoms. The major neuropathological hallmarks of AD are senile plaques, neurofibrillary tangles and neuronal loss. In particular, losses of glutamatergic, cholinergic and serotonergic neurons, as well as concomitant neurochemical alterations in specific brain regions, may underlie the clinical features of AD (Francis et al. 1993; Minger et al. 2000; Wilcock et al. 1982).

The *N*-methyl-D-aspartate (NMDA) receptors are thought to be critically involved in learning and memory. In AD, hypoactivity of NMDA receptors has been speculated to contribute towards the neurodegenerative process (Olney et al. 1997). Others have demonstrated a loss of coupling of postsynaptic cholinergic muscarinic M1 receptors from their G-proteins in AD neocortex (Flynn et al. 1991) as well as deficits of downstream signaling molecules such as protein kinase C (PKC) (Cole et al. 1988) in AD neocortex. There is also evidence from *in vitro* studies that potentiation of NMDA receptor function is regulated by agonists of G-protein-coupled receptors, including those for muscarinic receptors, in a pathway dependent on PKC and Src kinase (Ali and Salter 2001; Lu et al. 1998). Taken together, these results suggest that the disruption of M1-mediated signaling as well as associated NMDA receptor hypofunction may underlie the cognitive symptoms in AD.

Although there is some evidence that serotonergic deficits are correlated with cognitive decline, changes in serotonergic neurochemistry is thought to underlie many of the neuropsychiatric symptoms of AD, which are often more stressful for the caregivers

to cope compared with the cognitive decline (Chen et al. 1996). These observations are in line with many pre-clinical and clinical studies establishing the essential roles of serotonergic neurotransmission in mood and emotional states, especially in the hippocampus and neocortex (Barnes and Sharp 1999; Lanctot et al. 2001; Meneses 1999). Currently, the effect of functional polymorphisms of serotonin (5-HT) receptors, such as those of the gene promoter region of the serotonin (5-HT) transporter on receptor levels or behaviors is unknown. Therefore, my research aim to measure the M_1 receptors, NMDA receptors, and 5-HT transporters in the postmortem frontal and temporal cortices of two cohorts of well-characterized AD patients as well as controls. Neurochemical findings are then correlated with the rate of cognitive decline as well as behavioral changes to test the hypothesis that neurochemical alternations may underlie both cognitive decline and behavioral changes in AD. Moreover, the status of M_1/G-protein coupling in AD is measured and correlated with cognitive decline as well as with measurements of choline acetyltransferase (ChAT), protein kinase C (PKC) and Src kinase activities to investigate the possible interactions between M_1 receptor mediated signaling and NMDA receptor status. Besides, the effects of two functional gene polymorphisms (i.e. ApoE ε4 allele and LL genotype of the promoter region of 5-HTT) on the cholinergic and serotonergic systems, respectively, are examined.

This project will add to our understanding of the neurochemical basis of cognitive decline and behavioral symptoms in AD, and may suggest novel drug targets.

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SECTION 1

Introduction and Literature Review

CHAPTER 1

Alzheimer's Disease: Definition, Cost to Society, Pathologic Features

1.1 INTRODUCTION

In 1907, Dr Alois Alzheimer described the first case of dementia which now bears his name (Alzheimer 1907). In his report, he described the clinical symptoms of a middle-aged woman who had developed memory deficits and progressive loss of cognitive abilities. The patient also showed behavioral symptoms such as hiding objects in her apartment and believing that people intended to kill her. At her death, Dr Alzheimer did an autopsy on her brain and discovered amyloid plaques and neurofibrillary tangles in the neocortex and hippocampus. After this case was reported, the term Alzheimer's disease (AD) was given to this type of presenile dementia.

Now, the neuropathology of AD (amyloid plaques, neurofibrillary tangles [NFTs] and selective loss of neurons, will be discussed later in **Chapter 1**) is recognized in

senile, or late onset, dementia, of which one of the most prominent features is progressive loss of cognitive functions. Besides cognitive impairment, AD patients frequently exhibit behavioral and psychological symptoms of dementia (BPSD, IPA 1996). BPSD include both psychotic symptoms (e.g. hallucinations and paranoid/delusional ideation) as well as non-psychotic symptoms (e.g. aggression and wandering, affective disturbances, and anxieties/phobias, Cummings et al. 1994). BPSD occur frequently in AD and BPSD such as aggression and psychosis, which have negative impact on both the patients and the caregivers, are causing tremendous distress to the caregivers and these symptoms often lead to institutionalization of the patients (Gilley et al. 1991).

1.1.1 Clinical Course of Alzheimer's Disease

As the clinical heterogeneity of AD complicates differentiation from disorders other than AD with similar phenotypes (e.g., other progressive dementias), AD diagnosis has remained somewhat difficult. Nevertheless, the standardization of the clinical diagnosis of probable AD by the National Institute of Neurological and Communicable Disease and Stroke / Alzheimer's Disease and Related Disorders Association (NINCDS/ADRDA) criteria (McKhann et al. 1984) has improved diagnostic accuracy and allowed meaningful comparison of results of therapeutic trails and other clinical investigations.

According to the fourth edition of the Diagnostic and Statistical Manual of Mental Disorders of the American Psychiatric Association (1994), the definition of dementia is "the development of multiple cognitive deficits that include memory impairment and at least one of the following: aphasia, apraxia, agnosia, or a disturbance in executive functioning". Dementia represents a decline from a higher level of cognitive function

such that a demented patient conducts accustomed activities less well because of cognitive loss. Dementia is typically progressive, although the pattern of decline (for example, rate of cognitive decline and the extent of loss of different cognitive domains) may not be uniform. Although the pattern of cognitive decline may differ among patients, there are recognizable stages of cognitive dysfunction during the course of AD which may be roughly divided into mild, moderate, and severe stages.

The stages of AD as described below were summarized from Morris 1999 (Morris 1999). The initial symptoms are insidious and may not warrant medical attention for several years. The main feature in earliest AD is mild memory loss, manifested by repetition of questions or statements, misplacement of items, and failure to recall conversations. There is also imperfect recall of recent events or names of new acquaintances. In contrast, long term memory such as personal demographical information and other highly learned materials are minimally affected. Language disturbances include word-finding difficulty and hesitancy of speech. The mildly demented patient is usually capable of performing self-care (e.g., dressing and toileting) independently. Other personality changes such as passivity and disinterest may become evident, such as when the patient is more withdrawn from social settings, although they rarely have psychiatric disturbances.

As the patient progresses to the moderate stage, typically 4-7 years after disease onset, he or she becomes increasingly dependent on others. New information is rapidly forgotten and, though established memory may be recalled frequently, obvious inaccuracies are noted; for example, long-deceased persons may be discussed as if they still were living. Judgment and problem solving skills are impaired and driving and other

complex activities are relinquished by this stage. Language skills also deteriorate further with poor comprehension of spoken and written language. Some patients may displace disruptive behaviors such as agitation, restlessness, aggressive verbal and/or physical behavior, delusions, and hallucinations. Supervision of self-care is usually required at this stage as the patient neglect bathing and grooming as well as demonstrating poor table manners.

The severe stage is characterized by nearly complete dependence on caregivers for even basic functions. Only memory fragments remain, and accurate identification of relationships and names is lost. Comprehension is limited to the simplest spoken language and verbal output is limited to short phrases and repetition of words. Although troublesome behaviors may still be evident, eventually they disappear along with all semblance of the patient's personality. Complications such as extrapyramidal dysfunction, tonic-clonic seizures, falls, and incontinence are present. In the terminal stage, the patient is bedridden and uncomprehending, dysphagia and weight loss are common, and death is usually attributed to complications associated with chronic debilitation, such as pneumonia, urosepsis, and aspiration.

1.2 COST TO SOCIETY

AD is a progressive neurodegenerative disorder with a mean duration of around a decade between onset of clinical symptoms and death. With the advances in medicine, the proportion of elderly people in the population has been increasing steadily since the last few decades. AD, which affects an estimated 15 million people worldwide, is one of the most common causes of dementia in elderly people. The prevalence of dementia /

cognitive impairment in elderly Singaporean Chinese is estimated at between 5-8% (Lim et al. 2003), similar to rates in Europe (Berr et al. 2005) and the USA (GAO 2006). As mentioned, many AD patients also exhibit BPSD such as aggression and psychosis, which have negative impact on both the patients and the caregivers, and often lead to institutionalization of the patients. Therefore, the burden of the disease has become a tremendous problem to both caregivers and national economics. Studies in the USA have shown that the direct costs for the care of patients in 1991 were calculated at US $20.6 billion and the total cost was calculated to be $76.3 billion and that a large proportion of the cost come from late stage disease when patients are placed in nursing homes (Ernst and Hay 1994). The expense of nursing home care was estimated at $47,000 per patient per year (Rice et al. 1993); hence, treatments that result in delay of institutionalization by even one year could represent billions of dollars in healthcare savings. The first step in finding treatment for AD is to acquire better understanding of the etiology and pathophysiological mechanisms underlying cognitive dysfunction and neuropsychiatric behaviors in AD.

1.3 NEUROPATHOLOGICAL FEATURES IN AD

As mentioned before, amyloid plaques, NFTs and loss of various neurotransmitter-producing neurons are the prominent neuropathological features of AD. These features may underlie both cognitive and behavioral clinical features of the disease (Cummings et al. 1996; Cummings, 2000; Cummings and Kaufer 1996; Naslund et al. 2000; Tekin et al. 2001; Zweig et al. 1988). Descriptions of the neuropathological features of AD have been summarized by Terry et al. (1999).

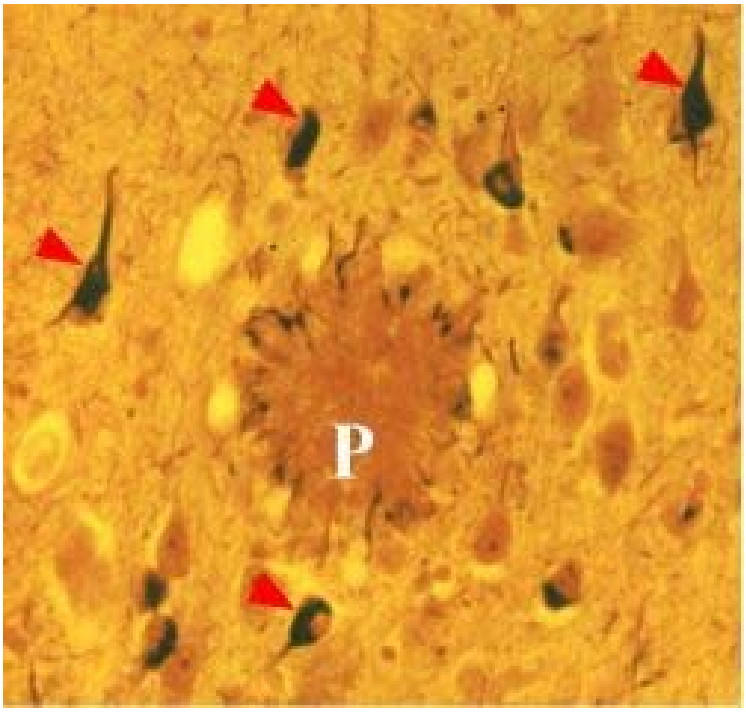
1.3.1 Amyloid Plaques

Amyloid plaques **(Figure 1.1)** are one of the major neuropathological hallmarks of AD, and are considered by many to play a critical pathogenetic role in the disease. Amyloid plaques are formed by extracellular accumulation of insoluble fragments of β-amyloid (Aβ) peptides which are 40 to 42 amino acids in length which in turn are derived from the proteolytic processing of a much larger amyloid precursor protein (APP) (see **Chapter 2** for the proteolytic processing of APP in detail). Amyloid plaques are grouped into three types: diffuse, neuritic, and burned out plaques. Diffuse plaques are mostly amorphous amyloid peptides without abnormal neurites. Neuritic plaques contain dense bundles of amyloid fibrils forming a filamentous amyloid core and are surrounded by dystrophic neurites which are mainly composed of paired helical filaments, laminated bodies, synaptic vesicles, mitochondria, and dense lysosomes suggesting that neurites are the debris of degenerated neurons. Burned out plaques consist of dense amyloid with reactive astrocytes without neurites. In addition to the amyloid fibrils and the abnormal neurites, amyloid plaques are surrounded by reactive microglia. Activated microglia have been implicated in amyloidogenesis. Perhaps activated microglia may be involved in the formation of filamentous amyloid which is derived from APP (Terry et al. 1964).

APP comprises a heterogeneous group of polypeptides that arise both from alternative exon splicing and from complex posttranslational processing, including N- and O-glycosylation, phosphorylation and sulfation, resulting in three major APP species with 770, 751, and 695 residues. The major difference between $APP_{751/770}$ and APP_{695} is that the former contains a 56-amino acid region homologous to the Kunitz family of serine protease inhibitors (KPI). Furthermore, APP_{770} has an additional 17-residue signal

peptide at the NH_2 terminus. Of the three APP species, the APP_{695} is neuron-specific, while the $APP_{751/770}$ forms are highly expressed in non-neuron cells, even though low levels are also found in neurons. At present, the physiological roles of APP are not clear and still under intense investigation. Studies using knockout mice which lack either APP, APLP1, or APLP2 have shown that the animals are viable with only minor neurological deficits (von Koch et al. 1997). Nevertheless, when two of the three proteins, either APP and APLP2, or APLP1 and APLP2 are deleted in knockout mice, resulting in premature death without histological abnormalities in any of the organs including brain. On the other hand, mutant mice with deletions at all three genes loci showed early lethality as well as high incidence of cortical dysplasia (Heber et al. 2000). Taken together, these data suggest that APP and related molecules play crucial roles in neurogenesis, but there is a certain degree of functional redundancy (von Koch et al. 1997). Further studies are needed to elucidate the role that APP and its derivatives after proteolytic processing (e.g. the insoluble form of Aβ) may play in AD.

Figure 1.1 Neuropathology of Alzheimer's disease. Neurofibrillary tangles (red arrow heads) and senile plaques (P) in postmortem brain. (Picture credit: http://www.meb.uni-bonn.de/neurologie /zellbiologie/img01.jpg)



1.3.2 Neurofibrillary Tangles (NFTs)

Unlike amyloid plaques, although NFTs **(Figure 1.1)** are critical lesions in AD, they are not specific to the disorder *per se*. NFTs have been found in a number of other neurological diseases such as postencephalitic Parkinson's disease and dementia pugilistica. Nevertheless, NFTs are quantitatively much higher in AD than normal aged brain. In AD brain, NFTs are commonly found in entorhinal, neocortex and hippocampus. NFTs consist of hyperphosphorylated microtubule-associated τ proteins which is usually required for microtubule assembly in axons (see **Chapter 2** for the process of NFTs formation in neuronal cells). There are six isoforms of τ proteins which are derived from alternative slicing of the same gene located on chromosome 17 (Goedert

et al. 1989; Lee et al. 1988). The finding of extraneuronal tangles may imply that the tangles are particularly toxic to neurons and are an important cause of neuronal death in AD. One mechanism could be that the formation of τ is accompanied by a gradual loss of microtubules which are normally stabilized by τ, resulting in disorganization and disintegration of neuronal cytoskeleton, as well as cell death (Buee et al. 2000).

1.3.3 Selective Loss of Neurons

In AD, a number of neurotransmitter systems are severely affected, including losses of cholinergic and serotonergic neurons, and associated neurochemical deficits. These will be described in detail in **Chapter 2** (cholinergic) and **Chapter 4** (serotonergic). Established preclinical and animal studies have shown the importance of cholinergic and serotonergic transmission in both memory and behavioral processes. Therefore, it is likely that neurochemical perturbations are a basis of clinical features in AD. Using postmortem brain tissues from two cohorts of longitudinally assessed AD patients, this thesis focuses on the correlations between cholinergic and serotonergic changes and the cognitive decline and BPSD to further elucidate the neurochemical and genetic bases of these clinical features in AD, as well as uncover additional associations between neurochemical changes and other molecules known to be involved in AD pathogenesis (e.g., ApoE, protein kinase C, Src kinase). Specifically, the aims of my thesis and the chapter which address them are:

1. To examine the effects of apolipoprotein *APOE* ε4 alleles on neurochemical alterations in a range of pre- and postsyanptic cholinergic markers in AD, including muscarinic M_1 and M_2 receptors, α4β2 nicotinic receptors*, M_1/G-

*Performed by our collaborators in the UK (see **Appendix I**)

protein coupling, choliner acetyltransferase (ChAT) and acetylcholinesterase (AChE)* activities (**Chapter 6**);

2. To uncover possible correlations between muscarinic M_1/G-protein uncoupling, cognitive decline (**Chapter 7**), protein kinase C and Src kinase activities, as well as glutamate *N*-methyl-D-aspartate (NMDA) receptors (**Chapter 8**);
3. To examine the effects of serotonin transporter 5-HTTLPR polymorphism on [^{3}H]citalopram binding parameters as well as anxiety behaviors (**Chapter 9**).

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CHAPTER 2

The Cholinergic System in the Central Nervous System

2.1 INTRODUCTION

AD is characterized by progressive loss of memory and cognitive decline, the presence of neuropsychiatric symptoms such as hallucination, aggression, and anxiety, as well as neuropathological features. The major neuropathological hallmarks of AD include amyloid plaques, neurofibrillary tangles and selective loss of neurons in discrete brain regions such as the neocortex and hippocampus. AD severely affects the cholinergic and serotonergic systems in the brain. Cholinergic neurons primarily innervate the neocortical and hippocampal regions, which are crucial for learning and memory

processes. In addition, cholinergic receptors such as muscarinic receptors are coupled to a number of G-protein subtypes and activate multiple signaling pathways which may further influence the process of learning and memory. Thus, cholinergic neuronal dysfunction may form the basis of learning and memory deficits in AD (Bartus et al. 1982). Deficits in cholinergic neurotransmission are also linked to hyperphosphorylation of τ protein as well as amyloidogenic processing of β-amyloid (Aβ) peptides in the brain (Hellstrom-Lindahl 2000). Taken together, the central cholinergic system plays an essential role in learning and memory processes and may be closely associated with AD neuropathology. In this chapter, the anatomy, function and neurochemistry of the central cholinergic systems are discussed, followed by a review of cholinergic neurochemistry in AD and its significance to clinical features of the disease. Knowledge gaps, which this thesis aims to address, are also discussed.

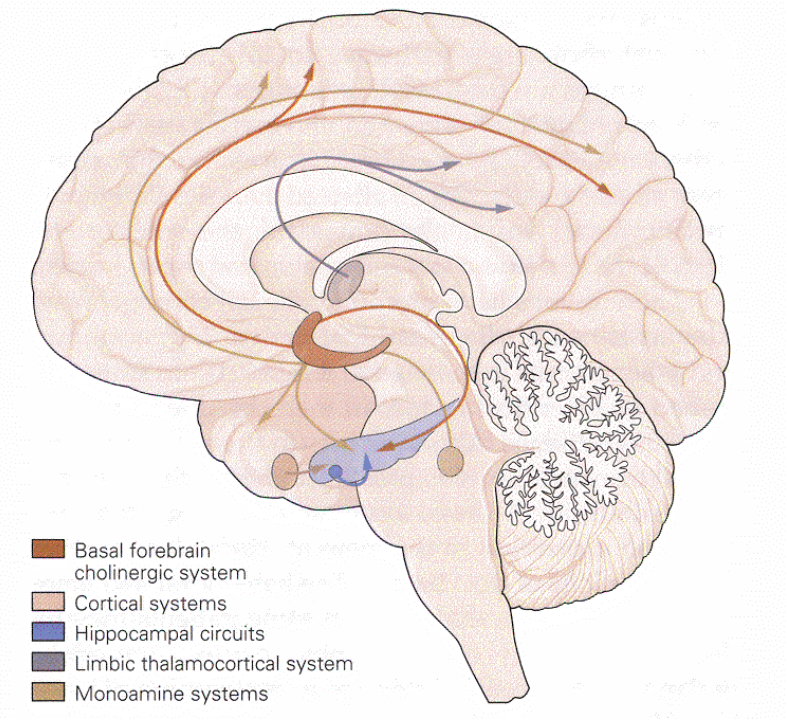
2.2 THE CHOLINERGIC SYSTEM IN MAMMALIAN BRAIN

2.2.1 Distribution of Cholinergic Neurons in the Brain

In the mammalian brain, the basal forebrain and pontine cholinergic neurons provide the primary cholinergic innervations to much of the cerebral cortex and brain stem region (See **Figure 2.1**). The basal forebrain cholinergic regions consist of the medial septum (MS), the vertical and horizontal diagonal bands of Broca (VDBB and HDBB), and the nucleus basalis of Meynert (NBM). While the neurons situated in the MS innervate predominately to the hippocampal formation, the neurons located in the VDBB and HDBB project to the anterior cingulate cortex and olfactory bulb, respectively. Additionally, the NBM is the source of cholinergic projections to the

amygdala as well as throughout the neocortex. Much research data have pointed to cholinergic circuits in the hippocampus and neocortex as playing essential roles in mediating learning and memory processes.

Figure 2.1 Cholinergic system in mammalian central nervous system. (Kandel et al. 2000)



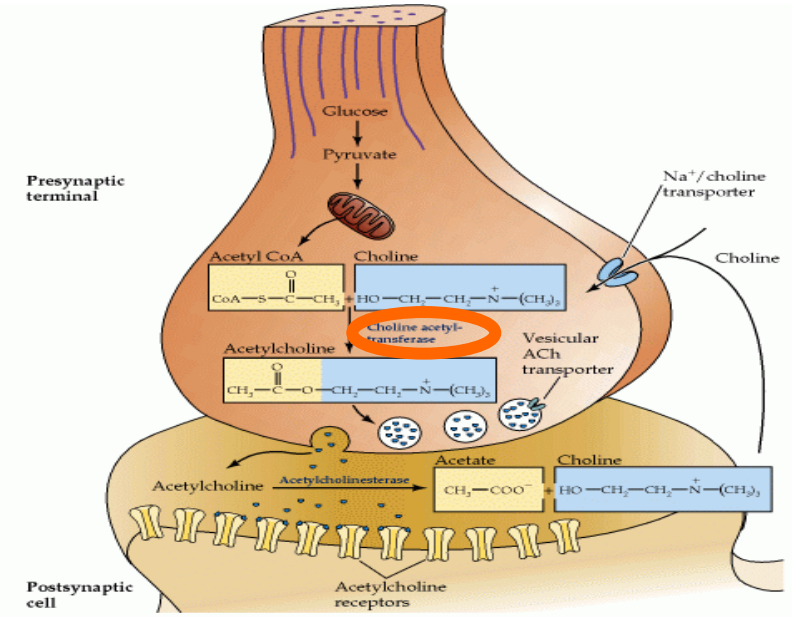
2.2.2 Cholinergic Pathways in the Brain

Cholinergic neurons are characterized by the expression of the enzyme choline acetyltransferase (ChAT), the enzyme that catalyzes the biosynthesis of acetylcholine (ACh). However the expression of acetyl- and butyrylcholinesterases (AChE / BChE), enzymes that catalyze the hydrolysis of ACh, is relatively less specific to cholinergic neurons (Darvesh et al. 2003; Mesulam et al. 1989; Mesulam and Geula 1988).

Biochemical studies which measure ChAT and AChE activities in human brain have demonstrated regional variations of enzyme activities, with the core limbic areas such as the amygdala and hippocampus having the highest ChAT and AChE activities while the visual cortex region has the lowest ChAT and AChE activities (Davies and Maloney 1976; Perry et al. 1977; Rossor et al. 1982). Both ChAT and AChE play important roles in the ACh metabolic pathway in the central nervous system.

ACh, synthesized in the terminals of cholinergic neurons, is one the major excitatory neurotransmitters in the brain. ACh can increase the intracellular membrane potential by reducing potassium ion conductance, leading to higher propensity to depolarization in cholinoceptive neurons. ChAT plays an essential role in ACh production in the brain by catalyzing the transfer of the acetyl group from acetyl coenzyme A to choline (See **Figure 2.2**). Thus, the activity of ChAT is a neurochemical marker for cholinergic innervation and status of the cholinergic neurons. Once ACh is produced, the neurotransmitter is packed into synaptic vesicles by vesicular ACh transporter (VAChT). After release into synaptic clefts, ACh is catabolized by acetylcholinesterase (AChE) resulting in the production of acetate and choline. Subsequently, choline is accumulated back into the presynaptic neurons via a high affinity Na^+/choline transporters and is used again for ACh synthesis. Therefore, AChE is important for determining the intensity and duration of cholinergic neurotransmission, and the enzyme regulates a number of cholinergic functions, including arousal, sensory processing, learning and memory (Taylor and Brown 1999).

Figure 2.2 Acetylcholine synthesis in cholinergic neurons. (Purves et al. 2001)



2.2.3 Cholinergic Receptors in Brain

Cholinergic signaling is mediated by two main groups of cholinergic receptors, the ion channel nicotinic receptors and the G-protein coupled muscarinic receptors.

2.2.3.1 Nicotinic receptors

Nicotinic receptors are ligand-gated heteropentameric ion channels. Nicotinic receptors primarily mediate conductance of Ca^{2+} ions. Currently five nicotinic receptor subunits have been identified, each of which exists in several subtypes (Steinbach and Ifune 1989). In mammalian brain, the α and β subunits combine with different configurations to form at least three pharmacologically distinct nicotinic receptors (nAChR), namely, α4β2, α3β4, and α7 nAChR (Giacobini, 1990; Newhouse et al. 1997).

Nicotinic receptors are not distributed uniformly in the brain, with the highest densities found in the hippocampus, followed by deeper layers of neocortex (Cimino et al. 1992; Rubboli et al. 1994).

2.2.3.2 Muscarinic receptors

Muscarinic cholinergic receptors are coupled to G-proteins, especially those belonging to the G_s and $G_{q/11}$ families, thus modulating multiple intracellular signal transduction pathways including those mediated by adenylate cyclase and phospholipase C (PLC). At present, five different muscarinic receptors (m1 – m5), each of which is the product of a different gene, have been identified. The m1-m3 receptors correspond to the pharmacologically characterized M_1-M_3 subtypes: M_1 and M_3 have relatively higher affinity for the antagonist pirenzepine, and low affinity for AFDX-116 or AFDX-384, while the M_2 subtype show the reverse affinities. Muscarinic receptors are distributed widely but heterogenously in human brain, with the highest densities found within the striatum and hypothalamus, followed by the hippocampus and cerebral cortex, and the lowest densities in the cerebellum (Cortes et al. 1987; Lin et al. 1986). In addition, higher densities of M_1 receptors are found in most parts of limbic and paralimbic regions, whereas M_2 receptors are commonly found in the primary sensory areas as well as parts of the primary motor cortex. The limbic and paralimbic localization of M_1 and M_3 receptors suggest that these receptors may be critically involved in learning and memory formation.

2.3 CHOLINERGIC SYSTEM IN THE CNS AND AD

2.3.1 Cholinergic Hypothesis

In AD, brain regions that are associated with higher mental functions e.g. the hippocampal formation, entorhinal cortex and neocortex, are more prone to neurodegeneration. As mentioned above, these areas are widely innervated by ChAT - containing neurons arising from the basal forebrain. It is well established that the basal forebrain cholinergic neurons are severely affected in AD (Whitehouse et al. 1982). Since such cholinergic neurons project widely to the hippocampus and cerebral cortex, they are thought to play important roles in learning and memory. The loss of these neurons may be involved in cognitive impairment in AD. Indeed, the integration of animal pharmacology, biochemistry, and clinical research have led to the proposal of the cholinergic hypothesis of AD (Bartus et al. 1982), which attributed the cognitive symptoms associated with AD to the cholinergic deficits. A series of human and primate studies have suggested the involvement of the cholinergic system in cognition. Drachman and Leavitt (1974) demonstrated that low doses of the cholinergic antagonist, scopolamine, resulted in cognitive deficits that were similar to those seen in elderly volunteers. Others have also shown that the pattern of cognitive deficits induced by cholinergic antagonism mimicked some of the cognitive deficits in AD patients (Bartus et al. 1985; Smith and Swash 1978). Furthermore, primate studies have shown that scopolamine induced cognitive deficits which closely matched the naturally occurring cognitive deficits in aged monkeys, while age-related memory losses in monkeys could be ameliorated by the anticholinesterase compound, physostigmine (Bartus 1979). Taken

together, findings from these studies suggest a direct relationship between central cholinergic receptors and cognitive function. The second line of evidence that gives support to the cholinergic hypothesis of AD is the finding of profound cholinergic deficits in AD. In addition to the loss of basal forebrain cholinergic neurons, neurochemical studies of AD have shown severe losses cholinergic neurons elsewhere in the cognitive brain, for example, loss of ChAT and AChE activities, reduction in ACh release and choline uptake, and loss of cholinergic (both nicotinic and muscarinic M_2 and M_4 receptors in the hippocampus and neocortex (Francis et al. 1985; Mash et al. 1985; Mulugeta et al. 2003; Perry et al. 1977; Perry et al. 1987; Sims et al. 1983; Wilcock et al. 1982). Postsynaptic M_1 receptor densities appear unchanged (Araujo et al. 1988; Mash et al. 1985). However, later studies reported losses of M_1 coupling to G-proteins, as well as associated deficits in phosphotidylinositide hydrolysis and protein kinase C (PKC) activities (Cole et al. 1988; Ferrari-DiLeo et al. 1995; Ferrari-DiLeo and Flynn 1993; Flynn et al. 1991).

2.3.2 Cholinergic System Association with Cognitive and Non-cognitive Features in AD

An increasing number of studies have demonstrated that cholinergic system dysfunction is associated with cognitive as well as non-cognitive features in AD. For example, the loss of ChAT activity is a reflection of the degeneration of cholinergic neurons in the CNS; thus, when ChAT activity reduction is correlated with dementia severity in AD (Perry et al. 1978; Wilcock et al. 1982), the loss of the CNS cholinergic neurons is associated with dementia severity (Wilcock et al. 1982). Moreover, Lai et al (2001) have demonstrated that the muscarinic M_2 receptor densities in frontal and temporal cortex are upregulated in AD patients with delusion and hallucinations. The

muscarinic M_1 receptor density, on the other hand, is unaltered in AD; thus, these results may provide an explanation why cholinergic replacement therapy results in improvement of BPSD including psychosis and suggest that M_2 receptor may be a potential drug target for BPSD in AD. Other cholinergic markers may not relate to neuropsychological behaviors as well as they relate to cognition. Procter et al (1992), for instance, have demonstrated that there is significant loss of ChAT activity in the cerebral cortex of AD patients both with and without aggressive symptoms. However, Minger et al (2000) have shown that the ratio of ChAT to dopaminergic D1 receptor correlates negatively to aggressive behavior in AD. These results suggest that central cholinergic system may not be directly associated with BPSD but may regulate other systems which do. **Table 2.1** summarizes the known deficits of pre- and postsynaptic cholinergic markers and their clinical correlates.

Table 2.1 Cholinergic changes in AD and their clinical correlates.

	Changes in AD	Clinical correlates
Cholinergic neurons & nerve terminals	*Postmortem* ↓ acetylcholine producing neurons in the BFC system (Davies and Maloney 1976; Whitehouse et al. 1982).	*Postmortem* Correlated with ↓ ChAT, thus indirectly with dementia severity (Wilcock et al. 1982).
ACh	*Biopsy* ↓ [^{14}C]ACh synthesis in FC and TC (Francis et al. 1985; Sims et al. 1983).	*Biopsy* ↓ [^{14}C]ACh synthesis correlates with cognitive impairment (Francis et al. 1985).
ChAT	*Postmortem* ↓ widespread: cerebral cortex , HP and basal nucleus (Araujo et al. 1988; Bowen et al. 1982; Davies 1979; Rossor et al. 1984). *Biopsy* ↓ neocortex (Sims et al. 1983).	*Postmortem* ↓ ChAT correlated with dementia severity (Perry et al., 1978; Wilcock et al. 1982) but ↓ cerebral cortex in AD patients both with and without aggressive symptoms (Procter et al. 1992)[1].
AChE	*Postmortem* ↓ (Davies, 1979; Garcia-Alloza et al. 2005; Perry et al. 1978). *Biopsy* ↓ neocortex (Sims et al. 1983).	*Postmortem* AChE / serotonin ratios correlated with predeath cognitive scores (Garcia-Alloza et al. 2005).
HACU	*Postmortem* ↓ FC and HP; ↔ caudate-putamen and cerebellum (Pascual et al. 1991).	Not reported.
Muscarinic receptors M_1	*Postmortem* ↔ FC and TC **(Lai et al. 2001)**; ↔ cerebral cortex (Mash et al. 1985); ↔ cortical, subcortical regions, but modestly ↑ in HP and ST (Araujo et al. 1988); uncoupled from G-proteins (Flynn et al. 1991).	*Postmortem* M1-G-protein uncoupling correlated with dementia severity **(Tsang et al. 2006)[2]**.
M_2	*Postmortem* ↓ in cerebral cortex (Mash et al. 1985); ↓ all cortical areas and HP, but ↔ in subcortical areas (Araujo et al. 1988).	*Postmortem* Delusion and hallucinations correlated with ↑ in FC and TC **(Lai et al. 2001)[2]**.
M_3	Not reported.	Not reported.
M_4	*Postmortem* ↓ in HP (Mulugeta et al. 2003).	Not reported.
M_5	Not reported.	Not reported.
Nicotinic receptors (nAChR)	*Postmortem* ↓ cortical areas and HP, but not in subcortical regions (Araujo et al. 1988)[3]. *Postmortem* specific ↓ $\alpha4\beta2$ populations in TC (Warpman and Nordberg 1995); ↓ $\alpha4$, but ↔ $\alpha3$ and $\alpha7$-containing nAChR in TC (Martin-Ruiz et al. 1999).	Not reported.

Table 2.1 (**previous page**) *Note*: ↓ = decreased; ↑ = increased; ↔ = no change; ACh = acetylcholine; AChE = acetylcholinesterase; ChAT = choline acetyltransferase; FC = frontal cortex; HACU = high affinity choline uptake; HP = hippocampus; ST = striatum; TC = temporal cortex
[1]Results may suggest that 5-HT deficits is more closely related than acetylcholine to aggression in AD (Procter et al. 1992).
[2] Correlations with longitudinally assessed clinical data.
[3] Use of [^{3}H]carbamylcholine which has limited specificity for nAChR subtypes.

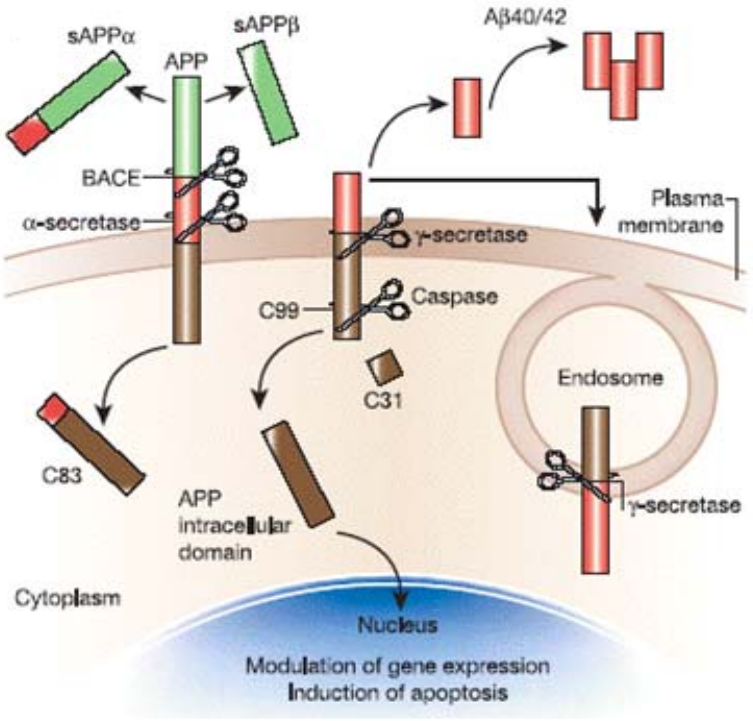
2.3.3 Cholinergic Neurotransmission, τ Phosphorylation and Aβ Processing

The major pathological hallmarks of AD are extracellular deposit of neuritic plaques (NPs), intracellular formation of neurofibrillary tangles (NFTs), and selective loss of neurons (Braak and Braak 1997; Dickson 1997; Jellinger and Bancher 1997). Furthermore, as mentioned above, AD severely affects the cholinergic system. Previous studies have suggested that cholinergic system function, Aβ production, and formation of NFTs are closely related.

Amyloid precursor protein (APP) processing follows two alternative pathways: the non-amyloidogenic and amyloidogenic pathways. Most of APP is cleaved by α-secretase within the Aβ domain (between positions 16 and 17), resulting in the release of a large, secretory N-terminal fragment - secreted APP (sAPPα) - into the extracellular space, and leaving the 83-amino-acid carboxyl-terminal (C83) fragment in the cell. This pathway is also known as non-amyloidogenic pathway because α-secretase cleave within the Aβ domain, thus precluding its formation. Alternatively, the amyloidogenic pathway involves the sequential cleavages by β-site APP-Cleaving-Enzyme (BACE) and γ-secretase at the N- and C-termini of the Aβ domain, respectively. After APP is cleaved by BACE, two peptides are generated: secreted sAPPβ and a 99-amino-acid C-terminal peptide. The subsequent cleavage of the C99 peptide by the γ-secretase complex which consists of presenilin, nicastrin, Aph-1 and Pen-2 (Xia and Wolfe 2003) liberates either a

40- or 42-amino acid Aβ peptide into the extracellular space and an amyloid intracellular domain (AICD) inside the cell. While the accumulation of insoluble Aβ40/42 peptides produce the neuritic plaques in the extracellular space, AICD may regulate expression of specific genes such as apoptotic genes (Leissring et al. 2002). Additionally, cleavage of C99/AICD by caspases generates a neurotoxic peptide (C31) (Lu et al. 2000, see **Figure 2.3**).

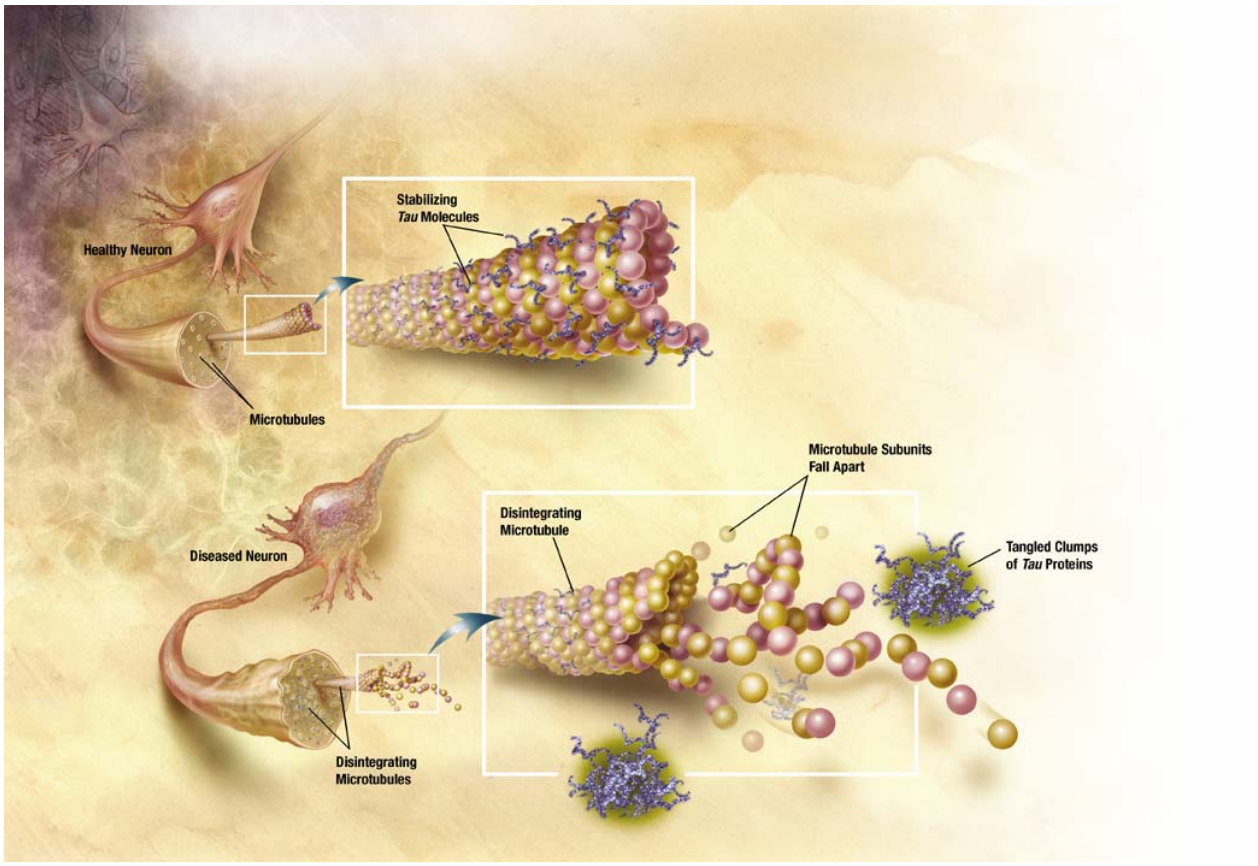
Figure 2.3 Proteolytic processing of APP. (Mattson, 2004)



NFTs are deposits of insoluble filaments and amorphous material in the cell body of a neuron, and when similar deposits are found in axons and dendrites, they are termed neuropil threads (NTs). Both NFTs and NTs are composed of paired helical filaments

(PHFs), which in turn consist mainly of τ protein. τ protein belongs to a family of proteins known as microtubule-associated proteins (MAPs) which binds to and stabilizes microtububles (MTs). τ protein is widely distributed throughout the central (CNS) and peripheral (PNS) nervous system, especially in the axons of nerve cells (Binder et al. 1985). τ is a phosphoprotein containing both proline-directed serine/threonine phosphorylation sites and non-proline directed phosphate acceptor residues (Morishima-Kawashima et al. 1995; Trojanowski and Lee 1994). *In vitro* studies have shown that mitogen-activated protein kinase (MAPK) (Drewes et al. 1992; Greenberg and Kosik, 1995), glycogen synthase kinase-3 (GSK3, Hanger et al. 1992; Mandelkow et al. 1992; Sperber et al. 1995), and cyclin-dependent kinase 5 (cdk5) (Baumann et al. 1993) phosphorylate τ at a number of the identified serine/threonine-proline residues. Phosphorylated τ proteins dissociate from MTs and accumulate in the perikarya. They may then associate with other intracellular chaperones, for example, sulfated glycosaminoglycans (SGs), RNA, and/or DNA, forming NFTs in the cell body of a neuron, or NTs in axons or dendrites, and/or around the cores of amyloid plaques (see **Figure 2.4**). The deposits of NFTs and NT have profound effects on neurons. For example, the aggregates can block normal trafficking, resulting in cell death. Additionally, when τ proteins are hyperphosphorylated, they cannot bind to MTs which become depolymerized, resulting in disruption of the neuronal cytoskeleton and interference of axonal transport which may result in neuronal degeneration (Vincent et al. 1994)**.**

Figure 2.4 Neurofibrillary tangles (NFTs) formation.
(Picture credit: http://www.alzheimers.org/rmedia/graphicslowres.htm)



Several lines of evidence suggest a close relationship among cholinergic system, Aβ, and NFTs. Perry et al. (1978) have reported that cholinergic depletion as measured by loss of ChAT activities correlated with senile plaque counts. More recent *in-vitro* studies have demonstrated that even very low concentrations (at picomolar to nanomolar ranges) of Aβ can inhibit ACh synthesis in SN56 cells derived from mouse basal forebrain cholinergic neurons (Pedersen et al. 1996) and ACh release in rat hippocampal and cortical slices (Kar et al. 1996). Moreover, Nitsch et al. (1992) have shown that in human kidney embryonic cells overexpressing muscarinic M_1 and M_3 receptors, activation of these receptors with carbachol, a non-selective agonist which activates both nicotinic and

muscarinic receptors, increased the release of soluble APP. Similarly, reduction of the levels of phosphorylated τ in PC12 cells which were transfected with the gene for muscarinic M_1 receptor was observed when the cells were treated with carbachol and AF102B (Sadot et al. 1996). In addition, Arendt et al. (1999) have demonstrated a strong association between cholinergic deficits and the cortical distribution of hyperphosphorylated τ in AD. Taken together, although these correlative studies cannot confirm the cause-effect relationships between cholinergic dysfunction and plaque and tangle formation, they do suggest that the cholinergic system, APP and τ interact during the AD process.

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CHAPTER 3

Impairment of G-protein Coupled Receptor Signaling in Alzheimer's Disease

3.1 INTRODUCTION

As mentioned in **Chapter 2,** one of the most consistently affected transmitter systems in AD is the cholinergic system in regions such as cerebral cortex and hippocampus. Findings of loss of cholinergic neurons in the basal forebrain have been supported by neurochemical findings of reductions in presynaptic cholinergic markers such as choline acetyltransferase (ChAT) activities. Since cholinergic neurons project to widespread areas of the cerebral cortex and are thought to play essential roles in learning and memory, the loss of these neurons may lead to cognitive impairment in AD.

Furthermore, Lu et al (1999) demonstrated that G-protein coupled acetylcholine receptors may potentiate glutamatergic *N*-methyl-D-aspartate recceptor (NMDAR) via a protein kinase C (PKC) dependent signaling pathway. Because NMDAR function, especially in mediating long term potentiation (LTP) is widely believed to be a mechanism in learning and memory, it is possible that cholinergic deficits may affect learning and memory processes via its effect on NMDAR.

Acetylcholine receptors can be classified into nicotinic receptors and muscarinic receptors. Muscarinic receptors belong to a group of seven-transmembrane spanning receptors which transduce their signals across membranes by interacting with GTP-binding proteins (G-proteins). Muscarinic receptors can be further divided into 5 subtypes – M_1 to M_5. While M_1, M_3 and M_5 receptors are coupled to $G_{q/11}$, M_2 and M_4 are coupled to the $G_{i/o}$ proteins. In brain, M_2 receptors are predominantly presynaptic autoreceptors while M_1 receptors are believed to be postsynaptic heteroreceptors. Using radioligand binding approaches, Araujo et al. (1988) have demonstrated that in AD muscarinic M_2 receptors are markedly reduced while M_1 receptors are unaltered. However, Flynn and colleagues (1991) later showed that muscarinic M_1 receptor is "uncoupled" to its G-proteins in AD. Other studies (Cole et al. 1988; Masliah et al. 1991) also found that PKC activities are significantly reduced in AD. Taken together, the findings suggest that the downstream signaling of cholinergic system in AD is impaired. This chapter provides a brief overview of the pathways and molecules involved in G-protein-coupled receptors (GPCR)-mediated signaling which may be affected in AD.

3.2 G-PROTEINS

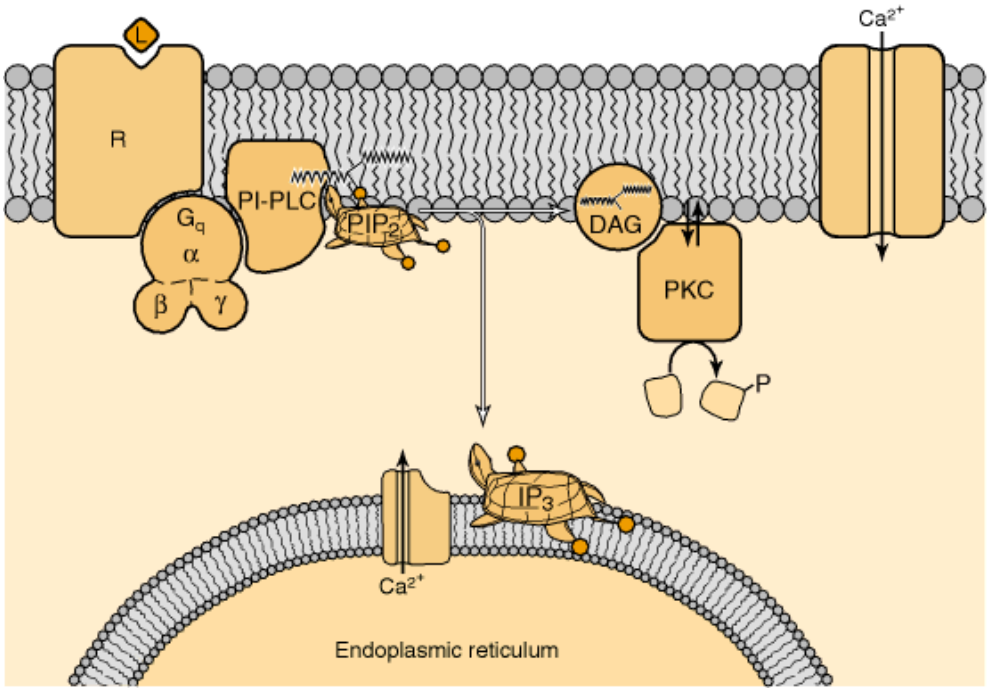
3.2.1 G-protein Structure and Activation

G-protein stands for guanine neucleotide dependent protein. In general, G-protein serves as intermediaries between GPCR (e.g. muscarinic M_1 and M_3 receptors) and downstream effectors (e.g. phospholipase C [PLC]). When G-proteins of the type associated with muscarinic receptors are inactive, they exist as complexes made up of three subunits - α, β, and γ subunits – with guanosine diphosphate (GDP) binding to the α subunit. Once GPCR binds to an agonist, the coupled G-protein undergoes conformational changes and exchanges GDP for guanosine triphosphate (GTP). Subsequently, the α subunit of the activated G-protein separates from the β and γ subunits and may activate downstream effectors. After the α subunit activates its target, the GTP is hydrolyzed to GDP by intrinsic GTPase activity, and the inactivated α subunit reunites with its β and γ subunits.

3.2.2 A Common $G_{q/11}$ G-protein Signaling Pathway

Figure 3.1 depicts a common signaling pathway for $G_{q/11}$-coupled receptors. Briefly, agonists bind to GPCR and subsequently activate PLC via G_q. The activated PLC cleaves phosphatidylinositol-4,5-bisphosphate (PIP_2) and yields inositol-1,4,5-triphosphate (IP_3) and diacylglyceral (DAG). The released IP_3 binds to specific IP_3 receptor in the endoplasmic reticulum and Ca^{2+} is liberated, causing its intracellular Ca^{2+} concentrations to rise. Ca^{2+} and DAG then activate protein kinase C (PKC) which in turn triggers multiple downstream signaling pathways. In addition, increased cytosolic Ca^{2+} levels cause Ca^{2+} membrane channel to open.

Figure 3.1 G-protein signaling pathway. (Siegel et al. 1999)



3.3 PROTEIN KINASE C (PKC)

3.3.1 PKC Structure

PKC is a proteolytically activated serine/threonine kinase (Nishizuka 1992). Since the discovery of PKC in 1977 (Takai et al. 1977), at least 12 PKC isoforms that are expressed by mammalian cells have been discovered. PKC isoforms can be classified into 3 main groups based on their different requirements for activators such as Ca^{2+} and DAG (Casabona 1997): calcium or conventional (cPKC) (α, βI, βII, γ), novel (nPKC) (δ, ε, η, θ), and atypical (aPKC) (ι/λ, ζ). The groups differ in cofactors requirements, tissue distribution, intracellular localization and potential substrate specificity. Two new isoforms of PKC (μ/PKD,ν) were added to the list later. Of all the mammalian PKC

isoforms, the γ, ε, θ, ι/λ PKC isoforms are specifically expressed in the CNS, whereas others are widely expressed in different cell types (see **Table 3.1**).

Table 3.1 Mammalian protein kinase C isoenzymes. (Battaini et al. 2001)

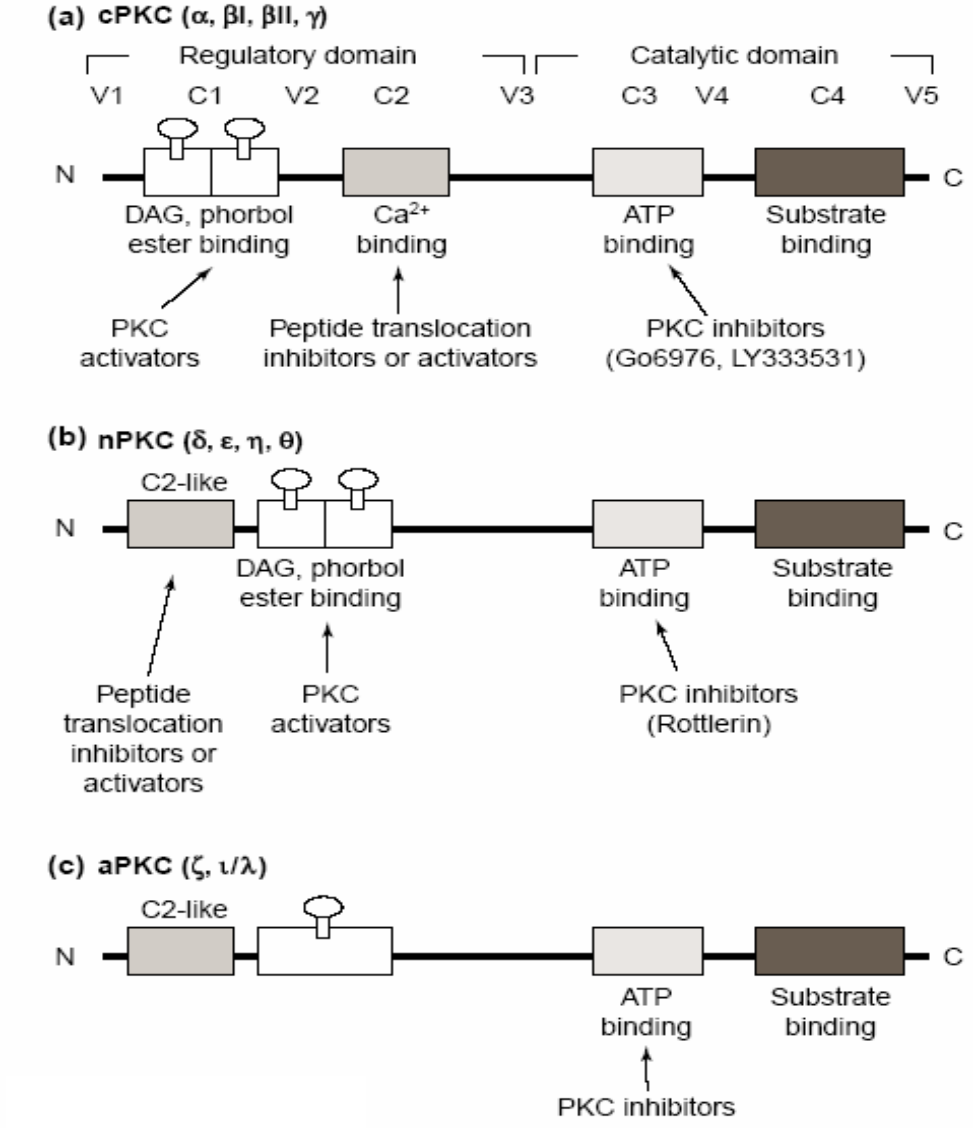
Type	*Activators*	*Isoenzyme*	*Distribution*
Conventional	Ca, PS, DAG	α	Widespread
		βI	Widespread (low levels)
		βII	Widespread
		γ	Brain, Spinal cord
Novel	PS, DAG	δ	Widespread
		ε	Brain, Hematopoietic tissues
		η	Heart, Lung, Skin
		θ	Hematopoietic tissues, Brain, Skeletal muscle
Atypical	PS, PI-3,4,5-P3	ζ	Widespread
		ι/λ	Brain, Kidney, Lung
Recently described	DAG, PI-4,5-P2	μ/PKD	Lung, Epithelial cells
		ν	Widespread

Ca = Calcium; PS = Phosphatidylserine; DAG = Diacylglycerol; PI-3,4,5-P3 = phosphatidylinositol-3,4,5-triphosphate, PI-4,5-P2 = phosphatidylinositol 4,5 diphosphate

PKC is a single polypeptide with an NH_2-terminal regulatory region and a C-terminal catalytic region. (See **Figure 3.2**) In general, all PKC isoforms share similar but non-identical domain structures. In the NH_2-terminal of cPKCs, for example, the regulatory region contains two conserved sequences - C1 and C2 - that are important for regulation of enzymatic activity. On the other hand, the nPKCs and aPKCs possess a C2-like domain. In both cPKCs and nPKCs, the C1 regions comprise a pseudosubstrate sequence and two cysteine-rich repeats that bind to DAG and phorbol esters (Ono et al. 1989), but there is only one cysteine-rich region in the aPKCs. Additionally, the C2 region which is present only in cPKCs is responsible for Ca^{2+} sensitivity and is involved in the translocation to membranes after the levels of Ca^{2+} increase in the cell. Two additional conserved regions – C3 and C4 - are found in the C-terminal catalytic region. The C3

domains are responsible for ATP binding while the C4 domains are important for protein substrate binding. Taken together, possession of the various domains allow PKC isoforms to respond to a wide array of signals from hormonal, neuronal and growth factor stimuli. All PKC isoforms except the μ/PKD and ν also contain a pseudosubstrate sequence which interacts with the substrate-binding region, thus keeping the enzyme in an inactive state. The mode of activation for μ/PKD and ν PKCs may be different from the other PKC isoforms since they lack the pseudosubstrate sequence; nevertheless, the activation of the conventional, novel, atypical as well as the μ/PKD and ν PKCs is regulated by phosphorylation-dependent pathways. PKC is mostly located in the cytosol where it is in inactive state. Upon external stimulation, PKC translocates to the plasma and/or nuclear membrane where it is bound by PKC anchoring proteins or RACKs (receptor for activated C-kinase) (MacDonald et al. 2001). Muscarinic receptors which couple to $G_{q/11}$ G-proteins (M1, M3 and M5), are known to activate PLC, leading to Ca^{2+} influx, intracellular release of Ca^{2+}, IP_3 and DAG, which in turn activates cPKC. In brief, 3-phosphoinositide-dependent kinase-1 (PDK-1) which is activated by inositol phospholipids phosphorylates the newly synthesized PKC enzymes as membrane-bound enzymes in the catalytic region. Subsequently, the phosphorylated PKC undergoes autophosphorylation and translocates to the cytosol. Although the cytosolic PKC is inactive, it is catalytically competent and is readily responsive to physiological activators.

Figure 3.2 Primary structure of PKC structure. (Way et al. 2000)



3.3.2 PKC Functions

PKC contributes to the function and maintenance of neurons in a number of ways, including the transduction of mitogenic signals of receptor tyrosine kinases (RTKs) as well as GPCRs (Gutkind, 1998; Luttrell et al. 1999). In neuronal cells, PKC is responsible for multiple neuronal signal transduction pathways by phosphorylating

specific substrates, resulting in increasing neuronal excitability in response to external inputs. In particular, PKC is involved in the control of ion channel function, desensitization of receptors and enhancement of neurotransmitter release, suggesting that PKC may regulate synaptic plasticity which in turn plays an important role in learning and memory processes (Tanaka and Nishizuka 1994). In addition, specific PKC isoforms may be involved in regulating apoptotic pathways. Although most PKC isoforms are antiapoptotic, some PKC isoforms such as PKC_{δ} is pro-apoptotic in a number of cell types (Dempsey et al. 2000), including neurons (Maher 2001). Therefore, PKC may play a part in the pathogenesis of neurodegenerative diseases, including AD.

3.4 NMDA RECEPTORS

3.4.1 NMDA Receptor Structure and Subunits

The *N*-methyl-D-aspartate (NMDA) receptor belongs to the ionotrophic glutamate receptor family, which also includes the α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor, and kainate (KA) receptors. NMDA receptors (NMDAR) possess unique features such as voltage-dependent block by Mg^{2+}, Ca^{2+} permeability, and slow deactivation kinetics (Cull-Candy et al. 2001). Since excitatory postsynaptic transmission is mediated by glutamate receptors, NMDAR is thought to play critical roles in synaptic plasticity events such as LTP which is a cellular mechanism in learning and memory in mammalian central nervous system.

NMDAR is made up of NR1, NR2, and NR3 subunits. The NR1 subunit can exist in 8 splice variants due to three independent sites of alternative splicing in the NR1 gene (Sugihara et al. 1992). The NR2 subunit family comprises four members (NR2A to

NR2D) which are encoded by separate genes. The NR3 subunit family consists of two splice variants, NR3A and NR3B (Cull-Candy et al. 2001).

Functional NMDARs exist as heterotetramers or heteropentamers which are assembled from any two obligatory NR1 splice variants interacting with two or three NR2 encoded subunits to form a functional NMDA receptor. While the NR2 subunits contain the glutamate-binding sites, NR1 subunits contain the glycine-binding sites which modulate the function of the receptor in the presence of glycine. Therefore, functional NMDARs contain at least 2 glycine-binding sites and 2 glutamate-binding sites on the ion channel complex, and both ligand binding sites have to be occupied for the channel to open. Similar to NR2 subunits, NR3 subunits do not form functional NMDAR alone. However, NR3 subunits can bind with the NR1/NR2 complexes and depress NMDAR responses (Das et al. 1998). Several studies have demonstrated that NMDAR channel properties are determined by NMDA subunit composition (Flint et al. 1997; Misra et al. 2000; Monyer et al. 1992; Takahashi et al. 1996). NMDARs also contain allosteric recognition sites that can modulate their function. These include the binding sites for polyamines (e.g. spermine and spermidine) and Mg^{2+}. There are regional distribution differences in NMDAR subunit compositions. In dorsal horn spinal neurons, NR1/NR2D and NR1/NR2B receptors are predominately located extrasynaptically and NR1/NR2A receptors are found in the primary inputs to mature cells (Momiyama 2000).

3.4.2 NMDA Receptor Functions

NMDARs are thought to play critical roles in learning and memory because of two unique characteristics. Firstly, NMDARs are highly permeable to Ca^{2+} and other cations; thus, activation of NMDARs is particularly effective in promoting Ca^{2+} entry.

Secondly, NMDAR channels are blocked by voltage-dependent Mg^{2+}. These properties of NMDARs are important for long-term potentiation (LTP) / long-term depression (LTD) which are thought to be the cellular substrate for learning and memory (Bliss and Collingridge 1993). Briefly, at normal membrane potentials, NMDAR channel is blocked by Mg^{2+}. However, if enough glutamate is released to activate the postsynaptic NMDAR channels after a conditioning train of stimuli, the voltage-dependent Mg^{2+} molecule is then removed from the NMDAR channels resulting in PKC activation by the influx of Ca^{2+}. Subsequently, the activated PKC phosphorylates AMPA receptors as well as other proteins that are involved in gene transcription in the postsynaptic cells. Activation of gene transcription in the postsynaptic neuronal cells may be essential for remodeling of synaptic structures, which lead to synaptic plasticity.

3.5 GPCR SIGNALING PATHWAY IN RELATION TO NEUROPATHOLOGICAL AND NEUROCHEMICAL ALTERNATIONS IN AD

The major neuropathological hallmarks of AD include senile plaques, neurofibrillary tangles (NFTs) and selective loss of neurons. The formation of senile plaques and NFTs has been discussed in detail in **Chapter 1 and 2**. As mentioned in **Chapter 2**, there is a strong association between cholinergic deficits and the cortical distribution of hyperphosphorylated τ and Aβ in AD. Furthermore, Qiu et al. (2003) has demonstrated that treatment with the cholinergic agonist carbachol increases sAPP release and reduces Aβ production in rat hippocampal slices and that M_1, not M_2 receptors, are responsible for the effects. Similarly, activation of PLC-linked M_1 and M_3 receptors is found to enhance the non-amyloidogenic cleavage of APP as well as reduce the levels of

phosphorylated τ (Hellstrom-Lindahl 2000). Although these correlative studies cannot confirm the cause-effect relationships between cholinergic dysfunction and Aβ and NFTs formation, findings from further studies (Cole et al. 1988; Kurumatani et al. 1998; Masliah et al. 1991; Smith et al. 1987) suggest that cholinergic system may affect the production Aβ and τ proteins via muscarinic M_1 receptors / PLC / PKC signalling transduction pathway.

Besides plaques and tangles, AD is also characterized by cortical atrophy and losses of glutamatergic and cholinergic neurons. Throughout all layers of the neocortex as well as in the hippocampal formation, glutamatergic pyramidal neurons which form extensive synapses with basal forebrain cholinergic neurons are known to be severely degenerated AD (Francis et al. 1993). Because of its proximity with cholinergic neurons, degeneration and hypoactivity of glutamatergic pyramidal neurons which mediate the bulk of excitatory neurotransmission and synaptic plasticity in the brain may underlie the cognitive symptoms of AD (Francis 2003).

A direct interaction between cholinergic and glutamatergic systems has been established by several studies in that both muscarinic and nicotinic receptors can activate glutamatergic pyramidal neurons, resulting in increasing glutamate release (Chessell et al. 1993; Dijk et al. 1995). Other studies have demonstrated the potentiation of NMDAR currents by muscarinic agonists in a PKC and Src-dependent manner (Ali and Salter 2001; Lu et al. 1999; Xiong et al. 1998), suggesting that muscarinic receptors may regulate NMDAR function. Furthermore, Markam and Segal (1992) have also shown that the potentiation of NMDAR currents by mAChR agonists requires activation of phosphoinositide hydrolysis. Additionally, results from an *in-vitro* studies showed that in

hippocampal CA1 pyramidal cells NMDAR is potentiated by genetically defined M_1 receptors, and that M_1 receptors colocalized with NMDARs at postsynaptic glutamatergic synapses (Marino et al. 1998). The results reveal a spatial relationship between M_1 receptors and NMDARs. Taken together, these findings suggest that cholinergic deficits in AD are linked to glutamatergic deficits via breakdown of intracellular signaling. However, until now it has been unclear whether the M_1/G-protein uncoupling correlates with cognitive decline in AD. This question will be addressed in **Chapter 7.** Furthermore, it is also not clear whether M_1/G-protein uncoupling is related to the PKC deficits seen in AD (Cole et al. 1988), or to the hypofunction of NMDARs. These issues will be investigated in detail in **Chapter 8**.

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CHAPTER 4

The Serotonergic System in the Central Nervous System

4.1 INTRODUCTION

Besides progressive loss of cognitive functions, AD patients frequently exhibit behavioral and psychological symptoms of dementia (BPSD). BPSD include both psychotic symptoms (e.g. hallucinations and paranoid/delusional ideation) as well as nonpsychotic symptoms (e.g. aggression and wandering, affective disturbances, and anxieties/phobias, Cummings et al. 1994), and cause tremendous distress to the caregivers, often lead to institutionalization of the patients. Based on extensive animal and preclinical research evidence, it is reasonable to hypothesize that both cognitive decline and BPSD arise as a result of altered neurochemistry in the AD brain, which is in turn a consequence of both the neurodegeneration processes as well as reactive synaptic plasticity to these processes.

The serotonergic system is also one of the most consistently affected neurotransmitter systems in AD. Animal studies have already shown that the serotonergic system is important for cognition as well as behavioral processes (Buhot

1997). For example, members of the 5-HT_1 class of receptors are involved in a wide range of behavioral and physiological effects including sensorimotor reactivity, feeding, aggression, sexual behavior and anxiety responses (Lucki 1992). Additionally, the serotonergic system has become a major target for many psychotropic medications such as selective serotonin reuptake inhibitor (SSRI) and neuroleptics. Preclinical and clinical data suggest the anxiolytic and antidepressant activity of 5-HT_{1A} receptor agonists (Charney et al. 1990; Handley 1995). These findings suggest that the serotonergic system may be involved in cognition and behavioral symptoms in AD. **Table 4.1** summarizes alternations of serotonergic neurons, 5-HT metabolites and 5-HT receptor subtypes as well as clinical correlations in AD. This chapter will present the current state of knowledge on serotonergic system alternations in AD using results from postmortem brain tissues.

Table 4.1 Serotonergic system changes and clinical correlates in AD.

	Second messenger	Changes in AD	Clinical correlates
Serotonergic neurons & nerve terminals		↓ neurons in RN (Aletrino et al. 1992; Chen et al. 2000; Wilcock et al. 1988; Zweig et al. 1988).	No correlation with behavioural change or cognitive decline (Chen et al. 2000); delusions and hallucinations correlated with ↓ neurons in dorsal RN (Forstl et al. 1994).
5-HT		↓ TC, HP and ST (Cross, 1990; Middlemiss et al. 1986; Reinikainen et al. 1990).	Rate of cognitive decline correlated with ↓ in FC (Lai et al. 2002); ↓ levels of 5-HT in cortical and subcortical regions in AD patients with psychotic behaviours compared with nonpsychotic AD patients (Zubenko et al. 1991); ↔ TC in AD patients with psychosis (Lawlor et al. 1995); ↓ cortical levels of 5-HT in patients with aggression (Palmer et al. 1988).
5-HIAA		↓ TC (Cross, 1990)	Anxiety correlated with ↓ in cortex, thalamus and putamen (Reinikainen et al. 1990).
5-HTT		↓ neocortex (Chen et al. 1996; Cross, 1990).	Depression correlated with ↓ in FC and TC (Chen et al. 1996); ↓ in TC in non-anxious AD patients only, anxiety correlated with LL genotype of the 5-HTTLPR functional polymorphism (Tsang et al. 2003)[1,6].
5-HT_1 receptors			
1A	↑ basal cAMP ↓ stimulated cAMP ↑K^+ conductance	↓ FC, TC, HP and AMG; (Bowen et al. 1983; Cross et al. 1984; Crow et al. 1984; Middlemiss et al. 1986); ↔ FC (Sparks, 1989)[2].	↓ TC with moderate / severe aggression (Lai et al. 2003); ↓ 5-HT to 5-HT_{1A} ratio in FC with higher rate of cognitive decline (Lai et al. 2002).
1B,1D	$1D_\alpha$ - ↑cAMP (via G_i)	↓ FC and TC (Garcia-Alloza et al. 2004).	Cognitive decline correlated with ↓ in FC only (Garcia-Alloza et al. 2004)[3].
1E, 1F	↓ cAMP	Not reported.	Not reported.
5-HT_2 receptors	*5-HT_2 receptors*-		
2A	↑ PI turnover ↑ Ca^{2+} influx ↓K^+ conductance	↓ neocortex (particularly TC), HP and AMG (Cross et al. 1984; Crow et al. 1984; Jansen et al. 1990); ↔ in FC and HP(Dewar et al. 1990).	Cognitive decline correlated with ↓ in TC (Lai et al. 2005); a functional polymorphism in 5-HT_{2A} receptor (102-T/C) correlated with the presence of visual and auditory hallucinations in AD patients (Holmes et al. 1998); ↔ in cerebral cortex in a group of patients without aggressive symptoms (Procter et al. 1992).

Table 4.1. (Cont'd)

		Changes in AD	Clinical correlates
2B		Not reported.	Not reported.
2C		Not reported.	A functional polymorphism in 5-HT_{2C} receptor (Cys23Ser) correlated with visual hallucinations in AD patients (Holmes et al. 1998).
5-HT_3 receptors	Direct ↑ cation conductance	↔ AMG and HP (Barnes et al. 1990).	Not reported [5].
5-HT_4 receptors	↑ cAMP (via G_s)	↔ FC and TC (Lai et al. 2003).	None (Lai et al. 2003).
5-HT_5 receptors	Unknown	Not reported.	Not reported.
5-HT_6 receptors	↑ cAMP	↓ FC and TC (Garcia-Alloza et al. 2004).	Overactivity correlated with ↓ in TC; aggression correlated with 5-HT_6 to ChAT ratio in both FC and TC (Garcia-Alloza et al. 2004)[1].
5-HT_7 receptors	Not reported	Not reported.	Not reported.

Table 4.1. *Note*: ↓ = decreased; ↑ = increased; ↔ = no change; 5-HIAA = 5-hydroxyindoleacetic acid; 5-HT = 5-hydroxytryptamine; 5-HTT = 5-HT transporter; 5-HTTLPR = 5-HTT gene promoter region; AMG = amygdala; FC = frontal cortex; HP = hippocampus; OC = occipital cortex; PC = parietal cortex; RN = raphe nucleus; ST = striatum; TC = temporal cortex; cAMP = cyclic adenosine 3',5'-monophosphate; G_i = inhibitory G protein; G_s = stimulatory G protein; K^+ = potassium ion; PI = phosphoinositide

[1]Correlations with longitudinally assessed clinical data.

[2]May be due to cross-reactivity of [^{3}H]5-HT for other receptor subtypes (Sparks, 1989).

[3]↓ 5-$HT_{1B/D}$ receptors could reflect serotonergic neuronal loss in AD, or may be plasticity responses to a deteriorated cholinergic system, as 5-$HT_{1B/D}$ receptors act as inhibitors of ACh release (Garcia-Alloza et al. 2004).

[4]Potential confounding by cross-reactivity to dopamine receptors (Blin et al. 1993).

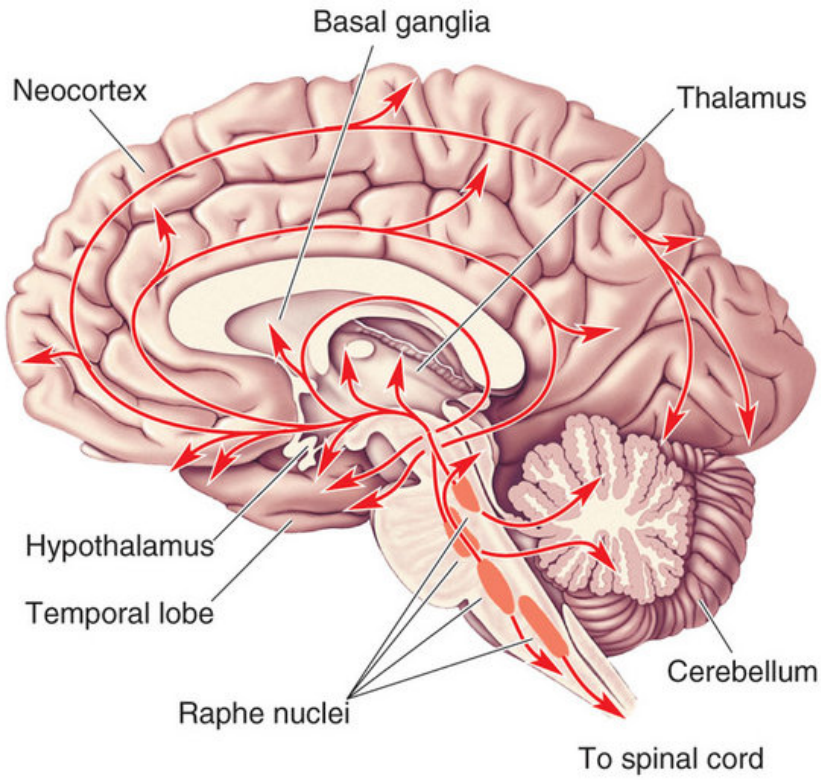
[5]5-HT_3 antagonist ondansetron also failed to improve cognition in AD in a controlled trial (Dysken et al. 2002).

[6]This study will be discussed in **Chapter 9** of this thesis.

4.2 SEROTONERGIC SYSTEM IN THE MAMMALIAN BRAIN

4.2.1 Distribution of Serotonergic Neurons in the Brain

Figure 4.1 Serotonergic system in the central nervous system.
(Picture credit: http://www.fi.au.dk/uk/jl/bc15/fig1512.jpg)

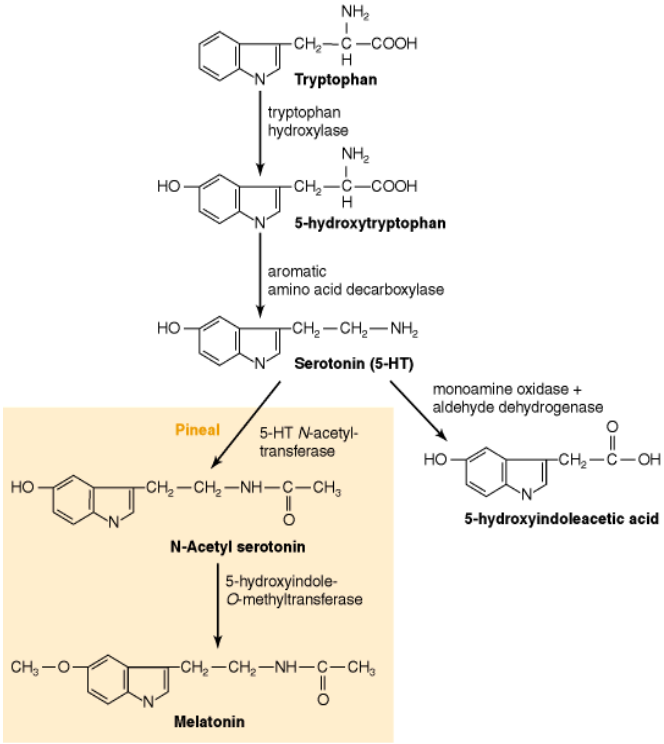


Serotonergic neurons project to widespread areas of the brain, and most of the cell bodies of serotonergic neurons are found in the raphe nuclei of the midline brainstem **(Figure 4.1)** (Palacios et al. 1990). The bulk of raphe nuclei consist of nine groups of serotonergic cell bodies (B_1 – B_9), and the largest group of serotonergic cells is B_7, which is continuous with a smaller group of serotonergic cells, B_6. Depending on the location of the cell bodies of 5-HT neurons and the areas that they innervate, the serotonergic cell

bodies can be divided into two main groups: dorsal and median raphe nuclei (Tork 1990). The B_6 and B_7 serotonergic cells together are considered as the dorsal raphe nuclei, and the B_8 serotonergic cells are considered as the median raphe nuclei. While fibres from the median nuclei project to the hippocampus, septum, and hypothalamus, the dorsal raphe nuclei innervate predominately to striatum. Since both dorsal and median raphe nuclei are responsible for projecting extensively to higher brain structures, the serotonergic system is thought to play an important role in the regulation of behavioral state and the modulation of more specific behaviors.

4.2.2 Biosynthesis and Metabolism of Serotonin

Figure 4.2 The biosynthesis and metabolism of serotonin. (Siegel et al. 1999)



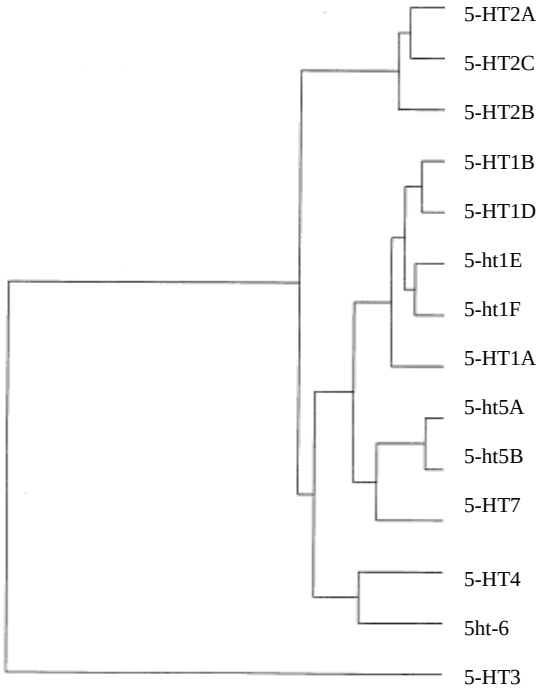
Serotonin (5-HT) is derived primarily from dietary tryptophan, and the process of 5-HT biosynthesis is relatively straightforward. Briefly, tryptophan is converted to 5-hydroxytryptophan by the action of tryptophan hydroxylase (an enzyme confined to 5-HT-producing cells). The 5-hydroxytryptophan is then decarboxylated to 5-HT, by amino-acid decarboxylase. Degradation of 5-HT occurs mainly through oxidative deamination, catalysed by mitochondrial monoamine oxidase (MAO), followed by oxidation to 5-hydroxyindoleacetic acid (5-HIAA). Alternatively, the released 5-HT is removed from synaptic cleft via an active 5-HT transporter (5-HTT, or 5-HT reuptake sites).

4.2.3 Serotonergic Receptors in the Brain

5-HT receptors are diverse **(Figure 4.3)**. At least 14 serotonin receptor subtypes are known to date. Among the seven 5-HT receptor families-5-HT_{1-7}-5-HT_1 (5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1E}, and 5-HT_{1F}) and 5-HT_2 (5-HT_{2A}, 5-HT_{2B}, 5-HT_{2C}) are further classified into various subtypes according to similar pharmacology and structure (Barnes and Sharp 1999). All serotonin receptors are G-protein-coupled metabotropic receptors, except for 5-HT_3 receptor which is an ion-gated channel receptor. All 5-HT_2 receptor subtypes are coupled positively to phospholipase C (PLC) and trigger the release of intracellular Ca^{2+}. 5-HT_{2A}, 5-HT_{2C}, 5-HT_3, and 5-HT_4 receptors are located postsynaptically where some are known to modulate ion flux to cause neuronal depolarization. Other 5-HT receptor subtypes like the 5-HT_{1A}, 5-HT_{1B} and possibly 5-HT_{1D} receptors are located on the 5-HT autoreceptors at the somatodendritic or nerve terminals. The 5-HT_1 receptors couple negatively to adenylate cyclase via G_i proteins. In CNS, 5-HT are excitatory on some neurons and inhibitory on others, depending on the 5-

HT receptor subtypes involved. It is becoming clear that some 5-HT receptors such as 5-$HT_{1B/D}$, 5-$HT_{2A/C}$, 5-HT_3 and 5-HT_4 receptors are also located on the nerve terminals of non-5-HT neurons where they appear to function as heteroreceptors, regulating neurotransmitter release. So far, very little is known about the locations and functions of 5-$HT_{5\text{-}7}$ receptors in CNS.

Figure 4.3 Dendrogram showing the evolutionary relationship between various human 5-HT receptor protein sequences (except 5-HT_{5A} and 5-HT_{5B} receptors which are murine in origin, Barnes and Sharp 1999).



4.3 SEROTONERGIC SYSTEM ALTERNATIONS IN AD

4.3.1 Serotonergic System Association with Cognitive and Non-cognitive Features

The evidence for serotonergic dysfunction in AD is based on the post mortem measurements in brain tissue of 5-HT metabolites and 5-HT receptor changes. Losses of 5-HT presynaptic markers such as 5-HTT (Chen et al. 1996; Cross 1990) and degeneration of raphe nuclei (Chen et al. 2000) are prominent in AD. Similarly, concentrations of 5-HT and metabolite (5-HIAA) in regions including temporal cortex, hippocampus and striatum were markedly reduced in AD (Cross 1990; Middlemiss et al. 1986; Reinikainen et al. 1990). Furthermore, 5-HT receptors are selectively affected in AD. Postmortem studies have demonstrated that the levels of 5-HT_{1A} receptor are significantly reduced in the frontal and temporal cortex, hippocampus and amygdala (Bowen et al. 1983; Cross et al. 1984; Crow et al. 1984; Middlemiss et al. 1986). However, the loss of 5-HT_{2A} receptors is greater than the loss of 5-HT_{1A} receptors in AD neocortex (Cross 1990). Furthermore, the levels of 5-$HT_{1B/1D}$ receptor are reduced in AD frontal and temporal cortex (Garcia-Alloza et al. 2004). On the other hand, the levels of 5-HT_3 and 5-HT_4 receptors do not seem to be affected in AD (Barnes et al. 1990; Lai et al. 2003). Currently, not much work has been done on other 5-HT receptors except for a recent study showing a reduction of 5-HT_6 receptors in the frontal and temporal cortex of AD patients (Garcia-Alloza et al. 2004).

Various studies have demonstrated that cognition and neuropsychological symptoms in AD are correlated with the levels of 5-HT and 5-HIAA. The reduction of 5-HT to 5-HT_{1A} ratio is correlated in frontal cortex is correlated with higher rate of cognitive decline in AD (Lai et al. 2002). A postmortem study also reported that

cognitive decline is correlated with the reduction of 5-$HT_{1B/1D}$ levels in the frontal cortex (Garcia-Alloza et al. 2004). There is also some evidence that serotonergic function may be involved in BPSD. Zubenko et al. (1991) have shown a greater loss of 5-HT in cortical and subcortical areas in AD patients with psychotic behaviors compared with nonpsychotic AD patients. Another study also reported that the reduction of 5-HT levels in AD cortical region was correlated with aggression (Palmer et al. 1988). 5-HIAA levels also associated with neuropsychological symptoms, with Reinikainen et al (1990) reporting that anxiety is associated with reduced levels of 5-HIAA in AD cortex, thalamus and putamen. Other postmortem studies have demonstrated that the loss of neocortical 5-HT transporters (5-HTT), which affects the turn-over rate of the released 5-HT in the synaptic cleft, was correlated with depression (Chen et al. 1996).

There are specific patterns of serotonin receptors alternations that may underlie the presence of different behavioral symptoms in AD. While the reduction of 5-HT_{1A} receptors in the temporal cortex was correlated with moderate / severe aggression (Lai et al. 2003), a loss of 5-HT_6 receptors in the temporal cortex was correlated negatively with overactivity (Garcia-Alloza et al. 2004). However, not all serotonin receptors are affected in AD. Lai et al (2003) reported that the levels of 5-HT_4 receptor in the frontal and temporal cortex in AD were unchanged and that 5-HT_4 receptor was not associated with any neuropsychological symptoms. More studies are needed to elucidate the status of other 5-HT receptors in AD as well as their putative roles in the clinical features of the disease.

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SECTION 2

Methodology

CHAPTER 5

Neurochemical Measurements in Alzheimer's Disease : General Overview and Methodology

5.1 SUBJECT RECRUITMENT, COGNITIVE AND BEHAVIORAL ASSESSMENTS

The postmortem brain tissues used in this thesis were obtained from two cohorts: one from Oxfordshire, United Kingdom (in collaboration with Professors Tony Hope and Margaret Esiri), and the other from the University of California, Los Angeles Alzheimer's Disease Research Center (UCLA-ADRC), Los Angeles, USA (in

collaboration with Professors Harry Vinters and Jeffrey Cummings). Both cohorts were recruited for longitudinal studies initiated in the UK and USA over several years, and all clinical, cognitive and behavioral assessments as well as collection of associated data were conducted by our overseas collaborators, who provided the clinical data as well as postmortem samples. In this section, an overview of the subject recruitment process, patient demographics as well as the range of instruments used in the clinical assessments will be given in order to facilitate better understanding and interpretation of our clinico-neurochemical correlation.

5.1.1 The Oxford AD Cohort

Introduction. Postmortem tissues from the Oxford cohort were obtained from 97 dementia patients recruited into a longitudinal study of behavior in dementia (Hope et al. 1997a; Hope et al. 1997b; Hope et al. 1999), of which 40 had autopsy follow-up. Tissues from 20-25 patients only were shipped to Singapore in batches as they became available and were used in studies described in **Chapters 6,7 and 9.** All available tissues from subjects with neuropathological confirmation of AD diagnosis at the time of a study were used, and there were no selection of subjects based on sex or dementia severity.

Recruitment. Community-based dementia subjects living at home in Oxfordshire, UK, with a caregiver (e.g., spouse or child) who was able to give detailed information about the patient were recruited into the study (Hope et al. 1997a; Hope et al. 1997b; Hope et al. 1999). The study had been approved by the Institutional Review Board (IRB) of the Greater Oxford Psychiatric Sector, and informed consent was obtained either the patients or the next-of-kin as appropriate, from the dementia patients. Initial assessments were carried out to confirm a diagnosis of dementia using the Diagnostic and Statistical

Manual of Mental Disorders, 4th Edition (DSM-IIIR, 1987) criteria using CAMDEX (Cambridge Mental Disorders of the Elderly Examination) (Roth et al. 1986), a cognitive examination designed to detect dementia in the elderly which includes the Mini-mental state examination (MMSE) (Folstein et al. 1975). Cognition was assessed every four months from study entry till death with MMSE, scores ranging from 0-30 (Folstein et al. 1975). Dementia severity was measured by using the mean of up to five MMSE scores before death so as to avoid floor effects, since some patients may live for extended periods of time after their MMSE scores had reached 0 (Lai et al. 2001). Patients were considered to have severe dementia (AD_{severe}) if the mean predeath MMSE was ≤ 9, while those with scores between 10 – 24 were deemed to have mild to moderate dementia ($AD_{mild/mod.}$). None of the subjects had scores > 24. The rate of cognitive decline per year was measured by the linear regression of serial MMSE scores on the time interval between recruitment and death, or when the MMSE first reached 0. Findings from clinical investigations, including physical examinations, drug histories, full blood counts and biochemical screening were then used to assess whether patients fulfill the NINCDS-ADRDA criteria (McKhann et al. 1984) for the diagnosis of probable AD. Subjects who (i) presented with a probable cause of dementia other than AD or vascular dementia (e.g., hypothyroidism, previous head injury); (ii) had drunk more than 30 units of alcohol per week for two years; and (iii) had caregivers who could not give an accurate account of the day-to-day behavior of the subject were excluded from the study. Brain tissues from up to 20 elderly controls who were not known to have dementia or any other neurological disease, and based in the same community as the AD patients were also obtained. Tissues were not available in all brain areas for all subjects.

Demographics. The 97 subjects (49 males, 48 females) were aged between 60-95 years (mean = 78; interquartile range = 75-83), and had a MMSE score from 0-26 (mean = 14; interquartile range = 10-20). The distribution of social class of subjects based on occupation were similar to that found in the population as a whole (Hope et al. 1997b).

Clinical Assessments. Behavioral changes in the subjects were assessed with Present Behavioural Examination (PBE) (Hope and Fairburn, 1992) every four months from study entry till death. The PBE is a caregiver directed, semi-structured interview with high inter- and intra-rater reliability carried out by one of two highly trained interviewers on home visits, and covers in detail the observable behavior and mental state of the subject over the previous four weeks. Assessment for a range of behaviors, including depression, anxiety, aggression and psychosis consists of a main question, and some have further 'nested' questions to elucidate the caregiver's positive response The majority of items are rated on a seven-point scale (0-6) based on the frequency reported by the caregiver (from 0, not present in the last 28 days; to 3, present in approximately 14 days of the last 28 days; to 6, present everyday). For behaviors such as anxiety, which I studied (see **Chapter 9**), changes were considered to be significant if there a minimum of two frequency ratings were >3, or there was at least one rating of >3 and two ratings or 1-3 (Chen et al. 1996). At each PBE interview, cognitive tests were performed on the patients, including the MMSE, which has a maximum score of 30 (0, severely impaired; 28-30, cognitively normal). Dementia severity was defined as the mean of a maximum of five MMSE scores before death, also to avoid floor effects. The mean follow-up for clinical assessments was 3.3 years (SD = 2.38, range 0.33-9.0).

Neuropathology and tissue collection. At death, informed consent was obtained from the family of both the patient and control subjects prior to the removal of brain. One hemisphere was processed for histological examination. The neuropathological diagnosis of AD was confirmed by the CERAD (Consortium to Establish a Registry for AD) criteria (Mirra et al. 1991). Ten-μm-thick paraffin embedded sections of the orbito-frontal gyrus (Brodmann area 11, BA11) and mid-temporal gyrus (BA21) were stained with methenamine silver and modified Palmgren stains, and semi-quantitatively scored for senile plaques (SP, 0-3 point scale) and neurofibrillary tangles (NFT, 0-4 point scale) by two neuropathologists (Prof. Margaret Esiri and Dr Brandon MacDonald, University of Oxford) blinded to the clinical information. Blocks of gray matter were removed from BA11 and BA21 of the other hemisphere and stored at -75°C. In addition, the pH of brain homogenates (as an indication of agonal state) was measured as previously described (Hardy et al. 1985).

5.1.2 The UCLA AD Cohort

Introduction. 22 prospectively assessed dementia patients who were recruited into the University of California, Los Angeles Alzheimer's Disease Research Center (UCLA-ADRC) were used in the study described in **Chapter 8**. Frozen brain chunks were shipped to Singapore on dry ice and stored at -70°C until use. All 22 subjects fulfilled the DSM-IV (1994) clinical criteria for dementia as well as the CERAD criteria (Mirra et al. 1991) for neuropathological diagnosis of definite AD. Selection of subjects for the study was based on tissue availability, and not on dementia severity or Braak stage (Braak and Braak 1991).

Recruitment. The clinical study was approved by the IRB of UCLA and informed consent was obtained from the subjects or their caregivers. Subjects with other associated neurodegenerative diseases or extensive cerebrovascular disease were excluded from the study. Clinical information including the duration of dementia and the most recent MMSE scores were obtained from the UCLA-ADRC longitudinal study database and patient charts.

Demographics. Demographic details for the 22 AD (12 males, 10 females), as well as 12 elderly controls (6 males, 6 females) are listed in **Table 5.1.** The mean age at death, storage time, and postmortem interval (PMI) were well-matched between AD subjects and controls (Student *t*-test; $p>0.05$). Among the AD subjects, the mean duration of dementia symptoms was 9.5 ± 1.1 years.

Table 5.1 Demographics of controls and AD subjects, UCLA cohort.

	Control	*AD*
Demographics		
Maximum Number of Cases	12	22
Age at Death (y)	73.2 ± 4	78.8 ± 2
Sex (M/F)	6 / 6	12 / 10
Storage (y)	4.5 ± 0.5	5.4 ± 0.7
PMI (h)	14.4 ± 3	14.2 ± 2

Table 5.1 Data are mean ± S.E.M. PMI, post-mortem interval.

Neuropathology and tissue collection. At death, informed consent was obtained from the family of both patients and control subjects prior to the removal of brain. The brains of patients were removed within 48 hours after death and fixed in 10% buffer

formalin for at least 4 weeks. One hemisphere was processed for histological examination. One-cm-thick coronal sections were made and blocks were taken bilaterally from eight brain regions commonly sampled in the CERAD protocol, including the left and right orbitofrontal gyrus (Brodmann area, BA, 11), superior and mid-temporal gyrus (BA 21/22), and CA1 region of the hippocampus, were evaluated for neuropathology. The orbital frontal cortex was used in my study of the neurochemical correlates of muscarinic signaling in AD (see **Chapter 8**), as this area is known to be an important regulator of learning and behavior (Van Hoesen et al. 2000). One subject showed evidence of Lewy bodies (LB), another had LB and superior frontal infarct, while two others had mutli-infarct or cingulated stroke. In addition, severity of disease as determined by Braak staging of the subjects showed that the majority (n=18) were in Braak V/VI, and only two were in stage IV/V, and another two in stage IV.

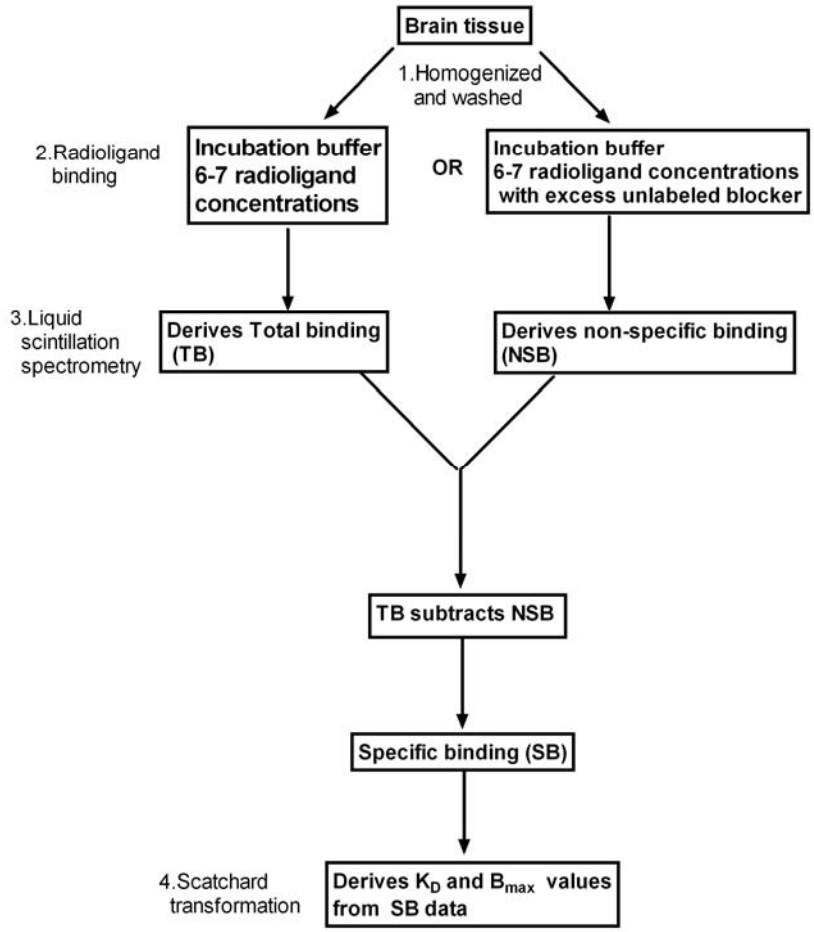
5.2 RADIOLIGAND BINDING ASSAYS

5.2.1 Overview

Saturation radioligand binding assay is a well-established approach to measuring neurotransmitter receptors in the postmortem brain. The neurochemical variables of interest are the affinity of binding of radioligand to its specific receptor sites (K_D, expressed in nM) and the density of binding (B_{max}, expressed in fmol radioligand bound / mg protein). Total binding (TB) is measured by incubating aliquots of brain membrane homogenates in appropriate assay buffer with a range of radioligand concentrations. which gives total binding (TB). Non-specific binding (NSB) is measured by parallel

series of incubation set up as above but in the presence of a saturating concentration of unlabeled ligand ("cold blocker") which will displace radioligand binding to specific sites. The specific binding (SB) value can then be obtained by subtracting the NSB value from TB value. **Figure 5-1** depicts a flow-chart of assay procedures with detailed description of each numbered step to follow.

Figure 5.1 Protocol for radioligand saturation binding assay.



5.2.2 Detailed Protocol

Most of the radioligand binding assays described in **Chapters 6-9** are based on minor modifications of published protocols (see references for the Chapters). In this thesis, the assay conditions have been optimized for single site, equilibrium binding. This section describes the conditions used. Chemicals and reagents were purchased from Sigma Aldrich Co. (St Louis, MO, USA) unless otherwise specified.

Brain tissue homogenization and washing. All studies involving human postmortem tissues described in this thesis were approved by the IRB of the Singapore General Hospital. Brain tissues obtained as described above **(Section 5.1)** were thawed on ice, dissected free of white matter and meninges, and homogenized with a Polytron Ultra-Turrax T8 homogenizer (IKA Labortechnik GmbH, 15 s at maximum setting) in 10 volumes of ice-cold 50mM Tris-HCl buffer, pH 7.4 containing 120mM NaCl and 5mM KCl. The homogenate was then centrifuged at 15,000 g, 4˚C, and the resultant supernatant was discarded and fresh buffer added to the pellet. The cycle of homogenization and centrifugation was repeated once more before the pellet was suspended in fresh buffer at a concentration of 30 – 50mg wet weight / ml. The washed plasma membrane preparation for radioligand binding studies was stored at -75˚C until use.

Radioligand binding. The washed plasma membrane preparations were thawed and diluted 1:3 vol./vol. with incubation buffer. If the incubation buffer was not identical to the buffer (50mM Tris-HCl, pH 7.4) already in the preparation, the thawed samples would be centrifuged and the pellet resuspended in incubation buffer (4x original volume). The choice of incubation buffer is based on previously published data on human or animal brains. For all radioligand binding assays the buffer pH was kept between 7.4–

7.7 according to published protocols. The radioligand concentration ranges were usually from K_D / 5 to around 5 times K_D*, which would label > 80% of available binding sites. Cold blockers used to define non-specific binding were usually a different ligand from the radioligand (except for MK-801 binding to NMDA receptors) and they were used at a concentration that was 100 - 1000 times of K_D. As shown in **Figure 5-2(A)** in the example of [^{3}H]pirenzepine binding to muscarinic M_1 receptors, both total and non-specific binding are correlated with radioligand concentration, while specific binding assumes a hyperbola and approaches a plateau with increasing radioligand concentration, indicating that the specific binding sites are saturable. In contrast, NSB varied linearly with radioligand concentration including its nonsaturable characteristics. The cold blockers used were usually non-selective ligands which recognize the class of receptors in which the receptor of interest belongs. This is preferable to using the unlabeled form of the radioligand in cases where the radioligand binding is not well characterized.

Incubation. Incubation time was based on previous work which reported extensive characterization data, including association constants (K_{on}). This ensures that the incubation period is adequate for binding to reach equilibrium. All the radioligands used here had K_{on} values in the range of 10^8 M^{-1} based on published data and our own results, indicating that an incubation time of at least 60 minutes would ensure equilibrium to be established.

*Initial K_D values were estimated from published data or determined from a trial assays.

Figure 5.2 [^{3}H]pirenzepine binding in human postmortem neucortex.

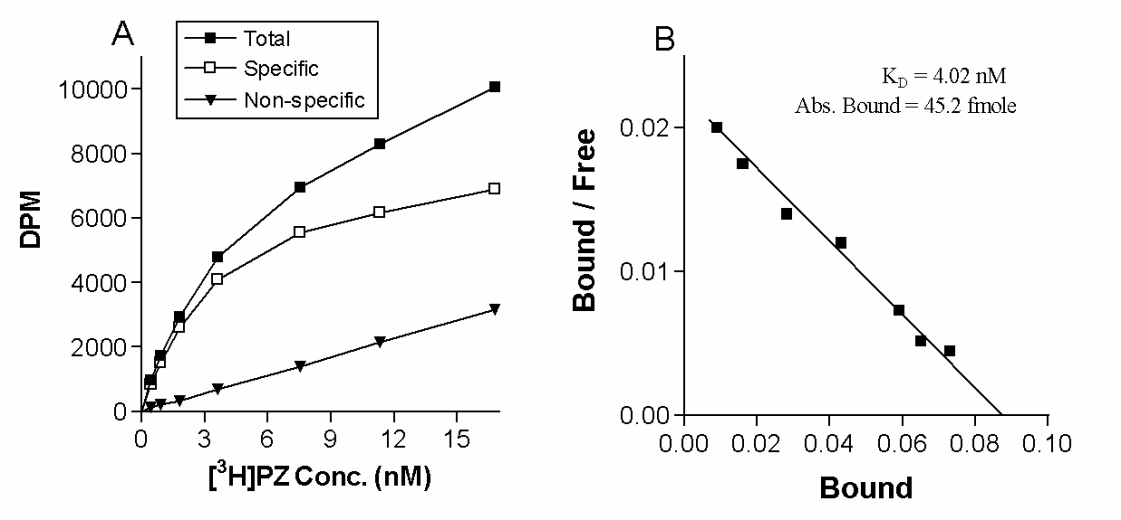


Figure 5.2 [^{3}H]Pirenzepine (PZ) binding in postmortem control and AD neocortex. **A**, representative saturation isotherm of [^{3}H]PZ binding in an AD frontal cortex. **B**, Scatchard plot of binding data presented in A with derived neurochemical parameters.

Terminating the binding assay. At the end of incubation period, the binding assays were terminated by rapid vacuum filtration through GF/B glassfibre filters (Whatman Ltd, UK) followed by a 8 second wash with ice-cold sodium phosphate buffer, pH 7.4 using a Skatron semi-automatic cell harvester (Molecular Devices Ltd. USA). The filters were pre-treated with 0.1 % polyethylenimine to reduce non-specific binding. The filters were then dried, and discs containing the filtered membrane were punched out into scintillation vials, and containing 2ml of scintillation fluid (Wallac HiSafe 2, Perkin Elmer Life Sciences, USA). Bound radioactivity was measured by liquid scintillation spectrophotometry (LSS) as described below. **Table 5.2** summarizes the binding conditions of the radioligands under study.

Table 5.2 Optimized conditions for saturation radioligand binding assays.

	M1	*M2*	*NMDA*	*5-HTT*
Radioligand (specific activity, in Ci/mmol)	[^{3}H]Pirenzepine (77.9)	[^{3}H]AFDX-384 (120-133)	[^{3}H]MK-801 (28.9)	[^{3}H]Citalopram (83)
Concentration Range (in nM)	0.5-15	0.5-15	0.5-30	0.1-12.0
Assay Buffer	50mM Sodium phosphate, pH 7.4	50mM Sodium phosphate, pH 7.4	50mM Tris-HCl, 250μM spermine, 25μM glycine, 20μM L-glutamate, pH 7.7	50mM Tris-HCl, pH 7.4
'Cold' Blocker	Atropine, 1μM	Atropine, 1μM	MK-801 maleate, 10μM	Fluvoxamine, 10μM (Tocris Ltd., UK)
Incubation Time (in min)	60	60	60	60
Incubation Temperature (in °C)	25	25	25	25

Liquid scintillation spectrophotometry. Measurements of bound radioactivity by LSS was performed with a Wallac 1414 beta counter at ~45 % efficiency. The data output in disintegrations per minute (dpm) were obtained from counts per minute (cpm) values for each vial after correction for radioactivity quenching.

Scatchard Transformation. Raw dpm data were entered into a PC-based software (EBDA and LIGAND) (McPherson 1985) for the computation of K_D and B_{max} values. EBDA performed the Scatchard transformations (see **Figure 5-2(B)**) while LIGAND is

an iterative curve fitting program which compared best fit to one-site or two-site binding. The software also computed Hill coefficients (N_H). For the calculation of receptor density, an aliquot of tissue preparation used in each binding assays was measured for protein content by the Coomassie Blue reagent (Pierce Biotech, Inc., USA) using bovine serum albumin as the standard.

5.3 MUSCARINIC M1 RECEPTOR-G-PROTEIN UNCOUPLING

5.3.1 Overview

The status of the M_1 receptor coupling to its G-protein was pharmacologically determined by [^{3}H]pirenzepine/carbachol competition assay according to previously published methods by Flynn et al. (1991) with minor modifications. The assay was performed by measuring the extent of inhibition of [^{3}H]pirenzepine binding to M_1 receptor by increasing concentrations of cholinergic agonist, carbachol. A parallel series of experiments was carried out by adding guanylyl-imidodiphosphate (GppNHp). GppNHp is a non-hydrolysable analogue of guanosine triphosphate (GTP) which keeps the G-protein in an "uncoupled" state. In principle, the M_1 receptor forms a ternary complex with its G-protein in the presence of an agonist, such as carbachol. When M_1 receptor is in a "coupled" state or in the presence of G-protein, M_1 receptor binds to carbachol with high affinity (i.e. the high affinity binding constant, K_i, is low). Alternatively, in the "uncoupled" state or in the absence of G-protein, M_1 receptors bind to carbachol with a lower affinity (i.e. the K_i value is high). Thus, the change of high affinity binding constant in the presence (Ki) and absence (K_{iG}) of G-protein will serve as an index of G-protein receptor coupling. For data analysis, the percentage specific binding data were plotted against log-transformed values of carbachol concentrations

with a non-linear regression curve-fitting software (Prism 3.0, GraphPad Inc. USA), and fifty percent inhibitory concentrations (IC_{50}) values were derived from the binding curves. The IC_{50} values were subsequently used to calculate inhibitory constants for carbachol binding in the absence (K_i) and presence (K_{iG}) of GppNHp using the Cheng and Prusoff's equation (Cheng and Prusoff 1973). The presence of excess GppNHp uncouples G-proteins from M_1 receptors, thus causes a 'right shift' of the carbachol displacement curve and the value for K_{iG} is significantly greater than that for K_i **(Figure 5.3A)**. However, in samples where G-proteins are endogenously uncoupled to M_1 receptors (higher K_i), addition of GppNHp results will not result in a similar shift **(Fig. 5.3B)** and the K_{iG} /K_i ratio will be closely to 1. Therefore, the ratio between K_{iG} and K_i provide a measure of the state of M_1/G-protein coupling in control and diseased brains.

Figure 5.3 M_1/G-protein coupling in controls and AD.

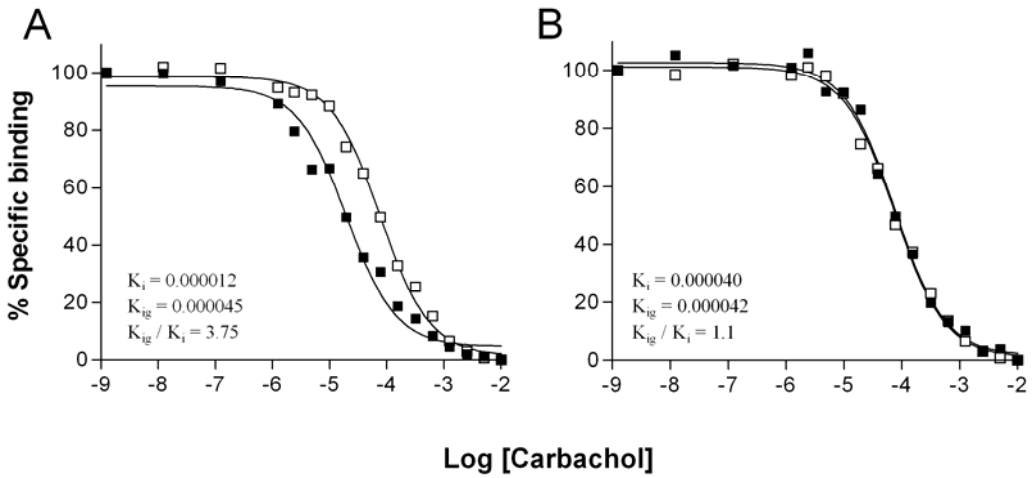


Figure 5.3 Carbachol competition for the % specific binding of [^{3}H]pirenzepine to M_1 receptors in the temporal cortex of a randomly selected control (**A**) and Alzheimer's disease (AD) patient (**B**). Best-fit curves were derived from non-linear aggression of data. Each data point denotes specific binding in the absence (■) and presence (□) of 0.2mM of GppNHp across log transformed carbachol concentrations.

5.3.2 Detailed Protocol

Aliquots of tissue preparation prepared as described in section **5.2.2** were incubated in duplicates with 3nM of [^{3}H]pirenzepine (specific activity 70-80 Ci/mmol, Perkin-Elmer Life Sciences, USA) and increasing concentrations of carbachol (10^{-9} to 10^{-2}M) in buffer (20mM Tris-HCl, 1mM $MnCl_2$, pH 7.4) for 150 min at room temperature. Specific binding was defined by binding difference in the absence and presence of 10μM atropine sulphate. A parallel series of competition assays were performed with and without addition of 0.2mM GppNHp. At the end of incubation period, the bound radioactivity was obtained by vacuum filtration measured by LSS as in **Section 5.2.2**.

5.4 DETERMINATION OF CHOLINE ACETYLTRANSFERASE (ChAT) ACTIVITY

5.4.1 Detailed Protocol

The method of measuring ChAT activity was modified from previously published method by Fonnum et al. (1975). In brief, after thawing the brain homogenates, the Triton-X detergent (1μl of 5% triton for every 50μl of tissue, i.e. the final concentration of Triton-X is 0.1%) was added to the sample and incubated for 10 minutes at room temperature. Then 10μl of reaction mixture (refer to **Table 5.3**) was added either to the sample tubes with brain homogenate (in triplicates, 4μl of homogenate per tube) or to the background tubes (in triplicates, 4μl of 50mM phosphate buffer per tube) and incubated for 10 minutes at 37°C. After incubation, 100μl of ice-cold phosphate buffer (10mM) was used to terminate the reaction and 1ml of Kalignost (0.5g sodium tetraphenylboron per 100ml acetonitrile) was immediately added to the sample or background tubes and vortexed for 10 second. The sample or background tubes were then centrifuged for 2

min. After centrifugation, 500µl of the acetonitrile layer was transferred to scintillant vial with 5ml of scintillation fluid (HiSafe2, Wallac) for measurement by LSS using a Beta counter. Homogenized brain tissue samples were kept at -30°C for subsequent protein determination with the Coomassie Blue reagent (Pierce Biotech, Inc., USA).

Table 5.3 Reagents for 300µl of reaction mixture for ChAT assay (dissolved in 50mM phosphate buffer, pH7.4).

Reagent	Reagent initial concentration	Reagent final concentration	Volume (in µl)
Choline chloride	140mM	10mM	30
NaCl	2.1M	300mM	60
Sodium EDTA	140mM	10mM	30
Eserine	1.4mM	0.1mM	30
Acetyl-CoA	2.5mM	0.54mM	90
[^{3}H]acetyl-CoA	-	-	30

5.5 DETERMINATION OF KINASE ACTIVITIES

5.5.1 Overview

The assay kit is based on phosporylation of a specific substrate peptide by the kinase under study using the transfer of the γ-phosphate of adenosine-5'-[^{32}P] triphosphate ([γ-^{32}P]ATP). The [^{32}P]phosphorylated substrate is then separated from the residual [γ-^{32}P] ATP using P81 phosphocellulose paper and quantified using LSS.

5.5.2 Protein Kinase C (PKC) Activity

Protein kinase C enzymatic assays were carried out using components of a commercially available PKC enzymatic assay kit (Upstate Biotechnology Inc., Lake Placid, NY) based on following reaction:

$$\text{QKRPSQRSKYL} + [\gamma\text{-}^{32}\text{P}]\text{ATP} \xrightarrow[\text{DAG, } Mg^{2+}]{\text{PKC, } Ca^{2+}\text{, PS,}} \text{QKRP}[^{32}\text{PS}]\text{QRSKYL} + \text{ADP}$$

The assay kit was based on [^{32}P]γ-phosphorylation of a specific substrate peptide (QKRPSQRSKYL) by PKC. In brief, 10µl of brain tissue lysate, was added to 50µl of incubation buffer containing the following (in final concentrations): 83µM substrate peptide, 0.3µM PKI (PKC inhibitor peptide), 3.3µM R24571 (CAMK inhibitor), 0.08mg/ml phosphatidyl serine, 0.008mg.ml DAG, 10µCi [γ-^{32}P]ATP (spec. act. 3000 Ci/mmol, Perkin Elmer Life Sciences), 12.5mM $MgCl_2$, 83µM unlabeled ATP, 20mM MOPS, 25mM β-glycerol phosphate, 1mM sodium orthovanadate, 1mM DTT, 1mM $CaCl_2$, 0.83mM EGTA, and incubated for 10 min at 30°C. A 25µl aliquot was then transferred onto the center of a numbered phosphor-cellulose paper square. The assay squares were washed with 0.75% phosphoric acid three times for 5 min each and once with acetone. These steps separated the phosphorylated substrate from the residual [γ-^{32}P]ATP. After washing, the assay squares were transferred to a scintillation vial, 5ml of scintillation mixture was added, and radioactivity was determined in a scintillation counter to calculate enzyme activity (in pmol [^{32}P]-phosphate incorporated into substrate / min / mg protein). In order to control for phosphorylation of endogenous proteins in the membrane lysate, parallel assays were carried out as above in the absence of substrate

peptide, and radioactivity (typically < 20%) is subtracted from total counts for the calculation of specific PKC activity.

5.5.3 Src Kinase Activity

The assay kit is based on phosphorylation of a specific substrate peptide [KVEKIGEGTYGVVYK] using the transfer of the [γ-^{32}-P]ATP by Src kinase as shown by the following equation:

$$\text{KVEKIGEGTYGVVYK} + [\gamma\text{-}^{32}\text{P}]\text{ATP} \xrightarrow[\text{Src kinase, } Mn^{2+}]{} \text{KVEKIGEGT}[^{32}\text{pY}]\text{GVVYK} + \text{ADP}$$

Src kinase activity assays were performed as for **5.5.2** except the reaction buffer consists of the following: 150µM substrate peptide, 50mM Tris-HCL, pH 7.2, 10µCi [γ-^{32}P]ATP, 30mM $MnCl_2$, 125µM ATP, 20mM MOPS, 25mM β-glycerol phosphate, 0.5mM sodium orthovanadate, 3mM DTT, 3mM EGTA, and incubated for 10 minutes at 30°C. 10µl substrate peptide, 10µl of Src reaction buffer, and 10µl of [γ-^{32}P]ATP stock were added to a microcentrifuge tube and incubated for 10 minutes at 30°C with agitation. 20µl of 40% trichloroacetic acid was then added to precipitate peptide, and a 25µl aliquot was transferred onto the center of a numbered P81 paper square. The assay squares were washed with 0.75% phosphoric acid five times for 5 minutes each and once with acetone and radioactivity was measured by LSS. In order to control for phosphorylation of endogenous proteins in the membrane lysate, parallel assays were carried out as above in the absence of substrate peptide, and radioactivity (typically 50-60%) was subtracted from total counts for the calculation of specific Src activity.

5.6 IMMUNOBLOTING

5.6.1 Detailed Protocol

Boiled brain homogenates in Laemmli sample buffer were electrophoretically separated on 7% polyacrylamide gels, transferred to nitrocellulose membranes, and blocked in 10mM phosphate buffered saline, pH 7.4 with 0.1% Tween 20 (PBST) / 5% skim milk for 1 hour before immunoblotting with a rabbit polyclonal antibody directed against the protein of interest (NR1 subunit of NMDA receptor) at the recommended dilution in PBST / 1% milk overnight at 4°C. Following washings in PBST / 1% milk and a 1 hour incubation with horseradish peroxidase conjugated goat anti-rabbit antibody (1:10,000, Jackson ImmunoResearch Inc.), immunoreactive bands on the membranes were visualized by enhanced chemiluminescence using the ECL system (Amersham Pharmacia Biotech), and quantified by an image analyzer (UVItec Ltd.). To ensure comparable sample loading across lanes, membranes were then stripped with stripping buffer (Promega Corp.), washed and reblotted with a mouse monoclonal anti-β-actin (1:5000, Sigma Aldrich Ltd., expected molecular weight 42 kDa), and the protocol described above was repeated with horseradish peroxidase conjugated goat anti-mouse antibody (Jackson ImmunoResearch Inc., USA). One lane for external standard consisting of known amounts of protein from an individual homogenate was included in each membrane for normalization of data.

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SECTION 3

Results and Discussions

CHAPTER 6

Effects of *APOE* ε4 Allele on Cholinergic Alterations in Alzheimer's Disease*

*Data presented in this chapter has been published: Lai MK, Tsang SW, Garcia-Alloza M, Minger SL, Nicoll JA, Esiri MM et al. Selective effects of *APOE* ε4 allele on presynaptic cholinergic markers in the neocortex of Alzheimer's Disease. *Neurobiology of Disease* (2006) **22**:555-561.

6.1 INTRODUCTION

The cardinal neuropathological features of AD include extracellular senile plaques (SP), intracellular neurofibrillary tangles (NFT) and neuronal degeneration. Of the several neurotransmitter systems known to be affected in AD, one of the earliest and most consistently reported are degeneration of basal forebrain cholinergic neurons (Davies and Maloney 1976; Whitehouse et al. 1982), accompanied by presynaptic neurochemical alterations including reduced choline acetyltransferase (ChAT) and acetylcholinesterase (AChE) activities, reduced acetylcholine release, as well as losses of muscarinic M_2 in the neocortex and hippocampus (Francis et al. 1985; Mash et al. 1985; Perry et al. 1977; Sims et al. 1983; Wilcock et al. 1982). Postsynaptic M_1 receptors appeared to be preserved in AD (Mash et al. 1985) but was subsequently shown to be defective in coupling to G-proteins (Flynn et al. 1991; Ladner et al. 1995). Other studies also reported deficits in postsynaptic signaling such as decreases in phosphoinositide hydrolysis and protein kinase C (PKC) activities (Cole et al. 1988; Ferrari-DiLeo and Flynn 1993; Jope et al. 1997) which are activated by M_1 and M_3 receptors (Caulfield 1993). There are also reports of deficits in nicotinic receptors (Perry et al. 1987; Warpman and Nordberg 1995). Taken together, these studies suggest a state of profound presynaptic as well as postsynaptic cholinergic dysfunction in AD that may underlie both the cognitive and behavioral symptoms of AD (Cummings and Kaufer 1996; Lai et al. 2001; Minger et al. 2000; Perry et al. 1978; Tsang et al. 2005; Wilcock et al. 1982).

AD is divided into familial and sporadic forms of disease according to family history. Both early onset (before 65 years of age) and late-onset (65 years and over) familial cases constitute up to 40 % of all AD (van Duijn et al. 1991). Several gene

mutations have been linked to familial forms of AD, such as those encoding amyloid precursor protein (APP), presenilin 1 and presenilin 2, which result in altered APP processing and increased production of amyloidogenic (plaque-forming) Aβ species (Selkoe 2001). For sporadic AD which constitutes the majority of cases, the major identified genetic risk factor is *APOE*, which encodes apolipoprotein E (apoE), a 34 kDa lipid carrier protein. The *APOE* gene is located on the long arm of chromosome 19 and exist as three polymorphic variants (Emi et al. 1988) (see **Table 6.1**).

Table 6.1 Polymorphisms in apoE.

E2	NH_2---------Cys_{112}----------Cys_{158}----------COOH
E3	NH_2---------Cys_{112}----------Arg_{158}----------COOH
E4	NH_2---------Arg_{112}----------Arg_{158}----------COOH

Of the three *APOE* alleles (ε2, ε3 and ε4 encoding apoE2, apoE3 and apoE4 isoforms respectively), the ε4 is dose-dependently associated with increased risk of developing AD compared with ε3 and ε2 (Corder et al. 1993; Poirier et al. 1993; Saunders et al. 1993). Clinical studies also indicate that the age of AD onset is inversely proportional to the number of ε4 alleles. On the other hand, ε3 and ε2 seem to provide some protection against AD. ApoE4 is different from apoE3 by a single amino acid change from cysteine to arginine at position 112, resulting in the formation of a salt bridge between Arg-61 in the amino terminus and Glu-255 in the carboxyl terminus, leading to a more compact structure than ApoE3 and resulting in ApoE4's preference for binding VLDL (very low density lipoproteins) to HDL (high density lipoproteins, Dong

and Weisgraber 1996). The most common combination of *APOE* alleles is ε3/ε3 (Hallman, et al. 1991). ApoE is expressed in various types of tissues such as atrocytes, macrophages and monocytes, but mainly in the liver and brain (Siest et al. 1995). The apoE protein is the major apolipoprotein in the central nervous system which is a key regulator of lipid metabolism; therefore, apoE plays a role in many biological processes involving lipid metabolism, including myelination, synaptic plasticity and repair, with the E4 being less effective than the E3 isoform (Mahley and Huang 1999; White et al. 2001). E4 has also been shown to facilitate Aβ deposition and τ hyperphosphorylation more potently than E2 and E3 *in vitro* (Holtzman et al. 2000; Tesseur et al. 2000). Additionally, AD patients with one or two ε4 showed higher plaque and, to a lesser extent, higher tangle burden than non-ε4 carriers (Nagy et al. 1995; Olichney et al. 1996; Schmechel et al. 1993). Finally, apoE may affect cholinergic neurons and signaling in an isoform-specific manner. For example, some studies demonstrated that ε4 positive AD patients had more extensive deficits in ChAT activities compared with non-ε4 carriers (Beffert and Poirier 1996; Poirier et al. 1995; Soininen et al. 1995;) while others did not find a linear relationship between ChAT activities and number of ε4 allele copies (Corey-Bloom et al. 2000; Svensson et al. 1997; Tiraboschi et al. 2004). The effect of apoE genotype on other cholinergic markers are less clear. M_1, M_2 and nicotinic receptors do not seem to differ between patients with or without ε4 (Poirier et al. 1995; Reid et al. 2001; Svensson et al. 1997). However, the radioligands used to label nicotinic sites in these studies are not subunit-specific, and it is not known whether apoE genotype is correlated with specific losses of nicotinic receptor subpopulations, such as those containing α4β2 subunits (Warpman and Nordberg 1995). Furthermore, although studies

have shown selective impairment of muscarinic receptor-mediated phosphoinositide hydrolysis by the E4 isoform (Cedazo-Minguez and Cowburn 2001), it is not known whether apoE affected the coupling of G-proteins to M_1 receptors in AD. In this study, we correlated *APOE* genotype with a range of pre- and postsynaptic cholinergic neurochemical markers (M_1 receptors, M_2 receptors, $\alpha4\beta2$ nicotinic receptors, M_1/G-protein coupling, ChAT activity and AChE activity) in the postmortem frontal and temporal cortex of a cohort of AD patients in order to test the hypothesis that deficits or alterations in cholinergic neurotransmission in the AD neocortex is influenced by *APOE* $\varepsilon4$ genotype.

6.2 MATERIALS AND METHODS

6.2.1 Patients and Neuropathological Assessments

Up to 40 AD subjects and 20 elderly controls were included in this study. The AD subjects were derived from an autopsied subset of a cohort of community-based dementia patients from Oxfordshire, UK enrolled in a longitudinal study of behavior in dementia (Hope et al. 1999). Subject recruitment, demographics and clinical assessment have been previously described in detail in **Chapter 5 (Section 5.1.1)**. Complete drug histories were recorded, and none of the subjects was on cholinomimetic medication. At autopsy, brains were harvested after informed consent had been obtained from the patients' relatives. One hemisphere was processed for neuropathological assessments, while the other was fresh-frozen for subsequent neurochemical assays. As mentioned in **Chapter 5 (Section 5.1.1)** all subjects fulfilled CERAD criteria for the neuropathological diagnosis of AD (Mirra et al. 1991), and were neuropathologically staged at Braak V / VI (Braak

and Braak 1991). Two patients (both of ε3/ε3 genotype) had evidence of mixed vascular dementia and AD. Paraffin-embedded blocks of frontal as well as temporal cortex were then stained with methenamine silver and a modified Palmgren stain for a blinded, semi-quantitative scoring of SP (0-3 points) and NFT (0-4 points), as previously described **(Chapter 5, Section 5.1.1)**. The selection of AD subjects as well as controls for the current study was based on postmortem-tissue availability; and not on *APOE* genotype. Not all neurochemical measures were performed in all participants due to shortage of tissue, and data for some of the neurochemical measurements were provided by our collaborators (AChE activities from Dr Maria Ramirez, University of Navarro, Spain; [^{3}H]epibatidine binding and brain pH from Dr Paul Francis, King's College London, UK and *APOE* genotyping from Prof James Nicoll, University of Southampton, UK). All other assays described here were performed by us.

6.2.2 Tissue Processing

Tissue processing and neurochemical assays were performed blind to clinical information. All chemicals and reagents were from Sigma-Aldrich Ltd. (St Louis, MO) unless otherwise stated. Brain tissue homogenate preparation has been described in **Chapter 5 (Section 5.2.2)**.

6.2.3 *APOE* Genotyping

APOE genotyping were determined with DNA extracted from brain tissues according to previously described methods as performed in Prof James Nicoll's laboratory (Nicoll et al. 1997).

6.2.4 Neurochemical Measurements

Radiochemical determination of ChAT activity has been described in **Chapter 5 (Section 5.4)**. AChE assay using a colorimetric method (Wang, et al. 1999) was modified as previously reported and performed in Dr Maria Ramirez's laboratory (Garcia-Alloza et al. 2005). Measurement of muscarinic M_1 and M_2 receptors was performed by saturation radioligand binding assays using [^{3}H]pirenzepine (PZ) and [^{3}H]AF-DX 384 (both from Perkin Elmer Life Sciences, Boston, MA), respectively, as detailed in **Chapter 5 (Section 5.2.2)**. [^{3}H]Epibatidine (Perkin Elmer Life Sciences, Boston, MA) for α4β2 subunit-containing nicotinic receptors have been previously described as performed in Dr Paul Francis' laboratory, using 250 nM unlabelled cytosine used to define non-specific binding (Gnadisch et al. 1999). Neurochemical parameters K_D (binding affinity, in nM) and B_{max} (binding density, in fmol/mg protein) for M_1, M_2 and α4β2 nicotinic receptors were derived from binding data by Scatchard analyses as described in **Chapter 5 (Section 5.2.2)**. Pharmacological determination of M_1 receptor coupling to G-proteins using a [^{3}H]PZ / carbachol competition assay was detailed in **Chapter 5 (Section 5.3)**.

Table 6.2 Demographic, disease and neurochemical variables in control and AD.

		AD	*Control*
Maximum Number of Cases[1]		40	20
Age at Death (y)		81.7 ± 1	77.3 ± 2
Sex (M/F)		20 / 20	13 / 7
Post-Mortem Interval (h)		37.7 ± 4	47.7 ± 7
pH		6.35 ± 0.07*	6.62 ± 0.09
ChAT (pmol/mg prot/h)	*Frontal*	3.5 ± 0.3 (36)**	6.3 ± 0.7 (16)
	Temporal	1.9 ± 0.3 (34)†	6.4 ± 0.3 (19)
AChE (% Control)	*Frontal*	72 ± 3 (17)†	103 ± 5 (9)
	Temporal	70 ± 3 (17)†	105 ± 3 (9)
[^{3}H]PZ K_D (nM)	*Frontal*	4.4 ± 0.2 (27)*	3.6 ± 0.2 (12)
	Temporal	4.5 ± 0.2 (27)	4.1 ± 0.4 (12)
[^{3}H]PZ B_{max} (fmol/mg prot)	*Frontal*	575 ± 13 (27)	601 ± 30 (12)
	Temporal	614 ± 22 (27)	594 ± 31 (12)
[^{3}H]AF-DX 384 K_D (nM)	*Frontal*	3.7 ± 0.2 (27)	4.2 ± 0.6 (12)
	Temporal	4.3 ± 0.2 (27)	4.2 ± 0.3 (12)
[^{3}H]AF-DX 384 B_{max} (fmol/mg prot)	*Frontal*	483 ± 21 (27)**	583 ± 30 (12)
	Temporal	558 ± 26 (27)	566 ± 42 (12)
[^{3}H]Epibatidine K_D (pM)	*Frontal*	45 ± 6 (31)	57 ± 8 (12)
	Temporal	91 ± 22 (30)	60 ± 24 (14)
[^{3}H]Epibatidine B_{max} (fmol/mg prot)	*Frontal*	50 ± 3 (31)*	65 ± 9 (12)
	Temporal	60 ± 5 (30)	80 ± 11 (14)
K_{iG} / K_i	*Frontal*	2.4 ± 0.3 (21)*	3.6 ± 0.2 (8)
	Temporal	2.1 ± 0.2 (25)**	6.1 ± 0.9 (6)
SP	*Frontal*	1.97 ± 0.1 (35)†	0.08 ± 0.08 (12)
	Temporal	2.31 ± 0.1 (35)†	0.15 ± 0.1 (13)
NFT	*Frontal*	1.74 ± 0.2 (35)†	0.08 ± 0.08 (13)
	Temporal	2.26 ± 0.2 (35)†	0.14 ± 0.1 (14)

Table 6.2 Data are mean ± S.E.M. ChAT, choline acetyltransferase; AChE, acetylcholinesterase; K_D, binding affinity constant; B_{max}, binding density; K_{iG} / K_i , measure of M1 / G-protein coupling (see text); SP, senile plaque score; NFT neurofibrillary tangle score.
[1]Not all neurochemical measures were available for all cases. The *N* values available for each neurochemical measure are listed in parentheses.
*Different from control $p < 0.05$; **Different from control $p < 0.01$; †Different from control $p < 0.001$.

6.2.5 Statistical Analyses

Data were tested for normality for the selection of parametric or non-parametric tests. Demographic, disease and neurochemical variables between controls and AD were compared with Student's *t*-tests. The effect of potential confounders on neurochemical variables, as well as the inter-correlations among variables were studied by Pearson's product moment or Spearman's correlation. The distribution of genotype frequencies between AD and controls were analyzed by Chi-square tests, while demographic and disease variables among the *APOE* genotypes in AD were compared by one-way analysis of variance (ANOVA) followed by Dunnett's *post-hoc* tests, as the number of subjects in the groups were not matched and equal variances could not be assumed. All statistical analyses were performed with the SPSS 10.0 for Windows software (SPSS Inc. USA), except for Chi-square tests, which were carried out with StatXact 3 for Windows (Cytel Software Corp. MA, USA).

6.3 RESULTS

6.3.1 Demographic, Disease and Neurochemical Variables in Control vs. AD

Table 6.2 shows that demographic variables were well-matched between the AD subjects and controls except for lower pH in AD, possibly an indication of more severe acidosis due to prolonged agonal state (Hardy et al. 1985). However, pH, as well as other demographic variables listed in **Table 6.2**, did not correlate with neurochemical variables (Pearson $p > 0.05$). As shown in **Table 6.2**, ChAT and AChE activities as well as M_2 and $\alpha4\beta2$ nicotinic receptor densities are reduced in one or both regions in AD, while M_1 densities remained unchanged. Except for higher K_D in [^{3}H]pirenzepine binding in the

AD frontal cortex (Lai et al. 2001), there were no differences in receptor binding affinities. There was minimal neuropathology in controls, contrasting with significantly higher SP and NFT scores in AD. The distribution of *APOE* alleles between controls and AD also appeared to be different **(Table 6.3)**; in fact, none of the controls in this cohort had the ε4/ε4 genotype. There was also no ε2/ε2 genotype in either AD or controls.

Table 6.3 Distribution of *APOE* genotypes in control and AD.

Genotype	***AD**** *N = 40*	***Control*** *N = 20*
ε2/ε3 or ε3/ε3	15 (38 %)	16 (80 %)
ε2/ε4 or ε3/ε4	21 (52 %)	4 (20 %)
ε4/ε4	4 (10 %)	0 (0 %)

*Significantly different from control, Pearson's Chi-Square ($\chi^2 = 10.04$) with Fisher's exact test, $p < 0.01$.

6.3.2 Effect of APOE Genotype on Demographic, Disease and Neurochemical Variables in AD

Demographic factors as well as postmortem conditions were not significantly different among AD patients with zero (ε3/ε3 or ε3/ε2), one (ε3/ε4 or ε2/ε4) or two ε4 alleles **(Table 6.4)**, although there is a trend towards lower estimated age of AD onset with increasing ε4 dose. In contrast, both dementia severity (estimated by the Mini-Mental State Examination score before death, Folstein et al. 1975) as well as temporal cortical senile plaque (SP) loads were higher in patients with two ε4 alleles compared to those with zero or one ε4, with similar trends for frontal cortical SP as well as neurofibrillary tangle scores in both regions. Finally, **Figure 6.1** illustrates the neocortical cholinergic variables across *APOE* genotypes in AD. Because the neurochemical

variables between controls with the ε4 allele and those without were not significantly different (Student's *t* tests, $p > 0.05$, data not shown), the mean control values were used as a baseline for comparisons with AD *APOE* genotype groups in order to study the effect of ε4 on the neurochemical alterations in AD.

Table 6.4 Effect of *APOE* ε4 allele on demographic and disease variables in AD.

		0 ε4	*1 ε4*	*2 ε4*
Age at Death (y)		83.7 ± 2	80.6 ± 1	80.5 ± 0.6
Age at Onset (y)		75.2 ± 2	71.7 ± 1.5	69.2 ± 3.2
Disease Duration (y)		8.5 ± 0.8	8.9 ± 1	11.3 ± 3
Sex (M/F)		7 / 9	12 / 9	2 / 2
Post-Mortem Interval (h)		34.0 ± 3	39.1 ± 7	44.7 ± 16
pH		6.38 ± 0.1	6.33 ± 0.1	6.34 ± 0.2
Predeath MMSE		4.5 ± 2	5.3 ± 2	0.0 ± 0*
SP	*Frontal*	2.23 ± 0.2	1.68 ± 0.2	2.67 ± 0.3
	Temporal	2.31 ± 0.2	2.21 ± 0.2	3.00 ± 0.0*
NFT	*Frontal*	1.92 ± 0.3	1.53 ± 0.3	2.33 ± 0.3
	Temporal	2.23 ± 0.3	2.21 ± 0.2	2.67 ± 0.3

Table 6.4 Data are mean ± S.E.M. for AD patients with no *APOE* ε4 allele (n = 13-15), 1 ε4 allele (n = 18-21) or two ε4 alleles (n = 3-4). MMSE, Mini-Mental State Examination; SP, senile plaque score; NFT neurofibrillary tangle score. *Significant different from the *0 ε4* and from the *1 ε4* groups, one-way ANOVA with *post-hoc* Tamhane's $p < 0.05$.

Figure 6.1 Effect of *APOE* ε4 allele on cholinergic neurochemical alterations in AD.

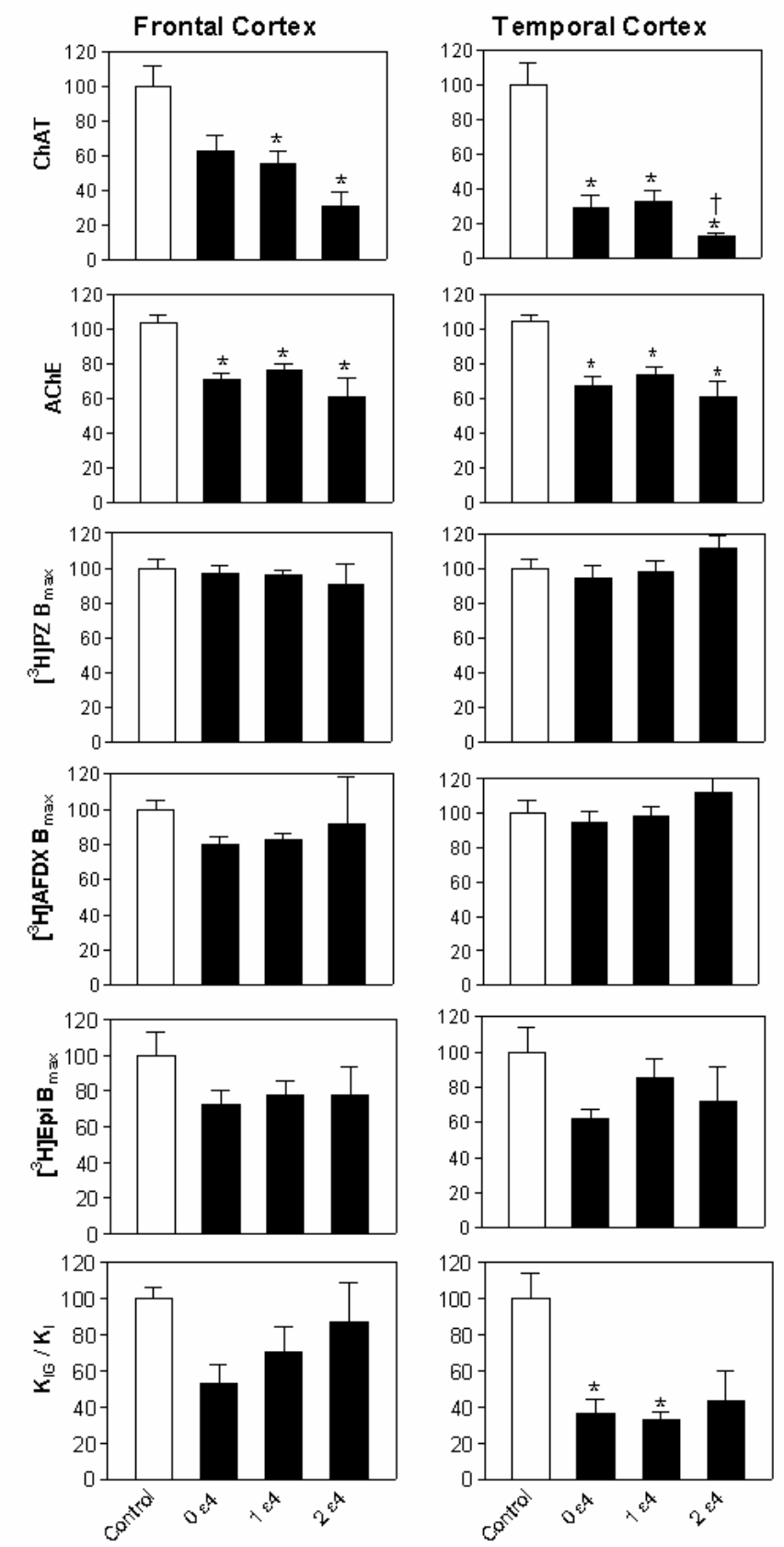

Figure 6.1 (Previous page) For each neurochemical measure, data are expressed as mean % control values ± S.E.M. for controls (n = 6-19), AD patients with no *APOE* ε4 allele (n = 6-13), one ε4 allele (n = 9-20) or two ε4 alleles (n = 2-4). ChAT, choline acetyltransferase; AChE, acetylcholinesterase; [^{3}H]PZ B_{max}, binding density of [^{3}H]pirenzepine (labels M1 receptors); [^{3}H]AFDX B_{max}, binding density of [^{3}H]AF-DX 384 (labels M2 receptors); [^{3}H]Epi B_{max}, binding density of [^{3}H]epibatidine (labels α4β2 nicotinic receptors); K_{iG} / K_i, measure of M1 / G-protein coupling (see text).
*Significantly different from controls, one-way ANOVA with post-hoc Dunnett's $p < 0.05$.
†Significantly different from 0 ε4 and 1 ε4 groups, one-way ANOVA with post-hoc Dunnett's $p < 0.05$.

6.4 DISCUSSION

The ε4 allele of *APOE* is at present the only well-established susceptibility gene for sporadic AD, with risk of disease development at a particular age increasing with ε4 allele dose. The widely replicable nature of these findings has led to intense research efforts toward uncovering the mechanisms by which ApoE4 affect the neurodegenerative process of AD. Here, using a well-characterized cohort of AD patients, we investigated whether inheritance of the ε4 allele was associated with cholinergic alterations using several pre- and postsynaptic neurochemical markers known to be affected in AD, including two markers (nicotinic α4β2 receptors and M_1/G-protein uncoupling) which, to our knowledge, have not been previously correlated with *APOE* genotypes.

6.4.1 Effects of APOE ε4 on ChAT Loss in AD

Although ChAT and AChE activities, muscarinic M_2 and nicotinic receptor densities as well as M_1 receptor coupling to G-proteins were all found to be reduced in one or both cortical regions (see **Table 6.2**), only losses in ChAT activities were associated with ε4 **(Figure 6.1)**. In the frontal cortex, ChAT activities were only significantly decreased in AD patients with at least one ε4. In the temporal cortex, although ChAT activities were reduced in all three genotype groups of AD patients when compared with controls, the ChAT values were most severely affected in patients with

two ε4 alleles, which were significantly lower than those with zero or one ε4. The particular vulnerability of the temporal cortex to neurodegeneration and cholinergic deficits in AD (Jobst et al. 1994; Wilcock et al. 1982) may explain the lower ChAT values we observed in this region. Importantly, our results suggest that presence of *APOE* ε4 does not directly lead to ChAT deficits, since temporal cortical ChAT was reduced even in AD patients without ε4; instead, ε4 may exacerbate the cholinergic denervation in both neocortical regions as indicated by loss of ChAT activities. These data are in agreement with some studies (Beffert and Poirier 1996; Poirier et al. 1995; Soininen et al. 1995), while others (Corey-Bloom et al. 2000; Svensson et al. 1997; Tiraboschi et al. 2004) failed to find significant effects of ε4 on ChAT. However, it is worth noting that in two of the negative studies (Corey-Bloom et al. 2000; Tiraboschi et al. 2004) cholinomimetic treatment status of the subjects was not reported. Given that cholinesterase inhibitor treatment in rodents are known to upregulate ChAT expression (Tayebati et al. 2004), the possibility that cholinomimetic treatment may confound the effect of ε4 on ChAT needs to be addressed. In the present study, all subjects were community-based patients who were never on cholinomimetic therapies, thus removing one potential confounder in the interpretations of ChAT data.

6.4.2 Lack of APOE ε4 Effects on Peri- and Postsynaptic Cholinergic Markers

In contrast to the ChAT results, neocortical AChE activity was reduced in AD but was not affected by *APOE* genotype (**Figure 6.1**). Unlike ChAT, which is expressed exclusively in cholinergic neurons in the brain, AChE is produced in cholinoceptive sites as well as non-cholinergic cells and released into the synaptic cleft (Grisaru et al. 1999; Taylor and Brown 1999). Thus, our data suggest that the reduced AChE in AD is an

indication of synapse loss rather than a consequence of *APOE* ε4-related cholinergic denervation. Similarly, the densities of M_1 and α4β2 nicotinic receptors, which are predominantly postsynaptic; as well as M_2 receptors, which could be localized to both pre- and postsynaptic compartments (Arroyo-Jiménez et al. 1999; Mash et al. 1985), were not significantly different among patients with different ε4 genotypes. Furthermore, although M_1/G-protein coupling was reduced in the temporal cortex, the extent of coupling loss again did not differ among the ε4 genotypes. Taken together, our results indicate that *APOE* ε4 may selectively influence pre-, rather than postsynaptic, alterations of cholinergic neurotransmission in AD.

In the frontal cortex, there was a nonsignificant trend towards recovery of K_{iG}/K_i values with increasing ε4 dose. Possible mechanisms for this observation are unknown and need to be further studied. However, one limitation of the current study is the relative small numbers of ε4/ε4 AD patients in the cohort **(Table 6.3)**, mainly due to the very low abundance of this genotype (1-3 % in the general population, reviewed in (Wilson et al. 1996). At present, we have failed to uncover an effect of ε4 on M_1/G-protein coupling. Taking into account the report by Cedazo-Minguez and Cowburn (2001) that apoE4 disrupts muscarinic receptor-mediated phosphoinositide hydrolysis in neuroblastoma cells, our M_1/G-protein coupling data suggest that either i) apoE4 targets signaling molecules downstream of G-protein (for e.g., phosphotidylinositol 3-kinase); or (ii) M_1/G-protein uncoupling is a postsynaptic response to presynaptic ChAT loss (see Potter et al. 1999), and is not directly affected by *APOE* ε4.

6.4.3 Possible Mechanisms of APOE ε4 Effects on ChAT Loss in AD

It is worthwhile to further study the mechanisms whereby apoE4 may affect cholinergic neuronal function. One possibility is that cholinergic neurons, with their reliance on cholesterol transport as well as phospholipid metabolism for synaptic plasticity and synthesis of acetylcholine in the brain, may be particularly vulnerable to the diminished affinity of apoE4 for receptor-mediated uptake (Poirier 1994; Poirier et al., 1995). Compared to the other isoforms, apoE4 also demonstrated lower activation of signaling molecules such as extracellular signal regulated kinase 1 / 2 (ERK1/2) which are involved in neurite outgrowth and synaptic plasticity (Qiu et al. 2004). Interestingly, Dubelaar et al. (2004) have shown that *APOE* ε4 is related to reduced metabolic activity in the nucleus basalis of Meynert (nbM) in both cognitively normal controls as well as prodromal early AD, suggesting that ε4 contribute to AD risk via a state of hypometabolism and reduced adaptive capacity in the nbM, perhaps years before the appearance of cognitive decline. It is possible that reduced ChAT activity would be one marker of nbM hypometabolism or cell loss, leading to cholinergic dysfunction which may further contribute to AD progression or by increasing amyloidogenic processing of precursor protein and Aβ in plaques (Hellstrom-Lindahl 2000). This postulate is supported by present findings that frontal and temporal ChAT activities were correlated with SP from the same regions in AD (Pearson's r = -0.376 [frontal], -0.408 [temporal], $p < 0.05$). In addition, SP scores were also higher in patients with two copies of ε4 compared to those with zero or one ε4 in the temporal cortex, with similar trends in the frontal cortex **(Table 6.4).** However, as this is a postmortem neurochemical study where

the majority of the subjects had severe disease at death, and further work using subjects with a wider range of disease severity is needed to corroborate the present findings.

In conclusion, using a cohort of well characterized, community-based AD patients, we show that the *APOE* ε4 allele does not lead to reduced ChAT *per se*, but is related to higher losses of ChAT activities in the neocortex compared to non *APOE* ε4 patients. Furthermore, *APOE* ε4 has limited or no effect on other neurochemical cholinergic markers such as AChE, M_1, M_2, α4β2 nicotinic receptors, and M_1/G-protein uncoupling. These findings suggest that presynaptic cholinergic function is selectively vulnerable to the effects of ε4, and the resultant exacerbation of cholinergic deficit may be related to AD risk via its effect on cognitive processes as well as Aβ deposition and plaque formation.

6.4.4 Further Studies

It is well established that the presynaptic components of the cholinergic neurotransmission including ChAT and muscarinic receptors are severely impaired in AD. Additionally, findings from this study have shown that *APOE* ε4 selectively affects presynaptic cholinergic function which may contribute to both the clinical and neuropathological features of AD and that *APOE* ε4 exacerbates the cholinergic neurotransmission dysfunction in AD by indirectly influencing the ChAT activity. However, the current view that only the presynaptic cholinergic neurotransmission is impaired has been challenged by findings from other postmortem studies. Clinical trials using cholinergic replacement or cholinomimetic therapies to treat AD patients have shown relatively modest or no improvements (Greenlee et al. 2001). One possible explanation may be that the postsynaptic signal transduction of cholinergic

neurotransmission is disrupted in AD and thus severely limit the ability of cholinergic agonist to activate downstream signaling. Flynn et al. (1991) have also shown that the postsynaptic muscarinic M_1 receptor is "uncoupled" from its G-protein in AD. In addition, other scientists have demonstrated that PKC activity is significantly reduced in AD (Cole et al. 1988; Masliah et al. 1991) But, whether M_1/G-protein uncoupling correlates with cognitive decline in AD is still not fully understood and will be addressed in the next chapter.

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CHAPTER 7

Effects of Impaired Coupling Muscarinic M_1 Receptors to G-proteins on Cognition in Alzheimer's Disease*

7.1 INTRODUCTION

As previously mentioned, AD is characterized neuropathologically by senile plaques, neurofibrillary tangles, and losses of various neurotransmitter-producing neurons. Of the transmitter pathologies seen in AD, cholinergic dysfunction occurs early in the disease process (Mesulam 2004) and is thought to underlie much of the characteristic cognitive and neuropsychiatric symptoms (Francis et al. 1999; Lai et al. 2001; Minger et al. 2000; Perry et al. 1981; Wilcock et al. 1982). Losses of basal

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forebrain cholinergic neurons, along with reduced neocortical choline acetyltransferase (ChAT) activity, acetylcholine (ACh) release and muscarinic M_2 receptor binding, constitute a state of presynaptic cholinergic deficit in AD (Francis et al. 1985; Mash et al. 1985; Perry et al. 1977; Whitehouse et al. 1982). In contrast, the predominantly postsynaptic M_1 receptors were originally thought to be unaltered (Mash et al. 1985), but subsequent studies showed that normal levels of radioligand binding to M_1 receptors contrasted with reduced M_1 immunoreactivity (Flynn et al. 1995). In addition, the ability of M_1 receptors to form high affinity agonist-binding complexes with guanine nucleotide-binding (G-) proteins was found to be impaired in AD (Flynn et al. 1991), along with reductions in phosphoinositide hydrolysis, phospholipase C (PLC) and protein kinase C (PKC) activities (Ferrari-DiLeo and Flynn 1993; Jope 1999; Masliah et al. 1991). Taken together, these findings suggest that M_1 or M_1-like receptors are altered in the AD cortex, resulting in defective coupling to G-proteins and hypoactivation of second messengers. This breakdown in intracellular signal transduction associated with M_1 receptors in the cortex is hypothesized to underlie the limited efficacy of cholinergic replacement therapies, since the ability of ACh or agonists to ameliorate deficits in cholinergic neurotransmission would have been severely limited (Flynn et al. 1991). However, it is presently unclear whether M_1/G-protein uncoupling is related to cognitive decline in AD. In this study, I measured M_1 receptor densities and coupling to G-proteins in the post-mortem neocortex of a cohort of longitudinally assessed AD patients, as well as in a group of age-matched controls. I then correlated the M_1 neurochemical parameters with clinical data.

7.2 MATERIALS AND METHODS

7.2.1 Patients, Clinical and Neuropathological Data

Up to 24 AD subjects were included in this study. The AD subjects were originally derived from an autopsied subset of a cohort of community-based dementia patients from Oxfordshire, UK, who were recruited for a prospective study of behaviour in dementia (Hope et al. 1999). The entry data, inclusion and exclusion criteria, natural history of these patients, and cognition assessment using Mini-Mental State Examination (MMSE) have previously been described in detail in **Chapter 5 (Section 5.1.1).** Dementia severity was measured by using the mean of up to five MMSE scores before death so as to avoid floor effects, since some patients may live for extended periods of time after their MMSE scores had reached 0 (Lai et al 2001). Patients were considered to have severe dementia (AD_{severe}) if the mean predeath MMSE was ≤ 9, while those with scores between 10 and 24 were deemed to have mild to moderate dementia ($AD_{mild/mod.}$). None of the subjects had scores >24. The rate of cognitive decline per year was measured by the linear regression of serial MMSE scores on the time interval between recruitment and death, or when the MMSE first reached 0. Complete drug histories were taken, and none of the subjects received cholinomimetics. As mentioned in **Chapter 5 (Section 5.1.1)**, all AD patients fulfilled the CERAD criteria for neuropathological diagnosis of AD. Selection of subjects for the current study was based on tissue availability, and not on dementia severity or Braak stage, and controls were well matched to subjects with regards to age, sex, and post-mortem delay.

7.2.2 Brain Membrane Homogenates

Tissue processing and neurochemical assays were performed blind to clinical information. All chemicals were from Sigma Aldrich (St Louis, MI) unless otherwise stated. Brain tissue homogenate preparation has been described in **Chapter 5 (Section 5.2.2)**.

7.2.3 Neurochemical Assays

The methods of pharmacological determination of M1 receptor coupling to G-proteins using [^{3}H]pirenzepine (PZ) / carbachol competition assay (see **Fig 7.2 A** and **B**) as well as measurement of muscarinic M_1 receptors using saturation radioligand binding assay were detailed in **Chapter 5 (Section 5.2 and 5.3)**. Radiochemical determination of ChAT activity has been described in **Chapter 5 (Section 5.4)**. Note that brain homogenates were not available from both regions in some subjects.

7.2.4 Statistical Analyses

Data were first tested for normality to determine the use of parametric or non-parametric tests. Neurochemical and demographic variables among the groups were compared by one-way analysis of variance (ANOVA) with *post-hoc* Tamhane's tests, since the number of subjects was not evenly matched and equal variances could not be assumed. Disease variables between the AD cognitive groups were compared by Student's *t* tests. Correlations of neurochemical variables with cognitive data, as well as inter-correlations among the neurochemical variables were performed by Pearson's product moment or Spearman's correlation, as appropriate. Results were considered statistically significant if $p < 0.05$.

7.3 RESULTS

7.3.1 Demographics

Table 7.1 shows that demographic variables such as age, sex, and post-mortem interval were well matched among the control, AD with mild / moderate dementia ($AD_{mild/mod.}$), and AD with severe dementia (AD_{severe}), except for brain pH, which was significantly lower in the AD_{severe} group. This is likely due to the prolonged agonal state of severely demented patients, resulting in brain acidosis (Hardy et al. 1985). However, brain pH and the other demographic variables listed on the table did not correlate with K_{iG} /K_i values in either controls or AD subjects (Spearman $p > 0.10$). Among the AD patients, entry and predeath MMSE scores were expectedly lower in the AD_{severe} compared to the $AD_{mild/mod.}$ group. Additionally, the rates of MMSE decline were significantly higher in the AD_{severe} group, while both disease duration (estimated duration between first symptoms and death) and follow-up (duration between study entry and death) were matched in the AD cognitive groups.

Table 7.1 Demographic and disease variables in controls and cognitive subgroups of AD patients.

	Control N = 12	AD (Mild / Moderate) N = 7	AD (Severe) N = 17
Demographic Variables			
Age, y	77 ± 3	81 ± 2	81 ± 2
Sex, M / F	5 / 7	5 / 2	9 / 8
Postmortem interval, h	47 ± 9	45 ± 9	35 ± 6
pH	6.48 ± 0.1	6.25 ± 0.09	6.02 ± 0.05*
Disease Variables			
Disease duration, y	--	8.4 ± 0.8	9.0 ± 1.2
Follow up, y	--	3.1 ± 0.2	3.4 ± 0.4
MMSE at study entry	--	20.7 ± 1.1	11.3 ± 1.4**
MMSE at predeath	--	13.1 ± 3.0	1.2 ± 0.7**
MMSE decline / year	--	2.9 ± 0.7	6.1 ± 0.8**

Table 7.1 Data are mean ± s.e.m. MMSE = Mini-Mental State Examination.
*Significantly different from control, ANOVA with post-hoc Tamhame's, $p < 0.01$.
**Significantly different from AD (mild/moderate), Student's *t* tests, $p < 0.01$.

7.3.2 M_1 Receptor Binding in AD: Correlation with Dementia

For the neurochemical variables, [^{3}H]pirenzepine binding affinity (K_D) and density (B_{max}) were unchanged in both frontal and temporal regions (**Figure 7.1C and D**). This contrasted with significantly reduced temporal cortical K_{iG}/K_i in both AD_{severe} and $AD_{mild/mod.}$ (**Figure 7.2C**). In the frontal cortex, K_{iG}/K_i was significantly reduced only in AD_{severe}, with intermediate values in $AD_{mild/mod}$ which were not significantly different from either control or AD_{severe} (**Figure 7.2C**).

Figure 7.1 [^{3}H]Pirenzepine (PZ) binding in postmortem control and AD neocortex.

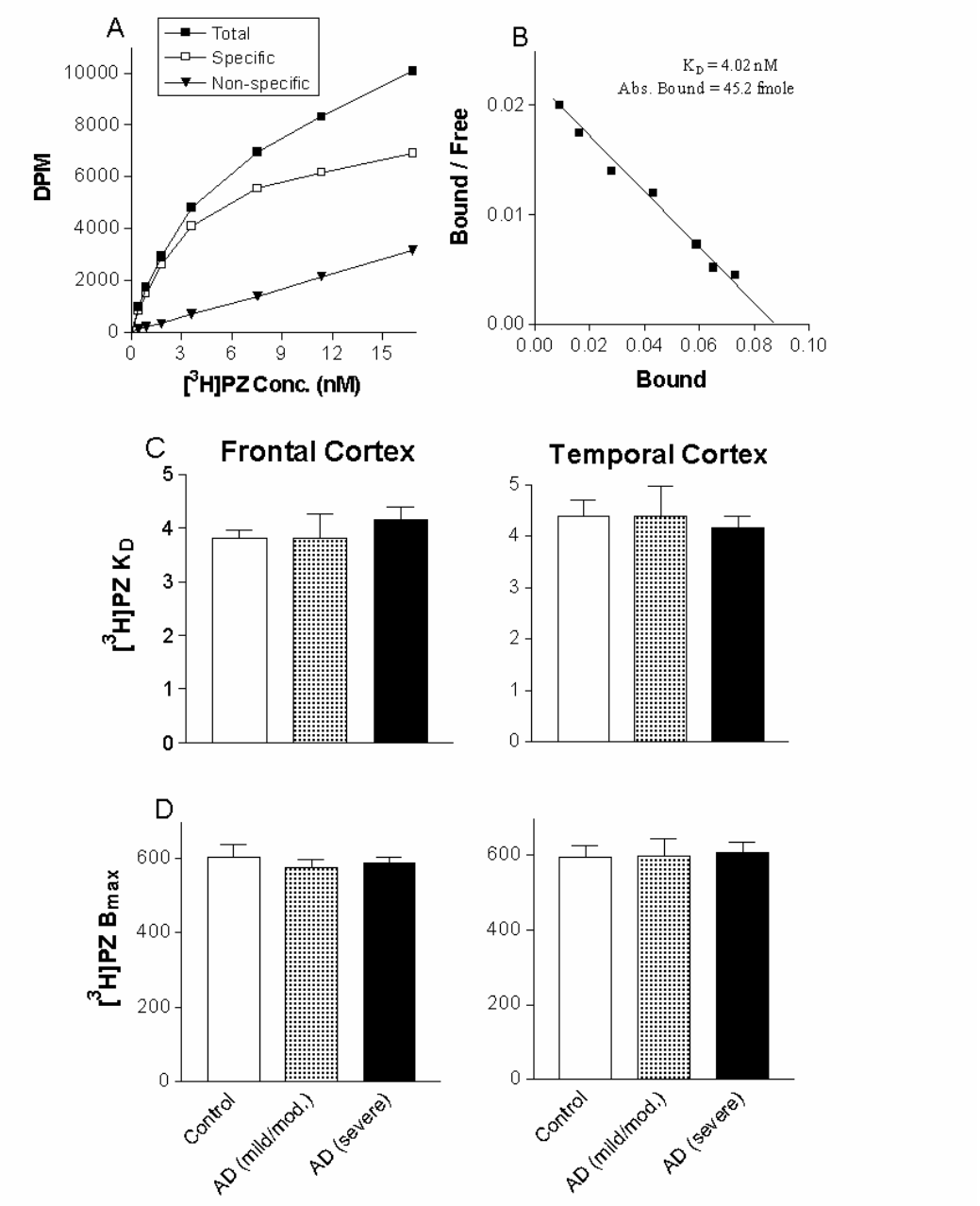

Figure 7.1 **(A)** representative saturation isotherm of [^{3}H]PZ binding in an AD frontal cortex. **(B)** Scatchard plot of binding data presented in A with derived neurochemical parameters. **(C)** and **(D)** mean ± s.e.m values of neocortical [^{3}H]PZ binding affinity (K_D, in nM) and M_1 receptor density (B_{max}, in fmol/mg protein), respectively, in control and AD cognitive groups.

Figure 7.2 Carbachol competition for the specific binding of [^{3}H]pirenzepine to M1 receptors in the neocortex of a randomly selected control (A) and AD patient (B).

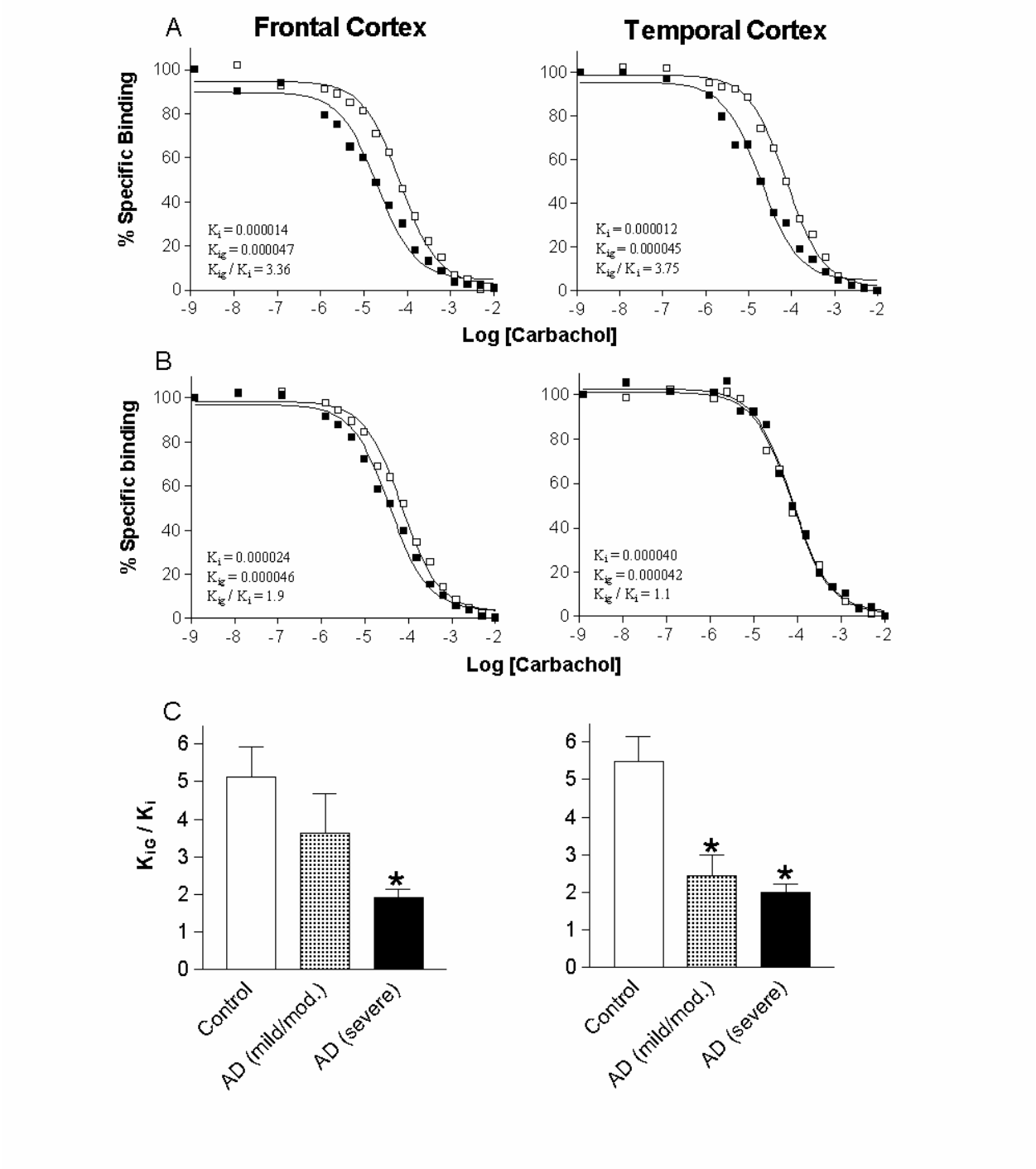

Figure 7.2 (**Previous page**) Best-fit curves were derived from non-linear regression of data. Each data point denotes specific binding in the absence (■) and presence (□) of 0.2 mM GppNHp across log transformed carbachol concentrations. **C**, mean ± s.e.m. values of K_{iG}/K_i in controls and cognitive groups of AD. *Significantly different from control, $p \leq 0.01$ one-way ANOVA post-hoc tests.

When the AD subjects were analyzed as a group, K_{iG}/K_i was significantly correlated with rate of MMSE decline in the frontal, but not temporal cortex (**Figure 7.3**). [^{3}H]Pirenzepine K_D and B_{max} did not correlate with MMSE decline in either region. Lastly, **Figure 7.4A** shows that ChAT activities were altered in AD cognitive groups in a pattern similar to K_{iG}/K_i (see **Figure 7.2C**), while **Figure 7.4B** demonstrates that K_{iG}/K_i values of all subjects correlated with ChAT activities in both brain regions.

Figure 7.3 Correlations of K_{iG}/K_i values with the rate of MMSE decline in AD patients using Spearman's test.

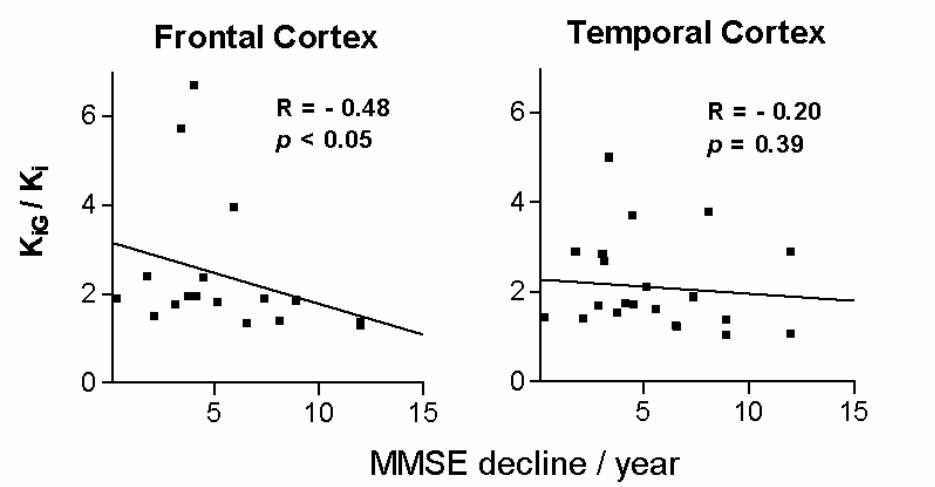

Figure7.3 Data are available for 18 patients in the frontal cortex and 22 patients in the temporal cortex.

Figure 7.4 ChAT activities in control and AD cognitive groups and correlations with K_{iG}/K_i values.

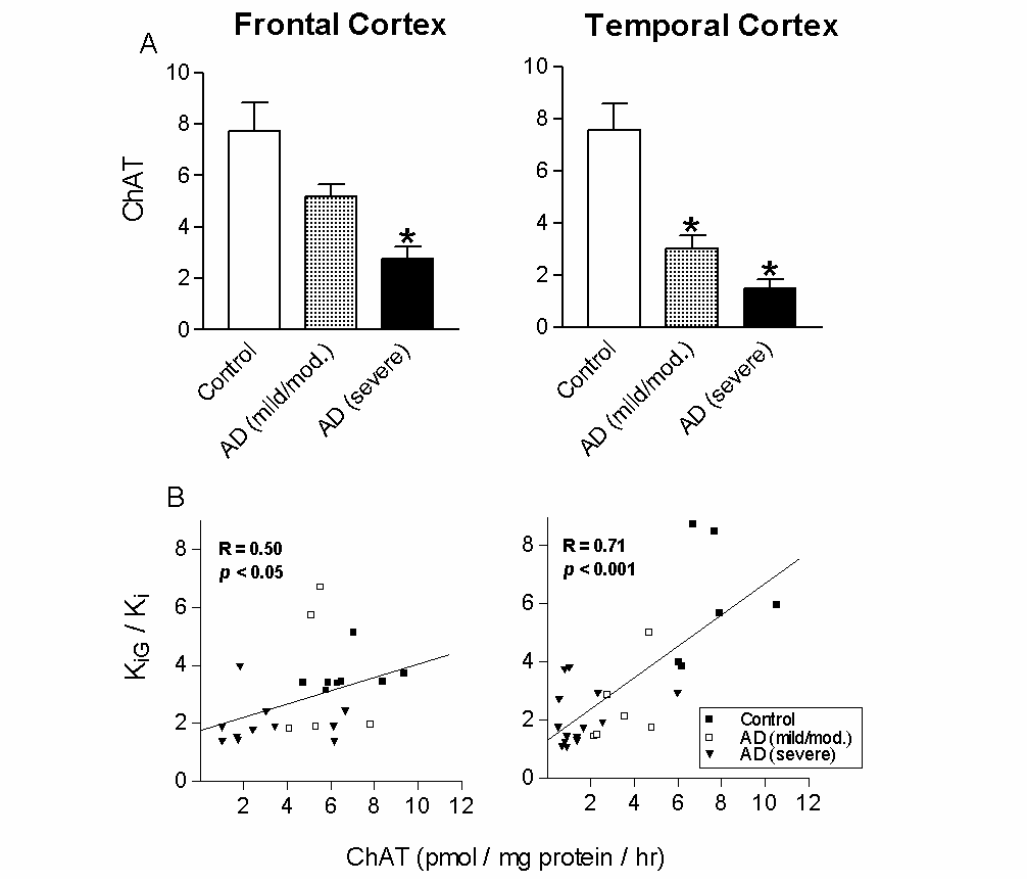

Figure 7.4 (A) ChAT data were available for 8 controls, 5 $AD_{mild/mod.}$ and 11 AD_{severe} patients in the frontal cortex, and for 6 controls, 6 $AD_{mild/mod.}$ and 14 AD_{severe} patients in the temporal cortex. *Significantly different from control, $p < 0.01$ one-way ANOVA post-hoc tests. **(B)** Spearman correlations of ChAT activities with K_{iG}/K_i values.

7.4 DISCUSSION

Muscarinic M_1 receptors, together with M_3 and M_5 subtypes, belong to the family of $G\alpha_{q/11}$-coupled seven-transmembrane domained receptors which are known to activate PLC and PKC mediated signaling pathways (Caulfield 1993). Postsynaptic M_1 receptors are found in high densities in the neocortex and hippocampus, where they mediate cholinergic neurotransmission in a range of CNS functions including learning and

memory (Anagnostaras et al. 2003; Hagan et al. 1987; Roldan et al. 1997). Furthermore, *in-vitro* study by Lu et al. (1999), has demonstrated that by activating signaling cascades involving PKC and Src kinases, muscarinic receptors can potentiate glutamatergic NMDA receptor function, whose regulation of long-lasting forms of synaptic plasticity is thought to be the major physiological substrate of memory consolidation (McBain and Mayer 1994).

Although cholinergic dysfunction is a well-known neurochemical feature in AD, investigations of its role in the cognitive features of AD have focused on presynaptic components (e.g., loss of ChAT activities, Minger et al. 2000; Perry et al. 1981; Wilcock et al. 1982). While previous studies had reported losses of postsynaptic M_1/G-protein coupling as well as impairment of associated downstream signal transduction (Ferrari-DiLeo and Flynn 1993; Flynn et al. 1991; Jope et al. 1997; Masliah et al. 1991), it was unclear whether the findings were related to cognitive decline in AD. Using post-mortem material from a cohort of longitudinally assessed AD patients, I now show that the extent of reduction in M_1/G-protein coupling is related to the severity of cognitive symptoms in AD. Specifically, I found that compared with controls, temporal cortical coupling was lower in AD patients with mild/moderate, as well as severe dementia, while frontal cortical coupling was significantly reduced only in the subgroup of AD patients with severe dementia. Furthermore, reductions of M_1/G-protein coupling in the frontal cortex (but not the temporal cortex) correlated with rate of cognitive decline. The basis of the observed regional difference is unclear. However, previous studies have demonstrated that cholinergic deficits selectively affect temporal and associated cortices in early stages of the disease (Jobst et al. 1994; Wilcock et al. 1982). It is thus possible that loss of

M_1/G-protein coupling in the temporal region occurs early in AD, and is extensive even in patients with mild to moderate symptoms. Therefore, the lack of correlation with cognitive symptoms in this region may simply reflect a dampened effect as the neurochemical deficits reached a stable, low level (a 'floor effect'). This postulate is supported by my finding that ChAT activities, a marker for cholinergic innervation, was also reduced in both cognitive groups in the temporal cortex; while in the frontal cortex, ChAT reduction was significant only in the severely demented group. However, because the majority of subjects in this longitudinal study were severely demented at the time of death, further studies on patients with a wider range of dementia severity are needed to confirm this postulate. In this regard, it may be of particular interest to investigate the state of M_1/G-protein coupling in subjects who have minimal cognitive impairment (MCI), since a significant proportion of such patients will eventually progress to AD (DeCarli et al. 2004; Morris et al. 2001).

My findings suggest that impairment of M_1 receptor-mediated signaling in the neocortex via uncoupling with its G-protein may be a novel neurochemical substrate of cognitive decline in AD. Compared to presynaptic cholinergic deficits, a signaling dysfunction in the postsynaptic M_1 receptors may be temporally more closely related to processes leading to cognitive impairment, and has implications in rational therapeutic strategies. For example, compounds which primarily act at the synaptic (e.g., cholinesterase inhibitors) or receptor (e.g., M_1 agonists) levels will be predicted in light of my findings to be of limited efficacy in ameliorating dementia in AD. Instead, therapeutic compounds acting at the level of M_1-activated signaling molecules such as PKC may show more benefit (Etcheberrigaray et al. 2004). However, the current results

have also raised important questions which need to be further studied. Firstly, the antecedent or causal events leading to M_1/G-protein uncoupling in AD are at present unclear. One hypothesis is that presynaptic cholinergic deficits precede M_1/G-protein uncoupling. Potter et al. (1999) reported that cholinergic deafferentation of the hippocampus by immunolesioning resulted in reductions of M_1-mediated norepinephrine release as well as M_1/G-protein coupling without affecting [^{3}H]-pirenzepine binding parameters. Using ChAT activity as a marker of cholinergic innervation, I show that neurologically normal controls with relatively intact cholinergic systems also had higher M_1/G-protein coupling compared with cholinergically denervated AD subjects (**Figure 7.4B**). I further showed that alterations of ChAT in cognitive groups of AD mirrored those seen in M_1/G-protein coupling. Therefore, my data suggest that like the cholinergic lesions, the uncoupling of M_1 receptors to their G-proteins may be an early event in the AD process. However, the molecular mechanisms relating ChAT alterations to M_1/G-protein coupling are unknown. Furthermore, since none of the patients in the current study were on cholinergic replacement therapies, it is not clear whether the use of cholinomimetics would also lead to restoration of M_1/G-protein coupling, and further studies are needed.

Another question arising from the present observations is the elucidation of molecular mechanisms underlying the uncoupling of M_1 receptors to G-proteins in AD. Studies showing preserved radioligand binding to M_1 receptors contrasting with reduced M_1 immunoreactivity (Flynn et al. 1995) suggest structural changes which do not interfere with ligand binding, but which may affect antibody recognition. Such structural changes may possibly include covalent modifications such as phosphorylation. Further

studies are needed to investigate whether aberrant phosphorylation or dephosphorylation of key residues on the M_1 receptor may alter its association with G-proteins. Another potential mechanism for the loss of M_1/G-protein coupling may be a reduction of $G\alpha_{q/11}$ proteins, although this has been refuted by studies which show that G-proteins, including the $G\alpha_q$ subtype, are generally preserved in the AD brain (García-Jiménez et al. 2003). A third possibility may be that G-protein function is altered in AD (for e.g., loss of GTPase activity), which may again involve covalent modifications (García-Jiménez et al. 2002).

In conclusion, the present study points to loss of M_1/G-protein coupling in the neocortex as a neurochemical substrate of cognitive decline in AD, and provides one explanation for the limited efficacy of cholinergic replacement therapies in ameliorating cognitive symptoms. Since M_1/G-protein uncoupling may occur early in AD, and represents an initial point of disruption in a signaling cascades which are involved in cognitive functions, therapies which aim to correct or circumvent deficits in M_1 mediated signaling may show improved efficacy in ameliorating clinical symptoms. However, more work is needed to elucidate both the causes and the mechanisms of G-protein uncoupling to M_1 receptors in the AD brain.

7.5 FURTHER STUDY

The findings from this study have demonstrated that M_1 uncoupling is correlated with cognitive decline in AD. One hypothesis is that the impairment of muscarinic M_1 receptor to its G-protein may affect NMDA receptor function. Indeed, Marino et al. (1998) has shown that activation of M_1 receptors potentiates NMDA receptor current and that M_1 receptors colocalize with NR1 subunit of NMDA receptors on the dendrites and

soma of hippocampal pyramidal neurons. Additionally, NMDA receptors are regulated by PKC (MacDonald et al. 2001) and Src kinase (Ali and Salter 2001). Taken together, results from these *in-vitro* studies suggest that there may be a functional link between M_1 and NMDA receptor. However, it is currently not clear whether M_1/G-protein uncoupling is associated with PKC deficits in AD and, more importantly, whether NMDA receptor hypofunction is correlated with both PKC and M_1/G-protein uncoupling. I will discuss my work on this question in the next chapter.

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CHAPTER 8

Effects of Impaired Coupling Muscarinic M_1 receptors to G-proteins on PKC Activity and NMDA Receptors Hypofunction in Alzheimer's Disease*

8.1 INTRODUCTION

Several neurotransmitter systems involved in cognitive processes are now known to be affected in Alzheimer's disease (AD), among which losses of cholinergic neurons in

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the basal forebrain are one of the earliest and most consistent findings (Davies and Maloney 1976; Whitehouse et al. 1982). Cholinergic neuronal loss was thought to underlie the clinical symptoms of AD and provided the rationale for cholinergic replacement or cholinomimetic pharmacotherapies (Bartus et al. 1982). To date, treatment with available acetylcholinesterase inhibitors (AChEIs) has yielded modest improvements in clinical rating scales, while clinical trials on agonists of G-protein coupled muscarinic receptors have not shown much promise (Greenlee et al. 2001; Mouradian et al. 1988). Limiting factors for these compounds include stage-dependent efficacy, unfavorable side effects (Greenlee et al. 2001) and the involvement of other acetylcholine receptors (AChRs, e.g., nicotinic, Paterson and Nordberg 2000) or transmitter systems (e.g., serotonergic, Buhot et al. 2000). Another possible reason is a disruption of AChR-mediated signaling. For instance, muscarinic M_1 receptors are the major cholinergic receptor subtype expressed in postsynaptic sites in the neocortex and are critically involved in the cholinergic regulation of cognitive processes. M_1 receptors are coupled to the $G\alpha_{q/11}$-family of guanine nucleotide-binding (G-) proteins and activate phospholipase C (PLC) and protein kinase C (PKC) mediated signaling cascades, which also involve Src family kinases (Caulfield 1993; Lu et al. 1999). Previous studies have shown that while M_1 receptor densities are preserved in the AD neocortex, the coupling of these receptors to their G-proteins is impaired (Flynn et al. 1991; Flynn et al. 1995; Warpman et al. 1993), suggesting a postsynaptic cholinergic dysfunction which may limit the efficacy of cholinomimetic therapies in ameliorating the cognitive deficits of AD.

Prominent neuronal loss occurs in areas other than the basal forebrain, especially affecting the glutamatergic pyramidal neurons of the neocortex and hippocampus

(Greenamyre et al. 1988). Glutamate is the primary excitatory neurotransmitter and is involved in most aspects of cognition and higher mental functions. In particular, the phenomenon of long term potentiation (LTP), a form of use-dependent synaptic plasticity mediated by the ionotropic *N*-methyl-D-aspartate (NMDA) subtype of glutamate receptors, is the leading cellular model of memory consolidation (Milner et al. 1998). NMDA receptors are Ca^{2+} channels with a voltage-dependent Mg^{2+} block, and are thought to be tetrameric complexes of NR1 and NR2A - 2D subunits in a 1:1 ratio (Ishii et al. 1993; Kutsuwada et al. 1992; Monyer et al. 1992). In AD, abnormalities in the expression and ligand binding properties of NMDA receptor subunits (Hynd et al. 2001; Hynd et al. 2004; Procter et al. 1989) may underlie the cognitive symptoms of AD. Interestingly, M_1 receptors colocalize with NR1 on the dendrites and soma of hippocampal pyramidal neurons, and activation of M_1 receptors potentiates NMDA receptor currents (Marino et al. 1998). Additionally, work done by Lan et al. (2001) has provided evidence that PKC activity may enhance the rate of membrane insertion of NMDARs. Currently it is not known whether M_1 uncoupling is associated with loss of PKC in AD and whether PKC loss accounts for reduction of NMDAR density in AD. NMDA receptors are regulated by PKC (Lan et al. 2001; MacDonald et al. 2001) as well as Src kinase (Ali and Salter 2001). However, there is much *in-vitro* evidence of muscarinic receptors regulating the NMDA receptor function (Francis et al. 1999), but the relevance in AD is not known. Here I report the first post-mortem evidence that M_1 uncoupling is actually associated with losses of both PKC activity as well as NMDA receptor density. Given the important roles played by PKC in a variety of neuronal function, and the importance of NMDA receptor in learning and memory, these results

provide a mechanism whereby M_1 uncoupling may contribute to the clinical and neuropathological features of AD, and provide the therapeutic rationale for PKC ligands in AD treatment.

8.2 MATERIALS AND METHODS

8.2.1 Patient Characteristics

The 22 subjects with AD and 12 controls were obtained from our collaborators from UCLA (Professors Harry Vinters and Jeffrey Cummings). Subject recruitment, demographical data have been described in detail in **Chapter 5 (Section 5.1.2)**. Selection of subjects for the current study was based on tissue availability, and not on dementia severity or Braak stage, and controls were well matched to subjects with regards to age, sex, and post-mortem delay (see **Table 5.1**).

8.2.2 Postmortem Tissue Processing

Tissue processing and neurochemical assays were performed blind to clinical information. Brain tissue homogenate preparation has been described in **Chapter 5 (Section 5.2.2)**.

8.2.3 Saturation Radioligand Binding Assays

All chemicals were of analytical grade and were purchased from Sigma Aldrich (St Louis, MI) unless otherwise stated. Measurement of muscarinic M_1 and NMDA receptors was performed by saturation radioligand binding assays using [^{3}H]pirenzepine (PZ) and [^{3}H]MK-801, respectively, as detailed in **Chapter 5 (Section 5.2.2)**.

8.2.4 M_1/G-protein Coupling

Pharmacological determination of M_1/G-protein interactions was carried out by [^{3}H]PZ / carbachol competition binding as previously described in **Chapter 5 (Section 5.3.2)**.

8.2.5 PKC and Src Kinase Activities

PKC and Src kinase enzymatic assays were carried out using components of a commercially available PKC and Src kinase enzymatic assay kits (Upstate Biotechnology Inc., Lake Placid, NY). Detailed protocols were described in **Chapter 5 (Section 5.5)**.

8.2.6 Immunoblotting

Immunoblotting of the NR1 subunit of NMDA receptor with a rabbit polyclonal antibody (1:1000, Chemicon, Inc., expected molecular weight of around 114 kDa) followed by stripping and normalization with β-actin densities have been described in **Chapter 5 (Section 5.6)**.

8.2.7 Statistical analyses

All statistical analyses were performed using the SPSS 11.0 for Windows software (SPSS Inc.). Data were first checked for normality for the selection of parametric or non-parametric tests. Demographic and neurochemical variables were compared between AD and controls by Student's *t*-tests, and correlations among these variables were performed using Pearson's product moment. Within the AD group, the effects of potentially confounding demographic or disease variables on neurochemical measures were investigated with Pearson's product moment or Student's *t*-tests as appropriate. Lastly, multiple regression using the 'stepwise' method was used to investigate possible relationships between the dependent variables (PKC activity, Src

activity and NMDA receptor density), other neurochemical variables and demographic factors. This allowed for the fact that M_1/G-protein coupling (K_{iG} /K_i) may be related to multiple variables and indicated the strongest correlate. For all analyses, the null hypothesis was rejected at $p < 0.05$.

8.3 RESULTS

8.3.1 Effects of Demographic and Disease Factors on Neurochemical Variables

Demographic factors including age, sex, post-mortem interval and storage interval in AD and controls were well matched **(Table 8.1)**. Furthermore, binding affinity (K_D) and density (B_{max}) of [^{3}H]PZ (M_1 receptors) and [^{3}H]MK-801 (NMDA receptors), NR1 immunoblot density as well as M_1/G-protein coupling status (K_{iG} /K_i) did not correlate with the demographic factors listed above (Pearson's $p > 0.05$, data not shown). Within the AD cohort, the presence of additional neuropathological findings i.e., LB and stroke (n = 4, see above), and duration of dementia symptoms (9.5 ± 1.1 y, range 3 -17 y) did not affect the neurochemical variables (Student's p and Pearson's $p > 0.05$, respectively, data not shown). However, Braak stage was negatively correlated with NR1 density (Pearson r = -0.55, $p = 0.03$), but positively correlated with Src activity (Pearson r = 0.47, $p = 0.04$). Therefore, Braak stage was included as a covariate in subsequent regression analyses.

Table 8.1 Demographic and neurochemical variables in AD subjects and controls.

	Control	*AD*
Demographics		
Maximum Number of Cases[1]	12	22
Age at Death (y)	73.2 ± 4	78.8 ± 2
Sex (M/F)	6 / 6	12 / 10
Storage (y)	4.5 ± 0.5	5.4 ± 0.7
PMI (h)	14.4 ± 3	14.2 ± 2
Muscarinic M1 Receptors		
K_{iG}/K_i	7.1 ± 0.9 (12)	4.8 ± 0.4 (22)*
[^{3}H]Pirenzepine K_D	5.3 ± 0.5 (11)	6.9 ± 0.5 (18)
[^{3}H]Pirenzepine B_{max}	601 ± 39 (11)	590 ± 39 (18)
NMDA Receptors		
[^{3}H]MK-801 K_D	9.9 ± 1.7 (11)	8.2 ± 0.9 (22)
[^{3}H]MK-801 B_{max}	300 ± 46 (11)	200 ± 23 (22)*
Protein Kinase Activities		
PKC	776 ± 107 (11)	354 ± 57 (22)**
Src	6.8 ± 1.4 (11)	7.0 ± 1.2 (21)

Table 8.1 Data are mean ± S.E.M. PMI, post-mortem interval; K_{iG}/K_i, measure of M1/G-protein coupling (see text); K_D, binding affinity constant (in nM); B_{max}, binding density (in fmol/mg protein). Specific PKC (protein kinase C) and Src kinase activities are in pmol phosphate/min/mg protein.
[1]Not all neurochemical measures were available for all cases. The *N* values available for each neurochemical measure are listed in parentheses. *Different from control $p < 0.05$. **Different from control $p < 0.01$.

8.3.2 Neurochemical Variables of Controls vs. AD

Table 8.1 shows that while M_1 receptor K_D and B_{max} are unchanged in AD, receptor coupling to G-proteins is impaired (reduced K_{iG}/K_i). Furthermore, PKC activity, NMDA receptor B_{max} as well as NR1 immunoblotting density are reduced in the AD

frontal cortex (**Table 8.1 and Figure 8.1B**). In contrast, Src activity and NMDA receptor K_D are unchanged.

Figure 8.1 NMDA receptor NR1 levels in AD subjects and controls.

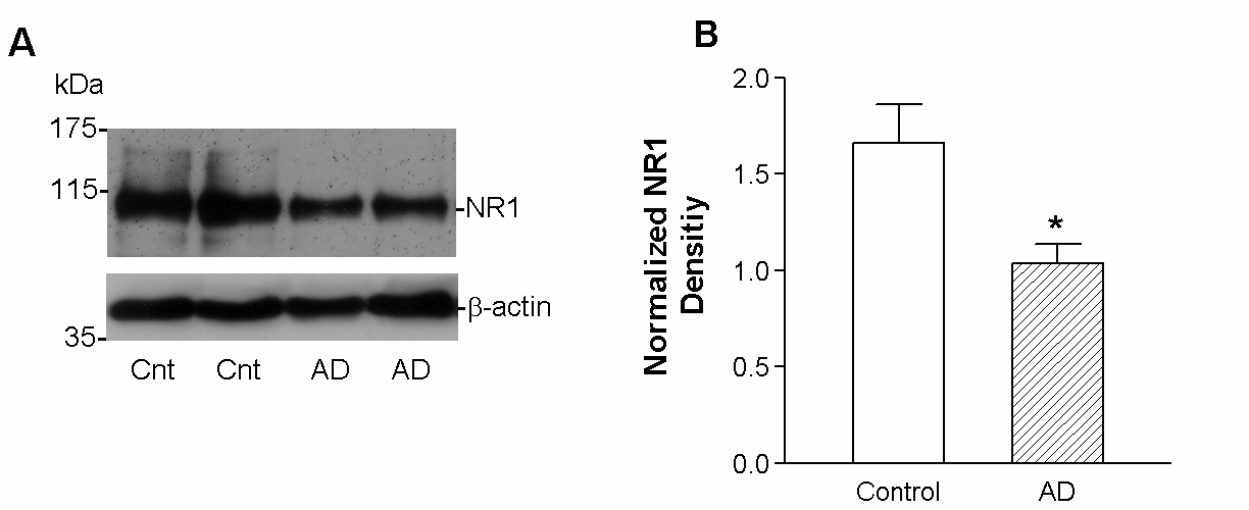

Figure 8.1 **(A)** representative immunoblot of NR1 (top lane) from brain membrane homogenates was stripped and reblotted with antibody against β-actin (bottom lane) for controls (Cnt) and AD. Molecular weight markers in kilodaltons (kDa) are as indicated. **(B)** bar graph of mean normalized NR1 immunoblot optical densities (in arbitrary units) for available control (n = 12) and AD subjects (n = 18). *Significantly different from control, $p < 0.05$ (Student's *t*-test).

8.3.3 Correlation of M_1/G-protein Coupling with NMDA Receptor Status and Kinase Activities

Figure 8.2 shows that K_{iG}/K_i values of all subjects correlated with PKC, but not Src kinase, activities in the frontal cortex, while **Figure 8.3** shows that K_{iG}/K_i correlated with both NMDA receptor indices ([^{3}H]MK-801 B_{max} and NR1 immunoblot density). Finally, stepwise multiple regression analyses showed that of the list of demographic / neurochemical variables (excluding the dependent variable) in **Table 8.1** plus Braak staging, K_{iG}/K_i was the strongest predictor for both PKC activity (adjusted $R^2 = 0.30$, $\beta = 0.59$, $p = 0.008$) and NR1 density (adjusted $R^2 = 0.26$, $\beta = 0.55$, $p = 0.015$).

Figure 8.2 Association of M_1/G-protein coupling with protein kinase activities.

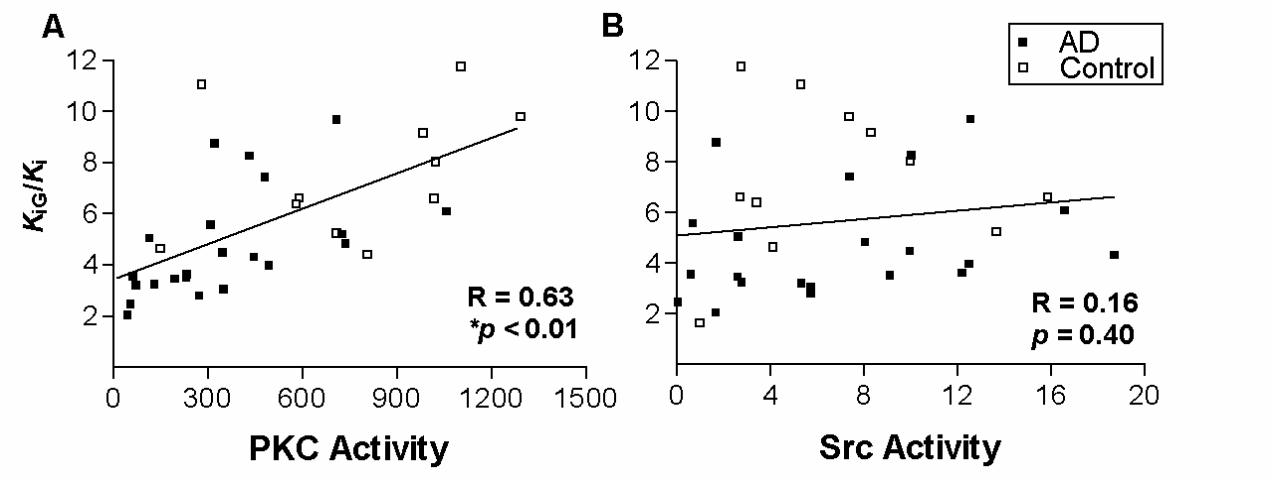

Figure 8.2 Correlation of K_{iG}/K_i, a measure of M1/G-protein coupling (see text) with **(A)** specific protein kinase C and **(B)** Src kinase activities, both in pmol phosphate/min/mg protein. *Significant Pearson correlation.

Figure 8.3 Association of M_1/G-protein Coupling with NMDA Receptor Measurements.

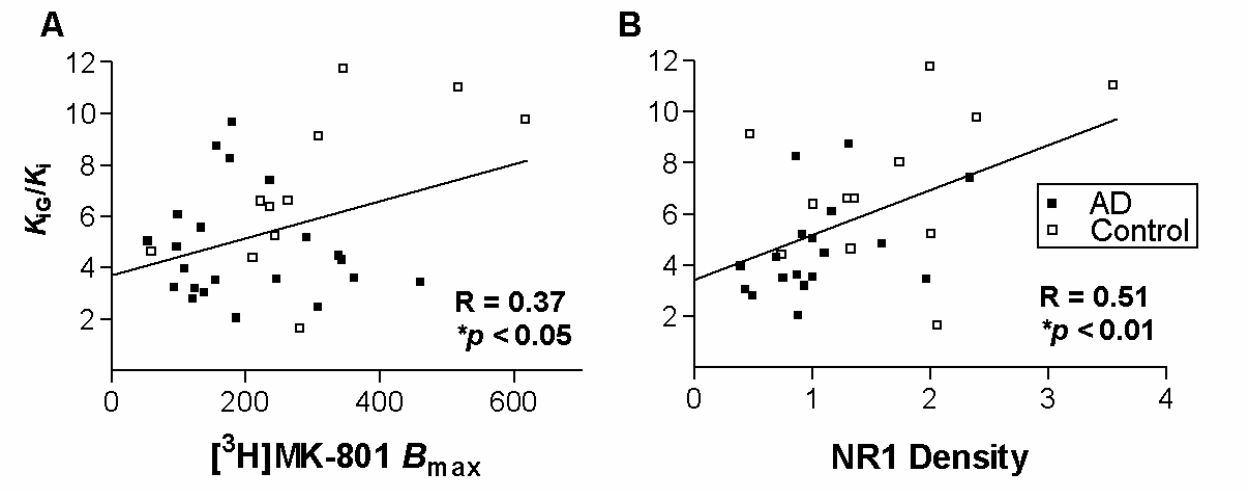

Figure 8.3 Correlation of K_{iG}/K_i with **(A)** [^{3}H]MK-801 B_{max} (binding density, in fmol/mg protein), and **(B)** normalized NR1 immunoblot optical densities (in arbitrary units). *Significant Pearson correlation.

8.4 DISCUSSION

Presynaptic cholinergic deficits are now well established findings in AD. Alterations of choline acetyltransferase (ChAT), acetylcholine levels and other presynaptic cholinergic markers are likely concomitant with basal forebrain neuronal loss as well as *APOE* ε4 allele as we have shown in **Chapter 6** (see also Lai et al. 2006). In turn, these presynaptic deficits may contribute to both the cognitive and behavioral symptoms of AD (Cummings and Kaufer 1996; Lai, et al. 2001; Minger et al., 2000; Perry et al. 1981; Wilcock et al. 1982). In contrast, although postsynaptic perturbations such as reduced muscarinic M_1/G-protein coupling and loss of nicotinic receptor densities have been reported (Flynn, et al. 1991; Warpman et al. 1993), little is known about the clinical relevance and neurochemical correlates of these changes. I have recently shown that attenuation of M_1/G-protein coupling is associated with dementia severity in AD (see **Chapter 7** in this thesis, Tsang et al. 2005). Using postmortem material from a separate, well characterized cohort of patients, it is found that the extent of loss of M_1/G-protein coupling is correlated with reductions of PKC activity as well as NMDA receptor density in AD frontal cortex. These correlations are not confounded by a number of demographic and disease factors under study. Therefore, the data link postsynaptic cholinergic dysfunction with two other substrates (PKC and NMDA receptor) known to play essential roles in cognitive processes (Milner et al. 1998; Sun and Alkon 2005) and provide further insight into the mechanisms by which impairment of M_1 mediated signaling may underlie the cognitive decline of AD.

The PKC family of isozymes are serine / threonine kinases which are critical signaling molecules in most cell types, serving to integrate a multitude of extracellular signals with downstream signaling events concerned with gene transcription, protein processing, synaptic plasticity, cell survival and other physiological processes (Newton 1995; Olariu et al. 2005; Tanaka and Nishizuka 1994). In neurons, these extracellular signals may be mediated by neurotransmitters like acetylcholine which acts on $G\alpha_{q/11}$-coupled receptors such as M_1 (Caulfield 1993). Interestingly, Etheberrigaray et al. (2004) showed that treatment with bryostatin 1, a PKC activator devoid of tumour promoting activity, improved maze performance in AD transgenic mice. In postmortem AD cortex, reductions of PKC level and activity have been reported (Cole et al. 1988; Wang et al. 1994), and the current data now suggest that this reduction may not simply reflect neuronal loss, but may be related to M_1 receptor dysfunction. However, the molecular mechanisms underlying down-regulation or deactivation of PKC in AD is still unclear. Inactive PKC is normally localized in the cytosol. Upon activation by diacylglycerol, Ca^{2+} and other signals released by the action of PLC (which is in turn activated by $G\alpha_{q/11}$), PKC translocates to the plasma or nuclear membrane via anchoring by RACK (receptor for activated C-kinase) proteins (MacDonald et al. 2001). Interestingly, RACK1 has also been found to be decreased in AD (Battaini et al. 1999). Therefore, more work is needed to clarify whether M_1 dysfunction is directly related to PKC hypoactivity by decreased activation, or by its effect on RACK and consequent perturbation of PKC translocation.

The loss of frontal NMDA receptors is another salient marker of neurodegeneration in AD and of potential significance to the clinical features of the

disease (Francis et al. 1993; Francis 2003). In this study, we measured NMDA receptors by [^{3}H]MK-801 binding as well as immunoblotting of the NR1 subunit. Because [^{3}H]MK-801 binding density (B_{max}) was expressed per unit weight of protein, the observed reduction in B_{max} would likely reflect specific loss of NMDA receptors, rather than generalized cortical atrophy. MK-801 (dizocilpine), a non-competitive NMDA receptor channel blocker with anticonvulsant properties, is known to bind within the channel pore region formed by the NR1/NR2A complex (Lynch et al. 1994; MacDonald et al. 2001). Therefore, specific [^{3}H]MK-801 binding can be considered to indicate functional NMDA receptor labeling. Nevertheless, since NMDA channel opening (hence, [^{3}H]MK-801 B_{max}) is also influenced by the concentrations of glutamate as well as regulators like glycine and polyamines (Mitchell and Anderson 1998), saturating concentrations of these compounds were added in the binding assays to maximize channel opening, and further measured levels of the obligatory NR1 subunits of NMDA receptor by immunoblotting. The two NMDA receptor indices were strongly correlated ($r = 0.58$, Pearson's $p = 0.001$) and showed a similar degree of reduction in AD (see **Table 8.1 and Figure 8.1B**), which suggest that overlapping pools of NMDA receptors were measured. However, although MK-801 binds with highest affinity to NR1/NR2A (Laurie and Seeburg 1994), which is the predominant NMDA receptor subtype in adult neocortex (Stephenson 2001), it is unclear whether reduced [^{3}H]MK-801 binding represents the loss of specific NMDA receptor populations in AD. Importantly, attenuation of M_1/G-protein coupling significantly correlated with both NMDA receptor indices, suggesting that impairment of M_1 mediated signaling is related to reduced levels of functional NMDA receptors.

What is the putative molecular mechanism linking M_1 signaling dysfunction with NMDA receptor alterations? The colocalization of M_1 with NR1 indicate a spatial relationship allowing physiological interactions between the two receptors (Marino et al. 1998), and preclinical studies have shown that activation of $G\alpha_{q/11}$-coupled receptors such as M_1 potentiates NMDA receptor currents, possibly via PKC dependent pathways (Lu et al. 1999; MacDonald et al. 2001; Marino et al. 1998). PKC is known to directly phosporylate regulatory regions of NR1 subunits and control trafficking and surface delivery of functional NMDA receptors (Lan et al. 2001; MacDonald et al. 2001; Scott et al. 2003; Tingley et al. 1997). Therefore, it is possible that impaired PKC activity associated with M_1/G-protein uncoupling underlie NMDA receptor deficits in AD, either by decreased delivery of subunits to the surface, or increased rate of endocytosis and degradative sorting. Alternatively, the NR1 gene promoter is induced by transcription factors such as Sp1 (Krainc et al. 1998; Liu et al. 2001; Okamoto et al. 2002) which is in turn regulated by a variety of signaling molecules including PKC (Chu and Ferro 2005). Therefore, the potential effects of M_1 mediated signaling on NMDA receptor gene expression should also be further studied. In contrast to PKC, activity of Src kinase, a non-receptor tyrosine kinase and another regulator of NMDA receptor function (Lu et al. 1999), was not altered in AD and did not correlate with M_1/G-protein coupling or NMDA receptor levels. One other study on postmortem kinase activities in AD also reported no change in Src (Vener et al. 1993). Therefore, Src activity may be resistant to AD changes, or compensatory mechanisms may exist. However, it should be noted that both PKC and Src kinase families include several closely-related enzymes with similar activities, and the enzymatic assays employed in this study may not be sensitive enough

to detect changes of specific kinase subtypes. Furthermore, although an effect of M_1/G-protein uncoupling on PKC and NMDA receptor alterations is biologically plausible, (Chu and Ferro 2005; Lu et al. 1999; MacDonald et al. 2001; Marino et al. 1998) the present correlational study is unable to verify a causal relationship between these neurochemical variables; other disease factors may also play a role. Indeed, the negative correlation of NR1 density with Braak stage (see **Results**) indicate that parenchymal neurodegenerative changes are at least partially responsible for NMDA receptor deficits, although stepwise regression analyses indicated a stronger correlation with M_1/G-protein uncoupling.

In conclusion, there is ample evidence from preclinical studies for physiological interactions among M_1 receptors, NMDA receptors and signaling molecules such as PKC and Src kinase in the regulation of cognitive processes, but the significance of these findings to AD is unclear. We now show that the extent of postsynaptic M_1 receptor uncoupling from G-proteins correlated with loss of PKC activity and NMDA receptor density in the postmortem frontal cortex of AD, suggesting that impaired M_1-mediated signaling may underlie cognitive deficits via effects on PKC and NMDA receptor function. This study provides a neurochemical basis for the limited success of AD therapies which increase synaptic acetylcholine availability or target M_1 receptors; instead, the data point to targets downstream of M_1/G-protein activation, and predict improved efficacy for compounds which regulate PKC (Etcheberrigaray et al. 2004) or NMDA receptors (Frankiewicz and Parsons 1999). However, more work is needed to provide molecular delineation of the specific kinase and receptor subtypes involved in disease processes of AD.

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CHAPTER 9

Neurochemical Alternations in Anxious Alzheimer's Disease Patients*

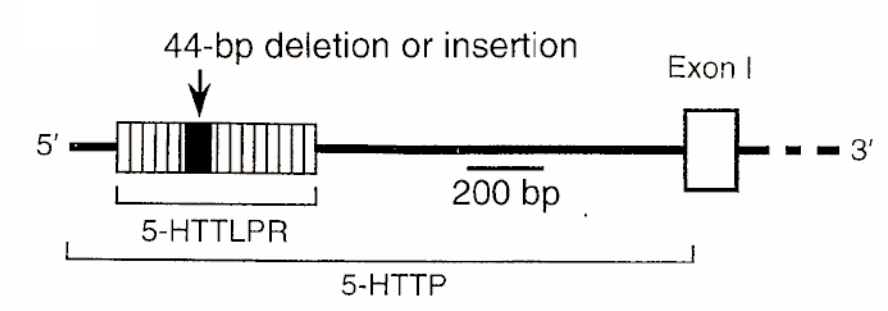
9.1 INTRODUCTION

Besides progressive cognitive impairment, Alzheimer's disease (AD) is also characterized clinically by the presence of neuropsychiatric behaviors such as depression, anxiety, and psychosis. Of these behavioral changes, anxiety is relatively prevalent, occurring in up to 50% of AD patients over the course of the disease (Hope et al. 1999; Mega et al. 1996). Neuropsychiatric behaviors like anxiety are clinically significant in AD in that they often lead to considerable caregiver distress and precipitate

* Data presented in this chapter has been published: Tsang SW, Lai MK, Francis PT, Wong PT et al. Serotonin transporters are preserved in the neocortex of anxious Alzheimer's disease patients. *NeuroReport* (2003) **14(10)**: 1297-1300.

institutionalization of the patient (Chen et al. 1996; Kaufer et al. 1998; Steele et al. 1990) However, the neurochemical basis of these behavioral changes is not well-studied. Because the serotonergic system is affected in AD, with findings of losses of serotonergic raphe neurons, serotonin (5-HT) levels, and cortical 5-HT transporters (5-HTT, Aletrino et al. 1992; Chen et al. 1996; Chen et al. 2000) which facilitate the uptake of serotonin, and because selective 5-HT re-uptake inhibitors (SSRIs) are effective in the treatment of anxiety disorders, I queried whether alterations of neocortical 5-HTT binding may underlie anxiety in AD. Furthermore, Heils et al.(1996) have discovered a 44-base pair insertion/deletion variant within the GC-rich repetitive sequence in the 5' promoter region of the 5-HTT gene (5-HTTLPR) **(Figure 9.1).** Functional studies have demonstrated that the basal transcriptional activity of the deleted or 'short' (S) variant is about 2.5 to 3 times lower than that of the inserted, or 'long' (L) variant, and furthermore, is associated with anxiety traits (Heils et al. 1996; Lesch et al. 1996). Therefore, I examined whether the 5-HTTLPR whose long (L) and short (S) variants are known to result in increased and decreased expression of the 5-HTT protein, respectively, may be associated with anxiety symptoms in AD.

Figure 9.1 Map of the 5-HTT gene promoter. The 5-HTTLPR comprises of a repetitive sequence with a 44-bp insertion / deletion as indicated by the black box (Lesch et al. 1996).



9.2 MATERIALS AND METHODS

9.2.1 Subjects Recruitment and Behavioral Assessment

The 34 AD subjects which comprised this study has been described in detail in **Chapter 5 (Section 5.1.1)**. Briefly, behavioral changes of the subjects were assessed every four months from recruitment to death (for a mean of 2.6 years) with the Present Behavioural Examination (PBE) (Hope and Fairburn 1992). Anxiety was assessed by behaviors and physical signs indicating inappropriate anxiety or fear. Rating for anxiety was on a 7-point score (0-6) based on frequency of occurrence reported by the caregiver. A subject was considered to have had significant anxiety if there were at least two ratings > 3, or one rating > 3 and two other ratings 1-3 over the course of the study (Chen et al. 1996). At death, informed consent was obtained from the families of patients and control participants for the removal of brain. Selection of AD subjects was based only on tissue availability and neuropathological confirmation of diagnosis by the CERAD criteria (Mirra et al. 1991). The 14 controls were neurologically normal, had no significant neuropathology and no history of psychiatric disease.

9.2.2 Saturation Binding

At postmortem, blocks of gray matter from orbito-frontal (BA11) and mid-temporal (BA21) gyri were dissected and processed as previously described in **Chapter 5 (Section 5.2.2)**. In addition, semi-quantitative scores for senile plaques (SP, score 0-3) and neurofibrillary tangles (NFT, score 0-4) were obtained from methanamine silver / modified Palmgren stained sections of BA11 and BA21. Saturation binding with [^{3}H]citalopram (Amersham Life Sciences, UK) has previously been described in **Chapter 5 (Section 5.2.2)**. Non-specific binding was determined in the presence of 10μM unlabelled fluvoxamine maleate (Tocris Cookson Ltd, UK).

9.2.3 5HTTLTR Genotyping

For 5-HTTLPR genotyping, DNA was extracted from 20 mg aliquots of cortical tissue using commercially available kits, and analyzed by polymerase chain reaction (PCR) according to previously reported methods (Heils et al. 1996) to generate the L (528-bp) and S (484-bp) fragments.

9.2.4 Statistical Analyses

Statistical analyses were performed with SPSS 10.0 for Windows. Relationships between [^{3}H]citalopram binding variables and potential confounders (demographic or disease factors) were studied with stepwise multiple regression. Binding parameters (K_D and B_{max}) of controls and AD behavioral groups were then compared by repeated measures analysis of variance (ANOVA, with brain area as the repeated measure), followed by post-hoc tests. Comparisons of binding parameters over 5-HTTLPR genotypes in the AD subjects were similarly studied. Analysis of genotype in controls was not attempted because of the small sample size, with only one individual having the LL genotype. Differences in binding parameters were considered significant if the *p* values for both the repeated measures ANOVA (effects of between-subject factor or interaction) and the *post-hoc* tests were < 0.05. Genotype frequencies in the behavioral groups of AD (anxious vs. non-anxious patients) were compared by Fisher's exact test.

9.3 RESULTS

9.3.1 Demographics

The AD subjects and controls were well matched with regards to age, sex, postmortem delay and tissue storage interval, with the exception of lower brain pH in the AD behavioral groups (probably due to prolonged agonal state, Hardy et al. 1985) **(Table 9.1).**

Table 9.1 Comparison of demographic and clinical features between controls and AD behavioral groups.

		Controls (n = 14)	AD - anxiety (n = 22)	AD + anxiety (n = 12)
Age, y		75 (4)	81 (1)	81 (1)
Sex, % male		50	50	58
Postmortem interval, h		47 (7)	38 (6)	49 (8)
Storage interval, mo		90 (3)	91 (3)	91 (3)
pH		6.7 (0.08)[a]	6.3 (0.08)	6.2 (0.11)
Disease duration, y		-	9.6 (0.9)	8.9 (1.5)
MMSE[b]		-	4.2 (1)	7.8 (3)
SP				
	BA11	-	2.8 (0.1)	3.0 (0.01)
	BA21	-	2.8 (0.1)	3.0 (0.01)
NFT				
	BA11	-	2.1 (0.4)	1.5 (0.3)
	BA21	-	3.0 (0.3)	2.3 (0.4)

Table 9.1 Data are mean (S.E.M.). MMSE, Mini-Mental State Examination [24]; SP, senile plaque scores; NFT, neurofibrillary tangle scores.
[a]Significantly different (one-way ANOVA $p = 0.003$) from AD – anxiety (Scheffé multiple means comparison $p = 0.014$) and AD + anxiety (Scheffé $p = 0.008$).
[b]Mean of last five MMSE scores before death used as a measure of dementia severity to avoid floor effects [3].

However, pH did not correlate with any of the binding parameters (stepwise multiple regression, $p > 0.05$) and thus was not entered as a covariate in subsequent analyses. Among the AD subjects, the binding parameters were not correlated with disease factors (disease duration, dementia severity, senile plaque and neurofibrillary tangle counts, stepwise multiple regression, $p > 0.05$), and there were no significant differences between the AD behavioral groups with regards to the disease factors (Student's or Mann-Whitney $p > 0.05$, **Table 9.1**). Because chronic psychotropic medication may affect central serotonergic activity, I also compared the binding parameters between subjects who were taking sedative-hypnotics (n = 8), neuroleptics (n = 10) or tricyclic antidepressants (n = 3) and those not on medication in the eight months before death. I found that of the medications, only sedative-hypnotic use was associated with increased [^{3}H]citalopram B_{max} in BA11 (adjusted $R^2 = 0.09$, $\beta = 0.34$, $p = 0.044$). Therefore, sedative-hypnotic use was entered as a covariate in subsequent analyses of AD subjects.

9.3.2 Saturation Binding Assays

There were no differences in affinity (K_D) of [^{3}H]citalopram binding among controls, non-anxious AD and anxious AD (respective mean values in nM [S.E.M.]: 2.72 [0.4], 3.67 [0.5], 4.04 [0.7] in BA11; and 3.38 [0.4], 3.51 [0.5], 4.91 [1.0] in BA21). In contrast, [^{3}H]citalopram binding density (B_{max}) was reduced in non-anxious AD patients compared to controls, while the B_{max} of anxious AD patients was preserved at around control values. However, this pattern of alteration reached post-hoc statistical significance only in BA21 **(Figure 9.3A).** When binding parameters in the AD patients were compared over the 5-HTTLPR genotypes, the B_{max} values were significantly higher

Figure 9.2A [^{3}H]Citalopram binding to 5HTT in controls and anxiety subgroups of AD.

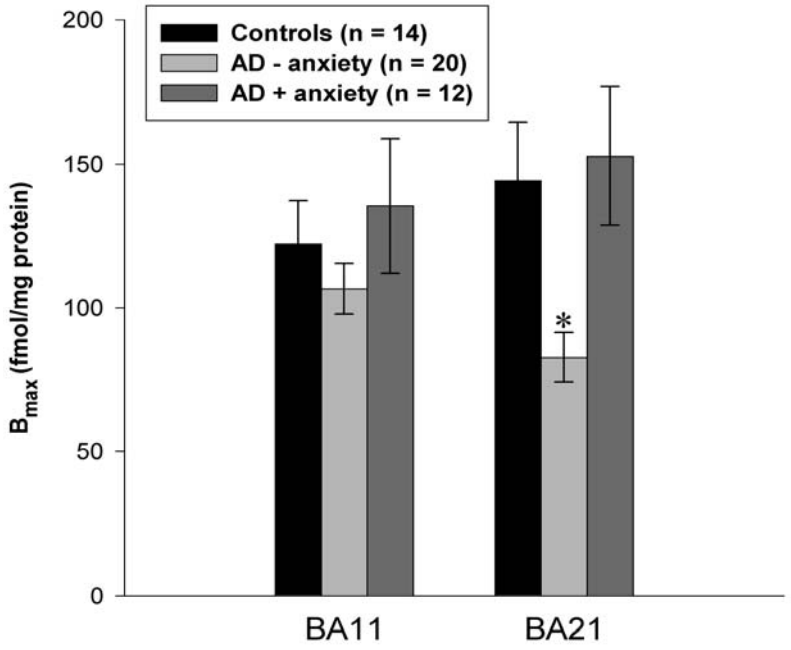


Figure 9.2B The effect of 5HTTLPR genotype on [^{3}H]Citalopram binding densities in AD.

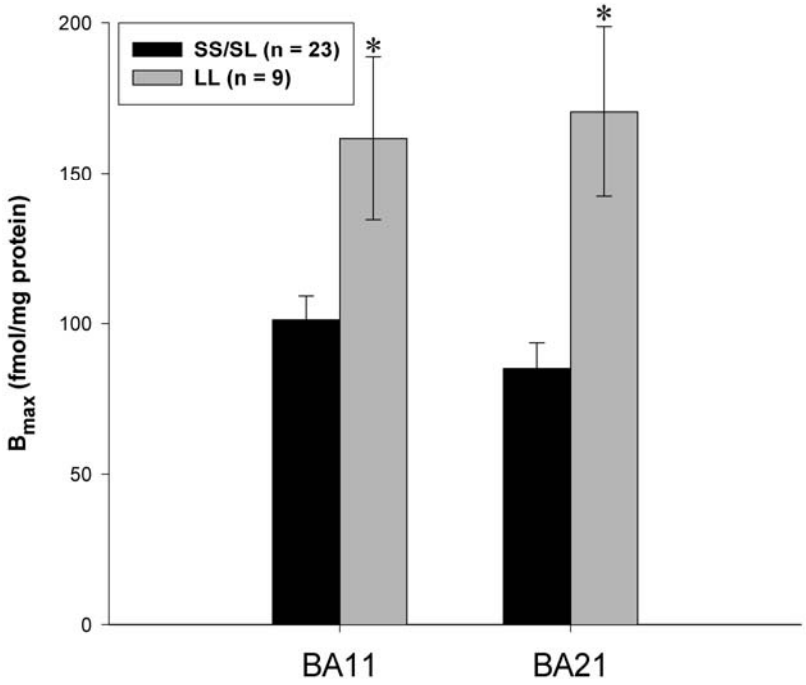


Figure 9.2 (Previous page) (A) Data are mean ± S.E.M. for [^{3}H]Citalopram binding to frontal cortex (BA11) and temporal cortex (BA21) in the controls and the AD behavioral groups with repeated measures ANOVA (2,43 *df*) for the effects of Group, $F = 4.02$, $p = 0.025$ and Interaction between Group and Brain area, $F = 2.58$, $p = 0.088$. Radioligand assays were not performed on two subjects due to shortage of tissue. *Significantly different (one-way ANOVA, $p = 0.006$) from Control (Scheffé $p = 0.033$) and AD + anxiety (Scheffé $p = 0.018$) values in BA21. **(B)** Data are mean ± S.E.M. for [^{3}H]Citalopram binding to frontal cortex (BA11) and temporal cortex (BA21) across genotype in AD subjects with repeated measures ANOVA (2,28 *df*; with sedative-hypnotic use as covariate) for the effect of Genotype, $F = 13.4$, $p = 0.001$ and interaction between Genotype and Brain area, $F = 2.04$, $p = 0.16$. *Significantly different from SS/SL (Student's t test, $p < 0.01$).

in the "LL", compared with "SS or SL", genotypes (with sedative-hypnotic use as covariate, **Figure 9.3B**). K_D was not significantly different between the genotypes (data not shown).

9.3.3 5-HTTLPR Genotype Distribution in AD Group

Finally, I compared 5-HTTLPR genotype distribution between the AD behavioral groups, and found higher proportions of anxious patients with the "LL" genotype versus the "SS or SL" genotypes **(Table 9.2)**, with an odds ratio of 6.25 (mid-p corrected 95% CI 1.14-37.04).

Table 9.2 Anxiety by *5-HTTLPR* genotype in AD. Indicated are number of patients. Fisher's exact $p = 0.031$.

Genotype	***Anxious***	***Non-anxious***
SS/SL	6	19
LL	6	3

9.4 DISCUSSION

At present, at least 14 pre- and postsynaptic 5-HT receptor subtypes are known to function as effectors of 5-HT signaling (Barnes and Sharp 1999). In contrast, 5-HT re-uptake from the synaptic junction is mediated by a single protein, the 5-HTT, which is critical in the regulation of the magnitude and duration of serotonergic activity (Lesch et al. 1996). Support for the involvement of 5-HTT in mood and emotional states comes in part from the efficacy of selective 5-HT reuptake inhibitors (SSRIs) in the treatment of depressive and anxiety disorders. This suggests that serotonergic deficits may form the neurochemical basis of some affective disorders, the symptoms of which can be ameliorated by blocking the reuptake of 5-HT and hence restoring serotonergic activity (Stahl 1998). In addition, a functional biallelic polymorphism of the gene promoter region of 5-HTT (5-HTTLPR) whose long (L) and short (S) variants determine increased or decreased 5-HTT expression, respectively, has been shown to be associated with anxiety-related personality traits (Greenberg et al. 2000; Lesch et al. 1996). These findings led me to examine whether the presence of anxiety in AD is related to 5-HTT levels in the postmortem neocortex; and if so, whether the 5-HTTLPR genotype may be a risk factor for the development of anxiety in AD via its influence on 5-HTT levels.

In agreement with previous reports of presynaptic serotonergic deficits (Aletrino et al. 1992; Chen et al. 1996; Chen et al. 2000), I found reduced densities of 5-HTT in BA21 of non-anxious AD patients and a trend towards reduction in BA11, which may be due in part to the relatively severe neurodegeneration in temporal cortex (Jobst et al. 1994; Wilcock and Esiri 1982;). However, the novel finding in this study is the relative preservation of 5-HTT sites in anxious AD subjects, who were also more likely to have the high activity LL genotype of 5-HTTLPR. I then showed that 5-HTT binding

is significantly higher in patients with the LL versus the SS/SL genotype. The SS and SL genotypes were analyzed as a group because of the phenomenon of "S dominance" (Lesch et al. 1996), whereby the heterozygous (SL) genotype displays 5-HTT activities which are similar to the homozygous (SS) genotype instead of intermediate values between SS and LL. Taken together, the data suggest that preservation of neocortical 5-HTT sites in anxious AD patients may be mediated in part by 5-HTTLPR driven, enhanced expression of 5-HTT on remaining serotonergic neurons. We further postulate that this preservation of 5-HT re-uptake activity concomitant to presynaptic serotonergic deficits (Aletrino et al. 1992; Chen et al. 1996; Chen et al. 2000;) may exacerbate the depletion of 5-HT in the synaptic junction and predispose the patient to developing anxiety symptoms.

This is, as far as I know, the first study which relates 5-HTTLPR with anxiety in AD, and the genetic data should be considered preliminary due to the relatively small sample size, perhaps inevitable in postmortem studies. Therefore, population stratification effects cannot be dismissed and may account for the atypically high odds ratio and a wide 95% CI. Furthermore, the present findings, although analogous to a recent study correlating the LL genotype with aggression in AD (Sukonick et al. 2001), is nevertheless at odds with data from the general population where the S allele has small but statistically significant contributions to anxiety-related personality traits (Greenberg et al 2000; Lesch et al. 1996). One possible reason for the discrepancy may be the presence of extensive cortical neurodegeneration and serotonergic deficits in AD which may lead to altered neurophysiological, neurochemical and behavioral manifestation of 5-HTTLPR compared to non-AD brains. For example, a loss of serotonergic neurons in the raphe nuclei is an established neuropathological feature of AD (Chen et al. 2000), and it

is possible that the presence of the L genotype would exacerbate this serotonergic deficit, leading to the occurrence of behavioral symptoms. Furthermore, a later study (Assal and Cummings 2004) did not find an association between 5-HTTLPR and neuropsychiatric symptoms in AD. One reason could be the different behavioral instrument used (Neuropsychiatric Inventory, NPI, Cummings et al. 1994) which uses different criteria and definitions for the behaviors. For example, the NPI does not have "anxiety" as a behavior but rather uses "agitation", which may lead to differences in the measurement of observable behavior. Secondly, unlike my study, Assal et al. (2004) did not use longitudinal measurement of behavior, which may lead to lower sensitivity of measuring intermittent manifestation of psychiatric symptoms.

9.5 CONCLUSION

This study demonstrates that [^{3}H]citalopram binding to 5-HTT is lost in the postmortem neocortex of non-anxious AD subjects, but preserved in subjects who manifested significant premortem anxiety. The preservation of neocortical 5-HTT sites, which may reflect synaptic plasticity mediated in part by genetic factors, is a putative neurochemical substrate of anxiety in AD by exacerbating the deficits in serotonergic neurotransmission. This study therefore provides the rationale for using SSRIs or serotonergic agonists as an alternative to benzodiazepines for the treatment of anxiety related symptoms in AD.

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SECTION 4

General Conclusions

CHAPTER 10

Concluding Remarks

10.1 SUMMARY OF MAJOR FINDINGS

In this thesis, three of the most severely affected neurotransmitter systems in AD, namely, the cholinergic, glutamatergic and serotonergic systems were investigated. Briefly, the muscarinic M_1 receptors, glutamatergic NMDA receptors, and serotonergic 5-HT transporters (5-HTT) were measured in the postmortem frontal and temporal cortices of two cohorts of well-characterized AD patients as well as controls **(Chapter 5).** Neurochemical findings were then correlated with the rate of cognitive decline as well as behavioral changes to test the hypothesis that neurochemical alternations may underlie both cognitive decline and behavioral changes in AD **(Chapter 7 and 9).** Moreover, the status of M_1 receptor coupling to G-protein in AD was measured and correlated with cognitive decline as well as with measurements of ChAT, PKC and Src kinase activities in order to investigate the possible interactions between M_1 receptor mediated signaling and NMDA receptor status **(Chapter 8).** Besides, the effects of two functional gene

Table 10.1 Summary of major findings.

	Status in AD[2]	Behavioral Correlates[3]
Receptors[1]		
1. M_1	M_1 receptors densities in frontal and temporal cortex were not altered.	
	Reduction of frontal cortical M_1/G-protein coupling only in severely demented AD patients.	Reduction of M_1 receptor coupling to G-protein was correlated with dementia severity.
	Loss of M_1/G-protein coupling was correlated with reductions in PKC activity and NMDA density, but not Src activity.	
2. NMDA	Reduction of NMDA receptors in the frontal cortex measured with [^{3}H]MK-801 binding as well as with immunoblotting.	
3. 5-HTT		Receptor density was reduced in temporal cortex of non-anxious AD but preserved in AD patients with anxiety.
Genetics		
1. ApoE ε4 allele	ε4 allele was dose-dependently correlated with higher losses of ChAT activities, but not with various other cholinergic markers.[4]	
	Double dosage of ε4 alleles was associated with more Aβ containing. senile plaques in the temporal cortex compared with 0/1 ε4 allele.	
2. 5HTTLPR	Significantly higher prevalence of LL genotype of the 5-HTTLPR functional polymorphism found in anxious AD patients.	LL genotype of the 5-HTTLPR functional polymorphism correlated with higher 5-HTT densities as well as with anxiety.
Enzyme activities		
1. PKC	PKC activity in neocortex was markedly reduced and correlated with extent of M_1/G-protein uncoupling as well as with NMDA receptor loss	
2. Src kinase	Src kinase activity in neocortex was not altered	

[1]The 5-HT transporter (re-uptake site) is termed a receptor for the purpose of this discussion.
[2]Receptor binding status of AD versus controls.
[3]Receptor binding status between behavioral groups of AD subjects.
[4]Includes neurochemical measurements performed by collaborators, see **Chapter 6** for details.

polymorphisms, i.e., *APOE* ε4 allele and LL genotype of the promoter region of 5-HTT, on the cholinergic and serotonergic systems, respectively, were examined in **Chapter 6 and 9** (refer to **Table 10.1** for a detailed summary of major findings arising from this thesis). In the sections below, I will list some of the limitations of the study approach, followed by measures taken to address the shortcomings or measures taken for quality control of the data. Lastly, I will discuss the insights into the neurochemistry of AD obtained, as well as suggestions for further studies.

10.2 LIMITATIONS

Postmortem artefacts. By using postmortem tissues to study the neurochemical changes in AD, we are limited to studying a single time point at a late stage of the disease process. Therefore, most, if not all, of the data are applicable only to severe AD. Interpretations in postmortem findings are also confounded by differences across studies in subject selection, postmortem delay, agonal state of the patients, tissue preparation, and exposure to psychotropic medications. To address these issues, I have tried to obtain tissues from comparable controls which closely match the AD subjects in demographic details, and also studied the effects of medication and other potential confounders on neurochemical variables statistically using correlational or comparative tests; and, where significant effects exist, I have included the confounders as covariants in subsequent regression analyses.

Cohort effects. All work from this thesis were based on either 20 – 25 patients from Oxford **(Chapter 6, 7, and 9)** or 22 patients from UCLA **(Chapter 8)**. Data was not available on all subjects for every receptor due to tissue shortage. However, using

similar cohorts of subjects to perform various radioligand receptor binding assays may lead to cohort effects which limit the generalizability of the results for the population. On the other hand, performing a range of neurochemical measurements on the same patients reduces variability from study to study.

10.3 QUALITY CONTROL

Anatomical consistency. Due to the established involvement of neocortex in behavior/cognition, we sampled two discrete regions from frontal cortex (BA11) and temporal cortex (BA21) to maintain anatomical consistence in sampling of patients.

Neuropathological consistency. Patients with evidence of neurodegenerative lesions suggestive of mixed diseases (e.g., Lewy bodies) or extensive neurovascular disease were excluded from the studies.

Sample selection and blinding. All available samples were used at the time of study, and there was no selection of subjects based on dementia severity, Braak stage, or presence of neuropsychiatric behaviors. Neurochemical measurements were performed blind to clinical information in order to minimize any potential bias.

10.4 INSIGHTS

Neurochemical alternations in AD are not a simple consequence of neurodegeneration but are summative expressions of complex, interacting processes involving neurodegeneration and synaptic plasticity. As can be seen from the preceding chapters, neurochemical changes are not universal, with subsets of AD patients displaying particular patterns of neurochemical changes which may in turn predispose them to increased risk of faster or more severe cognitive decline, or the development of

specific neuropsychiatric behaviors. Genetic variability adds another layer of complexity to these processes by interacting with neurochemical changes and further modifying synaptic plasticity.

One example that illustrates the above is the finding that the well established link between *APOE* ε4 and AD may be mediated by the allele's specific effects on presynaptic cholinergic markers such as ChAT activities, which may in turn exacerbate AD progression or cognitive impairment **(Chapter 6).** Furthermore, we showed the L* form of 5HTTLPR may predispose AD patients to manifest anxiety symptoms by upregulating 5-HTT levels **(Chapter 9).** Therefore, our studies provide putative mechanisms whereby genetic factors may modify AD progression and clinical features.

This thesis has also advanced a deeper understanding of the pathogenic mechanism of well established features in AD. For example, the loss of cholinergic neurons and associated deficits in presynaptic cholinergic transmission (e.g. reduction of both ChAT activities and M_2 receptor densities in the neocortex) are known since the 1970s and provided a basis for the "cholinergic hypothesis" of AD and subsequent development of cholinergic replacement therapies. In addition, findings of relatively preserved postsynaptic cholinergic markers (e.g., unchanged M_1 binding) led to therapeutic approaches which aimed to activate postsynaptic cholinergic signaling (e.g., M_1 agonists). However, as detailed in **Chapter 7**, these approaches have met with limited success, and Flynn et al. (1991) provided the first potential explanation by finding that M1 receptors, although not lost, were in fact uncoupled from their G-proteins. This suggested that deficits in postsynaptic M_1-mediated signaling may underlie the limited efficacy of cholinergic replacement therapies. Here, I have further studied M_1/G-protein

uncoupling in AD, and have, for the first time, provided a clinical relevance to this finding by showing that the extent of uncoupling correlated with dementia severity **(Chapter 7).** I have further shown that M_1/G-protein uncoupling correlated with loss of PKC activities as well as reductions of NMDA receptors. These findings provided a potential mechanism whereby M_1/G-protein uncoupling in AD interrupts its downstream signaling molecules such as PKC which in turn regulates NMDA receptor function **(Chapter 8).** In this regard, it is of interest to note that preclinical studies using AD rodent models have provided complementary data to my postmortem findings. For example, Potter et al. (1999) has shown that cholinergic immunolesioning, which models the presyanptic cholinergic deficit seen in AD, led to M_1 receptor uncoupling to G-proteins and reduces M_1 receptor function, confirming the close association between pre- and postsynaptic cholinergic neurotransmission. The data also suggest that alternate therapeutic targets which ameliorate or bypass M_1/G-protein uncoupling may show improved efficacy for AD, the proof of concept of which has been provided by Etcheberrigaray et al. (2004) using a non-tumorigenic PKC activator on AD animal models.

10.5 FURTHER STUDIES

Due to the limitations of postmortem studies, our findings should be validated by *in vivo* neuroimaging studies e.g., positron emission tomography (PET) studies employing [^{11}C] or [^{18}F] radioligands. Much progress has been made in the development of radioligands of sufficient specificity for individual receptor subtypes, which would render such approaches increasingly feasible in the near future. Moreover, cell-based *in*

vitro and animal studies will be useful for elucidating the molecular mechanisms linking muscarinic receptor signaling to NMDA receptor function and cognition. For example, M_1/G-protein uncoupling may be investigated by treating cultured neurons or cells expressing both muscarinic and NMDA receptors with uncouplers (e.g., GppNHp), and then investigating the effects of muscarinc agonists on NMDA-induced Ca^{2+} conductance. Furthermore, experimental cholinergic denervation could be performed on rodents (e.g., by intraventricular administration of 192-IgG conjugated with saporin neurotoxin) with subsequent measurements of neurochemical markers and learning (using water-mazes).

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SECTION 5

Appendices

Appendix I

Published Papers Arising from Thesis Work

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Selective effects of the *APOE* ε4 allele on presynaptic cholinergic markers in the neocortex of Alzheimer's disease

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The effects of the *APOE* ε4 allele on a range of pre- and postsynaptic cholinergic markers were studied in a cohort of community-based Alzheimer's disease (AD) patients. Compared with age-matched controls, the postmortem AD neocortex showed decreased choline acetyltransferase (ChAT) and acetyl cholinesterase activities, lower muscarinic M2, and nicotinic α4β2 receptor densities, as well as reduced M1 receptor coupling to G-proteins. However, the ε4 allele was dose-dependently correlated only with higher losses of ChAT activities. AD patients with two ε4 alleles also had more β-amyloid containing senile plaques in the temporal cortex compared to patients with 0/1 ε4. This study suggests that *APOE* ε4 selectively affects presynaptic cholinergic function which may contribute to the clinical and neuropathological features of AD.

Keywords: Alzheimer's disease; *APOE*; Choline acetyltransferase; Acetyl cholinesterase; Muscarinic receptors; Nicotinic receptors

Introduction

Alzheimer's disease (AD), the most common cause of dementia in the elderly, is characterized neuropathologically by extracellular β-amyloid (Aβ)-containing senile plaques (SP), intracellular neurofibrillary tangles (NFT) consisting of hyperphosphorylated forms of microtubule associated protein τ, and neuronal degeneration. Although degeneration in the source nuclei of several neurotransmitter systems has been found in AD, one of the earliest and most consistently affected is the basal forebrain cholinergic neuron (Davies and Maloney, 1976; Whitehouse et al., 1982). Accompanying the loss of cholinergic neurons are neurochemical alterations including reduced choline acetyltransferase (ChAT) and acetylcholinesterase (AChE) activities, reduced acetylcholine release, as well as losses of muscarinic M2 and nicotinic receptors in the neocortex and hippocampus (Francis et al., 1985; Mash et al., 1985; Perry et al., 1977; Perry et al., 1987; Sims et al., 1983; Wilcock et al., 1982). Postsynaptic M1 receptors appeared to be preserved in AD (Mash et al., 1985) but was subsequently shown to be defective in coupling to G-proteins (Flynn et al., 1991; Ladner et al., 1995). Other studies also reported deficits in postsynaptic signaling such as decreases in phosphoinositide hydrolysis and protein kinase C (PKC) activities (Cole et al., 1988; Ferrari-DiLeo and Flynn, 1993; Jope et al., 1997) which are activated by M1 and M3 receptors (Caulfield, 1993). Taken together, these studies suggest a state of profound presynaptic as well as postsynaptic cholinergic dysfunction in AD that may underlie both the cognitive and behavioral symptoms of AD (Cummings and Kaufer, 1996; Lai et al., 2001; Minger et al., 2000; Perry et al., 1978; Tsang et al., in press; Wilcock et al., 1982).

Various gene mutations have been linked to familial forms of AD, such as those encoding amyloid precursor protein (APP), presenilin 1 and presenilin 2, which result in altered APP processing and increased production of plaque-forming Aβ species (Selkoe, 2001). For sporadic AD which constitutes the majority of cases, the major genetic risk factor is *APOE* which encodes apolipoprotein E (apoE), a 34 kDa lipid carrier protein. Of the three *APOE* alleles (ε2,

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ε3, and ε4 encoding apoE2, apoE3, and apoE4 isoforms, respectively), the ε4 is dose-dependently associated with increased risk of developing AD compared with ε3 and ε2 (Corder et al., 1993; Poirier et al., 1993; Saunders et al., 1993). Much research effort has been expanded into elucidating the pathogenic mechanisms of E4 in AD. In addition to its role as a key regulator of plasma lipid levels, apoE may be involved in neuronal repair and plasticity in the CNS, with the E4 being less effective than the E3 isoform (Mahley and Huang, 1999; White et al., 2001). E4 has also been shown to facilitate Aβ deposition and τ hyperphosphorylation more potently than E2 and E3 in vitro (Holtzman et al., 2000; Tesseur et al., 2000). Additionally, AD patients with one or two ε4 showed higher plaque and, to a lesser extent, higher tangle burden than non-ε4 carriers (Nagy et al., 1995; Olichney et al., 1996; Schmechel et al., 1993). Finally, apoE may affect cholinergic neurons and signaling in an isoform-specific manner. For example, some studies demonstrated that ε4 positive AD patients had more extensive deficits in ChAT activities compared with non-ε4 carriers (Poirier et al., 1995; Soininen et al., 1995; Beffert and Poirier, 1996) while others did not (Corey-Bloom et al., 2000; Svensson et al., 1997; Tiraboschi et al., 2004). The effect of *APOE* genotype on other cholinergic markers is less clear. M1, M2, and nicotinic receptors do not seem to differ between patients with or without ε4 (Reid et al., 2001; Poirier et al., 1995; Svensson et al., 1997). However, the radioligands used to label nicotinic sites in these studies are not subunit-specific, and it is not known whether *APOE* genotype is correlated with specific losses of nicotinic receptor subpopulations, such as those containing α4β2 subunits (Warpman and Nordberg, 1995). Furthermore, although studies have shown selective impairment of muscarinic receptor-mediated phosphoinositide hydrolysis by the apoE4 isoform (Cedazo-Minguez and Cowburn, 2001), it is not known whether apoE4 affected the coupling of G-proteins to M1 receptors in AD. In this study, we correlated *APOE* genotype in a cohort of AD patients with a range of pre- and postsynaptic cholinergic neurochemical markers in the postmortem frontal and temporal cortex in order to test the hypothesis that deficits or alterations in cholinergic neurotransmission in the AD neocortex are influenced by *APOE* ε4 genotype.

Materials and methods

Patients and neuropathological assessments

A maximum of 40 AD subjects and 20 elderly controls were included in this study. The AD subjects were derived from an autopsied subset of a cohort of community-based dementia patients from Oxfordshire, UK enrolled in a longitudinal study of behavior in dementia (Hope et al., 1999). The inclusion/exclusion criteria as well as point-of-entry characteristics have been previously described in detail (Hope et al., 1997, 1999). Complete drug histories were recorded, and none of the subjects was on cholinomimetic medication. At autopsy, brains were harvested after informed consent had been obtained from the patients' relatives. One hemisphere was processed for neuropathological assessments, while the other was frozen for subsequent neurochemical assays. All subjects fulfilled CERAD criteria for the neuropathological diagnosis of AD (Mirra et al., 1991), and were neuropathologically staged at Braak V/VI (Braak and Braak, 1991). Two patients (both of ε3/ε3 genotype) also had evidence of vascular dementia. Paraffin-embedded blocks of frontal as well as temporal cortex were then stained with methenamine silver and a modified Palmgren stain for a blinded, semi-quantitative scoring of SP (0–3 points) and NFT (0–4 points), as previously described (Lai et al., 2001). Controls did not have dementia or other neurological diseases, did not meet CERAD criteria for AD diagnosis, and were staged at Braak 0-II. The selection of AD subjects as well as controls for the current study was based on postmortem-tissue availability; and not on *APOE* genotype. Data were not available for all neurochemical measures in all participants, and some neurochemical data for a subset of subjects have been previously reported (Garcia-Alloza et al., 2005; Lai et al., 2001; Minger et al., 2000; Tsang et al., in press).

Tissue processing

Tissue processing and neurochemical assays were performed blind to clinical information. All chemicals and reagents were from Sigma-Aldrich Ltd. (St Louis, MO) unless otherwise stated. Frozen brains were thawed on ice and blocks of gray matter (0.5–1.0 g) from the frontal (Brodmann Area, BA 9–11) and temporal (BA 20, 21) cortices were dissected free of meninges before homogenization in either of the following buffers for subsequent neurochemical assays: 10 mM/75 mM phosphate buffer (pH 7.4, ChAT/AChE assays) or 50 mM Tris–HCl buffer (pH 7.4, receptor binding assays). Each neurochemical measure was measured only in one frontal (BA 9, 10, or 11) and/or one temporal (BA 20 or 21) area. Brain pH as an indication of agonal status was determined as previously described (Hardy et al., 1985).

APOE genotyping

APOE genotyping was determined with DNA extracted from brain tissues according to previously described methods (Nicoll et al., 1997).

Choline acetyltransferase (ChAT) and acetylcholinesterase (AChE) determinations

Radiochemical determination of ChAT activity was based on minor modifications of the method by Fonnum (1975) as previously reported (Minger et al., 2000). AChE assay using a colorimetric method (Wang et al., 1999) was modified as previously reported (Garcia-Alloza et al., 2005).

Receptor binding assays

Saturation binding assays with [^{3}H]pirenzepine (PZ) for M1 sites, [^{3}H]AF-DX 384 for M2 sites, and [^{3}H]epibatidine for α4β2 subunit-containing nicotinic receptors have been previously described (Gnadisch et al., 1999; Lai et al., 2001). Briefly, seven or eight concentrations (0.5–15 nM) of [^{3}H]PZ (sp. act. 70–80 Ci/mmol) or [^{3}H]AF-DX 384 (sp. act. 120–133 Ci/mmol, both from Perkin Elmer Life Sciences, Boston, MA) were added to 100 μl aliquots of washed brain membrane homogenates in triplicates and incubated in a total volume of 0.5 ml sodium phosphate buffer (pH 7.4) for 60 min at 25°C. Nonspecific binding was measured by the addition of 1 μM atropine sulfate. Incubation was terminated by vacuum filtration and washing onto polyethylenimine-pretreated Whatman GF/B filters (Whatman plc, UK) with a Skatron cell harvester (Molecular Devices Ltd. USA) before transferring into vials for measurement of bound

radioactivity by liquid scintillation spectrometry using a Wallac beta counter. Assays for [^{3}H]epibatidine (0.0125–2.5 nM, sp. act. 56 Ci/mmol, Perkin Elmer Life Sciences, Boston, MA) binding (4 h at 22°C) in 50 mM Tris–HCl buffer, 20 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, and 1 mM $MgCl_2$ (pH 7.4) were as above, with 250 nM cytosine used to define nonspecific binding. The neurochemical parameters K_D (binding affinity, in nM) and B_{max} (binding density, in fmol/mg protein) were derived from binding data by Scatchard analyses followed by iterative curve-fitting (McPherson, 1985). Binding data consistently fitted with one-site binding with Hill constants around 1.0.

G-protein coupling to M1 receptors

Pharmacological determination of M1 receptor coupling to G-proteins using a [^{3}H]PZ/carbachol competition assay was based on previously reported methods (Tsang et al., in press). Briefly, aliquots of brain membrane homogenates were incubated in duplicate with 3 nM of [^{3}H]PZ and 12–15 concentrations of unlabelled carbachol (10^{-9} to 10^{-2} M) in Tris–HCl buffer (pH 7.4) for 150 min at room temperature. Specific binding was defined in the presence of 10 μM atropine sulfate. Parallel series of competition assays were performed with or without the addition of 0.2 mM guanylyl imidodiphosphate (GppNHp) which uncouples G-proteins from M1 receptors and reduces the affinity of M1 receptors for carbachol. Specific binding data from liquid scintillation spectrometry were plotted against log-transformed values of carbachol concentrations with a nonlinear regression curve-fitting software (Prism 3.0, GraphPad Inc. USA) to derive inhibitory constants for carbachol binding in the absence (K_i) and presence (K_{iG}) of GppNHp using Cheng and Prusoff's (1973) equation. The ratio of K_{iG} to K_i was then used as a measure of the state of M_1/G-protein coupling in control and diseased brains, with lower K_{iG}/K_i indicating less coupling.

Statistical analyses

Data were tested for normality for the selection of parametric or nonparametric tests. Demographic, disease, and neurochemical variables between controls and AD were compared with Student's *t* tests. The effect of potential confounders on neurochemical variables, as well as the inter-correlations among variables, was studied by Pearson's product moment or Spearman's correlation. The distribution of genotype frequencies between AD and controls was analyzed by Chi-square tests, while demographic and disease variables among the *APOE* genotypes in AD were compared by one-way analysis of variance (ANOVA) followed by Dunnett's post hoc tests, as the number of subjects in the groups was not matched and equal variances could not be assumed. All statistical analyses were performed with the SPSS 10.0 for Windows software (SPSS Inc. USA), except for Chi-square tests, which were carried out with StatXact 3 for Windows (Cytel Software Corp. MA).

Results

Demographic, disease, and neurochemical variables in control vs. AD

Table 1 shows that demographic variables were matched between the AD subjects and controls (Student's $P > 0.05$) except for lower pH in AD, possibly an indication of more severe acidosis due to prolonged agonal state (Hardy et al., 1985). However, pH, as well as other demographic variables listed in Table 1, did not correlate with neurochemical variables (Pearson $P > 0.05$). As shown in Table 1, ChAT and AChE activities as well as M2 and α4β2 nicotinic receptor densities are reduced in one or both regions in AD, while M1 densities remained unchanged. Except for higher K_D in [^{3}H]pirenzepine binding in the AD frontal cortex (Lai et al., 2001), there were no differences in receptor binding affinities (Table 1). There was minimal neuropathology in controls, contrasting with significantly higher SP and NFT scores in AD (Table 1). The distribution of *APOE*

Table 1
Demographic, disease, and cholinergic neurochemical variables in control and AD

	AD	Control
Maximum number of cases[a]	40	20
Age at death (year)	81.7 ± 1	77.3 ± 2
Sex (M/F)	20/20	13/7
Postmortem interval (h)	37.7 ± 4	47.7 ± 7
pH	6.35 ± 0.07*	6.62 ± 0.09
ChAT (pmol/mg prot/h)		
Frontal	3.5 ± 0.3 (36)**	6.3 ± 0.7 (16)
Temporal	1.9 ± 0.3 (34)***	6.4 ± 0.3 (19)
AChE (% control)		
Frontal	72 ± 3 (17)***	103 ± 5 (9)
Temporal	70 ± 3 (17)***	105 ± 3 (9)
[^{3}H]PZ K_D (nM)		
Frontal	4.4 ± 0.2 (27)*	3.6 ± 0.2 (12)
Temporal	4.5 ± 0.2 (27)	4.1 ± 0.4 (12)
[^{3}H]PZ B_{max} (fmol/mg prot)		
Frontal	575 ± 13 (27)	601 ± 30 (12)
Temporal	614 ± 22 (27)	594 ± 31 (12)
[^{3}H]AF-DX 384 K_D (nM)		
Frontal	3.7 ± 0.2 (27)	4.2 ± 0.6 (12)
Temporal	4.3 ± 0.2 (27)	4.2 ± 0.3 (12)
[^{3}H]AF-DX 384 B_{max} (fmol/mg prot)		
Frontal	483 ± 21 (27)**	583 ± 30 (12)
Temporal	558 ± 26 (27)	566 ± 42 (12)
[^{3}H]Epibatidine K_D (pM)		
Frontal	45 ± 6 (31)	57 ± 8 (12)
Temporal	91 ± 22 (30)	60 ± 24 (14)
[^{3}H]Epibatidine B_{max} (fmol/mg prot)		
Frontal	50 ± 3 (31)*	65 ± 9 (12)
Temporal	60 ± 5 (30)	80 ± 11 (14)
K_{iG}/K_i		
Frontal	2.4 ± 0.3 (21)*	3.6 ± 0.2 (8)
Temporal	2.1 ± 0.2 (25)**	6.1 ± 0.9 (6)
SP		
Frontal	1.97 ± 0.1 (35)***	0.08 ± 0.08 (12)
Temporal	2.31 ± 0.1 (35)***	0.15 ± 0.1 (13)
NFT		
Frontal	1.74 ± 0.2 (35)***	0.08 ± 0.08 (13)
Temporal	2.26 ± 0.2 (35)***	0.14 ± 0.1 (14)

Data are mean ± SEM. ChAT, choline acetyltransferase; AChE, acetylcholinesterase; K_D, binding affinity constant; B_{max}, binding density; K_{iG}/K_i, measure of M1/G-protein coupling (see text); SP, senile plaque score; NFT, neurofibrillary tangle score.

[a] Not all neurochemical measures were available for all cases. The *n* values available for each neurochemical measure are listed in parentheses.

* Different from control $P < 0.05$.

** Different from control $P < 0.01$.

*** Different from control $P < 0.001$.

Table 2
Distribution of *APOE* genotypes in control and AD

Genotype	AD $n = 40$*	Control $n = 20$
ε2/ε3 or ε3/ε3	15 (38%)	16 (80%)
ε2/ε4 or ε3/ε4	21 (52%)	4 (20%)
ε4/ε4	4 (10%)	0 (0%)

* Significantly different from control, Pearson's chi-square ($\chi^2 = 10.04$) with Fisher's exact test, $P < 0.01$.

alleles between controls and AD also appeared to be different (Table 2); in fact, none of the controls in this cohort had the ε4/ε4 genotype. There was also no ε2/ε2 genotype in either AD or controls.

Association of APOE genotype with demographic, disease, and neurochemical variables in AD

Demographic factors as well as postmortem conditions were not significantly different among AD patients with zero (ε3/ε3 or ε3/ε2), one (ε3/ε4 or ε2/ε4), or two ε4 alleles (Table 3), although there is a trend towards lower estimated age of AD onset with increasing ε4 dose. In contrast, both dementia severity (estimated by the Mini-Mental State Examination score [Folstein et al., 1975] before death) as well as temporal cortical senile plaque (SP) loads were higher in patients with two ε4 alleles compared to those with zero or one ε4, with similar trends for SP in the frontal cortex, as well as NFT scores in both regions (Table 3). Fig. 1 illustrates the neocortical cholinergic variables across *APOE* genotypes in AD. In control subjects, because the neurochemical variables between patients with the ε4 allele and those without were not significantly different (Student's t tests, $P > 0.05$, data not shown), the mean control values were used as a baseline for comparisons with AD *APOE* genotype groups in order to study the effects of ε4 on the neurochemical alterations in AD. Fig. 1 shows that of the neurochemical variables studied, only losses of ChAT activities showed a dose-dependent association with ε4 in both cortices; while AChE activities as well as temporal cortical K_{iG}/K_i values are reduced in AD but are not related to ε4 dose.

Discussion

The ε4 allele of *APOE* is at present the only well-established susceptibility gene for sporadic AD, with risk of disease development at a particular age increasing with ε4 allele dose. The widely replicable nature of these findings has led to intense research efforts toward uncovering the mechanisms by which ApoE4 affect the neurodegenerative process of AD. Here, using a well-characterized cohort of AD patients, we investigated whether inheritance of the ε4 allele was associated with cholinergic alterations using several pre- and postsynaptic neurochemical markers known to be affected in AD, including two markers (nicotinic α4β2 receptors and M1/G-protein uncoupling) which, to our knowledge, have not been previously correlated with *APOE* genotypes.

Association of APOE ε4 with ChAT loss in AD

Although ChAT and AChE activities, muscarinic M2 and nicotinic receptor densities as well as M1 receptor coupling to G-proteins were all found to be reduced in one or both cortical regions (see Table 1), only losses in ChAT activities were associated with ε4 (Fig. 1). In the frontal cortex, ChAT activities were only significantly decreased in AD patients with at least one ε4. In the temporal cortex, although ChAT activities were reduced in all three genotype groups of AD patients when compared with controls, the ChAT values were most severely affected in patients with two ε4 alleles, which were significantly lower than those with zero or one ε4. The particular vulnerability of the temporal cortex to neurodegeneration and cholinergic deficits in AD (Jobst et al., 1994; Wilcock et al., 1982) may explain the lower ChAT values we observed in this region. Importantly, our results suggest that presence of *APOE* ε4 does not directly lead to ChAT deficits, since temporal cortical ChAT was reduced even in AD patients without ε4; instead, ε4 may exacerbate the cholinergic denervation in both neocortical regions as indicated by loss of ChAT activities. These data are in agreement with some studies (Poirier et al., 1995; Soininen et al., 1995; Beffert and Poirier, 1996), while others (Corey-Bloom et al., 2000; Svensson et al., 1997; Tiraboschi et al., 2004) failed to find significant effects of ε4 on ChAT. However, it is worth noting that in two of the negative studies (Corey-Bloom et al., 2000; Tiraboschi et al., 2004) cholinomimetic treatment status of the subjects was not reported. Given that cholinesterase inhibitor treatment in rodents is known to upregulate ChAT expression (Tayebati et al., 2004), the possibility that cholinomimetic treatment may confound the effect of ε4 on ChAT needs to be addressed. In the present study, all subjects were community-based patients who were never on cholinomimetic therapy, thus removing one potential confounder in the interpretations of ChAT data.

Lack of APOE ε4 effects on peri- and postsynaptic cholinergic markers

In contrast to the ChAT results, neocortical AChE activity was reduced in AD but was not affected by *APOE* genotype (Fig. 1). Unlike ChAT, which is expressed exclusively in cholinergic neurons in the brain, AChE is produced in cholinoceptive sites

Table 3
Association of *APOE* ε4 allele with demographic and disease variables in AD

	0 ε4	1 ε4	2 ε4
Age at death (year)	83.7 ± 2	80.6 ± 1	80.5 ± 0.6
Age at onset (year)	75.2 ± 2	71.7 ± 1.5	69.2 ± 3.2
Disease duration (year)	8.5 ± 0.8	8.9 ± 1	11.3 ± 3
Sex (M/F)	7/9	12/9	2/2
Postmortem interval (h)	34.0 ± 3	39.1 ± 7	44.7 ± 16
pH	6.38 ± 0.1	6.33 ± 0.1	6.34 ± 0.2
Predeath MMSE	4.5 ± 2	5.3 ± 2	0.0 ± 0*
SP			
Frontal	2.23 ± 0.2	1.68 ± 0.2	2.67 ± 0.3
Temporal	2.31 ± 0.2	2.21 ± 0.2	3.00 ± 0.0*
NFT			
Frontal	1.92 ± 0.3	1.53 ± 0.3	2.33 ± 0.3
Temporal	2.23 ± 0.3	2.21 ± 0.2	2.67 ± 0.3

Data are mean ± SEM for AD patients with no *APOE* ε4 allele ($n = 13–15$), 1 ε4 allele ($n = 18–21$), or two ε4 alleles ($n = 3–4$). MMSE, mini-mental state examination; SP, senile plaque score; NFT, neurofibrillary tangle score.

* Significant different from the 0 ε4 and from the 1 ε4 groups, one-way ANOVA with post hoc Dunnett's $P < 0.05$.

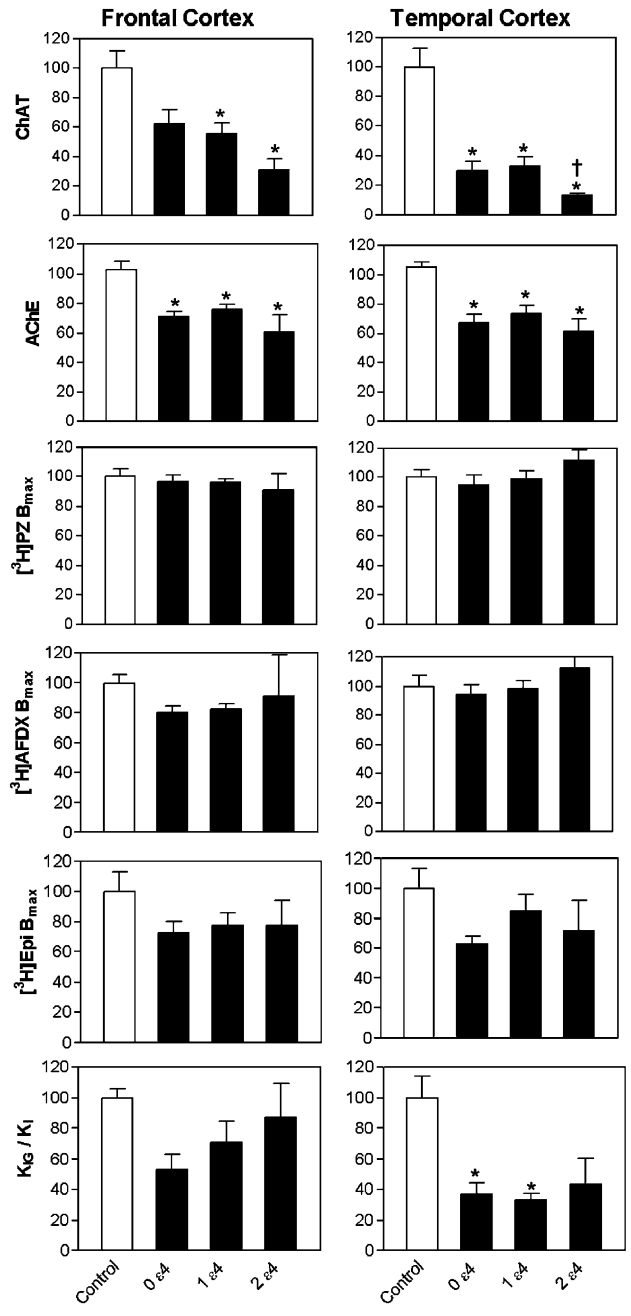

Fig. 1. Effect of *APOE* ε4 allele on cholinergic neurochemical alterations in AD. For each neurochemical measure, data are expressed as mean % control values ± SEM for controls ($n = 6–19$), AD patients with no *APOE* ε4 allele ($n = 6–13$), one ε4 allele ($n = 9–20$), or two ε4 alleles ($n = 2–4$). ChAT, choline acetyltransferase; AChE, acetylcholinesterase; [^{3}H]PZ B_{max}, binding density of [^{3}H]pirenzepine (labels M1 receptors); [^{3}H]AFDX B_{max}, binding density of [^{3}H]AF-DX 384 (labels M2 receptors); [^{3}H]Epi B_{max}, binding density of [^{3}H]epibatidine (labels α4β2 nicotinic receptors); K_{iG}/K_i, measure of M1/G-protein coupling (see text). *Significantly different from controls, one-way ANOVA with post hoc Dunnett's $P < 0.05$. †Significantly different from 0 ε4 and 1 ε4 groups, one-way ANOVA with post hoc Dunnett's $P < 0.05$.

as well as noncholinergic cells and released into the synaptic cleft (Grisaru et al., 1999; Taylor and Brown, 1999). Thus, our data suggest that the reduced AChE in AD is an indication of synapse loss rather than a consequence of *APOE* ε4-related cholinergic denervation. Similarly, the densities of M1 and α4β2 nicotinic receptors, which are predominantly postsynaptic, as well as M2 receptors, which could be localized to both pre- and postsynaptic compartments (Arroyo-Jiménez et al., 1999; Mash et al., 1985), were not significantly different among patients with different ε4 genotypes. Furthermore, although M1/G-protein coupling was reduced in the temporal cortex, the extent of coupling loss again did not differ among the ε4 genotypes. Taken together, our results indicate that *APOE* ε4 may selectively influence pre-, rather than postsynaptic, alterations of cholinergic neurotransmission in AD.

In the frontal cortex, there was a nonsignificant trend towards recovery of K_{iG}/K_i values with increasing ε4 dose. Possible mechanisms for this observation are unknown and need to be further studied. However, one limitation of the current study is the relative small numbers of ε4/ε4 AD patients in the cohort (Table 2), mainly due to the very low abundance of this genotype (1–3% in the general population, Wilson et al., 1996). At present, we have failed to uncover an effect of ε4 on M1/G-protein coupling. Taking into account the report by Cedazo-Minguez and Cowburn (2001) that apoE4 disrupts muscarinic receptor-mediated phosphoinositide hydrolysis in neuroblastoma cells, our M1/G-protein coupling data suggest that either (i) apoE4 targets signaling molecules downstream of G-protein (for e.g., phosphotidylinositol 3-kinase) or (ii) M1/G-protein uncoupling is a postsynaptic response to presynaptic ChAT loss (see Potter et al., 1999), and is not directly affected by *APOE* ε4.

Possible mechanisms of APOE ε4 effects on ChAT loss in AD

It is worthwhile to further study the mechanisms whereby apoE4 may affect cholinergic neuronal function. One possibility is that cholinergic neurons, with their reliance on cholesterol transport as well as phospholipid metabolism for synaptic plasticity and synthesis of acetylcholine in the brain, may be particularly vulnerable to the diminished affinity of apoE4 for receptor-mediated uptake (Poirier, 1994; Poirier et al., 1995). Compared to the other isoforms, apoE4 also demonstrated lower activation of signaling molecules such as extracellular signal regulated kinase 1/2 (ERK1/2) which are involved in neurite outgrowth and synaptic plasticity (Qiu et al., 2004). Interestingly, Dubelaar et al. (2004) have shown that *APOE* ε4 is related to reduced metabolic activity in the nucleus basalis of Meynert (nbM) in both cognitively normal controls as well as prodromal early AD, suggesting that ε4 contribute to AD risk via a state of hypometabolism and reduced adaptive capacity in the nbM, perhaps years before the appearance of cognitive decline. It is possible that reduced ChAT activity would be one marker of nbM hypometabolism or cell loss, leading to cholinergic dysfunction which may further contribute to AD progression or by increasing amyloidogenic processing of precursor protein and Aβ in plaques (Hellstrom-Lindahl, 2000). This postulate is supported by present findings that frontal and temporal ChAT activities were negatively correlated with SP from the same regions in AD (Pearson's $r = -0.376$ [frontal], -0.408 [temporal], $P < 0.05$). In addition, SP scores were also higher in patients with two copies of ε4 compared to those with zero or one ε4 in the temporal cortex, with similar trends in the frontal cortex (Table 3). However, it is also possible that the deleterious effects of *APOE* ε4 on ChAT may be mediated by increased deposition of neurotoxic Aβ species and subsequent neurodegeneration (Holtzman et al., 2000). As this is a postmortem neurochemical study where the majority of the subjects had severe disease at death, further work using animals as well as human

subjects with a wider range of disease severity is needed to clarify the pathogenic role of *APOE* ε4 in AD.

In conclusion, using a cohort of well characterized, community-based AD patients, we show that the *APOE* ε4 allele does not lead to reduced ChAT per se, but is related to higher losses of ChAT activities in the neocortex compared to non-*APOE* ε4 carriers. Furthermore, *APOE* ε4 has limited or no effect on other neurochemical cholinergic markers such as AChE, M1, M2, α4β2 nicotinic receptors, and M1/G-protein uncoupling. These findings suggest that presynaptic cholinergic function is selectively vulnerable to the effects of ε4, and the resultant exacerbation of cholinergic deficits may be related to AD risk via its effect on cognitive processes as well as Aβ deposition and plaque formation.

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Impaired coupling of muscarinic M_1 receptors to G-proteins in the neocortex is associated with severity of dementia in Alzheimer's disease

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Abstract

Impaired transmission of acetylcholine-mediated signaling by postsynaptic muscarinic M_1 receptors has been postulated to underlie the limited efficacy of cholinergic replacement therapies in Alzheimer's disease (AD). However, a clear relationship between the functionality of M_1 receptors and dementia severity has not been demonstrated. The present study aims to measure M_1 coupling to its nucleotide binding (G-) protein in the AD neocortex, and to correlate neurochemical findings with clinical features. A cohort of dementia patients was longitudinally assessed for cognitive decline, with postmortem neuropathological confirmation of AD diagnosis. Measures of M_1 receptor density, M_1/G-protein coupling and choline acetyltransferase (ChAT) activities were performed in the frontal and temporal cortex of 24 AD patients as well as in 12 age-matched controls. We found that M_1 receptor densities were unchanged in AD, which contrasted with significantly reduced M_1 coupling to G-proteins in severely demented AD patients. Loss of M_1/G-protein coupling in the frontal cortex, but not the temporal cortex, also correlated with the rate of cognitive decline. Additionally, correlations between M_1/G-protein coupling and ChAT activities were demonstrated in both regions. These results suggest that defective coupling of neocortical M_1 receptors to G-proteins is a neurochemical substrate of cognitive decline in AD. Based on its associations with ChAT deficits and dementia severity, we propose that M_1/G-protein uncoupling may have a significant role in the disease mechanism of AD and thus may be considered to be a potential therapeutic target.

Keywords: Muscarinic receptors; G-proteins; Alzheimer's disease; Cognition

1. Introduction

Alzheimer's disease (AD) is currently the commonest cause of dementia among those aged 65 years and above, and is characterized neuropathologically by senile plaques, neurofibrillary tangles, and losses of various neurotransmitter-producing neurons. Of the transmitter pathologies seen in AD, cholinergic dysfunction occurs early in the disease process [28] and is thought to underlie much of the characteristic cognitive and neuropsychiatric symptoms [13,22,29,32,37]. Losses of basal forebrain cholinergic neurons, along with reduced neocortical choline acetyltransferase (ChAT) activity, acetylcholine (ACh) release and muscarinic M_2 receptor binding, constitute a state of presynaptic cholinergic deficit in AD [12,24,33,36]. In contrast, the predominantly postsynaptic M_1 receptors were originally thought to be unaltered [24], but subsequent stud-

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ies showed that normal levels of radioligand binding to M_1 receptors contrasted with reduced M_1 immunoreactivity [8]. In addition, the ability of M_1 receptors to form high affinity agonist-binding complexes with guanine nucleotide-binding (G-) proteins was found to be impaired in AD [9], along with reductions in phosphoinositide hydrolysis, phospholipase C (PLC) and protein kinase C (PKC) activities [7,21,25]. Taken together, these findings suggest that M_1 or M_1-like receptors are altered in the AD cortex, resulting in defective coupling to G-proteins and hypoactivation of second messengers. This breakdown in M_1 mediated intracortical signal transduction is hypothesized to underlie the limited efficacy of cholinergic replacement therapies, since the ability of ACh or agonists to ameliorate deficits in cholinergic neurotransmission would have been severely limited [9]. However, it is presently unclear whether M_1/G-protein uncoupling is related to cognitive decline in AD. In this study, we measured M_1 receptor densities and coupling to G-proteins in the post-mortem neocortex of a cohort of longitudinally assessed AD patients, as well as in a group of age-matched controls. We then correlated the M_1 neurochemical parameters with clinical data.

2. Materials and methods

2.1. Patients, clinical and neuropathological data

The present neurochemical study was approved by the Institutional Review Board (IRB) in Singapore. All subjects described below were recruited under studies approved by the IRB in Oxford. At post-mortem, informed consent was obtained from the subjects' relatives prior to the removal of brains. The 24 AD subjects were derived from a cohort of community-based dementia patients from Oxfordshire, UK, who were recruited for a prospective study of behavior in dementia [18]. The entry data, inclusion and exclusion criteria and natural history of these patients have previously been described in detail [18,19,22]. Cognition was assessed every 4 months from study entry till death with the Mini-Mental State Examination (MMSE, scores ranging from 0 to 30 [10]). Dementia severity was measured by using the mean of up to five MMSE scores before death so as to avoid floor effects, since some patients may live for extended periods of time after their MMSE scores had reached 0 [22]. Patients were considered to have severe dementia (AD_{severe}) if the mean predeath MMSE was $\leq$9, while those with scores between 10 and 24 were deemed to have mild to moderate dementia ($AD_{mild/mod.}$). None of the subjects had scores >24. The rate of cognitive decline per year was measured by the linear regression of serial MMSE scores on the time interval between recruitment and death, or when the MMSE first reached 0. Complete drug histories were taken, and none of the subjects received cholinomimetics. At death, diagnosis of AD was confirmed neuropathologically by the CERAD criteria [30]. In addition, all AD patients in this study were in Braak stages V/VI [2].

2.2. Brain membrane homogenates

At death, brains were removed from the AD subjects as well as from 12 controls assessed to be free from neurological diseases. One hemisphere was processed for neuropathological assessment, while the other hemisphere was fresh frozen and stored at −75 °C. To obtain brain membrane homogenates, blocks of thawed tissue from frontal (Brodmann Area 11) and temporal (BA 21) cortex were dissected free of white matter and homogenized in Tris–HCl buffer (pH 7.4) as previously described [22] and stored at −75 °C.

2.3. Neurochemical assays

All chemicals were from Sigma Aldrich (St. Louis, MI) unless otherwise stated. Neurochemical assays were performed blind to clinical information. The state of M_1 receptor coupling to G-proteins was measured according to Flynn et al. [9] with modifications. Briefly, aliquots of brain homogenates were incubated in duplicates with 3 nM of [^{3}H]pirenzepine (spec. act. 70–80 Ci/mmol, Perkin-Elmer Life Sciences, USA) and increasing concentrations of carbachol (10^{-9} to 10^{-2} M) in buffer (20 mM Tris–HCl, 1 mM $MnCl_2$, pH 7.4) for 150 min at room temperature. Specific binding was defined by binding difference in the absence and presence of 10 μM atropine sulphate. Parallel series of competition assays were performed with and without the addition of 0.2 mM guanylyl imidodiphosphate (GppNHp), a non-hydrolyzable analogue of guanosine triphosphate (GTP). At the end of incubation, assays were terminated by vacuum filtration using a Skatron cell harvester (Molecular Devices, USA) onto Whatman GF-B glassfibre filters (Whatman BDS, UK), washed with ice-cold 50 mM sodium phosphate buffer (pH 7.4), dried, punched into tubes with scintillant (Wallac HiSafe 2, Perkin-Elmer Life Sciences, USA) and measured by scintillation spectrophotometry using a Wallac Beta-Counter. The percent specific binding data were plotted against log-transformed values of carbachol concentrations with a non-linear regression curve-fitting software (Prism 3.0, GraphPad Inc. USA), and fifty percent inhibitory concentrations (IC_{50}) values were derived from the binding curves. The IC_{50} values were subsequently used to calculate inhibitory constants for carbachol binding in the absence (K_i) and presence (K_{iG}) of GppNHp using Cheng and Prusoff's equation [4]. The presence of excess GppNHp uncouples G-proteins from M_1 receptors, and the ability of M_1 to form high affinity complexes with agonists like carbachol will be diminished, resulting in a 'right shift' of the carbachol displacement curve (Fig. 2A) and $K_{iG} > K_i$. However, in samples where G-proteins are endogenously relatively uncoupled to M_1 receptors (higher K_i), addition of GppNHp results in a smaller shift (Fig. 2B) and the K_{iG} to K_i ratio (or K_{iG}/K_i) will be relatively low. Therefore, K_{iG}/K_i provide a measure of the state of M_1/G-protein coupling in control and diseased brains.

Measurements of M_1 receptors were performed by saturation radioligand binding (Scatchard) assays with [^{3}H]pirenzepine as previously described [22] to derive binding density (B_{max}) and affinity (K_D) values using an iterative curve-fitting software [27]. Measurements of ChAT activity using a modification of the method of Fonnum [11] had been reported previously [29]. Protein determinations were performed with the Coomassie Blue reagent (Pierce Biotech Inc., USA). Tissues were not available from both regions in some subjects.

2.4. Statistical analyses

Data were first tested for normality to determine the use of parametric or non-parametric tests. Neurochemical and demographic variables among the groups were compared by one-way analysis of variance (ANOVA) with post-hoc Tamhane's tests, since the number of subjects was not evenly matched and equal variances could not be assumed. Disease variables between the AD cognitive groups were compared by Student's *t*-tests. Correlations of neurochemical variables with cognitive data, as well as inter-correlations among the neurochemical variables were performed by Pearson's product moment or Spearman's correlation, as appropriate. Results were considered statistically significant if $p < 0.05$.

3. Results

Table 1 shows that demographic variables were matched among the control, AD with mild/moderate dementia ($AD_{mild/mod.}$), and AD with severe dementia (AD_{severe}), except for brain pH, which was significantly lower in the AD_{severe} group (Table 1). This is likely due to the prolonged agonal state of severely demented patients, resulting in brain acidosis [17]. However, brain pH, as well as the other demographic variables listed on Table 1, did not correlate with K_{iG}/K_i values in either controls or AD subjects (Spearman $p > 0.10$). Among the AD patients, entry and predeath MMSE scores were expectedly lower in the AD_{severe} compared to the $AD_{mild/mod.}$ group. Additionally, the rates of MMSE decline were significantly higher in the AD_{severe} group, while both disease duration (estimated duration between first symptoms and death) and follow-up (duration between study entry and death) were matched in the AD cognitive groups.

For the neurochemical variables, [^{3}H]pirenzepine binding affinity (K_D) and density (B_{max}) were unchanged in both frontal and temporal regions (Fig. 1C and D). This contrasted with significantly reduced temporal cortical K_{iG}/K_i in both AD_{severe} and $AD_{mild/mod.}$ (Fig. 2C). In the frontal cortex, K_{iG}/K_i was significantly reduced only in AD_{severe}, with intermediate values in $AD_{mild/mod.}$ not significantly different from either control or AD_{severe} (Fig. 2C). When the AD subjects were analyzed as a group, K_{iG}/K_i was significantly correlated with rate of MMSE decline in the frontal, but not temporal cortex (Fig. 3). [^{3}H]Pirenzepine K_D and B_{max} did not correlate with MMSE decline in either region. Lastly, Fig. 4A shows that ChAT activities were altered in AD cognitive groups in a pattern similar to K_{iG}/K_i (see Fig. 2C), while Fig. 4B demonstrates that K_{iG}/K_i values of all subjects correlated with ChAT activities in both brain regions.

4. Discussion

Muscarinic M_1 receptors, together with M_3 and M_5 subtypes, belong to the family of $G\alpha_{q/11}$-coupled seven-transmembrane domained receptors which are known to activate PLC and PKC mediated signaling pathways [3]. Postsynaptic M_1 receptors are found in high densities in the neocortex and hippocampus, where they mediate cholinergic neurotransmission in a range of CNS functions including learning and memory [1,16,35]. Furthermore, previous studies [23] have shown that by activating signaling cascades involving PKC and Src kinases, muscarinic receptors can potentiate glutamatergic NMDA receptor function, whose regulation of long-lasting forms of synaptic plasticity is thought to be the major physiological substrate of memory consolidation [26].

Table 1
Demographic and disease variables in controls and cognitive subgroups of AD patients

	Control ($N = 12$)	AD (mild/mod.) ($N = 7$)	AD (severe) ($N = 17$)
Demographic variables			
Age (year)	77 ± 3	81 ± 2	81 ± 2
Sex (M/F)	5/7	5/2	9/8
Postmortem interval (h)	47 ± 9	45 ± 9	35 ± 6
pH	6.48 ± 0.1	6.25 ± 0.09	6.02 ± 0.05*
Disease variables			
Disease duration (year)	–	8.4 ± 0.8	9.0 ± 1.2
Follow-up (year)	–	3.1 ± 0.2	3.4 ± 0.4
MMSE at study entry	–	20.7 ± 1.1	11.3 ± 1.4**
MMSE at predeath	–	13.1 ± 3.0	1.2 ± 0.7**
MMSE decline (year)	–	2.9 ± 0.7	6.1 ± 0.8**

Data are mean ± S.E.M; MMSE = Mini-Mental State Examination.
* Significantly different from control, ANOVA with post-hoc Tamhame's, $p < 0.01$.
** Significantly different from AD (mild/moderate), Student's *t*-tests, $p < 0.0$.

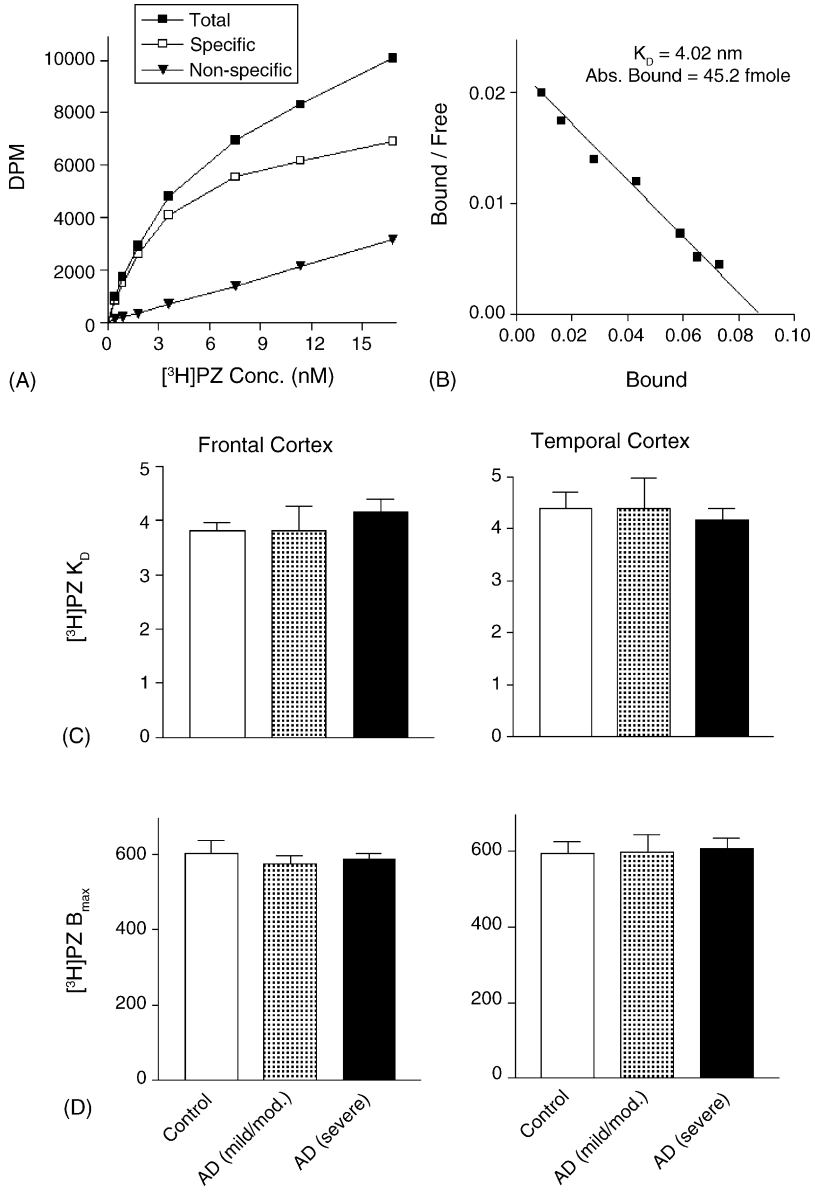

Fig. 1. [^{3}H]Pirenzepine (PZ) binding in postmortem control and AD neocortex. (A) Representative saturation isotherm of [^{3}H]PZ binding in an AD frontal cortex. (B) Scatchard plot of binding data presented in (A) with derived neurochemical parameters. (C and D) Mean ± S.E.M. values of neocortical [^{3}H]PZ binding affinity (K_D, in nM) and M_1 receptor density (B_{max}, in fmol/mg protein) in control and AD cognitive groups.

Although cholinergic dysfunction is a well-known neurochemical feature in AD, investigations of its role in the cognitive features of AD have focused on presynaptic components (e.g., loss of ChAT activities [29,32,37]). While previous studies had reported losses of postsynaptic M_1/G-protein coupling as well as impairment of associated downstream signal transduction [7,9,21,25], it was unclear whether the findings were related to cognitive decline in AD. Using postmortem material from a cohort of longitudinally assessed AD patients, we now show that the extent of reduction in M_1/G-protein coupling is related to the severity of cognitive symptoms in AD. Specifically, we found that compared with controls, temporal cortical coupling was lower in AD patients with mild/moderate, as well as severe dementia, while frontal cortical coupling was significantly reduced only in the subgroup of AD patients with severe dementia. Furthermore, reductions of M_1/G-protein coupling in the frontal cortex (but not the temporal cortex) correlated with rate of cognitive decline. The basis of the observed regional difference is unclear. However, previous studies have demonstrated that cholinergic deficits selectively affect temporal and associated cortices in early stages of the disease [20,37]. It is thus possible that loss of M_1/G-protein coupling in the temporal region occurs early in AD, and is extensive even in patients with mild to moderate symptoms. Therefore, the lack of correlation with cognitive symptoms in this region may simply reflect a dampened effect as the neurochemical deficits reached a stable, low level (a 'floor effect'). This postulate is supported

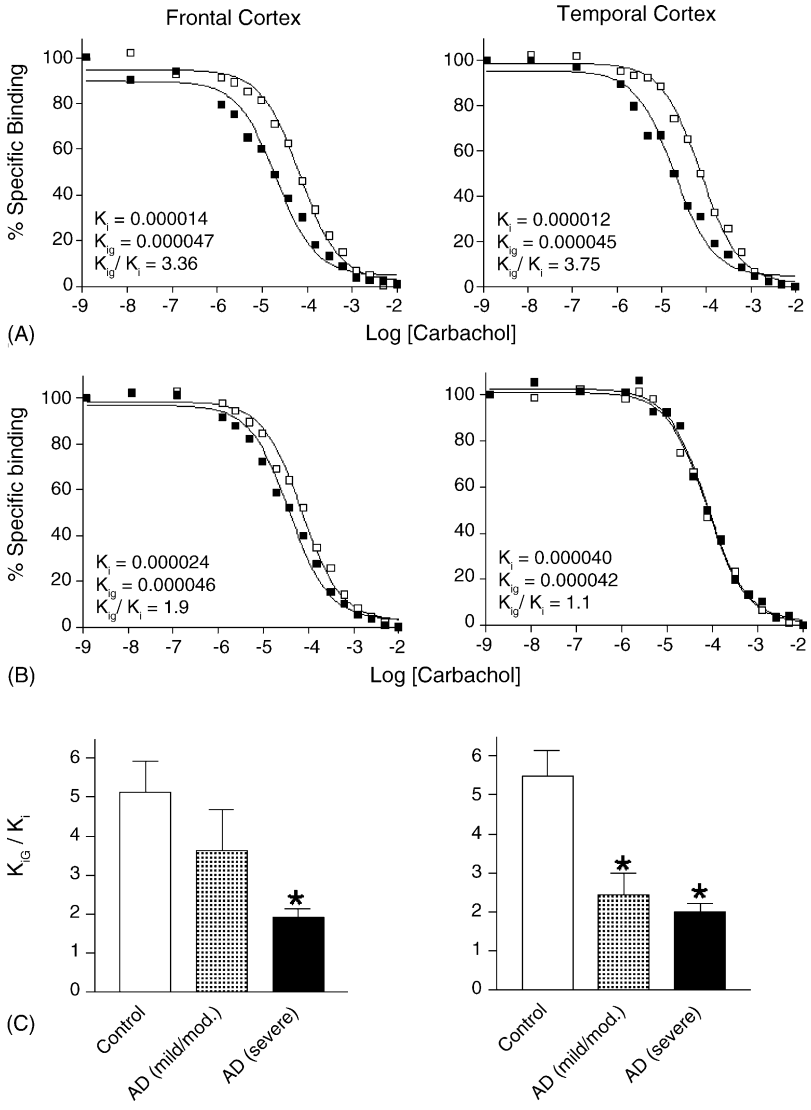

Fig. 2. Carbachol competition for the specific binding of [^{3}H]pirenzepine to M_1 receptors in the neocortex of a randomly selected control (A) and AD patient (B). Best-fit curves were derived from non-linear regression of data. Each data point denotes specific binding in the absence (■) and presence (□) of 0.2 mM GppNHp across log transformed carbachol concentrations. (C) Mean ± S.E.M. values of K_{iG}/K_i in controls and cognitive groups of AD. (*) Significantly different from control, $p \leq 0.01$ one-way ANOVA post-hoc tests.

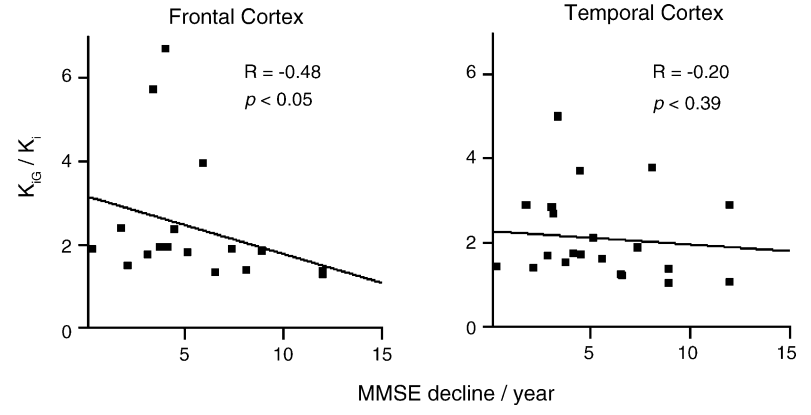

Fig. 3. Correlations of K_{iG}/K_i values with the rate of MMSE decline in AD patients using Spearman's test. Data are available for 18 patients in the frontal cortex and 22 patients in the temporal cortex.

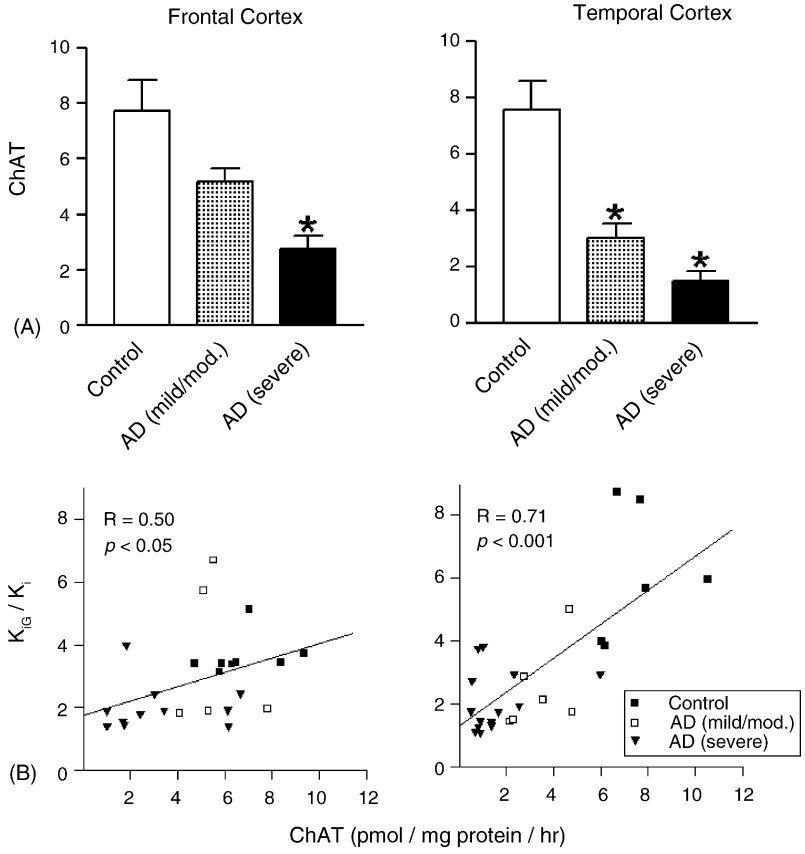

Fig. 4. (A) Mean ± S.E.M. values of choline acetyltransferase (ChAT) activity in control and AD cognitive groups. (B) Correlations of K_{iG}/K_i with ChAT activity in control and AD patients using Spearman's test. ChAT data were available for 8 controls, 5 $AD_{mild/mod.}$ and 11 AD_{severe} patients in the frontal cortex, and for 6 controls, 6 $AD_{mild/mod.}$ and 14 AD_{severe} patients in the temporal cortex. (*) Significantly different from control, $p < 0.01$ one-way ANOVA post-hoc tests.

by our finding that ChAT activities, a marker for cholinergic innervation, was also reduced in both cognitive groups in the temporal cortex; while in the frontal cortex, ChAT reduction was significant only in the severely demented group. However, because the majority of subjects in this longitudinal study were severely demented at the time of death, further studies on patients with a wider range of dementia severity are needed to confirm this postulate. In this regard, it may be of particular interest to investigate the state of M_1/G-protein coupling in subjects who have minimal cognitive impairment (MCI), since a significant proportion of such patients will eventually progress to AD [5,31].

Our findings suggest that impairment of M_1 receptor-mediated signaling in the neocortex via uncoupling with its G-protein may be a neurochemical substrate of cognitive decline in AD. Compared to presynaptic cholinergic deficits, a signaling dysfunction in the postsynaptic M_1 receptors may be temporally more closely related to processes leading to cognitive impairment, and has implications in rational therapeutic strategies. For example, compounds which primarily act at the synaptic (e.g., cholinesterase inhibitors) or receptor (e.g., M_1 agonists) levels will be predicted in light of our findings to be of limited efficacy in ameliorating dementia in AD. Instead, therapeutic compounds acting at the level of M1-activated signaling molecules, such as PKC may show more benefit [6]. However, an important limitation of the current correlational study is the inability to demonstrate a causal relationship between M_1/G-protein uncoupling and cognitive decline (Fig. 3). Since ChAT deficits correlated with M_1/G-protein uncoupling, and alterations of ChAT in cognitive groups of AD mirrored those seen in M_1/G-protein coupling (Fig. 4), it is possible that M_1 uncoupling is a secondary event preceded by presynaptic cholinergic deficits. Indeed, Potter et al. [34] reported that cholinergic deafferentation of the rodent hippocampus by immunolesioning resulted in reductions of M_1-mediated norepinephrine release as well as M_1/G-protein coupling without affecting [^{3}H]pirenzepine binding parameters. Nevertheless, the antecedent or causal events leading to M_1/G-protein uncoupling in the AD neocortex are at present unclear, and our data suggest that like the cholinergic lesions, the uncoupling of M_1 receptors to their G-proteins may be an early event in the AD process. Additionally, the molecular mechanisms relating ChAT alterations to M_1/G-protein coupling are unknown. Furthermore, since none of the patients in the current study were on cholinergic replacement therapies, it is not clear whether the use of cholinomimetics would also lead to restoration of M_1/G-protein coupling, and further studies are needed.

Another question arising from the present observations is the elucidation of molecular mechanisms underlying the uncoupling of M_1 receptors to G-proteins in AD. Studies showing preserved radioligand binding to M_1 receptors contrasting with reduced M_1 immunoreactivity [8] suggest structural changes which do not interfere with ligand binding, but which may affect antibody recognition. Such structural changes may possibly include covalent modifications, such as phosphorylation. Further studies are needed to investigate whether aberrant phosphorylation or dephosphorylation of key residues on the M_1 receptor may alter its association with G-proteins. Another potential mechanism for the loss of M_1/G-protein coupling may be a reduction of $G\alpha_{q/11}$ proteins, although this has been refuted by studies which show that G-proteins, including the $G\alpha_q$ subtype, are generally preserved in the AD brain [15]. A third possibility may be that G-protein function is altered in AD (for e.g., loss of GTPase activity), which may again involve covalent modifications [14].

In conclusion, the present study points to loss of M_1/G-protein coupling in the neocortex as a neurochemical substrate of cognitive decline in AD, and provides one explanation for the limited efficacy of cholinergic replacement therapies in ameliorating cognitive symptoms. Since M_1/G-protein uncoupling may occur early in AD, and represents an initial point of disruption in signaling cascades which are involved in cognitive functions, therapies which aim to correct or circumvent deficits in M_1 mediated signaling may show improved efficacy in ameliorating clinical symptoms. However, more work is needed to elucidate both the causes and the mechanisms of G-protein uncoupling to M_1 receptors in the AD brain.

Acknowledgments

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Disrupted muscarinic M_1 receptor signaling correlates with loss of protein kinase C activity and glutamatergic deficit in Alzheimer's disease

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Abstract

There are few studies on the clinical and neurochemical correlates of postsynaptic cholinergic dysfunction in Alzheimer's disease (AD). We have previously found that attenuation of guanine nucleotide-binding (G-) protein coupling to muscarinic M_1 receptors in the neocortex was associated with dementia severity. The present study aims to study whether this loss of M_1/G-protein coupling is related to alterations in signaling kinases and NMDA receptors. Postmortem frontal cortices of 22 AD subjects and 12 elderly controls were obtained to measure M_1 receptors, M_1/G-protein coupling, NMDA receptors as well as protein kinase C (PKC) and Src kinase activities. We found that the extent of M_1/G-protein coupling loss was correlated with reductions in PKC activity and NMDA receptor density. In contrast, Src kinase activity was neither altered nor associated with M_1/G-protein coupling. Given the well established roles of neuronal PKC signaling and NMDA receptor function in cognitive processes, our results lend further insight into the mechanisms by which postsynaptic cholinergic dysfunction may underlie the cognitive features of AD, and suggest alternative therapeutic targets to cholinergic replacement.

Keywords: Muscarinic receptors; Glutamate receptors; Protein kinase C; Src kinase; Neocortex

1. Introduction

Several neurotransmitter systems involved in cognitive processes are now known to be affected in Alzheimer's disease (AD), among which loss of basal forebrain cholinergic neurons is one of the earliest and most consistent findings [46]. Cholinergic neuronal loss was thought to underlie the clinical symptoms of AD and provided the rationale for cholinergic replacement or cholinomimetic pharmacotherapies [3]. To date, treatment with available acetylcholinesterase inhibitors (AChEIs) has yielded modest improvements in clinical rating scales, while treatment trials using muscarinic receptor agonists have not shown much promise [17,33]. Limiting factors for these compounds include stage-dependent efficacy, unfavorable side effects [17] and the involvement of other acetylcholine receptors (AChRs, e.g., nicotinic [36]) or transmitter systems (e.g., serotonergic [6]). Another possible reason is a disruption of AChR-mediated signaling. For example, muscarinic M_1 receptors are the major cholinergic receptor subtype expressed in postsynaptic sites in the neocortex and are critically involved in the cholinergic regulation of cognitive processes. M_1 receptors are coupled to the $G\alpha_{q/11}$-family of guanine nucleotide-binding (G-) proteins and activate

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protein kinase C (PKC) mediated signaling cascades, which also involve Src family kinases [8,26]. Previous studies have shown that while M_1 receptor densities are preserved in the AD neocortex, the coupling of these receptors to their G-proteins is impaired [12,13], suggesting a postsynaptic cholinergic dysfunction which may limit the efficacy of cholinomimetic therapies in ameliorating the cognitive deficits of AD.

In AD, prominent neuronal loss occurs in areas other than the basal forebrain, especially affecting the glutamatergic pyramidal neurons of the neocortex and hippocampus [16]. Glutamate is the primary excitatory neurotransmitter and is involved in most aspects of cognition and higher mental functions. In particular, the phenomenon of long term potentiation (LTP) mediated by the *N*-methyl-D-aspartate (NMDA) subtype of glutamate receptors, is the leading cellular model of memory consolidation [29]. NMDA receptors are Ca^{2+} channels formed from tetrameric complexes of NR1 and one of NR2A/2B/2C/2D subunits [22]. In AD, abnormalities in the expression and ligand binding properties of NMDA receptors [20,21,37] may underlie the cognitive symptoms of AD. Interestingly, M_1 receptors colocalize with NR1 on the dendrites and soma of hippocampal pyramidal neurons, and activation of M_1 receptors potentiates NMDA receptor currents [28]. Additionally, NMDA receptors are regulated by PKC [27] as well as Src kinase [2]. These data provide evidence of a functional link between M_1 and NMDA receptors. However, it is not known whether the loss of M_1/G-protein coupling is associated with changes in signaling kinases or NMDA receptors in AD. In this study, we correlated the neurochemical measurements of M_1 and NMDA receptors, PKC and Src kinase in the postmortem frontal cortex of a well characterized cohort of AD subjects and elderly controls.

2. Materials and methods

2.1. Clinical and neuropathological assessments

A total of 22 subjects with AD, as well as 12 controls, assessed at the University of California Los Angeles Alzheimer Disease Research Center (UCLA-ADRC) were included in this study, which was approved by the institutional review boards of UCLA and Singapore General Hospital. Relevant clinical information was obtained from the UCLA-ADRC longitudinal study database and patient charts. All 22 subjects met the Diagnostic and Statistical Manual of Mental Disorders, 4th Edition (DSM-IV) clinical criteria for dementia [1] as well as the CERAD criteria [31] for neuropathological diagnosis of definite AD. In addition, one subject showed evidence of Lewy bodies (LB), another had LB and superior frontal infarct, while two others had multi-infarct or cingulate stroke. Braak staging [5] of the AD subjects revealed that the majority ($n = 18$) were in Braak V/VI, and only two were in stage IV/V, and another two in stage IV. The elderly controls did not have neurological diseases and were found at postmortem to have minimal neurodegenerative changes (All Braak 0 except for one at stage II and another at stage IV). Selection of subjects for the current study was based on tissue availability, and not on dementia severity or Braak stage.

2.2. Postmortem tissue processing

Brains were collected at postmortem, and the orbitofrontal gyrus of the right hemisphere was dissected and stored frozen at −75 °C. Frozen brain chunks were later thawed on ice and dissected free of meninges before homogenization with an Ultra-Turrax homogenizer (IKA Labortechnik, 15 s maximum setting) in either (i) 10 mM sodium phosphate buffer, pH 7.4 containing 1.0 mM EDTA and protease inhibitors (Complete Mini, Roche Diagnostics) followed by dilution 1:1 and boiling in Laemmli sample buffer (Bio-Rad Laboratories) for immunoblotting; or (ii) 50 mM Tris–HCl buffer, pH 7.7 for radioligand binding/kinase assays, both in a final concentration of 50–100 mg tissue wet weight/ml.

2.3. Saturation radioligand binding assays

All chemicals were of analytical grade and were purchased from Sigma–Aldrich Ltd., unless otherwise stated. Neurochemical assays were performed blind to clinical information. M_1 and NMDA receptors were measured by saturation binding assays with [^{3}H]pirenzepine ([^{3}H]PZ, sp. act. 70–80 Ci/mmol) and [^{3}H]MK-801 (sp. act. 28.9 Ci/mmol, both from Perkin-Elmer Life Sciences), respectively, based on modifications of published methods [24,32] For [^{3}H]PZ, seven concentrations of radioligand (ranging from 0.5 to 15 nM) were incubated in triplicates with 100 μl aliquots of washed brain membrane homogenates in a total volume of 0.5 ml sodium phosphate buffer, pH 7.4 for 60 min at 25 °C. 1 μM atropine sulphate was added to some aliquots to define non-specific binding. At the end of incubation, homogenates were vacuum filtered onto polyethylenimine-pretreated Whatman GF/B glassfibre filters (Whatman plc). Dried filters were then punched into vials, and 2 ml aliquots of scintillant (Optiphase HiSafe 2, Perkin-Elmer Life Sciences) were added for measurement of bound radioactivity by liquid scintillation spectrophotometry. Assays for [^{3}H]MK-801 binding (0.5–30 nM, 60 min at room temperature) in 50 mM Tris–HCl buffer, pH 7.7, containing final concentrations of 250 μM spermine, 25 μM glycine, and 20 μM L-glutamate were as above, except that brain homogenates were preincubated for 60 min at 30 °C in 50 mM Tris–HCl buffer, pH 7.7 to facilitate degradation of endogenous glutamate before incubation with radioligand. Nonspecific [^{3}H]MK-801 binding was determined in the presence of 10 μM unlabelled MK-801 maleate. The parameters K_D (binding affinity, in nM) and B_{max} (binding density, in fmol/mg protein) were derived from binding data by Scatchard analyses followed by iterative curve-fitting. Binding data consistently fitted with one-site binding with Hill constants around 1.0.

2.4. M_1/G-protein coupling

Pharmacological determination of M_1/G-protein interactions was carried out by [^{3}H]PZ/carbachol competition binding as previously described [43]. Briefly, brain membrane homogenates were incubated in duplicate with 3 nM [^{3}H]PZ and 15 concentrations of unlabelled cholinergic agonist carbachol (10^{-9} to 10^{-2} M) in 50 mM Tris–HCl buffer, pH 7.4 for 150 min at room temperature. Non-specific binding was defined in the presence of 10 μM atropine sulphate, and parallel series of competition assays were performed with or without the addition of excess guanylyl imidodiphosphate (GppNHp, 0.2 mM), a non hydrolysable guanosine triphosphate (GTP) analogue which uncouples G-proteins from M_1 receptors and reduces the affinity of M_1 receptors for carbachol. Specific binding data were plotted against log-transformed values of carbachol concentrations by non-linear regression curve-fitting using Prism 3.0 software (GraphPad Inc.) to derive inhibitory constants for carbachol binding in the absence (K_i) and presence (K_{iG}) of GppNHp. The ratio of K_{iG} to K_i was then used as a measure of the state of M_1/G-protein coupling in control and diseased brains, with lower K_{iG}/K_i indicating less coupling.

2.5. PKC and Src kinase activities

The phosphotransferase activities of PKC and Src in brain membrane homogenates were measured with commercial kits (Upstate Biotechnology) according to manufacturer's instructions. Briefly, aliquots of brain homogenates were incubated with [γ-^{32}P]adenosine triphosphate (spec. act. 3000 Ci/mmol, Perkin-Elmer Life Sciences) and kinase-specific substrate peptide (QKRPSQRSKYL for PKC or KVEKIGEGTYGVVYK for Src) in supplied buffer containing the required activators, protease inhibitors and phosphatase inhibitors. Mixtures were then spotted onto phosphocellulose paper with washing and fixing, and transfer of [^{32}P]-phosphate to specific residues on the substrate peptide was quantified by scintillation spectrophotometry. Endogenous phosphorylation of homogenate proteins, defined as counts from measurements in the absence of substrate peptide, was subtracted from total counts to derive specific kinase activities on substrate peptides, and expressed in pmol phosphate/min/mg protein.

2.6. Immunoblotting

Boiled brain homogenates in Laemmli sample buffer were electrophoretically separated on 7% polyacrylamide gels, transferred to nitrocellulose membranes, and blocked in 10 mM phosphate buffered saline, pH 7.4, 0.1% Tween 20 (PBST)/5% skim milk for 1 h before immunoblotting with a rabbit polyclonal antibody directed against the NR1 subunit of NMDA receptor (1:1000, Chemicon, Inc., expected molecular weight of around 114 kDa) in PBST/1% milk overnight at 4 °C. Following washings in PBST/1% milk and a 1 h incubation with horseradish peroxidase conjugated goat anti-rabbit antibody (1:10,000, Jackson ImmunoResearch Inc.), immunoreactive bands on the membranes were visualized by enhanced chemiluminescence using the ECL system (Amersham Pharmacia Biotech), and quantified by image analyzer (UVItec Ltd.). To ensure comparable sample loading across lanes, membranes were then stripped with stripping buffer (Promega Corp.), washed and reblotted with a mouse monoclonal anti-β-actin (1:5000, Sigma–Aldrich Ltd., expected molecular weight 42 kDa), and the protocol described above was repeated with horseradish peroxidase conjugated goat anti-mouse antibody (Jackson ImmunoResearch Inc.). One lane for external standard consisting of known amounts of protein from an individual homogenate was included in each membrane for normalization of data.

2.7. Statistical analyses

All statistical analyses were performed using the SPSS 11.0 for Windows software (SPSS Inc.). Data were first checked for normality for the selection of parametric or non-parametric tests. Demographic and neurochemical variables were compared between AD and controls by Student's *t*-tests, and correlations among these variables were performed using Pearson's product moment. Within the AD group, the effects of potentially confounding demographic or disease variables on neurochemical measures were investigated with Pearson's product moment or Student's *t*-tests as appropriate. Lastly, multiple regression using the 'stepwise' method was used to investigate possible relationships between the dependent variables (PKC activity, Src activity and NMDA receptor density), other neurochemical variables and demographic factors. This allowed for the fact that M_1/G-protein coupling (K_{iG}/K_i) may be related to multiple variables and indicated the strongest correlate. For all analyses, the null hypothesis was rejected at $p < 0.05$.

3. Results

3.1. Effects of demographic and disease factors on neurochemical variables

Demographic factors including age, sex, postmortem interval and storage interval in AD and controls were well matched (Table 1). Furthermore, binding affinity (K_D) and density (B_{max}) of [^{3}H]PZ (M_1 receptors) and [^{3}H]MK-801 (NMDA receptors), NR1 immunoblot density as well as M_1/G-protein coupling status (K_{iG}/K_i) did not correlate with the demographic factors listed above (Pearson's $p > 0.05$, data not shown). Within the AD cohort, the presence of additional neuropathological findings, i.e., LB and infarct ($n = 4$, see Section 2.1.), and duration of dementia symptoms (9.5 ± 1.1 years, range 3–17 years) did not affect the neurochemical variables (Student's p and Pearson's $p > 0.05$, respectively, data not shown). However, Braak

Table 1
Demographic and neurochemical variables in AD subjects and controls

	Control	AD
Demographics		
Maximum number of cases[a]	12	22
Age at death (years)	73.2 ± 4	78.8 ± 2
Sex (M/F)	6/6	12/10
Storage (years)	4.5 ± 0.5	5.4 ± 0.7
PMI (h)	14.4 ± 3	14.2 ± 2
Muscarinic M_1 receptors		
K_{iG}/K_i	7.1 ± 0.9 (12)	4.8 ± 0.4 (22)*
[^{3}H]pirenzepine K_D	5.3 ± 0.5 (11)	6.9 ± 0.5 (18)
[^{3}H]pirenzepine B_{max}	601 ± 39 (11)	590 ± 39 (18)
NMDA receptors		
[^{3}H]MK-801 K_D	9.9 ± 1.7 (11)	8.2 ± 0.9 (22)
[^{3}H]MK-801 B_{max}	300 ± 46 (11)	200 ± 23 (22)*
Protein kinase activities		
PKC	776 ± 107 (11)	354 ± 57 (22)**
Src	6.8 ± 1.4 (11)	7.0 ± 1.2 (21)

Data are mean ± S.E.M. PMI, postmortem interval; K_{iG}/K_i, measure of M_1/G-protein coupling (see text); K_D, binding affinity constant (in nM); B_{max}, binding density (in fmol/mg protein). Specific PKC (protein kinase C) and Src kinase activities are in pmol phosphate/min/mg protein.

[a] Not all neurochemical measures were available for all cases. The *N* values available for each neurochemical measure are listed in parentheses.

* Different from control $p < 0.05$.

** Different from control $p < 0.01$.

stage was negatively correlated with NR1 density (Pearson $r = -0.55$, $p = 0.03$), but positively correlated with Src activity (Pearson $r = 0.47$, $p = 0.04$). Therefore, Braak stage was included as a covariate in subsequent regression analyses (see below).

3.2. Neurochemical variables of controls versus AD

Table 1 shows that while M_1 receptor K_D and B_{max} are unchanged in AD, receptor coupling to G-proteins is impaired (reduced K_{iG}/K_i). Furthermore, PKC activity, NMDA receptor B_{max} as well as NR1 immunoblotting density are reduced in the AD frontal cortex (Table 1 and Fig. 1B). In contrast, Src activity and NMDA receptor K_D are unchanged (Table 1).

3.3. Correlation of M_1/G-protein coupling with NMDA receptor status and kinase activities

Fig. 2 shows that K_{iG}/K_i values of all subjects correlated with PKC activities in the frontal cortex (A) as well as with both NMDA receptor indices [^{3}H]MK-801 B_{max} (C) and NR1 immunoblot density (D). K_{iG}/K_i did not correlate with Src kinase activities (B). Finally, stepwise multiple regression analyses showed that of the list of demographic/neurochemical variables (excluding the dependent variable) in Table 1 plus Braak staging, K_{iG}/K_i was the strongest predictor for both PKC activity (adjusted $R^2 = 0.30$, $\beta = 0.59$, $p = 0.008$) and NR1 density (adjusted $R^2 = 0.26$, $\beta = 0.55$, $p = 0.015$).

4. Discussion

Presynaptic cholinergic deficits are now well established findings in AD. Alterations of choline acetyltransferase (ChAT), acetylcholine levels and other presynaptic cholinergic markers are likely concomitant with basal forebrain neuronal loss, and may contribute to both the cognitive and behavioral symptoms of AD [10,24,30,47]. In contrast, although postsynaptic perturbations such as reduced muscarinic M_1/G-protein coupling and loss of nicotinic receptors have been reported [13,19], little is known about the clinical relevance and neurochemical correlates of these changes. We have recently shown that attenuation of M_1/G-protein coupling is associated with dementia severity in AD [43]. Using postmortem material from a separate, well characterized cohort of patients, we now report that the extent of loss of M_1/G-protein coupling is correlated with reductions of PKC activity as well as NMDA receptor density in AD frontal cortex. These correlations are not confounded by a number of demographic and disease factors under study. Therefore, our data link postsynaptic cholinergic dysfunction with two other substrates (PKC and NMDA receptor) known to play essential roles in cognitive processes [29,40] and provide further insight into the mechanisms by which impairment of

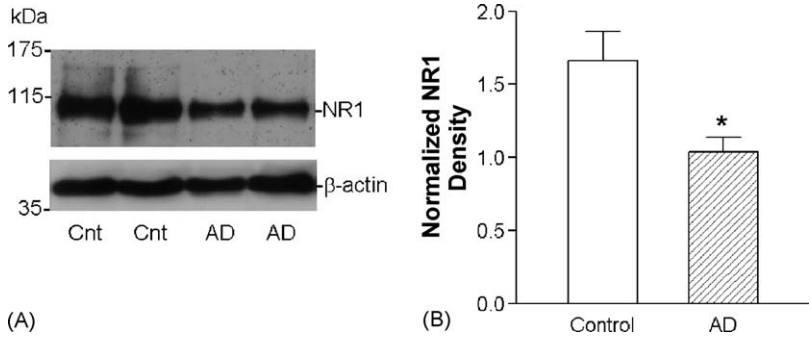

Fig. 1. NMDA receptor NR1 levels in AD subjects and controls. (A) Representative immunoblot of NR1 (top lane) from brain membrane homogenates was stripped and reblotted with antibody against β-actin (bottom lane) for controls (Cnt) and AD. Molecular weight markers in kilodaltons (kDa) are as indicated. (B) Bar graph of mean normalized NR1 immunoblot optical densities (in arbitrary units) for available control ($n = 12$) and AD subjects ($n = 18$). *Significantly different from control, $p < 0.05$ (Student's *t*-test).

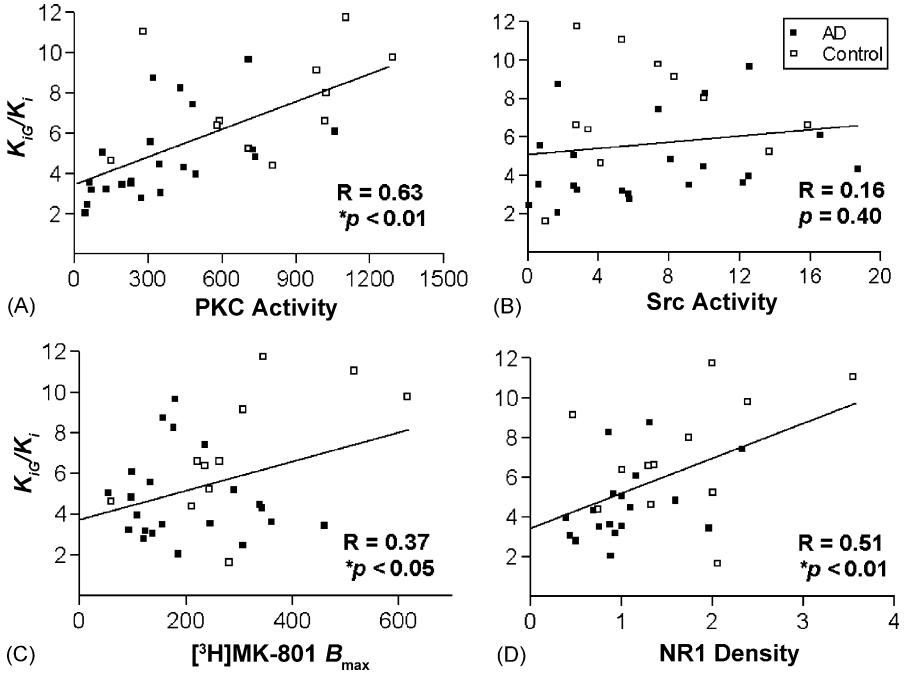

Fig. 2. Association of M_1/G-protein coupling with protein kinase activities and NMDA receptors. Correlation of K_{iG}/K_i, a measure of M_1/G-protein coupling (see text) with specific (A) PKC and (B) Src kinase activities (both in pmol phosphate/min/mg protein), (C) [^{3}H]MK-801 B_{max} (binding density, in fmol/mg protein), and (D) normalized NR1 immunoblot optical densities (arbitrary units). *Significant Pearson correlation.

M_1 mediated signaling may underlie the cognitive decline of AD.

The PKC family of isoenzymes are serine/threonine kinases which are critical signaling molecules in most cell types, serving to integrate a multitude of extracellular signals with downstream signaling events concerned with gene transcription, protein processing, synaptic plasticity, cell survival and other physiological processes [34,35,41]. In neurons, these extracellular signals include neurotransmitters like acetylcholine which are mediated by $G\alpha_{q/11}$-coupled receptors such as M_1 [8]. Interestingly, Etcheberrigaray et al. [11] showed that treatment with bryostatin 1, a PKC activator devoid of tumor promoting activity, improved maze performance in AD transgenic mice. In postmortem AD cortex, reductions of PKC level and activity have been reported [45], and our data now suggest that this reduction may not simply reflect neuronal loss, but may be related to M_1 receptor dysfunction.

Currently, the molecular mechanisms underlying down-regulation or deactivation of PKC in AD are still unclear. Inactive PKC is normally localized in the cytosol. Upon activation by diacylglycerol, Ca^{2+} and other signals released by the action of phospholipase C (which is in turn activated by $G\alpha_{q/11}$), PKC translocates to the plasma or nuclear membrane via anchoring by RACK (receptor for activated C-kinase) proteins [27]. Interestingly, RACK1 has also been found to be decreased in AD [4]. Therefore, more work is needed to clarify whether M_1 dysfunction is directly related to PKC hypoactivity by decreased activation, or by its effect on RACK and consequent perturbation of PKC translocation.

The loss of frontal NMDA receptors is another salient marker of neurodegeneration in AD and of potential significance to the clinical features of the disease [14]. In this study, we measured NMDA receptors by [^{3}H]MK-801 binding as well as immunoblotting of the NR1 subunit. Because [^{3}H]MK-801 binding density (B_{max}) was expressed per unit weight of protein, the observed reduction in B_{max} would likely reflect specific loss of NMDA receptors, rather than generalized cortical atrophy. MK-801 (dizocilpine), a non-competitive NMDA receptor channel blocker with anticonvulsant properties, is known to bind within the channel pore region formed by the NR1/NR2A complex [27]. Therefore, specific [^{3}H]MK-801 binding can be considered to indicate functional NMDA receptor labeling. Nevertheless, since NMDA channel opening (hence [^{3}H]MK-801 B_{max}) is also influenced by the concentrations of glutamate as well as regulators like glycine and polyamines [32], we added saturating concentrations of these compounds in the binding assays to maximize channel opening. We further measured levels of the obligatory NR1 subunits of NMDA receptor by immunoblotting. These two NMDA receptor indices were significantly correlated ($r = 0.58$, Pearson's $p = 0.001$) and showed a similar degree of reduction in AD (see Table 1 and Fig. 1B), suggesting that similar pools of NMDA receptors were measured. It should be noted, however, that although MK-801 binds with highest affinity to NR1/NR2A [25], which is the predominant NMDA receptor subtype in adult neocortex [39], it is unclear whether reduced [^{3}H]MK-801 binding represents the loss of specific NMDA receptor populations in AD. Importantly, attenuation of M_1/G-protein coupling significantly correlated

with both NMDA receptor indices, suggesting that impairment of M_1 mediated signaling is related to reduced levels of functional NMDA receptors.

What are the putative molecular mechanisms linking M_1 signaling dysfunction with NMDA receptor alterations? The colocalization of M_1 with NR1 indicate a spatial relationship allowing physiological interactions between the two receptors [28], and preclinical studies have shown that activation of $G\alpha_{q/11}$-coupled receptors such as M_1 potentiates NMDA receptor currents, possibly via PKC dependent pathways [27,28]. PKC is known to directly phosphorylate regulatory regions of NR1 subunits and control trafficking and surface delivery of functional NMDA receptors [27,38,42]. Therefore, it is possible that impaired PKC activity associated with M_1/G-protein uncoupling underlie NMDA receptor deficits in AD, either by decreased delivery of subunits to the surface, or increased rate of endocytosis and degradative sorting. Alternatively, the NR1 gene promoter is induced by transcription factors such as Sp1 [23] which is in turn regulated by a variety of signaling molecules including PKC [9]. Because the present techniques are not able to differentiate membrane surface expressed NMDA receptors from intracellular receptor pools, which may far exceed the former [18], further studies are needed to determine whether PKC deficits affect NMDA receptors via changes in protein sorting or receptor gene expression, or both.

In contrast to PKC, activity of Src kinase, a non-receptor tyrosine kinase and another regulator of NMDA receptor function [2], was not altered in AD and did not correlate with M_1/G-protein coupling or NMDA receptor levels. One other study on postmortem kinase activities in AD also reported no change in Src [44]. Therefore, Src activity may be resistant to AD changes, or compensatory mechanisms may exist. However, because Src is involved in multiple signaling events in a number of physiological processes, the present techniques may not be sensitive enough to detect specific changes in interactions between Src and NMDA receptor in M1-mediated signaling. It should also be noted that both PKC and Src kinase families include several closely related enzymes with similar activities, and the enzymatic assays employed in this study are unable to distinguish specific kinase subtypes. Furthermore, although an effect of M1/G-protein uncoupling on PKC and NMDA receptor alterations is biologically plausible [8,9,27,28], the present correlational study does not verify a causal relationship between these neurochemical variables; other disease factors may also play a role. Indeed, the negative correlation of NR1 density with Braak stage (see Section 3) indicate that parenchymal neurodegenerative changes are at least partially responsible for NMDA receptor deficits, although stepwise regression analyses indicated a stronger correlation with M_1/G-protein uncoupling. Interestingly, Caccamo et al. [7] showed that a novel M_1 agonist improved both plaque and tangle burden as well as spatial learning in a triple transgenic ($3\times$ Tg) AD mouse model. However, the M_1/G-protein coupling status in these animals is unknown, and it is possible that the $3\times$ Tg does not model the disruption of M1 signaling seen in AD. Importantly, these results suggest that M1 agonists maybe efficacious in AD if M_1 receptors could be re-coupled to their G-proteins.

In conclusion, there is ample evidence from preclinical studies for physiological interactions among M1 receptors, NMDA receptors and signaling molecules such as PKC and Src kinase in the regulation of cognitive processes, but the significance of these findings to AD is unclear. We now show that the extent of postsynaptic M_1 receptor uncoupling from G-proteins correlated with loss of PKC activity and NMDA receptor density in the postmortem frontal cortex of AD, suggesting that impaired M_1 mediated signaling may underlie cognitive deficits via effects on PKC and NMDA receptor function. This study provides a neurochemical basis for the limited efficacy of AD therapies which increase synaptic acetylcholine availability or target M_1 receptors; instead, our data point to targets downstream of M_1/G-protein activation, or to strategies which restores the coupling of G-proteins to M_1 receptors, and predict improved efficacy for compounds which regulate PKC [11] or NMDA receptors [15]. However, more work is needed to provide molecular delineation of the specific kinase and receptor subtypes involved in disease processes of AD.

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Serotonin transporters are preserved in the neocortex of anxious Alzheimer's disease patients

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Densities of serotonin transporters (5-HTT) in the postmortem neocortex of behaviorally assessed Alzheimer's disease (AD) patients and aged controls were measured by radioligand binding with [^{3}H]citalopram. It was found that 5-HTT sites in the temporal cortex of AD patients with prominent antemortem anxiety were unaltered compared with controls, but were reduced in non-anxious AD subjects. Furthermore, homozygosity for the high activity allele of a functional polymorphism in the 5-HTT gene promoter region (5-HTTLPR) was associated with both increased [^{3}H]citalopram binding and occurrence of anxiety in the AD subjects. Since serotonin-synthesizing neurons are known to be lost in the AD cortex, this study suggests that the preservation of 5-HTT may exacerbate serotonergic deficits and underlie anxiety symptoms in AD. *NeuroReport* 14:1297–1300 © 2003 Lippincott Williams & Wilkins.

Key words: Alzheimer's disease; Anxiety; Neocortex; Polymorphisms; Serotonin transporter

INTRODUCTION

Late-onset Alzheimer's disease (AD) is the most common cause of dementia in the elderly, affecting 5–10% of those aged > 65 years. Besides cognitive impairment, AD is characterized clinically by the presence of neuropsychiatric behaviors. Of these behavioral changes, anxiety is relatively prevalent, occurring in up to 50% of AD patients over the course of the disease [1,2]. Neuropsychiatric behaviors like anxiety are clinically significant in AD in that they often lead to considerable caregiver distress and precipitate institutionalization of the patient [3–5]. However, the neurochemical basis of these behavioral changes is not well-studied. Because the serotonergic system is affected in AD, with findings of losses of serotonergic raphe neurons, serotonin (5-HT) levels, and cortical 5-HT transporters (5-HTT) [3,6,7], and because selective 5-HT reuptake inhibitors (SSRIs) are effective in the treatment of anxiety disorders, we queried whether alterations of neocortical 5-HTT binding may underlie anxiety in AD. In addition, we examined whether a functional polymorphism in the 5-HTT gene promoter region (5-HTTLPR) [8,9] whose long (L) and short (S) variants result in increased and decreased 5-HTT expression and 5-HT reuptake, respectively, may be associated with anxiety in AD.

MATERIALS AND METHODS

The 34 AD subjects in this study were part of 100 community-based dementia patients in Oxfordshire, UK who were recruited for a longitudinal study of behavior in dementia [1,10]. Behavioral changes of the subjects were assessed every 4 months from recruitment to death (for a mean of 2.6 years) with the Present Behavioural Examination (PBE) [11], a standardized, caregiver-directed interview covering in detail the observable behavior and mental state of the patient over the previous 4 weeks. Anxiety was assessed by behaviors and physical signs indicating inappropriate anxiety or fear. Rating for anxiety was on a 7-point score (0–6) based on frequency of occurrence reported by the caregiver (from 0, absent in the last 28 days; 3, present in 14 days of the last 28 days; to 6, present every day). A subject was considered to have had significant anxiety if there were at least two ratings > 3, or one rating > 3 and two other ratings of 1–3 over the course of the study [3]. At death, informed consent was obtained from the families of patients and control participants for the removal of brain. Selection of AD subjects was based only on tissue availability and neuropathological confirmation of diagnosis by the CERAD criteria [12]. The 14 controls were neurologically normal,

had no significant neuropathology and no history of psychiatric disease.

At postmortem, blocks of gray matter from orbitofrontal (BA11) and mid-temporal (BA21) gyri were dissected from one hemisphere and processed for homogenization as described previously [13], while the other hemisphere was formalin fixed for neuropathological diagnosis. In addition, semi-quantitative scores for senile plaques (SP, score 0–3) and neurofibrillary tangles (NFT, score 0–4) were obtained from methanamine silver/modified Palmgren stained sections of BA11 and BA21. Saturation binding with [^{3}H]citalopram (sp. act. 83.0 Ci/mmol, Amersham Life Sciences, UK), which has previously been reported to label 5-HTT with high specificity and nanomolar affinity [14], was based on modifications of published methods [15]. Briefly, brain homogenates were thawed, diluted 1:1.5 vol/vol in incubation buffer (50 mM Tris–HCl, pH 7.4) and 100 µl aliquots were added to seven concentrations of [^{3}H]citalopram (0.1–12 nM) in triplicates and incubated for 60 min at 25°C in a total volume of 0.5 ml. Non-specific binding was determined in the presence of 10 µM unlabelled fluvoxamine maleate (Tocris Cookson Ltd, UK) and constituted around 30% of total binding. An aliquot of the homogenate was used for protein determination. Incubation was terminated by rapid filtration with a Skatron cell harvester (Skatron AS, Norway) through 0.1% polyethylenimine-treated Whatman GF/B filters (Whatman BDS, UK), and membrane-bound radioactivity was measured by liquid scintillation spectrometry. The EBDA and LIGAND software [16] were used to calculate binding affinity (K_D, nM) and density (B_{max}, fmol/mg protein). In all cases, the Scatchard plots were best fitted to single binding sites with Hill coefficients ranging from 0.98 to 1.01.

For 5-HTTLPR genotyping, DNA was extracted from 20 mg aliquots of cortical tissue using commercially available kits, and analyzed by polymerase chain reaction (PCR) according to previously reported methods [9] to generate the L (528 bp) and S (484 bp) fragments.

Statistical analyses were performed with SPSS 10.0 for Windows. Relationships between [^{3}H]citalopram binding variables and potential confounders (demographic or disease factors) were studied with stepwise multiple regression. Binding parameters (K_D and B_{max}) of controls and AD behavioral groups were then compared by repeated measures ANOVA (with brain area as the repeated measure), followed by *post-hoc* tests. Comparisons of binding parameters over 5-HTTLPR genotypes in the AD subjects were similarly studied. Analysis of genotype in controls was not attempted because of the small sample size, with only one individual having the LL genotype. Differences in binding parameters were considered significant if the *p* values for both the repeated measures ANOVA (effects of between-subject factor or interaction) and the *post-hoc* tests were < 0.05. Genotype frequencies in the behavioral groups of AD (anxious *vs* non-anxious patients) were compared by Fisher's exact test.

RESULTS

The AD subjects and controls were well matched with regards to age, sex, postmortem delay and tissue storage interval, with the exception of lower brain pH in the AD behavioral groups (probably due to prolonged agonal state [17], Table 1). However, pH did not correlate with any of the binding parameters (stepwise multiple regression, $p > 0.05$) and thus was not entered as a co-variate in subsequent analyses. Among the AD subjects, the binding parameters were not correlated with disease factors (disease duration, dementia severity, senile plaque and neurofibrillary tangle counts, stepwise multiple regression, $p > 0.05$), and there were no significant differences between the AD behavioral groups with regards to the disease factors (Student's or Mann–Whitney $p > 0.05$, Table 1). Because chronic psychotropic medication may affect central serotonergic activity, we also compared the binding parameters between subjects who were taking sedative-hypnotics ($n = 8$), neuroleptics ($n = 10$) or tricyclic antidepressants ($n = 3$) and those not on medication in the 8 months before death. We found that of the medications, only sedative-hypnotic use was associated with increased [^{3}H]citalopram B_{max} in BA11 (adjusted $R^2 = 0.09$, $\beta = 0.34$, $p = 0.044$). Therefore, sedative-hypnotic use was entered as a co-variate in subsequent analyses of AD subjects.

There were no differences in affinity (K_D) of [^{3}H]citalopram binding among controls, non-anxious AD and anxious AD (respective mean values ± s.e.m.: 2.72 ± 0.4, 3.67 ± 0.5, 4.04 ± 0.7 nM in BA11 and 3.38 ± 0.4, 3.51 ± 0.5, 4.91 ± 1.0 nM in BA21). In contrast, [^{3}H]citalopram binding density (B_{max}) was reduced in non-anxious AD patients compared to controls, while the B_{max} of anxious AD patients was preserved at around control values. However, this pattern of alteration reached *post-hoc* statistical significance only in BA21 (Fig. 1). When binding parameters in the AD patients were compared over the 5-HTTLPR genotypes, the B_{max} values were significantly higher in those of LL genotype than in SS or SL genotypes (with sedative-hypnotic use as co-variate, Fig. 2). K_D was not significantly different between the genotypes (data not shown).

Table 1. Comparison of demographic and clinical features between controls and AD behavioral groups.

	Controls ($n = 14$)	AD, no anxiety ($n = 22$)	AD, anxiety ($n = 12$)
Age (years)	75 ± 4	81 ± 1	81 ± 1
Sex (% male)	50	50	58
Post mortem interval (h)	47 ± 7	38 ± 6	49 ± 8
Storage interval (months)	90 ± 3	91 ± 3	91 ± 3
pH	6.7 ± 0.08[a]	6.3 ± 0.08	6.2 ± 0.11
Disease duration (years)	–	9.6 ± 0.9	8.9 ± 1.5
Mini-Mental State Examination[b] [24]	–	4.2 ± 1	7.8 ± 3
Senile plaques			
BA11	–	2.8 ± 0.1	3.0 ± 0.01
BA21	–	2.8 ± 0.1	3.0 ± 0.01
Neurofibrillary tangles			
BA11	–	2.1 ± 0.4	1.5 ± 0.3
BA21	–	3.0 ± 0.3	2.3 ± 0.4

Data are mean ± s.e.m.
[a]Significantly different (one-way ANOVA, $p = 0.003$) from AD without anxiety (Scheffé multiple means comparison $p = 0.014$) and AD + anxiety (Scheffé $p = 0.008$).
[b]Mean of last five MMSE scores before death used as a measure of dementia severity to avoid floor effects [3].

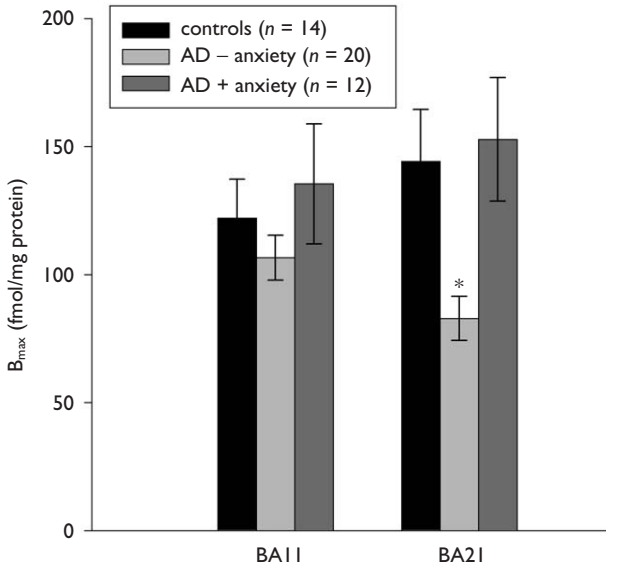

Fig. 1. Data are mean ± s.e.m. for [^{3}H]citalopram binding to frontal cortex (BA11) and temporal cortex (BA21) in the controls and the AD behavioral groups with repeated measures ANOVA (2,43 df) for the effects of group ($F = 4.02$, $p = 0.025$) and group × brain area interaction ($F = 2.58$, $p = 0.088$). Radioligand assays were not performed on two subjects due to shortage of tissue. *Significantly different (one-way ANOVA, $p = 0.006$) from control (Scheffé $p = 0.033$) and AD + anxiety (Scheffé $p = 0.018$) values in BA21.

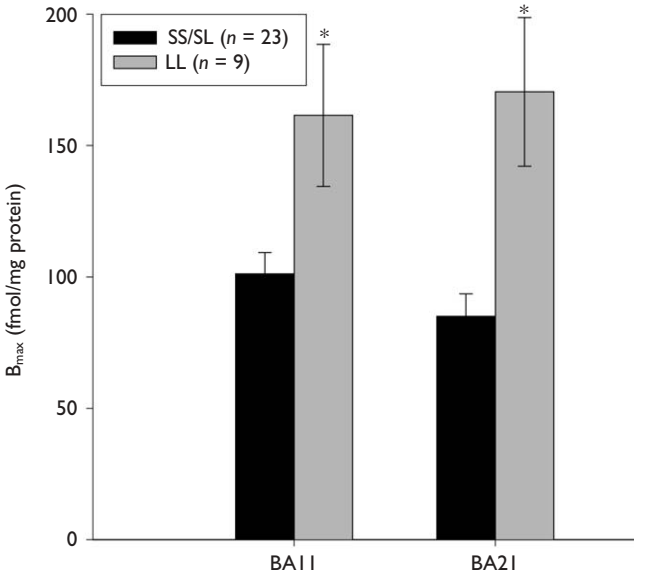

Fig. 2. Data are mean s.e.m. for [^{3}H]citalopram binding to frontal cortex (BA11) and temporal cortex (BA21) across 5-HTTLPR genotype in AD subjects with repeated measures ANOVA (2,28 df; with sedative-hypnotic use as co-variate) for the effect of genotype ($F = 13.4$, $p = 0.001$ and genotype × brain area interaction ($F = 2.04$, $p = 0.16$). *Significantly different from SS/SL (Student's t-test, $p < 0.01$).

Finally, we compared 5-HTTLPR genotype distribution between the AD behavioral groups, and found higher proportions of anxious patients with the LL genotype than in those of SS or SL genotypes (Table 2), with an odds ratio of 6.25 (mid-p corrected 95% CI 1.14–37.04).

Table 2. Anxiety by 5-HTTLPR genotype in AD.

Genotype	Anxious	Non-anxious
SS/SL	6	19
LL	6	3

Data are number of patients. Fisher's exact $p = 0.031$.

DISCUSSION

At present, at least 14 pre- and postsynaptic 5-HT receptor subtypes are known to function as effectors of 5-HT signaling [18]. In contrast, 5-HT reuptake from the synaptic junction is mediated by a single protein, the 5-HTT, which is critical in the regulation of the magnitude and duration of serotonergic activity [8]. Support for the involvement of 5-HTT in mood and emotional states comes in part from the efficacy of selective 5-HT reuptake inhibitors (SSRIs) in the treatment of depressive and anxiety disorders. This suggests that serotonergic deficits may form the neurochemical basis of some affective disorders, the symptoms of which can be ameliorated by blocking the reuptake of 5-HT and hence restoring serotonergic activity [19]. In addition, a functional biallelic polymorphism of the gene promoter region of 5-HTT (5-HTTLPR) whose long and short variants determine increased or decreased 5-HTT expression, respectively, has been shown to be associated with anxiety-related personality traits [8,20]. These findings led us to examine whether the presence of anxiety in AD is related to 5-HTT levels in the postmortem neocortex and, if so, whether the 5-HTTLPR genotype may be a risk factor for the development of anxiety in AD via its influence on 5-HTT levels.

In agreement with previous reports of presynaptic serotonergic deficits [3,6,7], we found reduced densities of 5-HTT in BA21 of non-anxious AD patients and a trend towards reduction in BA11, which may be due in part to the relatively severe neurodegeneration in temporal cortex [21,22]. However, the novel finding in this study is the relative preservation of 5-HTT sites in anxious AD subjects, who were also more likely to have the high activity LL genotype of 5-HTTLPR. We then showed that 5-HTT binding is significantly higher in patients with the LL *vs* the SS/SL genotype. The SS and SL genotypes were analyzed as a group because of the similar levels of 5-HTT activity in the SS and SL genotypes previously demonstrated for 5-HTTLPR (S dominance [8]). Taken together, the data suggest that preservation of neocortical 5-HTT sites in anxious AD patients may be mediated in part by 5-HTTLPR driven, enhanced expression of 5-HTT on remaining serotonergic neurons. We further postulate that this preservation of 5-HT reuptake activity concomitant to presynaptic serotonergic deficits [3,6,7] may exacerbate the depletion of 5-HT in the synaptic junction and predispose the patient to developing anxiety symptoms.

This is the first study which relates 5-HTTLPR with anxiety in AD, and the genetic data should be considered

preliminary due to the relatively small sample size, perhaps inevitable in postmortem studies. Therefore, population stratification effects cannot be dismissed and may account for the atypically high odds ratio and a wide 95% CI (see Results). Furthermore, the present findings, although analogous to a recent study correlating the LL genotype with aggression in AD [23], is nevertheless at odds with data from the general population where the S allele has small but statistically significant contributions to anxiety-related personality traits [8,20]. One possible reason for the discrepancy may be the presence of extensive cortical neurodegeneration and serotonergic deficits in AD which may lead to altered neurophysiological, neurochemical and behavioral manifestation of 5-HTTLPR compared to non-AD brains.

CONCLUSION

This study demonstrates that [^{3}H]citalopram binding to 5-HTT is lost in the postmortem neocortex of non-anxious AD subjects, but preserved in subjects who manifested significant premortem anxiety. The preservation of neocortical 5-HTT sites, which may reflect synaptic plasticity mediated in part by genetic factors, is a putative neurochemical substrate of anxiety in AD by exacerbating the deficits in serotonergic neurotransmission. This study therefore provides the rationale for using SSRIs or serotonergic agonists as an alternative to benzodiazepines for the treatment of anxiety related symptoms in AD.

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