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AMYLOSOMES AND MICROTUBULES IN THE HUMAN BRAIN: RELATIONSHIP TO AGING AND THE PATHOGENESIS OF ALZHEIMER'S DISEASE

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ABSTRACT

There is evidence that amyloid deposition within the brain is of major importance in the pathogenesis of Alzheimer's disease (AD), but the mechanism of this deposition is not known, and several theories are currently under great debate. The present study of intraneuronal changes in the human brain with age and disease suggests a new explanation for amyloid plaque formation in the pathogenesis of AD.

This work indicates that intraneuronal inclusions originally observed by Rees (1975), may contain amyloid peptide (Aβ), the beta-pleated form of which is the major component of the amyloid plaques in AD. We have therefore called them amylosomes. Using the techniques of histology, immunohistochemistry, computerized morphology, as well as protein isolation and analysis, we examined the distribution, size, density and chemical composition of amylosomes in the human brain throughout life. Amylosomes appear during early childhood and remain constant in size and number until advanced old age. Their protein content and immunostaining characteristics indicate they contain Aß, and their dendritic location in regions where AD plaques form suggested a possible role in Aß transport from the brain to the CSF. We therefore isolated and quantified human brain microtubule proteins (MT) and the dendritic microtubule-associated protein 2 (MAP_2), assessed their ability to polymerize *in vitro*, quantified the MAP_2 mRNA with age, and related these results to the apolipoprotein E (APOE) genotype, which is known to modulate the risk of AD. There was an age-related decrease in MT proteins and MAP_2 and in their ability to polymerize, that was accelerated in individuals with APOE $\epsilon 4$ allele(s), the group at greatest risk of AD. These results are consistent with the proposal that amylosomes play a role in Aß metabolism, and an age-related decrease in the dendritic MT transport system could lead to retention of amylosomes within the brain, with deposition of the Aß and plaque formation as a consequence. This theory can account for most of the important features of AD, and resolves a number of contradictory hypotheses, as well as suggesting relevant areas for further investigation. A better understanding of the pathogenesis will hopefully reveal strategies for prevention and treatment of this common and presently incurable disease.

RÉSUMÉ

Il existe d'amples évidences que le dépôt d'amyloïde au niveau du cerveau joue un rôle important dans la pathogénèse de la maladie d'Alzheimer. Cependant le mécanisme impliqué dans cette accumulation reste incertain malgré plusieurs théories proposée. L'étude des changements intraneuronaux au niveau du cerveau humain associée avec le vieillissement et la maladie, propose un nouveau mécanisme responsable de la formation de plaques dans la maladie d'Alzheimer.

Les présentes données indiquent que les inclusions intraneuronales décrites par Rees (1975), peuvent contenir le peptide amyloïde (Aß), dont la configuration en pli ß est un des éléments formant les plaques d'amyloïde dans la maladie d'Alzheimer. Nous y référons sous le nom "d'amylosomes". A l'aide des techniques d'histologie, d'immunohistochimie, de morphologie assistée par ordinateur ainsi que l'isolation et l'analyse de protéines, nous avons examiné la distribution, la taille, la densité et la composition des amylosomes dans des cerveaux humains d'âges différents. Les amylosomes font leur apparition tôt pendant l'enfance et leur taille et nombre demeurent constant jusqu'à un âge adulte avancé. Leur contenu en protéines et leurs caractéristiques immunohistochimiques indiquent la présence de Aß. De plus leur distribution au niveau dendritique dans les régions démontrant la formation de plaques de type Alzheimer suggère un transport de Aß du parenchyme cérébral au liquide céphalo-rachidien. Nous avons procédé à l'isolation et la quantification des protéines tubulines humaines et la protéine dendritique associée aux microtubules (MT) 2 (MAP_2). Apres l'étude de leur capacité de polymerisation *in vitro* et la quantification de l'ARNm de MAP_2 en fonction de l'age nous avons relié les résultats au génotype d'apolipoprotein E (APOE), qui module le risque de la maladie d'Alzheimer. Nos résultats démontrent qu'il existe une diminution des protéines MT et MAP_2 ainsi qu'une diminution de leur capacité de polymerisation associée à l'âge. Cette diminution est accélérée chez les individus porteur de l'allèle(s) APOE E4, qui représentent le groupe à plus haut risque pour la maladie d'Alzheimer.

Ces résultats supportent l'hypothèse que les amylosomes jouent un rôle dans le métabolisme de Aß. Une diminution reliée à l'age du système de transport de MT au niveau des dendrites pourrait permettre l'accumulation des amylosomes au niveau du cerveau ayant comme conséquence le dépôt de Aß suivi de la formation de plaques. Cette hypothèse tient compte des changements importants de la maladie d'Alzheimer et permet de résoudre un nombre de théories contradictoires. De plus, elle soulève de nouvelles avenues à fin de recherche. L'élucidation de la pathogenèse soulèvera de nouvelles stratégies à fin de prévenir et traiter cette maladie commune présentement incurable.

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LIST OF ABBREVIATIONS

Aβ	Beta amyloid
ACh	Acetylcholine
AChE	Acetylcholinesterase
AD	Alzheimer's disease
ANOVA	Analysis of variance
apo	Apolipoprotein
APO	Apolipoprotein gene
APP	Beta amyloid precursor protein
Avg.	Average
BBB	Blood brain barrier
BSA	Bovine serum albumin
CAT	Choline acetyltransferase
CERAD	Consortium to establish a registry for Alzheimer's disease
CNS	Central nervous system
CSF	Cerebrospinal fluid
CT	Computerized topography
CTF	C-terminal fragment
CVD	Cerebrovascular dementia
DEPC-H_2O	diethyl pyrocarbonate (treated water)
EM	Electron microscopy

FAD	Familial Alzheimer's disease
GTP	Guanosine 5'-triphosphate
GVD	Granulovacuolar degeneration
H&E	Haematoxylin and Eosin stain
HPLC	High performance liquid chromatography
Hr	Hour
MAP	Microtubule-associated protein
MID	Multiple infarct dementia
Min	Minute
MOPS	3-[N-morpholino]propanesulfonic acid
MRI	Magnetic resonance image
MT	Microtubule
NAC	Non-Aβ component of Alzheimer's Disease amyloid
NACP	Non-Aβ component of Alzheimer's Disease amyloid precursor gene
NFT	Neurofibrillary tangle
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PD	Parkinson's disease
PET	Positron emission topography
PHF	Paired helical filament
PNS	Peripheral nervous system
PVDF	Polyvinylidene difluoride microporous membrane

SD	Senile dementia
SD	Standard deviation
SDAT	Senile dementia Alzheimer type
SDS	Sodium dodecylsulphate
SE	Standard error
SP	Senile plaque
SPECT	Single-photon emission computer topography
TBS	Tris-buffered saline
VLDL	Very low density lipoprotein
Yr	Year

INTRODUCTION

Alzheimer's disease(AD) is a chronic, progressive, dementing illness of unknown etiology. It is increasing in frequency, and has now become one of the greatest challenges, not only to the medical sciences, but also to our whole society. Progress in documenting the cellular, biochemical, and genetic basis of AD has been very rapid, especially in the past 10 years. However, contradictions and questions remain in almost every aspect of the studies, as well as in our general understanding of the disease. Although aging, the influence of apolipoprotein E (protein, apoE; gene, APOE) and the deposition of beta amyloid (Aβ), as well as many other factors are all strongly associated with AD, their roles in the pathogenesis, especially their relative importance and potential interaction, are matters of considerable debate. At present the popular theories about the pathogenesis have actually raised more questions than they have answered. This could reflect the complexity to be expected if there are many different genes and environmental risk factors underlying the pathogenesis of AD (Hyman & Terry, 1994; Mayeux, 1996; Bernstein et al, 1996; Blass, 1996).

In 1975 Rees described a membrane bound structure within the dendrites of cortical neurons. Since then there have been only a few related studies (Hara & Misugi, 1980; 1982; Averback, 1983; Hara, 1986), mainly focused on the histology and some morphometric features of these intraneuronal structures. Because their distribution in the human brain occurs in a pattern similar to that of the amyloid plaques in AD we decided

to examine them further. Our initial study showed that they may contain Aβ, the constituent of the amyloid plaques, and we therefore named them amylosomes (Zorychta, 1991; Richardson, 1992). Understanding the physiological role and the dynamics of amylosomes in human brain might help to explain the development of amyloid plaques in aged brains, and in AD.

To pursue these aims, we first carefully documented the spatial distribution of amylosomes in the human brain, and their number and size throughout the lifespan. We felt that conventional morphometry by traditional microscopy would be too tedious and unreliable, based on the disparities shown in some of the previous studies (Hara & Misugi, 1980; 1982; Averback, 1983; Hara, 1986). We therefore decided to use a computer-aided morphometry system with an Image Analysis Program 1.44 (NIH), which allowed reproducible images and measurements to be stored and further processed statistically.

The results of these histological and morphometric studies indicated that intracellular transport may play a role in the metabolic pathway of amylosomes. We then decided to study the microtubule (MT) transport system in human brain by evaluating MT and microtubule associated protein$_2$ (MAP_2), a dendrite-specific MT associated protein, to detect possible changes during the lifespan. Previous studies of brain MT have mainly been conducted on animal tissue or cultured cells (Matus, 1988; Vaila, 1990; Hirokawa, 1991; Tashiro, 1991; Fifkova,1992), because most methods require

fresh tissues, which are difficult to obtain from human brains. In addition, methods such as the polymerization-depolymerization procedure (Shelanski, et al. 1973), the cycle procedure combined with DEAE-dextran (Yan et al, 1985), or the taxol-dependent procedure (Valle, 1982) are not only complex and tedious, they often yield protein products of different molecular weights, in various ratios (Valle et. al., 1986; Sparkman, 1992), making it difficult to quantify and to compare samples. A newer method developed by Sparkman (Sparkman, 1992), with some modification, allows isolation and reliable quantification of MT proteins from frozen human brains, and also facilitates the study of MT polymerization *in vitro*. We therefore decided to use this method.

APOE4, the recently discovered risk factor for late onset AD (Strittmatter et al, 1993a) has gained more and more attention in epidemiological studies (Saunders et al. 1993; Roses, 1994), biochemical and *in vitro* studies (Strittmatter et al., 1993b; Ma et al., 1994; Sanan et al, 1994; Nathan, et al. 1994; Wisniewski et al., 1994; Roses et al, 1996), and immunohistochemical studies (Namba et al, 1991; Rebeck et al., 1993; Naslund et al., 1995). We believe that APOE4 is neither "necessary nor sufficient", but like many other risk factors, possibly acts through some commonly shared pathogenic process which leads to the development of amyloid plaques. Therefore APOE genotyping was performed to assess the possible influence of apoE4 on MT. Although ApoE has not been considered as a MAP, recent reports such as the dendritic disintegration in APOE knockout mice (Masliah et al, 1995) and apoE isoform-specific effects on MT stabilization (Nathan, 1995; Roses et al, 1996) are actually in agreement with our

original prediction and our results.

What has mainly been pursued and achieved in this work has been to find or delineate: a) that neuronal inclusions which have not previously attracted attention in research on Alzheimer's Disease, may contain the amyloid peptide that constitutes the amyloid plaques in AD; b) based on their dendritic location, distribution in human cerebral cortex, and the relatively constant size and density over the lifespan, there may be a dendritic pathway for removal of these inclusions; c) there are age-related declines in the quantity of human brain tubulin proteins and MAP_2 , as well as their ability to polymerize into MT *in vitro*; d) the MAP_2 decline with age is regulated partly at the transcriptional level; e) these decreases with age are accelerated in humans with APOE $\epsilon 4$ allele(s). We have combined these observations into a hypothesis regarding the pathogenesis of AD. This theory proposes amylosomes to be the source of the amyloid in the plaques deposited in both aged and AD brains, and the age-related decline of dendritic MT function to be a fundamental process in the pathogenesis. This theory integrates several previous hypotheses, provides explanations for most of the important features of AD, and offers potential guidance for future pharmacological intervention. The ultimate goal is not only to aim at symptomatic relief, as most drugs presently available do, but also to target and modify the pathogenesis.

2. REVIEW OF BASIC CONCEPTS RELATED TO ALZHEIMER'S DISEASE

2.1. BACKGROUND AND HISTORICAL FACTORS

2.1.1. Naming of the disease

The early history of Alzheimer's disease (AD) is very poorly documented, which has raised questions on the merit of naming the disease after an individual investigator at the beginning of this century (Amaducci et al., 1986; Berrios, 1990; O'Brien, 1996). The history of "Alzheimer's disease" (AD) can be traced back to 1901, when a female patient, Auguste D., came under the care of the German physician, Alois Alzheimer at the Frankfurt Hospital for the Mentally Ill and Epileptics. Alzheimer diagnosed that Auguste D. suffered from progressive cognitive impairment, speech and perception problems, hallucinations, delusions, and psychosocial incompetence. She continued to deteriorate and died in 1906 from septicaemia secondary to bed sores. An autopsy revealed that her brain was atrophied. Silver-impregnation stains showed that the brain was studded with abnormal neurofibrillary tangles (NFT), and the "miliary foci", which we now call senile plaques (SP), as well as cerebral arteriosclerosis. Alzheimer first reported Auguste D's symptoms and pathology in November 1906 in Tübingen at a meeting of the South East German Psychiatrists, and published it in a brief paper in 1907 (Alzheimer, 1907; Amaducci et al., 1986; O'Brien, 1996).

The "miliary foci" were first identified by Blocq and Marinesco in an elderly epileptic patient in 1892, and later by Redlich in two senile dementia cases in 1898. The original name was replaced by the term "senile plaques", after a more extensive description by Fischer in 1907 (Amaducci, 1986; Berrios, 1990). Identification of the NFT has been attributed to Alzheimer (Amaducci,1986; Beach,1987), although it had been described earlier by others (Defelip, 1988; Berrios, 1990). Both NFT and SP had also been linked specifically to "senile dementia", a term which was already in use in psychiatric literature in the 19th century, before Alzheimer's report. Nor was the description of dementia in people younger than 65 a novelty in 1906. Thus Alzheimer did not actually describe anything novel in terms of either the clinical syndrome or neuropathological observations.

There were two rival schools of European neuropathology involved in finding the two characteristic lesions, and in naming the disease: Alzheimer, Bonfiglio and Perusini, at the Psychiatric and Neurological Clinic in Munich directed by Kraepelin, and Fischer, at the German Psychiatric Clinic in Prague directed by Pick. They contributed respectively to describing NFT and SP. Each played down the significance of the other's finding, and they were rivals for the eponym (Bonfiglio, 1908; Amaducci, 1986). Alzheimer himself, his colleagues and the members of the rival camp had all originally believed that they were simply observing an atypical form of senile psychosis, rather than a new disease (Perusini, 1911; Amaducci, 1986; Berrios,1990). It was Emil Kraepelin who codified the dementia with the salient clinical and pathological features described by

Alzheimer, as a separate entity in the 8th edition of his authoritative textbook "Psychiatie: ein Lehrbuch für Studierende und Ärzte" in 1910. It is of importance to note that, at the time, only 4-5 cases had been documented. Of four cases reported by Perusini in 1909, two had been previously reported, ie. one was Alzheimer's first case, the other was Bonfiglio's reported in 1908. It is also worth mentioning that some features of the two cases were changed, ie. postmortem results no longer showed arteriosclerotic lesions (Berrios,1990). AD as baptized by Kraepelin, was thereafter accepted as a presenile dementia with onset before the age of 65. The justification for making this nosological distinction is debatable. However because of Kraepelin's great reputation and authority, the dogma he created endures.

2.1.2. Confusion in the semantics and concepts

In addition to the above nosological uncertainty between AD and SD, many biological and medical terms we now constantly use, such as, senile dementia (SD), aging, senile dementia of Alzheimer type (SDAT), lack a unified common meaning in both lay and scientific societies. These are examples of codified definition lagging behind accepted usage. The meanings need to be clarified, as do the terminologies employed in this thesis.

Since the time of Avicenna, in medical science, the senile period was considered to begin at age sixty (Grmeck, 1958). There is a long history during which human

mental decline in the form of senile dementia has been observed over the ages. In the time of Hippocrates, incompetent behaviour in the elderly was recognized, and probably considered a natural part of aging, since he did not include it among his mental disorders. The term "dementia" was first introduced by Celsus, a Roman writer on medical subjects, in his book "De Medicina" in the first century A.D. It was described as a mental disease in old age in the second century A.D. by Galen, who had achieved the apex of Greek medicine with his description of the pulmonary circulation (Torack, 1983; Cohen, 1983). In 1797, Pinel gave the first medical definition for the term "demence", and later his student, Esquirol, differentiated 3 types of dementia: acute, chronic, and the senile form that was established slowly and caused by advanced age (Lipowski, 1980).

Brain atrophy was described by Wilks in 1864, as a constant pathological feature of senile dementia, which thereafter was considered an organic mental disorder. However there is still no consensus on the cause(s) of the atrophy, nor has the concept of age-related neuronal loss been universally accepted (Huag, 1984; Wickelgren, 1996). A more precise definition of senile dementia was created by including Redlich's SP (1898), Alzheimer's NFT (1906), and Simchowicz' granulovascular degeneration, or GVD (1910) (Torack, 1983). The term "presenile dementia" was introduced by Binswanger in 1898, and later enhanced by Alzheimer's report in 1906 with the subsequent naming of AD in 1910. Since then senile dementia with unknown etiology has been commonly referred to as "AD", however AD has also been preferred by some to designate only the

presenile dementia with onset before age 65.

Since they show similar pathological and clinical features to those Alzheimer described in his case report, and proposed differences have mostly been reported using relatively small sample sizes and are not qualitative (Katzman, 1976; McKhan, 1985; Hyman, 1996), it seems questionable and highly confusing, to distinguish the eponyms "AD" from "SD", or early onset AD from late onset AD, not to mention the AD-aging dichotomy (see chapter 2.2.3.5). The confusion was further confounded, when "senile dementia of Alzheimer type" (SDAT), a more circumscribed term than "SD" was used by some people to imply the presence of Alzheimer-type neuropathological changes, and when the terminology "presenile dementia and senile dementia simple type" was suggested by WHO (1977), strongly implying a dichotomy between dementia occurring in the "presenium" and that occurring in the "senium". These distinctions are controversial rather than convincing, and the problem is further magnified by the existence of a broader class of dementing illnesses, such as Pick's dementia, and multi-infarct dementia.

To avoid confusion regarding disease entities and clinical syndromes, the terminology used in this thesis is defined to be consistent with the usage preferred by the majority of investigators attempting to advance our present understanding of the disease.

"SD" and "presenile dementia" are used, if unavoidable, to refer to dementia with

onset late in life, generally after age 65, and with onset before age 65 respectively, the origin of which may be, but is not limited to, AD. However we do not imply a distinction between AD and SD.

"AD" is used to denote the disorder characterized by age-associated cognitive decline of gradual onset and progressive course, with Alzheimer-type neuropathological changes in the brain. No distinct age of onset is implied.

"Dementia of Alzheimer type" (DAT) is only used to refer to "probable" AD (McKhann, 1984; Mira, 1993), in which the disorder is diagnosed by clinical criteria, but not neuropathologically verified.

2.2. CURRENT CONCEPTS

2.2.1. Epidemiology of the disease

According to the US Department of Health, Education and Welfare (1978), Jorm (1990), and Statistics Canada (1992), the percent of elderly people in the population is rising dramatically. At present, 10.6% of the Canadian population is age 65 or over, this is expected to rise to 14.5% by 2011, and 21.8% by 2031 (Statistics Canada, 1990; 1991; 1992). It is estimated that 4-5% of the US population over 65 years old have severe dementia and 10% have mild to moderate impairment (Katzman, 1976; Tery, 1983; Breteler, 1992). These numbers are positively correlated with age. For example,

while it is about 0.1% in those aged 55-64, the prevalence of severe dementia is 1% in those between 65-70, 15-30% in those who are 80, 40-50% in those who are 90 (Gottfries, 1985; Evans et al, 1989; Larson et al., 1992), and over 50% in centenarians (Powell, 1994; Ebly, 1994), although some (Morgan et al, 1993; Skoog, et al, 1993; Ebly et al, 1994) have reported a levelling off of the prevalence rate in the very old. When the life expectancy extends into the late nineties by the year 2040, a large portion of our population will develop dementia at some point in their lives (Hagnell, 1981; Seegmiler,1989; Mortimer, 1995, CNN, 1997).

AD is presently recognized as the most frequent cause of dementia in adults, accounting for 50% to 80% of all cases (Tomlinson, 1970; Terry & Katzman, 1983; Larson et al, 1984; Kokmen et al., 1988; Brayne et al, 1995). Although the figures are not precise, it is estimated that AD presently affects 2 to 4 million Americans (Jorm, 1987; Advisory Panel on AD/NIH, 1992; Ernst et al. 1994), and is expected to affect 14-25 million by the middle of the next century (Marx,1996; CNN, 1997). The age-specific prevalence of AD rises from 0.3-3% in individuals who are 60-69 years old, to become 2-5% at 70-79 years, 10-12% at 80-89 years, and over 28% at 90 years and above (Monique et al., 1992, Corrada et al., 1995, Marx, 1996). AD now ranks as the 4th most common cause of death in developed nations (Reisberg,1983; Francis, 1989).

Comparisons of dementia across populations and cultures, although controversial, have demonstrated large differences. It has been claimed that in Asia, the aging

population is starting to resemble that of developed countries (Holden, 1996). However, unlike North America and western Europe, Russia, Japan and China have repeatedly claimed to have higher rates of vascular dementia than AD (Gavroilov, 1977; Karasawa, 1982; Li et al, 1989; Jorm, 1991). China was reported to have the lowest rates, claiming that only 1.86% of aged people were demented and of these only 0.07-0.66% had AD (Kuang, 1984; Yang, 1988). However, in cooperation with American universities recent reports (Zhang et al, 1990; Shen, 1994) show comparable statistics for the prevalence of dementia in people over 65, and the percent of demented individuals with AD, in China and in western countries.

Some investigators have found that AD occurs more often in women (Rorsman et al., 1986; Yoshitake et al. 1995), particularly the very old (Fratiglioni et al, 1993), while others (Copeland et al., 1992; Letenneur, 1994; Prencipe, 1996) did not detect a difference between the sexes. Many aspects of our lifestyle have been implicated as risk factors, such as low education (Zhang et al., 1990; Katzman, 1993; Cobb et al. 1995), late maternal age (Rocca et al. 1991; Foster et al. 1995), alcohol and cigarette consumption (Fratiglioni et al. 1993; Plassman et al. 1995), and environmental exposure to various agents such as aluminum, silicates (Edwardson et al, 1986; Doll, 1993), or solvents (Fratiglioni et al, 1993; Kukull et al, 1995). Some features of the individual health/medical history, have also been considered as risk factors, including thyroid disease (Breteler et al, 1991; Ewins et al, 1991), and medically treated depression (Jorm et al, 1991; Anonymous, 1994) but the evidence is often controversial or inconsistent

(Graves et al, 1991; Monique et al. 1992; Caroline et al., 1994; van Duijn, 1996).

So far, these epidemiological studies and the variations in AD prevalence rates reported worldwide have not led to important clues to the etiology, and inconsistencies are thought to be largely due to methodological differences. Nevertheless, one consistent finding is the dramatic increase in incidence with age (Larson et al., 1992; Corrada et al., 1995; van Duijin, 1996). It seems that the risk factors for AD are ubiquitous (van Duijin, 1996).

2.2.2. Clinical and pathological features of AD

2.2.2.1.Clinical and pathological features

A uniform natural history of AD (Bachman et al. 1993; Becker et al., 1994; Jost & Grossberg, 1995) may not exist (Boersma & Eefsting, 1995; Whitehouse & Deal, 1995). Generally, the initial symptoms of AD are often so subtle that the onset and details of its early course are difficult to establish. The most common initial symptoms are impaired memory, difficulty with problem solving, and failure to respond to changes in the environment with customary speed and accuracy. Various psychotic and neurological as well as extraneuronal changes may emerge through the course of the illness (Reisberg, 1983; Pirozzlol, 1989; Scott, 1993). As the disease progresses, the memory loss and disorientation become more severe, perception disorders arise, and

focal cortical signs such as aphasia and apraxia appear. Eventually, the patient loses insight, no longer attempts to compensate for the impaired mental function, and deteriorates to a stage of complete helplessness. The heterogeneity of AD exists at genetic, biochemical, neuropathological, and clinical levels. Subgroups of AD have been proposed, such as Mayeux's (1985) four groups: benign, myoclonic, typical and extrapyramidal. Other categories sometimes utilized include presenile and senile forms (Roth, 1878; Seltzer, 1983; Bondareff, 1983, 1987), familial and sporadic forms (Sjogren, 1952; Folstern, 1981); and benign and malignant forms (Mayeux, 1985).

The brains of AD patients vary in appearance from those with normal dimensions or mild atrophy to those with marked atrophy and ventricular dilatation. The atrophy, although general, is often more apparent in the basal and medial limbic portion of the temporal lobe, the frontal lobe and post-central parietal region. The primary projection areas such as the sensorimotor cortex and calcarine gyrus, the brain stem, cerebellum and spinal cord are largely preserved. Some additional changes, such as arteriolosclerosis, atherosclerosis, frank infarctions, hippocampal atrophy, and pale substantia nigra (SN), are seen usually in older patients (Brun, 1976: Miller, 1977; Mirra, 1993; Golomb et al, 1993, Kalaria, 1996).

Microscopically, the changes are usually confined to the cerebral cortex and the limbic grey matter, particularly in the basal and medial limbic portion of the temporal lobe, the frontal lobe and post-central parietal region. NFT and SP are deemed as

histological hallmarks (Terry, 1963, 1964; Wisniewski, 1983; Braak et al. 1991, 1996; Feany & Dickson, 1996), although they are not specific to AD, nor does the extent of either of them always correlate with cognitive impairment (Delaere et al, 1990; Dickson et al, 1994). Their presence is considered compulsory for the pathological diagnosis, and for practical purposes the SP is the more significant. The diagnosis of AD is often based on the extensive presence of SP, even without a significant amount of NFT, but not vice versa.

Histocytochemically, NFT are demonstrated by silver stain as thickened and tortuous fibrils in the neuronal cytoplasm, and are most frequent in large pyramidal neurons of the hippocampus, parahippocampal gyrus and of layers III and V in the cortex. They are also found in distal dendrites as neuropil threads that are associated with amyloid plaques (Terry, 1978; Brun, 1976; Bergeron, 1989; Mirra, 1993). They consist of either single, straight or more commonly, paired helical filaments (PHF), about 10nm wide and 500 to 2,000 nm in length, with a half-periodicity of 80nm. (Kidd, 1963; Terry, 1963; Wischik et al, 1985; Crowther, 1991). The PHF have been demonstrated to possess all the properties of the amyloid substance, ie. a cross-beta structure visualized by X-ray diffraction; an 8-12 nm fibrillar structure at the EM level; a green to yellow birefringence under polarized light with Congo Red staining; an insolubility and/or resistance to high ionic buffer or to proteolysis on exposure to guanidine, urea, or conditions such as heating to 100°C with SDS. (Iqbal et al, 1984; Kirschner et al, 1986; Delacourt & Defossez, 1986; Ghiso et al, 1994) .

SPs, demonstrated by H&E, silver, thioflavine S, or Bielschowsky stains, usually are discrete spherical structures with a diameter from a few to 200 μm. They contain abnormal distended neurites, typically surrounding a central core containing substances also possessing the property of amyloid. The major constituent of this amyloid is a 39-43 amino acid polypeptide, called amyloid beta protein (Aβ) (Glenner & Wong, 1984; Masters et al, 1985; Selkoe, 1994a). The SPs also contain inorganic material such as aluminosilicate, as well as many biochemical fragments, including complement, amyloid P component, α 1-antichymotrypsin, glycosaminoglycans, cytokines and apolipoproteins. (Snow et al, 1987; Picken et al, 1990; Duong, 1989; Wisniewski et al, 1992; McGeer et al, 1994; Cornwell et al, 1996). They are often accompanied by astrocytes, microglia and macrophages. (Wisniewski, 1985; Candy, 1986; Abraham, 1988; McGeer, 1995). Several morphological types of SPs have been reported, and suggested to represent stages in an evolution of SP (Wisniewski, 1983; Bergeron, 1989; Probst et al, 1991; Mirra et al, 1993), however, this is debatable (Cataldo, 1996). SPs are most frequent in layer II and III of the cerebral cortex (Pearson, 1985; Tomlinson, 1989; Mirra et al, 1993).

The nature and evolution of NFT and SP remain elusive. Generally, it is not clear whether their presence within nerve cells or neural tissue leads to impaired function, or whether they simply represent some byproducts of other pathological processes that lead to the damage and dysfunction of cells and tissues. Nor is it clear if, or how, the two are pathogenically related (Tomlinson, 1989; Probst, 1991; Perry, 1993; Mann, 1995; Wisniewski et al, 1996), and it is possible that they develop independently of each other

(Terry, 1987; Shaw, 1988; Probst, 1989; Kato, 1989; Engel et al, 1992; Braak et al. 1994).

Other pathological features have been consistently observed in the AD brain, including congophilic (amyloid) angiopathy (Glenner, 1981; Bergeron, 1987, 1989; Vinters, 1992), granulovacuolar degeneration (Ball, 1977), and dendritic changes (Flood & Coleman, 1986; Paula-Barbosa et al. 1987; Masliah et al., 1991; Einstein et al. 1994). Additional abnormalities are still being added to the list of Alzheimer's pathologies, such as selective loss of neuronal populations and decreased activities of their transmitter systems, altered regional metabolism (Davies, 1979; Bondareff, 1982; Curcio, 1984; Price et al., 1986; D'Amato, 1987; Nitsch et al., 1992; Klunk et al., 1992; Growdon, 1995), and/or occurrence of Lewy bodies in the cortex or substantia nigra (Dickson, 1987; Hansen, 1989; 1990), selective cerebral atrophy (Golomb et al., 1993; Cuenod et al, 1993), spongiosis and gliosis of the cerebral cortex (Gustafson et al. 1977; Brun, 1981), as well as some extraneuronal or peripheral manifestations (Joachim, et al. 1989; Nordberg et al, 1990; Scott, 1993; Algotsson et al, 1995; Scinto et al, 1995).

2.2.2.2. Diagnosis of AD

The clinical and pathological features are not always correlated, nor are they specific to AD. They vary in magnitude or stage, and they overlap with those found in the brain during "normal aging" and in other dementias (Ball, 1980; Brown, 1984;

Henderson, 1986; Arriagada et al.1992a), as well as some nondementing illnesses (Iqbal & Wisniewski, 1983; Engel et al., 1992; Reed et al, 1994; Al-Chalabi et al. 1996). There have been continuing efforts to find biological markers that could serve as diagnostic tools. Some of the parameters tested include the CSF content of choline acetyltransferase (CAT), acetylcholinesterase (AChE) (Davies & Maloney, 1976; Perry, 1977; Whitehouse et al, 1981; Beal et al, 1986); tau protein (Flament et al, 1989; Vandermeeren, 1993; Vigo-Pelfrey et al. 1995; Goedert, 1996; Rosler et al. 1996), APPs and/or Aβ (van Nostrand et al, 1992; Southwick et al, 1996); the ApoE genotype and/or phenotype (Strittmatter et al, 1993; Saunders et al., 1993; Poirier et al, 1993; Rosler et al. 1996), as well as extraneuronal markers, including pupillary dilation tests, lymphocyte receptors, skin vasodilation and Aβ deposition. (Nordberg et al, 1990; Scott, 1993; Hyeinonen et al, 1994; Algotsson et al, 1995; Scinto et al, 1995). There have also been continuing efforts to find surrogate markers (Growdon, 1995) to aid diagnosis. Attempts have been made to detect structural alterations in the hippocampus and amygdala (Jack et al, 1992; Golomb et al, 1993; Cuenod et al, 1993) by CT and MRI, to detect regional decreases in cerebral blood flow and glucose metabolism (Frackowiak et al, 1981; Duara et al, 1986; Grady et al, 1990) by functional neuroimaging with PET, single-photon emission computed topography (SPECT) and functional MRI (fMRI), and to detect regional neurochemical abnormalities (Nitsch et al. 1992; Klunk et al., 1992) by magnetic resonance spectroscopy (P^{31} MRS).

However, without knowing the fundamental pathogenic process(s) and

mechanism(s) underlying all the observed pathologies, or the interplay between them, and without even a clear definition of the essential pathological lesions, it has not been possible to locate diagnostic markers that could be practically and reliably applied. (Heinonen et al, 1994; Growdon, 1995; Vigo-Pelfrey et al, 1995; Southwick et al, 1996; Anonymous, 1996; Seuber, 1996) and none of the methods tested has sufficient sensitivity and specificity for diagnosis. Lacking a clear definition and reliable biological markers, the diagnosis of the disease is therefore still made by excluding other causes of dementia, and is confirmed by autopsy, while the neuropathological diagnosis itself is by no means absolute, and still requires clinical confirmation (Mckhann, 1984; Growdon, 1984; Tarn, 1986, Mirra, 1993; Seubert, 1996).

Criteria for AD diagnosis recommended by the National Institute of Neurological and Communicative Disorders and Stroke and the AD and Related Disorders Association (NINCDS-ADRDA) (Mckhann, 1984), although qualitative only, are still widely used. There have been some efforts to diagnose AD based on age-adjusted quantitative morphometric criteria, such as those promulgated by Tomlinson (1976) and his Newcastle group, by Khachaturian (1985) and his American panel, and by Ball (1988) and his team in Ontario, as well as the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) (Morris et al, 1989; Mirra et al, 1991; 1993). These vary in sample taken, staining techniques employed, pathological lesions targeted, as well as the morphometry and quantitative standards used. None of them effectively deal with the coexistence of other pathological entities that are very commonly seen in AD. Moreover, it seems

questionable to use the age of death to adjust the morphometric parameters, since it is possible that duration of the disease and /or the age of onset, rather than age at death has more effect on the severity of the lesions. All of these investigators found quantitative morphological differences between AD and aging, however they all had difficulty in establishing reliable demarcations between normal aging and the very early stage of AD, and thus could not define a set of minimal diagnostic criteria for AD. Depending on the method and the sample population chosen, the correlation between clinical and pathological diagnosis was between 43% to close to 90% (Sulkava, 1983; Wade, 1987; Joachim, 1988; Klatka et al. 1996). In summary, there are no universally accepted reliable criteria for either the clinical or the pathological diagnosis of AD and both rely on procedures which are still largely empirical.

2.2.3. Etiology and pathogenesis of the disease

The risk factors for AD are heterogeneous (Mann,1981, 1995; Jorm, 1985; Liberini et al, 1996; Marx, 1996; Hyman, 1996; Blass, 1993; 1996), the etiology is generally unknown, and so is the pathogenesis. Despite evidence to the contrary, many still consider it part of the normal aging process (Tomlinson, 1970; Jorm, 1987; Van Dras & Blumenthal, 1992; 1993; 1995; Forbs & Hirdes, 1993, Mortimer, 1995). Numerous risk factors, genetic alterations, altered autoimmune mechanisms, and various biopathological processes have been associated with the decline in cognitive functioning and development of Alzheimer's type encephalopathy. However, few of these have been

securely established as relevant to formation of the major pathological lesions, such as NFT, SP, cerebral amyloid angiopathy, or dendritic and synaptic alteration. So many hypotheses on the etiology and pathogenesis have been proposed, it is almost impossible to exhaust all of them, and it is difficult to summarize them in a way that is relevant to the present understanding of the disease, particularly to the pathogenesis. Generally, every one of the theories proposed has been contradicted or challenged by others.

2.2.3.1. Cholinergic system degeneration and AD

Disease-oriented research usually begins with clinical descriptions, then progresses to pathological correlations, and biochemical subtyping (Roses et al., 1996). Involvement of the cholinergic system in Alzheimer's pathogenesis was originally reported (Pope et al, 1964) from biopsies of human brain tissue, and later claimed by others to be a primary event in AD (Davies & Maloney, 1979; Perry, 1986).

The cholinergic theory has several lines of supporting evidence. Extensive studies on both animal and human brains have established that the brain cholinergic system plays an important role in memory and learning (Deutsch, 1971; Drachman & Leavitt, 1971; Bartus et al. 1982; Collerton D, 1986; Miyajima, 1996). Activities of the two enzymes predominantly involved in the CNS cholinergic system are markedly reduced in the cerebral cortex of AD patients and in animal models of dementia (Hope et al, 1964; Perry & Perry, 1980; Savill et al, 1993; Miyajima et al, 1996). These are CAT, the

enzyme responsible for the synthesis of acetylcholine (ACh), and AChE, an enzyme responsible for hydrolysing ACh at the cholinergic synapse (Nachmansohn & Machado, 1943; Korey et al., 1951; Fonnum, 1970; Nachmansohn, 1972; Silver, 1974). The degree of enzyme reduction is correlated with the impairment in cognitive function (Perry & Perry, 1980; Wilcock, 1982; Collerton, 1986), and with the extent of the morphological lesions, NFT and SP, in both topography and severity (Perry et al, 1978; Perry & Perry, 1980). Lastly, pharmacological administration of physostigmine, an AChE inhibitor, arecoline, a muscarinic cholinergic direct agonist, or choline and lecithin, the precursors of ACh, have been reported to enhance learning and memory in animal models of dementia, normal human subjects of various ages, and in selected AD patients. (Davis et al, 1978; Etienne et al, 1978; Drachman & Sahakian, 1980; Asthana et al, 1995; Nitta et al, 1996).

However, the contradictory evidence is equally strong and includes the following observations: Cognitive functioning and memory relies not only on the cholinergic system, but also on multiple neuronal pathways (Bennet, 1977; Shepherd, 1988). Many neuronal systems, as well as extra neuronal systems are defective in AD, as well as in other diseases, such as PD, multi infarct dementia (MID) and chronic alcoholism (Carlsson, 1983; Price et al, 1986; Joachim, et al. 1989; Scott, 1993; Rabey et al, 1996). There has been a consensus emerging from more recent studies, that in some regions, particularly the hippocampus, normal human aging is associated with a progressive decline in CAT, AChE and the number of nicotinic receptors, which can not be clearly

distinguished from that seen in AD (Bartus et al, 1982; Carlsson, 1983; Wilcock, 1995). Strictly controlled studies have shown a heterogeneity of clinical response, or absence of clear benefit from administering various cholinomimetics to geriatric patients with and without AD (Aarsland D et al, 1995; Poirier et al, 1995). All of these findings indicate that degeneration of the cholinergic system is not likely to be a primary event in AD, and it is simply one of the many neuronal systems that are altered by a still unknown etiology during the course of this slowly progressive pathogenesis.

2.2.3.2. Tau protein and Neurofibrillary tangles

Over the past several years significant progress has been made in unravelling the mechanisms that may lead to the formation of NFT, one of the two pathological hallmarks of AD. These are largely based on identifying the molecular composition of tangle PHF, particularly, the tau protein in PHF. Tau protein belongs to the large family of microtubule associated proteins (MAPs), which are co-purified when MT are repolymerized from isolated brain homogenate. It is an abundant protein in the central and peripheral nervous system, and is found in concentrated amounts almost exclusively in neuronal axons (Binder et al, 1985; Matus, 1988; Avila, 1990; 1991; Aberts et al, 1994). It represents a group of six protein isoforms, encoded by a single gene on chromosome 17 and generated by developmentally regulated alternative mRNA splicing (Goedert et al, 1989; 1996; Mukaetova-Ladinska & Roth, 1995). Tau binds MT at tandem repeats of 31-32 amino acids in the carboxyl terminal half of the proteins

(Goedert, 1989; 1996). It promotes MT assembly *in vitro*, and stabilizes MT *in vivo* against depolymerization (Weingarten et al, 1975; Matus, 1988; Lu & Wood, 1991). Although its role is controversial (Sparkman, 1991; 1993; Bondareff et al, 1995; Lai et al., 1995; Goux et al, 1995, 1996), tau protein has been found in PHF, and it has even been claimed to be the major or sole component of PHF (Delacourt & Defossez, 1986; Iqbal et al, 1989; Lee et al, 1991; Goedert et al., 1988; 1989; 1996). It has been hypothesized that the hyperphosphorylation of tau, and presumably the subsequent formation of PHF, leads to neuronal degeneration (Brundke-Iqbal, et al, 1986; Iqbal et al, 1989; Goedert, 1989, 1996), and even to amyloid plaques (Perry, 1993; Shin et al, 1993; Lee & Trojanowski, 1994), as the core process in the pathogenesis of Alzheimer's disease.

This hypothesis has several lines of supporting evidence. The PHF accumulated in neurons as NFT, or in distal dendrites as neuropil threads, are seen primarily and most frequently in the hippocampal and parahippocampal gyrus (Terry, 1978; Brun, 1976; Bergeron, 1989; Mirra, 1993), where they evolve gradually in a stereotyped pattern following the input and output of the hippocampus (Braak & Braak, 1991; 1996). These progress from layer II islands of the entorhinal cortex, where the multimodal cortical-cortical projections terminate, to the hippocampus, the subiculum, and into isocortical areas, particularly the temporal and parietal cortices, as well as other neocortical areas. Precise quantification of this staging system is not yet settled, however qualitative staging is generally correlated with the duration and/or the degree of dementia (Gray, 1982;

Bancher et al, 1993; Arriagada et al, 1992a; Lee & Trojanowski, 1994; Braak et al, 1993; 1996), as well as with the topographical distribution and or severity of other pathological features of AD (Terry et al, 1964; Ball, 1988). The phosphorylation of tau not only precedes (Bancher et al, 1991; Braak et al, 1994), but also is necessary for the neurofibrillary changes to occur (Lee et al, 1991), and all six isoforms of CNS tau proteins have been found to be the major, or the sole, building block of the PHF (Delacourt & Defossez, 1986; Lee et al, 1991, 1994; Goedert et al.,1988, 1989, 1996). The PHF-tau is excessively phosphorylated and far more resistant to proteolysis than its normal counterpart (Lee et al, 1994; Goedert et al, 1994). Numerous tau protein kinases, which phosphorylate tau *in vitro* at various sites of the tau peptides have been identified (Goedert et al, 1992; Biernat et al, 1993; Wisckit et al, 1995; Mandelkow et al, 1996), and in accordance, the activity of enzymes that dephosphorylate tau were found to be decreased in certain brain areas in AD (Goedert et al, 1992; Drewes et al, 1993; Wisckit et al, 1995; Gooch & Stennett, 1996). Phosphorylated tau also shows a reduced binding affinity for MT (Bramblett et al, 1992; Biernat et al, 1993; Mandelkow, 1996), which would lead to MT destabilization, or it may self-assemble *in vitro* into PHF-like filaments (Delacourte & Defossez, 1989; Crowther, 1992; Wille et al, 1992), resembling the aggregation of globulins, prealbumin, or insulin into an amyloid substance (Glenner, 1980; Ghiso, 1994). Both of these possibilities could lead to the obstruction of intraneuronal transport, the disruption of compartmentalization, and impaired relocation of mRNA for translation within different neuronal domains, so that neurons degrade and die. The release of PHF-tau from dying neurons into the extracellular space

has been shown to induce the codeposit of Aβ and other proteins that are frequently found in NFT and SP (Shin et al., 1993; Lee & Trojanowski, 1994).

All of these observations have been interpreted to suggest that tau phosphorylation initiates the formation of PHF which then leads to the formation of one or both of the two histological hallmarks of AD. This theory has been recently encouraged by a newly developed ApoE theory (Strittmatter et al,1993; Poirer et al,1993; Roses,1994; 1995; Roses et al,1996) which is presumably also rooted in tau phosphorylation. Therefore a strong school of pathogenesis has formed referred to as ApoE-Tauist (see 2.2.3.4.).

However, one encounters "maddening inconsistencies" (Binder, 1996) in this tau enigma, as various lines of evidence exist against the Tau/PHF theory. The involvement of the neocortex by NFTs is a highly heterogeneous process in the course of dementia (Price et al, 1991; Giannakopoulos et al, 1995). NFT can also occur in normal aged brains (Price et al,1991; Arriagada et al, 1992b), and even in brains during middle age (Braak et al, 1994). They also appear in certain diseases without the deposition of amyloid plaques, eg. Guam-Parkinsonism, hydrocephalus, or schizophrenia with neuroleptic treatment (Wisniewski et al, 1979; 1994; Gentleman et al, 1991; Kiuchi et al, 1991; Love et al, 1995). Neither PHF accumulation nor the decreased level of normal tau protein correlate with the extent of amyloid deposits in AD or Down's syndrome (Mukaetova-Ladinska et al, 1993; 1994; Giannakopoulos et al, 1995). The amyloid deposits can develop independently and/or much earlier than NFT (Iwatsubo et

al, 1995; Solkeo, 1994b; 1996). Tau protein is a phosphoprotein and its phosphorylation is regulated developmentally (Goedert et al, 1989; 1996; Mukaetova-Ladinska & Roth, 1995) with the highest level of phosphorylated tau protein found in the first postnatal week (Arioka et al, 1993). It is also seen to increase in the CSF in conditions other than AD (Seubert, 1996). Adult human brain tau protein is phosphorylated at the same sites as the PHF-tau present in AD (Matsuo et al, 1994; Gaver et al, 1994), and this can occur physiologically, such as under stress conditions, but does not lead to PHF (Papasozomenos & Su, 1991; Korneyev et al, 1995).

Immunocytochemical and biochemical studies have shown that PHFs contain neurofilament proteins, MAP2, ubiquitin (Perry, 1985; Delacourt & Defossez, 1986; Mori et al, 1987; Yen, 1987), and also nonprotein components, such as glycan and glycolipids (Emory et al, 1987; Sparkman et al, 1991; Goux et al, 1996; Wang et al, 1996). The protein components are largely confined in the "fuzzy coat" which constitutes about 17% in mass, of the PHFs (Wischik et al, 1988; Sparkman, 1991; Goux et al, 1995, 1996). Immunoreactivity of antibodies against Tau is lost after proteolytic stripping of the "fuzzy coat" on PHF (Wischik et al, 1988), and intact PHF remain more clearly defined morphologically after the embedded tau is released by sonication in formic acid (Wischik et al, 1985; Caputo et al, 1992), which indicates that components other than tau may be responsible for the integrity of the PHF, particularly the proteinease resistant core, of which protein components are less than 5% (Sparkman, 1991; Goux et al, 1995). Very few molecules of tau in PHF are actually

hyperphosphorylated (Lai, 1995; Lovestone et al, 1996), and they remain unchanged in the extent of phosphorylation, irrespective of the duration of the disease and magnitude of neurofibrillary changes (Mukaetova-Ladinska et al, 1993; 1994). The repeat region of tau is sufficient to form PHF-like structures in the absence of phosphorylation (Wille et al, 1992; Crowther et al, 1992), as are many naturally occurring amphiphile structures, some of which are indigenous constituents of brain cellular membrane, including glucose, galactose, cerebrosides, sphingosides and gangliosides, which are able to form proteinaceous sheets, concentrically layered tubules, and helical filaments (Sparkman, 1991; Fuhrhop & Helfrich, 1993; Wilson et al, 1995; Goux et al, 1995; 1996).

All this evidence suggests that Tau may not be a major component of PHF. Tau phosphorylation is neither necessary nor sufficient for the formation of PHF. Neither tau polymer nor the polymerization process itself can be assumed to be neurotoxic at this stage (Binder, 1996). None of the other mechanisms that propose to account for tau aggregation and insolubilization, such as cross-linking of protein monomers by transglutaminases (Montejo de Garcini et al, 1986); modification by glycation end products (Yan et al, 1994; Wang et al, 1996), and the C-terminal cleavage at glutamate-391 (Novak et al, 1993), have offered a complete explanation for the initiation of NFT. Moreover, even the particular role of the NFT itself in the whole process of AD is still not clear. It could be regarded as a nonspecific alteration of neuronal cytoskeletal proteins, that might be initiated in many different pathological settings (Kiuchi et al,

1991; Love et al, 1995 Shankar et al, 1989), including head trauma, viral disease, Down's syndrome, Guam-Parkinsonism, stroke, and theoretically in multiple forms of brain atrophy (Seubert, 1996). Furthermore, there is still no link between tau and APP, whose gene mutations and abnormal metabolism are associated with Aβ deposition in familial AD (Selkoe, 1994; 1996; Games et al, 1995). Thus there is a central element missing in this theory of pathogenesis.

2.2.3.3 APP/β-amyloid theory

Identifying the biochemical nature of the amyloid deposition in AD has been, and still is, a major focus in research on the disease. By the early 1970's, amyloid plaques had been isolated from AD brains and shown to be proteinaceous (Nikaido et al, 1970, 1971). One great advance was the isolation and sequencing of a 4-kilodalton (kDa), 39/40 amino acid peptide from meningovascular amyloid (Glenner & Wong, 1984), and shortly after, a similar peptide with 42/43 amino acids from the amyloid plaques (Masters et al, 1985), that we now call beta-amyloid protein (βA4 protein or Aβ). Provision of the amino acid sequence of Aβ soon led to cloning the cDNA encoding part or all of its precursor, referred to as the β-amyloid precursor protein (βAPP, or APP), which was a large polypeptide of 695 amino acids, containing a single hydrophobic stretch near its carboxyl terminus (C-terminus) and having the properties of a membrane-spanning domain (Goldgaber et al, 1987; Kang et al, 1987; Tanzi et al, 1987). Thereafter, APP and Aβ have been extensively studied from all possible aspects, including molecular

genetics, biochemistry, immunohistochemistry (Wisniewski et al, 1992; 1994; Golde & Younkin, 1995; Solkeo, 1994; 1996, Yankner, 1996) transgenic animal modelling (Sisodia et al, 1995; Games et al, 1995), as well as physiochemistry (Lansbery, 1992; 1996; Nandi, 1996). All of this has led to a predominant theory, that deposition of Aβ is the causative agent in AD pathology, and abnormal metabolism of APP/Aβ has a central role in the pathogenesis (Hardy & Higgins, 1992; Selkoe, 1994a; 1994b; 1996; Selkoe et al, 1996; Yankner, 1996).

Structure and expression of the APP. APP designates a complex group of type I integral membrane glycoproteins, 100-140 kDa in relative molecular mass, with the large N-terminal domain located outside of the membrane, and small C-terminal domain inserted into the membrane. The ~39 to ~43-residue Aβ is a proteolytic fragment of APP, that begins with 28 residues (Aβ1-28) external to the single transmembrane domain and extends 11-15 residues (~Aβ39, ~Aβ43) into that domain. A single gene encoding the human APP is located on chromosome 21, and generates more than 10 APP isoforms through alternative splicing of APP mRNAs. Four major isoforms, APP695, 714, 751 and 770, contain the Aβ sequence, APP 770 and APP751 contain a 56-amino acid insert in the extramembranous region with about 50% homology to the Kunitz family of serine protease inhibitors (KPI-like motif) (Goldgaber et al, 1987, Kang et al, 1987; Kitaguchi et al, 1988; Tanzi et al, 1988; Konig et al, 1992). APP is expressed in virtually all neural and nonneural mammalian tissues, with the highest levels of APP and its

transcripts in brain and kidney, while the lowest, barely detectable level, is in the liver (Selkoe et al, 1987; Tanzi et al, 1988; Yamada, 1989). The expression pattern is similar between species. In humans APP770 is the most widely and abundantly expressed, while APP695 shows particularly high levels in neurons within the brain (Selkoe, 1994b; Yankner, 1996). A key question arises with these discoveries: how can Aβ, a hydrophobic, putatively membrane-anchoring portion of the APP, be cleaved, released from the cell, and accumulate as amyloid deposits?

A variety of postranslational modifications of APP including sulfation, phosphorylation, and both N- and O-linked glycosylation (Weidemann A et al, 1989; Hung AY & Selkoe, 1994b) have been characterized in cultured cells, and presumably occur during APP trafficking into the endoplasmic reticulum and through the Golgi (Selkoe, 1994b; Selkoe et al, 1996). All of these postranslational modifications are either independent of (glycosylation, Selkoe, 1994b), or only indirectly linked (phosphorylation, Suzuki et al, 1992; Hung et al, 1993; Selkoe, 1994b) to the regulation of APP metabolism.

Cellular processing of APP and physiological production of Aβ. There appear to be at least two major pathways for APP metabolism. The first, α-secretory pathway (Sisodia et al, 1990; Esch et al, 1990), involves cleavage by a protease referred to as "α-secretase" within the Aβ sequence (Aβ 16) outside of the membrane, which releases a large soluble ectodomain (referred to as α-APPs) (Selkoe, 1996) and leaves behind a

small membrane-associated ~10kDa C-terminal fragment (CTF). Both α-APPs and the ~10kDa CTF have been recovered from the brain and CSF (Pasternack et al, 1992). It has been shown, using site-specific mutagenesis (Wang et al, 1991; Zhong et al, 1994) that this protease is not like most endoproteases which are sequence-dependent, but it rather seems to act a specific distance from the membrane and prefers a bulky, hydrophobic amino acid residue next to the peptide bonds it cleaves (Hooper et al,1995). This relative nonspecificity of α-secretase indicates a novel type of endoprotease with a cleavage specificity regulated more by secondary structure of the substrate and distance from the membrane than by the primary sequence, or it may represent a combined activity of multiple proteases acting at or near the same site (Zhong et al, 1994; Golde & Younkin, 1995). The APP cleavage in this pathway can occur on the cell surface or intracellularly (Sisodia, 1992; Haass et al, 1992), but the precise location of intracellular processing has not been defined yet. Since the α-secretase cleaves APP on the carboxyl site of lys at Aβ16, releasing α-APPs ending at Aβ15 or Aβ16, and CTF beginning at Aβ17, it presumably precludes the formation of Aβ. This led to the early conclusion that Aβ is not derived from normal APP metabolism (Sisodia et al, 1990).

This conclusion was abandoned following the observation (Chen et al, 1990) that APP contains a conserved Asn-Pro-Thr-Tyr sequence in its cytoplasmic tail. This is known to mediate the endocytosis of cell-surface proteins via clathrin-coated-pit internalization, with subsequent targeting to the endolysosomal system, indicating an alternative pathway for APP metabolism. It was then suggested that APP is internalized

and trafficked to endo/lysosomes for degradation (Haass et al, 1992; 1993; Golde et al, 1992) or rapidly recycled to the cell surface (Yamazaki et al, 1996). Within this pathway, a "β-secretase" may cleave intact APP at precisely the N-terminus of the Aβ segment (Seubert et al, 1993; Koo & Squazzo, 1994). This results in truncated APPs (referred to as β-APPs), and a $\sim$12 kDa C-terminal fragment, which may then undergo "γ-secretase" cleavages within the hydrophobic transmembrane domain that physiologically release the 40- or 42-residue Aβ. Soluble Aβ has already been found in CSF of both healthy individuals and those with AD (Seubert et al, 1992; Shoji et al, 1992). The lysosomal proteinases, cathepsins D,B, and S, together with other lysosomal hydrolases, as well as their endogenous inhibitors cystatins, have all been found in SPs, and are suggested to be involved in the generation of SPs and NFTs (Bernstein et al, 1990; 1996; Cataldo et al, 1991; Ii et al, 1993). However, endo/lysosomes may also contain full-length APP, and multiple forms of CTFs, some of which are large enough to contain the amyloidogenic portion. This has been documented by various experiments including inhibition of endoso/lysosomal processing, cell surface APP labelling, and purification of late endo/lysosomes. (Haass et al, 1992; Golde et al, 1992; Siman et al, 1993). The fact that APP can be normally cleaved at various sites near (Estus et al, 1992; Wolozin et al, 1992) or within (Cheung et al, 1994; Zhong et al, 1994) the Aβ sequence, creating different CTFs, indicates that APP processing is highly complicated, and may involve several pathways with a variety of enzymes.

Many questions raised by these findings are yet unanswered including the

following: Since the inclusion of residues beyond Aβ 40 markedly enhances amyloidogenicity (Hilbich et al, 1991; Golde & Younkin, 1995), it is important to understand the proteolytic mechanism leading to production of Aβ with different C-termini, particularly the one with γ-secretase. This enzyme should be capable of cleaving CTFs or APP holoprotein in situ within the membrane, while no known endoprotease has been shown to be able to do this. The proteases involved in the generation of Aβ have not been identified, including the α-, β-, or γ-secretases, and the cellular compartment where the proteolytic events occur is not certain (Haass et al, 1993; Selkoe, 1994b; 1996). Recent finding of at least two secreted APPs that do not end at residue 16 of Aβ (Anderson et al, 1992; Seubert et al, 1993), and the demonstration of Aβ17-42 as a major species present in diffuse plaques (Golde & Younkin, 1995), indicates that the secretory pathway, including α-secretase may also be involved in the production of some of the Aβ that deposits in the AD brain. It is therefore difficult to interpret these numerous findings about APP cellular processing, leaving only the general impression that Aβ could be continually derived from normal APP metabolism, and secreted from cells by several possible exocytic and endocytic pathways.

Normal functions of APP/Aß and neurotoxicity of Aβ. The physiological functions of APPs and Aβ have not been defined. It has been surmised, based on the KPI-like motif that APP751 and APP770 contain, that α-APP molecules may function in human plasma as an inhibitor of factor XIa of the coagulation cascade (Smith et al, 1990). It has also been postulated, on the basis of cell culture studies, that APPs

containing other than the KPI motif may function as a growth promoting or autocrine molecule (Saitoh et al, 1989, Small et al, 1996), a neuroprotective molecule (Mattson et al, 1993), or a mediator of cell-cell or cell-substrate interactions (Schubert et al, 1989; Qiu et al, 1995; Small et al, 1996). However, homozygous APP-knockout mice surprisingly show no evidence of developmental deficit or neuronal degeneration (Zheng et al, 1995). This may be due to compensation by the existence (Wasco et al, 1992; 1993) of proteins homologous to APP, amyloid precursor-like proteins (APLP).

Because Aß is the major constituent in one of the primary AD lesions, the SP, work on the biological effects of Aβ has revolved largely around its toxicity. The observation that fetal human neurons and other cultured cells continuously release significant amounts of Aβ indicates a possible physiological function for Aβ in soluble form. This concept has been supported by finding a neurotrophic effect of Aβ on cultured neurons when it is added to the aqueous culture medium (Whitson et al, 1989) or when it is incorporated as a matrix molecule (Koo et al, 1993). Nevertheless, it is still difficult to draw conclusions on the physiological function of either APPs or Aβ. Although the interpretation is controversial, neurotoxic effects of Aβ and Aβ fragments have been documented both *in vitro* and *in vivo* (Yankner et al, 1990; Pike et al, 1991). Similar toxic properties of Aβ-containing APP CTFs (Yankner, 1989; Fukuchi et al, 1992), as well as abnormally metabolized APP (Yankner, 1989; Hyashi et al, 1992) have also been reported. The debate about this issue, which has triggered plenty of controversy and hostility beyond the level of pure scientific interest, ie "commercial commitments and

priorities for discovering the amyloid field" (Marx, 1992), is still in progress.

APP/Aβ in the pathogenesis of AD. There is mounting evidence that APP and/or Aβ play a key role in AD pathogenesis. Some of the pertinent information is as follows: Aβ deposition in the brain is the earliest pathological change preceding the formation of NFT, neuronal death and clinical symptoms (Braak & Braak, 1991; Ohgami et al, 1991; Mann, 1986; 1995). The APP gene is located on chromosome 21, and Down's syndrome (DS) individuals who have trisomy 21 almost invariably develop clinical and pathological features of AD if they live over the age of 30 (Mann et al, 1986; Iwatsubo et al, 1994). This indicates that the APP gene is likely to be the locus, or one of the loci responsible for the AD pathology in DS. All four known genetic alterations underlying familial AD have been found to increase the production or deposition of Aβ in brains (Selkoe, 1997). Several missense mutations have been detected close to the Aβ region of APP in several families with autosomal dominant early onset AD (Goate et al, 1991; Suzuki et al, 1994). A one point mutation (G-C) causing a single amino acid substitution (Glu-Gln) at position 22 of the Aβ has been detected in a rare form of autosomal dominant cerebral β-amyloidosis. This is the hereditary cerebral hemorrhage with amyloidosis- Dutch type (HCHWA-D) (Hardy, 1992), which is marked by severe Aβ deposition in the cerebral vessel walls, and large numbers of amyloid plaques in the brain parenchyma. Transfected cell lines expressing some of these mutations *in vitro* have demonstrated a marked increase in production of Aβ and or ~12 kDa Aβ-containing CTFs (Citron et al, 1992; Cai et al, 1993; Suzuki et al, 1994). More

compelling evidence has come from the recent achievement of a transgenic mouse model overexpressing one of the mutations (APP717) associated with familial AD (Games et al, 1995). These mice have large amounts of Aβ40 and Aβ42 in the extracellular fluid of the brain and exhibit diffuse and fibrillar Aβ deposits in the neuropil, accompanied by dystrophic neurites and reactive microglia and astrocytes. *In vitro* studies have demonstrated Aβ cytotoxicity on neurons (Pike et al, 1993; Lorenzo & Yankner, 1994), astrocytes (Pike et al, 1994), microglia (Meda et al, 1995), endothelial cells (Thams et al, 1996) and human cerebral smooth muscle cells (Van Nostrand, 1996). *In vivo* neurotoxicity has been documented by microinjecting Aß into adult rat brain (Frautschy et al, 1991; May et al, 1992), particularly in its aggregated or beta-pleated state (Pike et al, 1993; Lorenzo & Yankner, 1994; Howlett et al. 1995). Recently an APP epitope distal to Aβ has been found in PHF, and a synthetic peptide containing this region binds to tau *in vitro* causing it to become fibrillogenic (Giaccone et al, 1996).

Assigning a crucial role to Aß in the pathogenesis of AD also raises many questions, such as:

What is the cellular origin of the Aβ deposited in plaques, around vessels, neurons (Selkoe et al, 1989; Cork et al, 1990), astrocytes or microglia (Siman et al, 1989; Wegial & Wisniewski, 1990; Wisniewski & Wegial, 1993; Haass et al, 1991), and in vascular smooth muscle cells (Tagliavini et al, 1990), plasma or CSF (Selkoe, 1989; Seubert, 1992)?

Why does the Aβ only deposit in certain areas of the brain, while APP is widely

expressed in almost all neural and non-neural cells?

Why do some normal aged individuals (Katzman et al, 1988; Arriagada et al, 1992), or some younger patients with non-dementing illness (Engle et al, 1992) have a similar pattern of SP in the brain?

Why do some transgenic mouse models overexpressing a mutant APP gene associated with AD show either no Alzheimer-like pathology (Lamb et al, 1993; Hsiao et al, 1995; Malherbe et al, 1996) or only a small quantity of Aβ-immunoreactive deposit (Quon et al, 1991; Moran et al, 1995)? Why do the successful models, (Games et al, 1995) which do produce amyloid plaques, not develop any NFT?

Assuming that some transgenic models support the hypothesis that excessive accumulation of soluble, and then aggregated Aβ40 and Aβ42 peptides can initiate AD changes, what is the mechanism(s) underlying the different APP mutations associated with the accumulation and deposition of Aβ in other early onset AD families? What accounts for the AD majority who have no APP mutation?

What accounts for the absence of Aβ deposition in the fetal brain, which is physiologically releasing a high quantity of Aβ throughout gestation (Selkoe, 1994b)?

Why do Aβ fibrils induce synapse loss and neurodegenerative changes in culture without forming plaques (Lorenzo & Yankner, 1994; Howlett et al. 1995)?

What is the role of each of the numerous biochemical fragments that have been detected in the amyloid plaques, besides the aggregated Aβ, such as apolipoprotein E (Namba et al, 1991; Wisniewski & Frangione 1992), APP, α-antichymotrypsin (Picken et al, 1990), IgG, complement proteins, cytokines (McGeer et al,1994), amyloid P (Duong, 1989),

glycosaminoglycans (Picken et al, 1990), Sp40, (Ghiso et al, 1993) and inorganic materials such as aluminosilicate (Candy et al, 1992; Kawahara et al, 1994), and zinc (Bush et al, 1994)? The complete molecular composition of the fibrillar structures in the SP, the mechanisms of their assembly and role in the disease have yet to be explained.

This list of questions all related to one of the AD-associated genes, clearly illustrates the current need for a unifying hypothesis to explain the pathogenesis of AD. In addition to the APP gene, and the ApoE gene, implicated recently in the late-onset form of familial AD (Strittmatter et al, 1993) (reviewed below), at least two additional genes have been implicated to date in early-onset AD. One associated with the majority (St.George-Hyslop et al, 1992; Schellenberg et al, 1992) of early-onset FAD has been recently identified to be S182 on chromosome 14 (Sherrington et al, 1995); the other which is highly homologous to S182, STM2 (second transmembrane gene), has been identified on chromosome 1 in Volga German families with early onset FAD (Levy-Lahad et al, 1995). S182 and STM2 have been recently termed presenilin 1 and 2(SP1, SP2) respectively in anticipation of a gene family that causes early-onset FAD (AD Collaboration Group, 1995; Wasco et al, 1995). Encoded by these two genes are homologous 467- or 448-residue polypeptides, whose sequences suggest a structure with 7-9 transmembrane domains. Their functions are unknown, presumably involving intracellular protein trafficking, because of the high homology to two integral membrane proteins from C. Elegans SEL-12 and SPE4 (Levitan & Greenwald, 1995). We do not currently understand the mechanisms by which the normally occurring apoE

polymorphisms, and the two non-APP genes in which at least 27 missense mutations have been reported (Yankner, 1996; Selkoe, 1996), can predispose subjects to AD.

2.2.3.4 Apolipoprotein E and AD

The finding of apolipoprotein E4 (apoE, protein; APOE, gene) as a major risk factor for late-onset AD (Strittmatter et al, 1993; Roses, 1994) is one of the great progressions in recent AD research. Until then, little attention had actually been given to its metabolism in the brain, because it was widely believed that apoE mRNA was not expressed in neurons, nor was any apoE located in neurons (Strittmatter & Roses, 1995; Roses et al, 1996). Attempts to understand the role of apoE in the pathogenesis of AD have opened up new fields of basic neuroscientific inquiry with potential ramifications beyond AD. Although the underlying mechanism by which apoE is associated with AD is still hypothetical, and findings are somewhat controversial, APOE appears to be a major susceptibility gene determining the rate of AD expression, particularly the late-onset form, while the Aβ burden has been suggested to be merely "tautologically defined as relevant to AD" (Roses, 1994), and simply a secondary consequence dependent on APOE genotype and duration of disease (Strittmatter et al, 1993; Roses, 1994; Roses et al, 1996; Mahley et al,1996).

Apolipoprotein E. ApoE is a 299-amino acid protein with a molecular weight of 34 kD, which was first identified as a component of triglyceride-rich lipoproteins in

serum (Shore & Shore, 1973). ApoE is produced in many organs, mostly in the liver, brain, spleen, and kidney and has long been known to play a key role in plasma cholesterol and triglyceride metabolism through its ability to interact with lipoprotein receptors, particularly the low density lipoprotein (LDL) receptors (Mahley,1988; Mahley et al,1996).

Polymorphism. Using isoelectric focusing (Utermann et al,1980) and two-dimensional electrophoresis (Zannis & Breslow,1981), apoE was recognized to be polymorphic, with three major isoforms referred to as apoE2, E3, and E4, which are products of three alleles, $\epsilon 2$, $\epsilon 3$, and $\epsilon 4$ at a single gene locus. There are therefore six phenotypes, homozygous E2/2, E3/3, and E4/4 and heterozygous E2/3, E2/4 and E3/4, determined by expression of any two of the three alleles. The most common allele is $\epsilon 3$, and the most common phenotype is E3/3; therefore apoE3 is considered to be the ancestral isoform, and apoE2 and apoE4 are variants. The three isoforms differ by one or two amino acid substitutions at positions 112 and/or 158: Apo E3 contains a Cys and an Arg at positions 112 and 158 respectively, while E2 contains a Cys, and E4 contains an Arg at both positions (Davignon et al, 1988; Mahley, 1988).

Biosynthesis, structure, and metabolism. The human APOE is 3.7 kb in length and contains four exons on the long arm of chromosome 19. The apoE mRNA is 1163 bp in length and is found mostly in the liver which produces 2/3 to 3/4 of the plasma apoE. Significant levels also occur in the brain, spleen, lung, adrenal, ovary, kidney and

muscle (Elshoubagy et al, 1985; Mahley, 1988). According to the location, hepatic parenchymal cells, macrophages, astrocytes or smooth muscle cells are primarily responsible for apoE production in different organs (Lin et al, 1986; Pitas et al, 1987; Mahley, 1988). Physicochemical studies have identified two independently folded structural domains that can influence the properties of each other, one on the N-terminus from residues 1-191 that interacts with the LDL receptor, and the other on the C-terminus from residues 216-299, that contains major lipoprotein binding determinants (Aggerbeck et al, 1988; Weisgraber, 1990; 1994; Dong et al, 1994). Besides the LDL receptor, apoE also binds to other cell surface receptors, including the LDL related protein, VLDL receptor, and the scavenger receptor. ApoE isoforms differ in their interactions with the LDL receptor and in binding to cholesterol-containing lipid particles. ApoE3 and E4 bind the LDL receptor with much higher affinity than E2. Defective receptor binding has been blamed in some Type III hyperlipoproteinemias in individuals homozygous for apoE2 (Mahley,1988). ApoE2 and E3 display a preference for high density lipoprotein (HDL) particles, while apoE4 preferentially binds to triglyceride-rich LDL, VLDL and IDL particles (Weisgraber, 1990; 1994; Dong et al, 1994) . This isoform-specific preference is postulated to be caused by the Cys-Arg interchange at position 112, which distinguishes apoE2 and E3 from E4, and influences the lipoprotein class to which the C-terminal domain binds (Weisgraber, 1990). This seems to explain the observation that individuals with apoE4 have higher plasma levels of cholesterol and LDL, and are at higher risk for atherosclerosis than individuals with apoE3 or E2 (Davignon et al, 1988).

Metabolic pathways involving apoE can be summarized in three categories (Mahley, 1988). The first is an "endocrine-like" function: transport of lipids between cells of different organs, such as transport of chylomicrons from the intestine to the liver and peripheral tissues, transport of VLDL remnants from the liver to peripheral tissues, and transport of cholesterol from peripheral tissues to the liver. The second is a "paracrine-like" function: redistribution of lipids among cells within an organ or tissue, for example apoE being produced by various cells within the tissue is capable of targeting a lipid complex within the local environment, and can facilitate uptake by cells including the neuronal processes in the CNS and PNS (Pitas et al, 1987; Poirier et al, 1995). Thirdly, apoE secreted by macrophages has an "autocrine-like" function, by capturing lipids from the environment, and delivering them back to the macrophages where they are stored. ApoE also has functions unrelated to lipid transport. It modulates immunoregulation by mediating lymphocyte activation and the proliferation of lymphocytes that contain LDL receptors. It also supports peripheral nerve regeneration by acting as a neurotrophic or neurotropic factor (Ho et al, 1976; Mahley, 1988).

ApoE in the brain. A series of observations indicate a significant neurobiological role for apoE. The brain is second only to the liver in apoE mRNA content, where it is synthesized and secreted primarily by astrocytes (Elshourbagy et al. 1985; Pitas et al, 1987). ApoE has been recently detected immunohistochemically within tangle-bearing and tangle-free nerve cells (Strittmatter et al, 1993; Han et al, 1994) indicating a role in neuronal metabolism and degeneration. The major apoE receptor on neurons is the LDL

related receptor (Rebeck et al, 1993). ApoE-containing lipoproteins are found in the CSF, and appear to play a major role in lipid transport in the CNS. ApoE, through an LDL-mediated pathway, is involved in the metabolism and reutilization of lipid in the growth and maintenance of myelin and axonal membranes (Ignatius et al, 1986; Poirier et al, 1994). ApoE levels dramatically increase after peripheral nerve injury, and it appears to participate both in the scavenging of lipids released from degenerating axons and in the redistribution of these lipids for neuritic sprouting and remyelination (Ignatius et al, 1986; Rothe & Muller, 1991).

ApoE and AD. The standard positional cloning methodologies which have revealed gene mutations on chromosome 21, 14 or 1 in early onset AD families, have been confounded by an unclear mode of inheritance in late-onset AD. Using excessive allele sharing methods in which an assumption regarding the mode of inheritance is not required (Weeks & Lange,1988) and the same highly polymorphic genetic markers used in classical linkage studies, a linkage to chromosome 19q13.1-13.3 region has been found (Pericak-Vance et al, 1991). The apoE gene is located in the middle of this relevant region of chromosome 19q. By studying the binding of proteins by Aβ, apoE has been fished from CSF as one of many proteins exhibiting irreversible binding (Strittmatter et al, 1993). The relationship of APOE to AD has been confirmed by finding a significant increase in APOE ϵ4 allele frequency in autopsy-confirmed sporadic AD patients, in a consecutive series of 80 probable or possible AD compared to their spouses, and in the first affected twin in a group of 62 twin pairs (Saunders et al, 1993), as well as in

various ethnic and racial populations (Sakoda et al, 1994; Mayeux et al, 1994) around the world. The APOE ϵ4 allele was found to increase the risk and lower the mean age of onset, while the APOE ϵ2 allele decreases the risk and increases the mean age of onset (Corder et al, 1993; 1994; Saunders et al, 1993). APOE thus appears to be a major susceptibility gene for AD. The fact that no linkage disequilibrium with a nearby gene or marker has been found (Mayeux et al, 1993; Yu, 1994), provides further support for the theory, which has since been extended to account for some early onset AD as well (Okuizumi et al, 1994; van Duijn et al, 1994).

Research on the possible biochemical mechanism by which the normal polymorphism of APOE alters the predisposition to AD has become a hot field. There are several lines of supporting evidence for a role of apoE in the etiopathogenesis of AD. ApoE has been found immunohistochemically in both the SPs and in NFTs (Namba et al, 1991; Wisniewski et al, 1992; 1995), and has also been shown to bind to soluble Aβ *in vitro* (Wisniewski et al, 1993). ApoE3 irreversibly binds with tau *in vitro* (Strittmatter et al, 1994). It also binds to MAP2c (Huang et al, 1994), which presumably (Strittmatter et al, 1994; Roses, 1994; Roses et al, 1996) can then prevent tau from being hyperphosphorylated, and self-assembling into PHF. This binding does not occur with apoE4. ApoE4 irreversibly binds to Aβ peptide *in vitro* more rapidly than does E3. This is parallelled by similar differences *in vivo* and Aβ immunoreactivity of plaques is greater in AD patients who are homozygous for APOE ϵ4 (Schmechel et al, 1993). The brains of APOE knockout mice (Masliah et al, 1995) show decreased synaptic density,

and vacuolization of dendrites with fragmented MT-like structures. ApoE4 destabilizes MT *in vitro* (Nathan et al, 1995) with an isoform-specific effect on MT function. All of these observations have led some apoE investigators to renounce an initial and causative role for Aβ deposition (Hardy & Higgins, 1992; Selkoe, 1994a; 1994b; 1996. Yankner, 1996) in AD. They view the disease expression as a function of APOE genotype (Roses, 1994; 1996), presumably through an action on "chaperoning Alzheimer's amyloids" (Wisniewski et al, 1992; Fragione et al, 1994), or through its isoform-specific interactions with proteins found in NFTs and SPs, particularly, tau and MAP2c (Roses, 1994; 1996; Strittmatter & Roses, 1996). More radical viewpoints suggest that phenotypic definitions of AD need to be re-evaluated to incorporate genetic information on APOE (Strittmatter & Roses, 1996).

As is the case for all current hypotheses on AD, the apoE-tau theory presents numerous gaps and contradictions. The association of APOE4 with AD has been disputed by some recent epidemiological studies (Lannfelt et al, 1994; Sobel et al, 1995), or limited to ages below 70 (Asada et al, 1996; Blacker et al, 1997; Evans et al, 1997), with regard to effect on the age of onset (Blacker et al,1996). An increased risk for AD has been reported in some APOE2 populations (van Duijin et al, 1995; Maestre et al, 1995). Many individuals who inherit one or even two copies of the APOE ϵ4 escape AD, even at very old age, and conversely many AD patients do not carry the ϵ4 allele (Bird, 1995; Blacker et al, 1996). About 60% (Tanzi et al, 1996) of late-onset AD patients are not accounted for by the APOE ϵ4 factor, and they can still develop the full-

blown AD phenotype, including those who are APOE ε3/3 homozygous. Other genes, such as APOC (Kamino et al, 1996) and a non-Aβ component of AD amyloid (NAC) precursor gene (NACP) (Xia et al, 1996) have been recently reported to interact with or overwhelm the effect of APOE.

The apoE theory places an emphasis on a critical permissive role for apoE4 in tau hyperphosphorylation-NFT formation (Roses, 1994). However, NFT are known to also occur in a range of neurodegenerative diseases other than AD, with no apparent relationship to apoE genotype. Down's syndrome also shows no segregation with regard to apoE genotype (Sunders et al, 1993) and all patients develop robust NFT. Moreover, as mentioned above, the roles of tau phosphorylation in NFT formation, and of NFT itself in AD, have been seriously challenged (Wischik et al, 1988; Sparkman, 1991; Goux et al, 1995, 1996; Lai, 1995; Lovestone et al, 1996; Anderton & Lovestone, 1996). All of this suggests that APOE ε4 is a risk factor for AD, but not a causative one. The mechanisms by which apoE4 leads to earlier development of AD, and enhanced phenotypical expression of Alzheimer's pathology remain elusive.

2.2.3.5. Aging and AD

It is generally accepted that age is the most important risk factor for AD (Zhang et al, 1990; Blass, 1993; Letenneur, 1994; Macera et al, 1995; Fratiglioni et al, 1997). Whether the relationship between AD and aging is an accelerated/exaggerated aging

process, or a qualitatively different disease process, is still a matter of debate. Fundamental questions involved in this debate are basically: a) What is aging, a normal biological process or a disease? and b) What are the biopathological and/or psychological differences between aging and AD?

Aging *The definition of biological aging*. Aging is poorly understood at present (Evans, 1988; Thompsome & Foes, 1990). Traditionally, in order to separate biological from pathological events, aging was regarded as changes in cells or organs that were "intrinsic, irreversible, inevitable, progressive and universal" (Rowe & Schneider, 1990; Thompson; 1990). Disease, in contrast, was generally caused by extrinsic agents with biological aging only conferring a state of increased vulnerability, and was avoidable through preventive and therapeutic measures. In light of modern scientific knowledge, these kinds of definitions are not satisfactory in distinguishing normal biological aging from the pathological event of a disease process. It is now recognized that humans undergo a multiplicity of aging phenomena deriving from a diversity of mechanisms (Evans, 1988; Horvath & Davis, 1990; Mooradian & Wong, 1991; Finch et al,1997), occurring in various organs, tissues and cells. Aging-related changes have also been identified in DNA, RNA and proteins, including post-translational modification and the emergence of antigenic alterations (Mooradian & Wong, 1991; Vijg & Wei, 1995). The rate of biological aging may be under genetic control, and some aspects of cellular aging may well have pathogenic significance. Therefore a newer definition of aging as, "a process of progressive, generalized impairment of function, resulting in an increasing

age-specific death rate" (Kirkwood, 1994) seems to be one of the most satisfactory (Wilcock, 1995). Rather than attempting to define the aging process in terms of specific biochemical or other processes, which are probably numerous and diverse, it takes a more holistic view and describes it, at the level of the individual, as an increasing accumulation of degenerative changes, and at the population level, as an age-related death rate.

The cause(s) of aging. These remain elusive, although numerous theories have been proposed, which could be summarized as follows:

Population level

1) General - the evolutionary theory of aging (Kirkwood, 1977)
2) Specific - The disposable soma theory

Organ level

1) Neural and endocrine theories (Korenchevsky & Jones, 1947; Finch, 1987)
2) Immunological theories (Walford, 1969)

Cellular and molecular level

1) Programmed senescence theory (Hayflick, 1968)
2) Free radical theory (Beal, 1995; Harmon, 1955)
3) Protein error catastrophe theory (Orgel, 1963; Gershon & Gerson, 1976)
4) Somatic DNA mutation (Szilard, 1959; Macieira-Coelho, 1995).

Many of these theories, besides being debatable themselves, overlap, interact or are similar. Some, such as the free radical theory, can overlap or form the basis of others, including immunological and neuroendocrine ones. Moreover, none have been rigidly defined, or mathematically quantified (Vija & Wei, 1995; Kritchevsky et al, 1996). These facts, taken together with the generally accepted notion that aging is not a single unifying process explainable by a single mechanism, make it of minimal use to discuss aging on the basis of the various theories proposed. It appears of more value to assess the numerous factors that could lead to the variety of deteriorating processes that together form aging.

Aging - disease dichotomy. As mentioned above, it is generally accepted that biological aging can be distinguished from diseases of the aged (pathological aging) on the basis that the latter are not genetically programmed, but are caused primarily by external factors, including risk factors such as diet, smoking, and environmental toxins, and are therefore preventable. Moreover, no single disease is "universal", or present in all species or in all aged subjects (Blumenthal & Premachandra, 1990). There exists a "separatism" slogan "aging is not a disease", which emphasizes that biological aging and pathological aging are essentially independent phenomena, although they may impinge on one another, for example an older individual who loses the ability to function probably does so because a specific disease accelerates or is superimposed on the aging processes. Furthermore, the "separatists" see the association between the two, such as the increase in prevalence of age-related diseases, simply as a natural selection, and

argue that aging processes are either relatively unimportant or even non-existent. That is, time is merely a risk factor for various diseases. As an analogy (Forbes & Hirdes, 1993), an object placed in the middle of traffic will eventually be struck by a vehicle, and the odds of being struck will increase with time. The damage caused by being struck can not be attributed to an aging process, nor can the increased prevalence of collisions among objects placed there for a longer time.

However, this aging-disease dichotomy has long been challenged in gerontology (Forbs & Gentleman, 1973; Evans, 1988; Von Dras & Blumenthal, 1992; Forbs & Hirdes, 1993; Blumenthal, 1995). Many molecular, cellular or tissue alterations seen in disease initiate in benign form as age-dependent phenomena in nondiseased individuals, and could be regarded as harmless before they progress to a certain degree. Mild osteoporosis, stenosis, or hypertension are unlikely to be clinically significant, and many people with greater than 75% arterial stenosis (Forbes & Hirdes, 1993) may suffer no clinical events, although they are considered to have disease and are at a relatively greater risk for such events. In contrast many identified age-related changes in molecular, cellular or tissue function (Mooradian & Wong, 1991; Vijg & Wei, 1995) may well have pathogenic significance. At which point, based on what criteria can we define them as diseases? Many age-related diseases, including diabetes, osteoporosis, atherosclerosis, and cancer, like aging itself, are multifactorial in nature, and fulfill at least some of the criteria that define biological aging. Smoking-induced shortening of life may progress along a similar biological pathway as that of natural aging, implicating a

commonality between the effects of risk factors and those of genes/biological processes associated with intrinsic aging. The "unionists" believe there is continuum between biological aging and age-related diseases, and that "to draw a distinction between disease and normal aging is to separate the undefined from the undefinable." (Evans, 1988). There is no doubt that such attempts are sometimes impossible (Blumenthal, 1993; 1995) or provide very little insight.

Brain aging and AD. This is a particular example of the ongoing general debate on aging: is AD an accelerated aging or a separate disease entity? AD was originally considered (Roth, 1967; Hubard, 1981; McGeer, 1984) and is still believed by some, (Von Dras & Blumenthal, 1992; Mortimer, 1995) to be an acceleration/exaggeration of normal aging. This "unionist" concept is based on the following observations:

From a biopathological perspective:

a) Virtually all of the lesions associated with AD, including NFT, SP, GVD, amyloid angiopathy, and neurochemical deficits, are, to some degree, commonly seen in non-demented older individuals as age-dependent phenomena (Tomlinson et al, 1970; Ball, 1977; Bartus et al, 1982; Henderson, 1986; Von Dras & Blumenthal, 1992; Morris et al, 1991; Mirra, 1993).

b) Normal aged and demented subjects demonstrate similarities in brain atrophy (Tomlinson et al, 1970; Ball, 1980; Golomb et al, 1993), regional vulnerability to neuronal depletion (Ball, 1977; 1980; Horvath & Davis, 1990; Van Hoesen & Solodkin, 1995), and a similar (Arriagada et al. 1992a; Mirra et al, 1993; Van Hoesen & Solodkin,

1995) distribution pattern and combination of AD-type lesions.

c) The immunological mechanism of maintaining homeostasis is a general physiological process for removing senescent cells. Some immune manifestations have long been recognized in aging. These include the increasing IgG response elicited by antigens (Kay, 1990) from many types of senescent cell, the presence of a specific neuronal binding antibody (Walford, 1969; Franceschi et al, 1989), and the gliosis which is commonly present in the aged brain at sites of neuronal depletion, considered to be a local immune response evoked by antigenic changes in aging neurons (Blumenthal, 1988). These antibodies are also present in the AD brain, and some bind to amyloid plaques or to cerebral vessels (Kay, 1990) suggesting consistent molecular and physiological processes between normal aging and AD.

d) Molecular studies on the genesis of AD lesions are also congruent with the concepts of biological aging. The abnormal tau phosphorylation that is believed to be associated with the formation of NFT (Delacourt & Defossez, 1986; Lee et al, 1991, 1994; Goedert et al., 1988, 1989, 1996), is also considered as an age-related change in protein synthesis. The increasing APP mRNA expression demonstrated in cellular senescence (Adler, 1991) indicates that amyloid deposition in AD may at least in part be due to a more general rise in synthesis of the precursor with age, and that toxic effects of Aß may be responsible for at least some neuronal loss in normal aging and AD.

From a psychological perspective:

a) Cognitive deficits take place as a function of chronological age in the absence

of identified illness. It is proposed that if a continuum exists in the loss of cognitive function between normal aging and AD, then psychometric tests should show a frequency distribution curve that is highly skewed and unimodal (Brayne & Calloway, 1988; Goodwin, 1991; Forbes & Hirdes, 1993). Various studies have been performed on a variety of cognitive functions with age, including the five major aspects (Salthouse, 1982): memory and language (Sullivan et al, 1986; Light, 1990); problem solving (Flicker et al, 1986; Grady et al, 1988); spatial abilities (Browers et al, 1984; Henerson et al, 1989); speed of responding (Mahurin et al, 1986; Carella, 1990) and sensory process (Joss et al, 1988; Freedman et al, 1987; Carella, 1990). These results all showed a continuity between aging and AD.

b) Intelligence has been found to decline with advancing age (Schaie & Hertzog, 1983; Botwinick, 1977; Labouvie-Vief, 1985; Mortimer, 1995), although the extent and significance is controversial (Schaie, 1974; Von Dras & Blumenthal, 1992), and interpretive arguments exist regarding which specific intellectual functions decline and at what age the decline begins. The pattern of decline in AD is similar to that found in normal aging, but is more pronounced and accelerated (Botwinick, 1977; Storandt & Hill, 1989).

In summary, the "unionism" concept (Von Dras & Blumenthal, 1992; Blumenthal, 1995; Mortimer, 1995) emphasizes the parallel biopathological and psychological changes seen in both aging and AD. The fact that no unequivocal features distinguish AD from normal aging is interpreted to support a shared process for declining

integrity of the cognitive-neurobiological system in later life.

In contrast, a strong "separatist" movement emphasizes the differences, and considers AD as distinct from an acceleration of normal aging, mainly on the following grounds:

a) Some epidemiological reports indicate the increasing prevalence of AD may plateau at about age 95 and over (Skoog et al, 1993; Wernicke & Reischies, 1994).

b) Twin studies (Kumar et al, 1991; Rapoport et al, 1991) do not show a concordance in development of AD between monozygotic twins, as there should be (Wilcock, 1995), if the same intrinsic aging mechanism leads to AD.

c) There is no consistent evidence (Whatley & Anderton, 1990), that AD involves a generalized increase in susceptibility to aging changes in other tissues, such as increased incidence of certain types of neoplasia, autoimmune disorders or increased frequency of chromosomal aberrations (Martin, 1977).

d) There are some qualitative differences in the neuropathological and neurochemical features. These include topographic involvement (Bell & Coleman, 1981; West et al, 1994) tau abnormalities (Feany & Dickson, 1995; Goedert, 1996; Wang et al, 1996), and the type of immunoreactivity on PHF-containing neurons (Mena et al, 1992; Liberini et al, 1993) Many quantitative differences in the lesions (Tomlinson, 1982; Braak et al, 1996) also exist between non-demented elderly and AD patients, with some centenarian brains having no pathological changes at all (Mizutani & Shimada, 1992).

e) The heterogeneity in AD subjects at the genetic, biochemical, neuropathological and

clinical levels, and the lack of uniform presentation of the symptoms and signs in AD in a way that mirrors the normal aging process, implies that in at least some of the AD variations, the etiology of the disorder is unlikely to be simply an acceleration of normal aging.

It is not the purpose of this thesis to judge these opposing points of view. The two sides seem to be derived largely from the different preferences and/or interpretations emphasized by the two camps with regard to definitions and to processes that occur in aging and AD. It is worth noting that the "separatist" view has a political origin (Rabins, 1988; Adelman, 1995; Blumenthal, 1995) with implications beyond the purely scientific (Rabins, 1988). In the present situation, the definitions of both viewpoints are actually indefinite, and the basic biological process(es) and mechanism(s) of both are largely unknown. Therefore, this thesis will take the position that the overlap between normal aging and AD does imply some mechanisms shared by the two, no matter how they are semantically defined, and it is not possible to classify AD with certainty as accelerated aging or as a totally separate disease. Recognizing aging as the principal risk factor for most important diseases, AD among them, will allow major biological mechanisms of aging to be identified, and aid in unravelling the pathogenesis of AD.

2.2.3.6. Other risk factors and hypotheses

Numerous other risk factors and hypotheses for the pathogenesis of AD have also

been proposed. They can briefly be summarized in the following five categories:

1) Viruses have long been suspected as an etiological factor in AD (Gibbs & Gajdusek, 1978; Goudsmit et al, 1980). However, this concept is highly controversial, and viruses have not been clearly demonstrated in human AD brain by any of several techniques (Itzhaki, 1994). The olfactory pathway has been considered a possible route of entry, in a manner similar to the possible transmission of prion disease (Price, et al, 1993; Nandi, 1996).

2) Aluminum and silicon toxicity (Landsberg et al, 1992; Doll et al, 1993) have been reported to have a possible and controversial influence on the conformation of both amyloid and NFT (Nikaido, 1972; Edwardson, 1993; Scott, 1993; Kawahara et al, 1994). It has also been suggested that these compounds may affect neuronal metabolism and membrane integrity by accelerating APP proteolytic processing (Claugberg & Joshi, 1993), or altering calcium channels (Wakui et al, 1990).

3) Repeated head trauma as a contributor to AD (Rasmussen, 1995; van Dujin, 1995), is another issue of longstanding debate. Head trauma has recently been found to induce Aβ deposition as an acute phase response to injury (Roberts et al, 1994), and the extent of the response may be modified by the APOE4 allele (Mayeux et al, 1993).

4) A calcium hypothesis (Siesjo, 1995; Khachaturian, 1995) has been recently linked to the concept of Aβ-induced apoptosis. Calcium influx is proposed to account for neuronal death (Su et al, 1994; Cotman et al, 1995).

5) Miscellaneous factors suggested in AD pathogenesis include occupational exposure to solvents including benzene, toluene, phenols, alcohols and ketones

(Fratiglioni et al, 1993; Kukull et al, 1995). A history of various health problems or diseases such as thyroid disease (Breteler et al, 1991; Ewins et al, 1991), magnesium deficiency (Glick, 1990), depression (Jorm et al, 1991; van Duijin, 1996), and late maternal age at birth (Whalley et al, 1982; Forster, 1995) have all been reported as possible risk factors.

All of these risk factors have been proposed as a result of epidemiological data, retrospective studies of patient histories, autopsy findings, and/or *in vitro* experiments. No firm conclusions can be drawn from any of these voluminous studies. The suggestions are often controversial and the data is frequently inconsistent (Graves et al, 1991; Monique et al. 1992; Caroline et al., 1994; Itzhaki, 1994; van Duijin, 1996) and therefore difficult to interpret. In terms of their etiological role in AD, none of these factors has yet been distinguished as a causative agent, a secondary effect, or simply an epiphenomenon. Their relative importance and possible interplay in the pathogenesis, particularly the genesis of SP and/or NFT are not known.

2.2.4. Key questions related to the study of Alzheimer's Disease

Significant progress has been made in our understanding of AD, however the etiology of the disease remains an enigma. Controversy and uncertainty exist in all aspects of AD research, particularly with respect to the pathogenesis. A brief review of the history and current situation is therefore provided, as a preface to the aims of the

present study.

AD has been an enigma from the beginning. Even the scientific merit of naming it after Alzheimer is questionable (Amaducci et al, 1986; Berrios, 1990; O'Brien, 1996). There were two European neuropathology schools at the time of the original studies, Kraepelin's where Alzheimer worked, and Pick's. These contributed in describing neurofibrillary tangles (NFT) and senile plaques (SP) respectively, and were rivals for the name of the disease. Although there was nothing novel in the clinical syndrome and neuropathological findings of Alzheimer's report, Kraepelin created the eponym of "AD" fairly quickly thereafter when only a few cases had been published, and furthermore, their autopsy findings of arteriosclerotic lesions were quietly dropped. Thus the boundaries of AD were left unclear from the beginning. Not even the early cases, including Alzheimer's, fit well with the original description, or with the current operational definitions (DSM-III-R, CERAD). The concept of AD is being continually modified with more and more pathological and clinical features being associated with the disease.

The confusion in the semantics and concepts inherited from this historical beginning have created subsequent difficulty in understanding the observations, designing the studies, and interpreting the results. Various risk factors have been proposed in light of their statistical association with the pathological and/or clinical manifestations, which themselves were assembled and defined as part of AD without a clear, unified definition.

The data and concepts drawn from various studies are therefore not always reliable, nor can they always be meaningfully interpreted with a high degree of certainty. AD therefore, like the proverbial elephant, has been described in a number of ways (Blass, 1996): as a cytoskeletal disorder, a cerebral amyloidosis, a premature loss of neurons/synapses, a disorder of lysosomal enzymes, of free radical metabolism, of impaired glucose/energy metabolism, of cerebral calcium homeostasis, of neurotransmission, of signal transduction, or a disease of apoE $\in 4$ allele. All of these are in some ways right, but incomplete in the eyes of the beholders. As mentioned above, many fundamental aspects are still being constantly debated, most notably whether or not AD is different from senile dementia, whether AD is an accelerated aging with the rate increasing, or levelling off after age 90, and whether the characteristic lesion is NFT, SP, neuronal/synapse loss, or some other feature which does or does not correlate with the dementia. Other debates question whether or not the primary causative factor is a virus, tau/cytoskeleton abnormality, amyloid deposition, aging, or ApoE and whether or not the possible biological marker for diagnosis is CSF tau, APP, Aβ, CAT, AChE, skin vasodilation, Aβ deposition, or pupillary dilation, and whether an effective pharmacological agent is a cholinergic agonist, a neurotrophic factor, or a hydrophobic agent (Merlini et al, 1995; Nandi, 1996) coupled with covalent iodide.

These uncertainties have led numerous investigators to question whether AD really exists, (Watson, 1992; Engel et al, 1992; Blumenthal, 1993; 1995), and if so, in what definition? If the concept of AD was expanded to become as broad as that of chronic

renal or heart failure, there would be no distinct "AD", and what has been assembled as "AD" could be considered as a group of different disorders, some of which might share etiological factors and/or pathogenic processes, while others might simply be separate disease processes which happen to coincidentally occur in old age.

Not surprisingly, subgroups or heterogeneity of the disease have been suggested to deal with the controversy and the inconsistency. However, similar questions exist regarding the AD subgroups that have been proposed using clinical, pathological, or etiological differentiation. For example, what is the logical basis to separate AD into early (presenile)- and late-onset (senile) forms by age of onset before or after 65, when the onset of the disease is subtle, gradual, variable and difficult to differentiate? (Crook, 1983; Branconnier & DeVitt, 1983; Morris et al, 1989, Pirozzlol, 1989; Mortimer, 1995). The onset of AD is often impossible to reliably determine and even the diagnosis may be easily affected by various factors, such as accuracy of patient and family histories, educational backgrounds, occupations, economic conditions, and access to medical consultation. One can also question the logical basis and practical advantage of separating AD from accelerated aging, if indeed there is a continuum of fundamental processes linking the two. In reality, therefore the conceptual criteria for demonstrating the existence of disease subtypes have not been clearly elucidated (Jorm, 1985; Mohr et al, 1990; Mortimer, 1995). Heterogeneity may simply represent different stages in a disease progress, or alternatively, may indicate combinations of various pathogenic factors, or separate and distinct etiological processes.

Being recognized as a heterogenous disorder (Mohr et al, 1990; Schellenberg, 1995; Wallin et al, 1996), AD is poorly defined by the present system linking a clinical pattern of dementia with a "particular pattern of scarring of the brain" (Blass, 1996). Current data indicate strongly that a variety of mechanisms can lead to the formation of these characteristic scars, some may have more importance among different individuals or ethnic/geographic populations, and some may be a common pathway in the pathogenesis, being shared or acted upon by others. Advances in understanding AD are more likely to come from studies that focus on and clarify the particular mechanisms which lead to these characteristic lesions. Diagnostically, Aβ plaque deposit in the brain is the characteristic feature of AD (Wisniewski, 1983; Hardy & Higgins, 1992; Solkeo, 1994a,b; 1996; Yankner,1996). The fundamental question is therefore to determine the mechanism by which Aβ, presumably a normal proteolytic fragment of APP, can accumulate, beta-pleat and deposit in the form of amyloid plaques, with a particular topographic distribution in the aged human brain.

Mutations in APP SP1/SP2 genes promote the characteristic Aβ deposits (Selkoe, 1994a,b, Rogaev et al, 1995; Schellenberg et al, 1995), but identifying the locus and the nature of these mutations is not sufficient, since they are not necessary, or sufficient to cause the disease. Even in some archetypical monogenetic diseases, such as Tay-Sachs disease (GM2 gangliosidosis), the absence of hexosaminidase B activity, which usually leads to brain damage in utero, can be accompanied by motor neuron disease or by no clinical abnormality at all (Navon et al, 1976; Johnson, 1981). Thus other genetic factors

and environmental influences play a role in the clinical expression of these mutations (Blass, 1996).

Therefore it is more important to determine the functional consequences of these mutations on the protein (APP) synthesis, structure, function, and behaviour, which may indicate a central step in the pathogenesis. Possible interactions with the products of other AD associated genes, such as APOE or NACP must also be investigated (Corder et al, 1993, Xie et al, 1996; Barinaga,1997). There is a long chain of cellular processing of APP and its derivative Aβ, which may extend beyond the tissue of origin. Any factor(s) which significantly alter(s) a key point in this long complex metabolic chain, may lead to the accumulation of Aβ in the brain, and the possibility of subsequent (Taubes, 1996; Bernstein et al, 1996) beta-pleating and amyloid plaque formation. In other words, the Aβ deposits could be derived from gradually accumulated defects in this chain, beginning, but not limited to the APP gene integrity and function.

A unifying hypothesis is needed to explain AD, as emphasized by many investigators (Hyman & Terry, 1994; Khachaturian, 1995). Rather than attempting to account for each and every observation, it appears more logical to emphasize the possible existence of an imbalanced cellular pathway of APP metabolism, as a central step in the "pathogenic core" (Hyman & Terry, 1994; Blass, 1996), be it "primary", "causative" (Joachim et al, 1992; Hardy et al, 1992) or "secondary" (Perry, 1993; Roses, 1994; Wisnewski et al, 1993; 1996). While not expecting a complete explanation of all the

pathological and clinical features as well as the subtle molecular events from each experiment on AD, we think it is reasonable that a hypothesis on the pathogenic core should incorporate the divergent pieces of data into a coherent story. It should explain how the amyloid plaque is generated, unless this observation can be convincingly argued as an epiphenomenon which happens to coincidentally occur in old age (Jorm, 1985; Wallin et al, 1996). It should provide explanations that are plausible on the basis of well established biological processes to answer the following questions: a) what is the cellular origin of the Aβ deposit in the amyloid plaques? b) what is the cascade of events preceding and/or leading to accumulation and beta-pleating of the Aβ, a peptide derived from presumably normal proteolysis? c) why does the amyloid deposition show characteristic features, with a constant spherical shape of the SPs, and consistent topographic involvement of the brain? d) why do amyloid plaques deposit as an age-related event? e) how are the other risk factors, such as aging, apoE, and brain trauma associated with the development of the disease? The work in this thesis has been designed to study such a hypothesis on the pathogenesis of AD.

3. AIMS OF THE PROJECT

The purpose of this Ph.D research was to investigate possible factors in the pathogenesis of AD by testing several new and interrelated hypotheses, initially based on the studies of a specific category of intraneuronal structure found in the human brain (Rees, 1975). Experiments were designed to explore the physiological role of this intraneuronal structure, which we have called the amylosome, by studying its histological features and chemical composition in the human brain, and to examine the possible relationship between amylosomes and the pathogenesis of AD. This research consists of the following specific aims:

1) to identify the intracellular location, the distribution and the morphological features of amylosomes in normal human brains throughout the lifespan.

2) to evaluate the chemical composition of amylosomes, particularly the possible Aβ content.

3) to determine the morphometric characteristics, density (number per mm^2) and size (diameter in μm) of the amylosomes in human brains over the entire lifespan.

4) to investigate the possible changes in MT and MAP_2, as well as MAP_2 mRNA in normal human brains throughout the adult lifespan.

5) to determine the effect of APOE genotype on human brain MT and MAP_2 content throughout the adult lifespan.

4. WORKING HYPOTHESIS & RATIONALE

4. 1.WORKING HYPOTHESIS

We proposed that:

a) The intraneuronal structure first described by Rees may contain the beta amyloid peptide(Aβ), the major constituent of SP in AD, and may represent a part of the endosomal/lysosomal pathway by which the APP and its amyloidogenic fragments are normally metabolized.

b) As the APP is normally degraded and its amyloidogenic fragments are produced, these membrane-bound inclusions, or amylosomes, may be continually formed and removed from neurons along dendrites to the perivascular space or cortical surface where they are released into CSF.

c) Any change which alters the balance between the normal production of amyloidogenic peptides and their removal, in particular, a decline in dendritic transport of amylosomes, would lead to the accumulation of the amylosomes in the brain. These may eventually break into the neuropil to form amyloid plaques, with AD as a consequence.

d) The decline of dendritic transport is likely to be an age-related general phenomenon in the normal population, and to be a fundamental process in the pathogenesis of AD, while apoE and many other risk factors, may interact with this central process by affecting MT function.

Therefore we believe that amylosomes are a possible source of plaque amyloid in AD, and that dendritic transport plays a key pathogenic role in the formation of amyloid plaques in AD, as well as in aged human brains.

4.2.RATIONALE

Studies on AD have been focused on identifying the APP gene mutations and the abnormal metabolism of APP (Selkoe, 1994a, b; 1996; Yankner, 1996), ever since Aβ was isolated from cerebral congophilic angiopathy (Glenner & Wong, 1984) and from amyloid plaques (Masters et al. 1985). However the mechanism of Aß deposition, and its cellular origin remain elusive. Multiple etiological factors and pathogenic processes, such as primary cholinergic system degeneration, APP gene mutation, aging, environmental toxins, neurotrophic factor deficiency, neuronal MT dysfunction, virus infection, oxidative stress, immune dysfunction, brain trauma, and recently the apoE4, have all been indicated in the pathogenesis of AD, but their relative importance and potential interaction in the pathogenesis is a matter of considerable debate (Selko, 1994a; Roses, 1994; 1996a; 1996b; Yankner, 1996; Blass, 1996; Adams, 1997). In particular,

the cellular origin and mechanism of Aß accumulation in human brains, and the method of deposition as amyloid plaques remain unknown.

Lacking an understanding of the pathogenesis of AD and a consensus on the basic concepts of AD and aging, theories derived from various individual observations are often contradictory. There are few correlations between risk factors and the pathological changes and the clinical symptoms, and the results from some *in vitro* studies are often contradictory to each other, or narrowly limited, making it difficult to draw a meaningful explanation for the complicated pathogenic tangles of AD. The observations that: a) there are multiple genetic defects and polymorphism in both the APP gene (Yankner, 1996; Hyman, 1996) and non-APP genes (Han, 1995; Xia, 1996; Barinaga, 1997) in some familial and sporadic AD, b) there are significant links between many risk factors, particularly aging, and apoE and the pathogenesis, and c) there are heterogeneous biological, pathological, and psychological alterations associated with these risk factors in AD, have led to a reasonable conclusion (Hyman & Terry, 1994; Barinaga, 1997) that the "crucial black box that links these" to the eventual development of AD is still missing. Therefore, "It is a challenge to the field to develop biologically plausible, testable hypotheses." Additional genes and other risk factors contributing to the pathogenesis are expected (Mayeux, 1996; Hyman, 1996; Xia, 1996; Roses, 1996, Blass, 1996; Adams, 1997; Barinaga, 1997) to be eventually found.

We now know a few causative genes and many risk factors, but we do not know

their function, and how they produce the disease, nor do we know the etiology for most AD cases. We have documented many biological and pathological features associated with AD, but we do not know which ones are actually pathogenic, and which ones are just consequential effects, nor do we know the sequence of pathological events. A rough establishment of the basic pathway by which at least one verified etiological agent leads to disease manifestations is therefore called for (Blass, 1996; Adams, 1997). This should give insight into the key pathological events during disease progression, where and how other causes or risk factors may interact and/ or enter the pathway, and sites of possible intervention. It would also help to delineate some separate yet converging pathways.

The membrane-bound intraneuronal structure initially described by Rees (1975), which has not raised any interest in the field of AD research, first attracted our attention because it has a distribution pattern in human brain similar to that of amyloid plaques in AD. Our initial studies indicated that these intraneuronal inclusions may contain beta amyloid peptide, the major constituent of the amyloid plaques in AD. Therefore amylosomes might play a role in the normal cellular processing of APP and its amyloidogenic fragment, Aβ, as well as its abnormal deposition in the brain.

It seems reasonable to presume that the presence of amylosomes in human cerebral cortex is normal and physiological, rather than abnormal and pathological, considering their appearance in the general population and in very young children. Considering their amyloidogenic content and the large amount of precursor turnover,

which is estimated as 80-100mg/day production of APP in human brain (Miller et al, 1992), it is reasonable to also presume that an alteration of their normal metabolic pathway could lead to the accumulation of these vesicles containing amyloidogenic peptide within the brain, with eventual rupture into the neuropil, and beta-pleating of their contents to form the amyloid plaques. It is also reasonable to presume that some of the alterations, be it at a molecular, a cellular or a functional level, would be age-related phenomena in the general population, considering that aging is the principal risk factor for most important diseases (Vijg & Wei, 1995; Blumenthal, 1993, 1995), AD in particular. The various morphological and biochemical changes in AD, particularly the SP, NFT, amyloid angiopathy, and dendritic alterations, are all commonly seen in non-demented elderly (Coeman & Flood, 1987; Mountjoy, 1988; Arriagada et al, 1992; Braak et al, 1996). It is therefore reasonable to explore the possibility, that one of the relevant age-related alterations might be a decline of MT integrity and function, considering its fundamental role in cell biology (Alberts et al, 1994) and some observations indicating its decline with age (Rao & Cohen, 1990; Raes, 1991; Klowen & de Aguilar, 1995; Macieira-Coelho, 1995). Multiple risk factors may act upon, or interact to alter this normal process which then leads to the common pathogenic product, amyloid plaques. This theory might provide a reasonable link and explanation for proposed risk factors, observed pathological changes, and a variety of clinical manifestations, as well as outline their relative importance and potential interaction in the pathogenesis.

Technically, using the traditional methods of morphological evaluation to delineate the intraneuronal inclusions over the lifespan in the human brain would not only be tedious, but also unreliable or unrepeatable, as disparities already exist between the few previous studies (Averback, 1982, Hara, 1986). We therefore decided to study a large number of samples using standardized histological sections, and to use computer-assisted morphometry, available at the Pathology Department of the Montreal General Hospital. To study intracellular transport requires a reliable method to assess MT function in the human brain with age. Previous studies of brain MT proteins have been mainly conducted on animal tissues or cultured cells (Matus, 1988; Vaila, 1990; Hirokawa, 1991; Tashiro, 1991; Fifkova, 1992; Ruben et al. 1996), because most methods require fresh tissues which are difficult to obtain from human brains, and most available methods are not only complex and tedious, they often yield protein products of different molecular weights in various ratios, making it difficult to quantify and compare. A newer method developed by Sparkman (1992), with some modification, allows isolation and reliable quantification of MT proteins from frozen human brains, and also facilitates the study of MT polymerization *in vitro*. Using this method, we would be able to assess the MT function in the human brain, through a major portion of the lifespan, by quantifying the MT proteins α and β tubulin and the predominantly dendritic microtubule associated protein MAP_2 from a large number of autopsy samples available from the Pathology Department at the Montreal General Hospital. The MAP_2 mRNA levels over the lifespan and the possible influence of apoE4 on the levels of MT could also be assessed, since the cDNA probe (Shafit-Zagardo, 1994) and apoE genotyping techniques (Main, 1991,

Nalbantoglu et al, 1994) have recently become available.

In summary, the present knowledge of AD indicates the existence of some basic pathogenic process that has yet to be determined. Previous work indicates the possible role of amylosomes, a type of cerebral neuronal inclusion, in the pathogenesis of AD and the material and techniques required to test this hypothesis are accessible. Should the results be supportive, it might be possible to uncover, at least part of the "black box" relating various risk factors and pathogenic processes, and to provide a "plausible, testable" explanation for the pathogenesis of AD. Such a theory could also offer guidance for eventual development of pharmacological intervention, not only for symptomatic relief, but also for the prevention and treatment of AD.

5. EXPERIMENTAL DESIGNS

5.1. BRAIN TISSUE ACQUISITION AND PROCESSING

Human brain samples were collected during 230 autopsies performed at the Montreal General Hospital, and 5 additional samples were obtained from the Douglas Hospital Brain Bank. Criteria for case selection included absence of documented neurological disorder during life, and no diagnosed abnormality during autopsy or subsequent histological examination of the brain. The brains were fixed with 10% formalin, and cut coronally into blocks of 1.5-2 cm thickness, which were then processed according to standard histochemical techniques (McManus, 1960; Adams, 1965). Some samples were quickly frozen and kept at -60°C or -80°C for subsequent biochemical studies.

Histological slides were prepared from different areas of the brain according to standard histological techniques. Multiple sections of the cortex and hippocampus, areas of the brain most vulnerable to changes in Alzheimer's Disease (Tomlinson, 1976; Khachaturian, 1985; Mirra et al, 1991; 1993), were always included. A total of 222 brains of different ages were included in the pathological assessment and morphometry study, and 55 brains were also sampled for biochemical analysis, which included quantification of MT, MAP_2 , MAP_2 mRNA, and APOE genotyping. All general information, including the number of the samples, the age-grouping, the distribution of

age and sex, and the postmortem delay of the brains used for the histology and morphometry or the biochemical analysis have been summarized in Table 1 or Table 2 in Section 6.1 and Section 6.2., respectively.

5.2. CHARACTERISTICS OF AMYLOSOMES

5.2.1. Light and electron microscopy

The H&E (Haematoxylin and Eosin) stain, which has been widely used histologically, was employed for neuropathological diagnosis and morphometric analysis. For morphometry, H&E stained slides were obtained from the frontal, parietal, occipital and temporal lobes including the hippocampal area. They were first thoroughly screened with the light microscope under 25x eyepiece graticules to identify similar areas, from which images under a 40x lens were subsequently transferred onto a computer image analyzing system. Any major lesions detected during this process were recorded, and other stains, such as modified Bielschowsky, Congo Red or periodic acid-Schiff were used, when needed to further identify the lesions. In order to reveal the intracellular location of the amylosomes, EM was performed on samples surgically removed during biopsy of the cerebral cortex.

5.2.2. Morphometry

The computer image analyzing system consisted of One Scanner with OfotoTM scanning software (Light Source Inc, Greenbrea, CA), the NIH Image 1.41 program (NIH Research Services Branch, Bethesda, MA) and Light resolution CCD camera PULNIX TM-80 (Pulnix America Inc, Sunnyvale, CA). Morphometric analysis was performed on a total of 222 brains, without identifying any general information on the patient or the neuropathological diagnosis in advance.

The images used for measurement under the 40x lens were chosen while screening the slides from the surface to the deep cortical areas, and then from left to right in order not to count the same area repeatedly, and moving of the slides was done without focusing, in order to avoid unintentional bias in the selection. In accordance with methods used by others (Hara, 1986; Lennar, 1990), with some modification, the density of amylosomes was expressed as number per square millimeter, and the size of amylosomes was expressed as the diameter in micrometers. The density was calculated from the total number of amylosomes captured in 32 images of 0.033 mm^2 ($\approx 1 mm^2$) from each case, with 8 images from each lobe (frontal, parietal, temporal and occipital), and the size of amylosomes was based on the mean diameter of all the amylosomes captured.

5.2.3. Immunohistochemistry

Immunohistochemistry was performed with the Streptavidin/Biotin affinity

immunostaining system (Shandon-Lipshaw, Pittsburgh, PA) with diaminobenzidine (DAB) as chromogen. The brain sections from each lobe were first deparaffinized, and exposed to 90% formic acid for up to 24 hours in order to increase the immunoreactivity (Mann et al, 1990; Davies et al, 1993; Perlmutter et al, 1995). A solution of 3% hydrogen peroxide (provided in the kit) was applied to quench endogenous peroxidase activity. The slides were incubated with Protein blocking agent (provided in the kit) at room temperature for 5 min, and then with the primary antibodies in a humid chamber at room temperature for 1 hour. Control sections were incubated with the secondary antibody only to verify lack of crossreactivity. The antibodies used were Monoclonal Mouse Anti-Human beta-Amyloid (Dako) diluted to 1:100, and Polyclonal Rabbit Anti-β amyloid (Boehringer Mannheim), diluted to 20μg/ml.

5.2.4. Isolation of the amylosomes

A 50 gram aliquot of cortex was taken during each of 24 autopsies performed less than 6 hours postmortem, after preliminary dissection to remove meninges and blood vessels. All of these cases were under 58 years of age, with the majority being in the fourth decade of life. The tissue was homogenized in a Polytron homogenizer in 2 volumes of phosphate buffer (0.15 M, 10mM EGTA, pH 6.2) and sieved through a nylon mesh under vacuum. Homogenates were centrifuged in a Sorval 600 B centrifuge at 1,100 g for 10 minutes at 4°C. The pellet was collected, placed on a discontinuous sucrose gradient of 1.5, 1.9 and 2.3 M respectively, and spun in a SW 28 Beckman

swinging bucket head at 122,000 g for 30 minutes, at 4°C. The layer at the interface 1.9-2.3 M sucrose was collected and spun in a Beckman JA20 rotor at 18,000 g for 10 minutes. The pellet was then further purified with 80% iso-osmotic Percoll (Phamacia Biotechnology, Uppsala, Sweden) in a Ti&O Beckman head at 93,000 g for 30 minutes, and the area between density 1.087 and 1.12, as determined by beads, was collected. This was diluted with PBS at 1:1, and centrifuged in a T70 Beckman head at 126,000 g for 90 min to remove the Percoll. The resulting pellet containing the amylosomes was diluted with 10 volumes of PBS and recovered by centrifugation at 30,0000 g for 20 min.

5.2.5. Composition analysis of the amylosomes

The amylosomes obtained from the above isolation procedure were broken by sonication, solubilized with 90% formic acid, and their contents were purified by size exclusion chromatography with Superose (Pharmacia) and then further purified with reverse phase HPLC (High performance liquid chromatography) with a C4 column and buffer A being 15% B with 0.5% TFE (trifluroethanol), and buffer B being 5% TFE and 95% acetonitrile. We also tried other solvent systems to keep the contents of the amylosomes in solution. The solvents tried were tetrahydrofuran, hexafluroisopropanol, 6M guanidinium thiocyanate trifluroethanol (Masters et al, 1985, Barrow et al, 1991), and guanidine HCL (Joachim et al, 1988). The results were not consistently satisfactory, and others have also reported similar difficulty in keeping the beta amyloid peptide in solution (Halverson et al, 1990).

The HPLC peaks were collected and immunoblotted on Immobilon (Millipore Co, Bedford, MA), and those reacting positively with the anti-beta amyloid antibodies were subjected to amino acid composition analysis. Antibodies used were against synthetic Aβ peptide 1-14 (Dr.Miller), 17-23 (Dako), and 16-32 (Dr.Greta Huber of Basel).

To compare the amylosomes with plaque amyloid from cases of AD, plaques were isolated from the cerebral cortex of AD brains according to the technique reported by Masters et al (1985), and also subjected to reverse phase HPLC.

5.3. ISOLATION AND ANALYSIS OF MICROTUBULES AND ASSOCIATED PROTEINS

5.3.1. Isolation of MT proteins

Brain tissues, which were removed at autopsy and quickly frozen to -60°C were subsequently used for the analysis of MT proteins. From each sampling area, including the frontal, parietal, temporal and occipital lobes, segments of brain were first dissected into small pieces less than 1mm in thickness, and meningeal tissue and small blood vessels were removed, along with the white matter, as much as possible. The isolation of MT was performed using D. Sparkman's method (Sparkman, 1992) with some modification. In each case, 7.5 g of the tissue and 2.5ml/g of cold MT glycerol assembly buffer were homogenized with a Polytron homogenizer, and centrifuged at 100,000G in a Beckman Ti 70 rotor at 4°C for 1 hour. The supernatant excluding the fat pad on the surface was carefully transferred, and then taxol (Sigma) and Guanosine 5'-Triphosphate (GTP) (Sigma) were added to achieve a concentration of 10 μM and 1mM respectively. The solution was incubated in conical tubes at 37°C for 45 min, and then slowly layered over a 2 ml pad of the glycerol buffer which was prewarmed to 37°C, and finally centrifuged at 50,000 g at 25°C for 45 min in a Beckman SW 28 rotor. The supernatant and the pads were aspirated and the MT pellets were resuspended in warm glycerol buffer with a final volume of 250 μl.

Preparation of the MT glycerol assembly buffer: A solution containing 0.1M piperazine-N, N-bis (2-ethanesulfonic acid) (PIPES) (Sigma), pH 6.95, 2mM EDTA, 0.1 mM $MgCl_2$, and 4M glycerol (Fisher) was prepared in advance. At the time of the experiment, 2mM Phenylmethylsulfonyl fluoride (PMSF) (Sigma), 20mM benzamidine-HCl (Sigma) and 10μg/ml aprotinin (Sigma) were added to the buffer just before use.

5.3.2. Protein assay by spectrophotometry

Isolated MT protein was quantified with a PU8610 UV/VIS Kinetics spectrophotometer (Unicam Inc) using a 1 μl aliquot of MT sample diluted with distilled H_2O. The total quantity of MT protein (gram of MT isolated per gram of tissue used) was calculated with a formula derived from a standard curve. The curve was created from spectrophotometric readings at 595nm using two sets of serial concentrations of bovine serum albumin (BSA) (Sigma). The linear range of the assay for BSA is 1.2 to 10 μg/ml. A new standard curve was created for each set of MT samples being measured, in order to make sure that test readings were within the linear range of the standard, and to minimize variation between sets of samples.

Preparation of the BSA and MT samples: Two sets of serial concentrations of BSA in dH_2O were prepared, at 0, 1, 2, 5, 10 and 20 μg/ml respectively. MT samples were diluted to 5% with dH2O, and 20 μl of each sample was then used for quantification. A volume of 200 μl Protein Assay Dye Reagent (BIO-RAD Laboratories,

Richmond, CA) was added to each BSA concentration and to each sample MT preparation, and an interval of at least 5 min was allowed before measurement. (maximum interval 1 hour).

5.3.3. Gel electrophoresis

Electrophoresis was performed with the Mini-PROTEAN® II CELL (BIO-RAD). In each case, 1.25 μl of MT sample was prepared with the MT sample buffer, loaded into one gel well, and electrophoresed by sodium dodecylsulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) (Laemmli, 1970) with a separating gel, 10% and a stacking gel, 5% in 300 ml of the electrode buffer at 200V, at room temperature, for 42 min. Prestained and non-prestained low molecular weight markers (BIO-RAD) were used to monitor the electrophoresis and to indicate the molecular weight of the protein profiles. A 2 μg aliquot of BSA was loaded on each gel as a standard for the quantification. To be stained, the gels were put in fixative (40% methanol/10% acetic acid). To be electrophoretically transferred, the gels were placed in equilibration buffer (the transfer buffer).

Preparation of the Acrylamide/bis stock: Acrylamide (Bio-Rad) 14.6g, and N'N'-bis-methylene-acrylamide (BIO-RAD), 0.4g, were dissolved in 50ml of dH_2O, filtered and stored at 4°C in dark.

Preparation of the 10% separating gel (one gel): In the first step, 4 ml of dH_2O, 2.5 ml of 1.5M [Hydroxymethyl] Amino-methane (Tris) (Sigma), 100 μl of 10% SDS, and 3.4 ml of the acrylamide/bis stock, were mixed and degassed for 15 min. In the second step, just prior to use, 50 μl of 10% ammonium persulfate and 5 μl of TEMED (BIO-RAD) were freshly added in.

Preparation of the 5% stacking gel: Initially, 5.8ml of dH_2O, 2.5ml of 0.5 M Tris-HCl, 100 μl of 10% SDS, and 1.62ml of the acrylamide/bis stock were mixed, and degassed for 15 min. Then 50 μl of 10% ammonium persulfate and 10 μl of TEMED were added before use.

Preparation of the MT sample: From the 250 μl of isolated MT, 5 μl were removed and diluted with 95 μl MT sample buffer. The samples were then heated to 100°C for 5 min, and 25 μl of each preparation was loaded on the gel.

Preparation of the MT sample buffer: Initially, 50mM Tris-HCL, pH 6.8, containing 5% glycerol, and 1% SDS, was prepared as a stock, and 10mM Dithiothreitol (DTT) (BIO-RAD) was added just before use.

Preparation of electrode buffer: Tris base 15g/l, Glycine 72g/l, SDS 3g/l, pH 8.3, was stored at 4°C as a stock solution. This was warmed to 37°C before use if precipitation occurred. Before use, 60ml of the stock solution was diluted with 240 ml

H_2O for each electrophoretic run.

5.3.4. Western blotting and Protein detection

To identify the protein profiles, the gels were first equilibrated in the transfer buffer for 20 min, and trans-blotted onto a polyvinylidene difluoride (PVDF) microporous membrane (Immobilon-P) (Millipore Co, Bedford, MA) with MiniTrans-Blot® cell (BIO-RAD) in the transfer buffer at 100 V for 42 min with an ice box placed inside the chamber to prevent the temperature from rising during the transfer. The membranes were pre-soaked sequentially in 100% methanol for 1-3 sec, water for 1-2 min, and then the transfer buffer for 2-3 min, before use. The blots were blocked with Tris-buffered saline (TBS) (50mM Tris-HCl, pH7.5, 150mM NaCl) with 3% non fat milk powder for 1 hour, and incubated with primary antibodies diluted in 10mM Tris-HCL, pH 8.2, 200mM NaCl (TBS-200) with 0.1% Tween 20 for 1 hour at 25°C. The antibodies were mouse monoclonal antibody against α-tubulin (Sigma) at 1:10,000 dilution, mouse monoclonal antibody against ß-tubulin (Sigma) at 1:6,000 dilution, and mouse monoclonal antibody against MAP_2 (Boehringer Mannheim) at 1:10,000 dilution. The blots were washed 3 times with TBS-200 plus 0.1% Tween 20, 10 minutes each time, and then incubated with alkaline phosphatase-conjugated rabbit anti-mouse immunoglobulin (Sigma) at 1:1,000 diluted in TBS-200 without Tween 20, followed by 3 washes in TBS-200 plus Tween 20. The color was developed in Bromochloroindolyl Phosphate/NitroBlue Tetrazolium (BCIP/NBT) (Harlow, 1988).

Preparation of the transfer buffer: A solution of 50mM Tris-base, 384mM glycine, 0.01% SDS, and 10% methanol, was prepared in advance and kept at 4°C. A volume of 350 to 400 ml was required for each transfer.

Preparation of the BCIP/NBT: Three solutions, containing 0.5g of NBT dissolved in 10ml of 70% dimethylformamide, 0.5 g BCIP dissolved in 10 ml of 100% dimethylformamide and alkaline phosphatase buffer (100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris, pH 9.5) were kept as stock at 4°C. Prior to use, 66 μl of NBT stock was added to 10ml of alkaline phosphatase buffer, this was mixed well and 33 μl of BCIP stock was added. The resulting solution was used within 1 hour.

5.3.5. Laser densitometry

Protein profiles on the electrophoretic gels were demonstrated by staining with Coomassie brilliant blue (Bio-Rad). The α-, ß-tubulins and MAP_2 were quantified by laser densitometry with USB SciScan 5000 automated scanning system (United States Biochemical Co, Cleveland, OH). A 2 μg BSA solution was used as a standard for each SDS-PAGE for densitometry on each set of samples. The quantities of the protein profiles were expressed as an optical density percentage of the standard.

Coomassie Blue staining: Gels in fixative were stained with Coomassie Blue (BIO-RAD) for 1/2 hour, and destained with 40% MeOH/10% HOAc) to remove

background for 1-2 hour.

5.3.6. MT polymerization *in vitro* and electron microscopy

EM was used to examine the polymerization of the MT *in vitro*. MT polymerized from a second set of specimens carried through the above procedure were immediately fixed, or frozen overnight and then brought back to 37°C for 30 min, then fixed for 15 min in 1% glutaraldehyde in glycerol buffer at 37°C. Samples were absorbed onto 400-mesh formvar nickel grids that were carbon coated and freshly discharged prior to use. The grids were negatively stained by 1% aqueous uranyl acetate, and were viewed by Philips CM10 electron microscopy.

5.4. ISOLATION AND ANALYSIS OF MAP_2 mRNA

5.4.1. Isolation of total RNA

Total RNA was extracted with a single-step RNA isolation procedure, Guanidium isothiocyanate (GIC)-phenol-chloroform extraction. For each sample, about 2 grams of cortical tissue were homogenized with a Polytron homogenizer in 3 ml GIC solution in a 14-ml polypropylene tube (round bottom with cap, sterile/Gamma irradiated) (Becton Dickinson Labware, Lincoln Park, NJ). The following reagents were added to the homogenate sequentially: 400 μl of 2M Sodium acetate, pH4, 4ml of water-saturated

phenol, and 800 μl of chloroform: iso-amyl alcohol (49:1). The samples were mixed thoroughly by inversion after the addition of each reagent. The final mixture was vortexed again for 10 second, cooled on ice for 20 min, and centrifuged in a Beckman JA-20 rotor at 10,000 g for 20 min at 4°C. The aqueous phase (about 4 ml) containing RNA was transferred into a fresh tube, precipitated with an equal volume of isopropanol at -20°C for at least 1 hr, and then centrifuged again in a JA-20 rotor at 10,000 g for 20 min at 4°C. The RNA pellets were slightly vortexed in 1 ml of 75% ethanol, incubated for 10 min at room temperature to dissolve residual guanidium, and finally centrifuged at 10,000 g for 20 min at 4°C. The pellets were then air-dried and dissolved in diethyl pyrocarbonate treated water (depc-H_2O). The MAP_2 mRNA analysis was performed on a total of 48 brain samples. General information on the samples used is listed in Table 3 in section 6.3.

Preparation of the GIC solution: A solution of 4M Guanidium isothiocyanate, 25 mM Sodium acetate, 0.5% N-lauroylsarcosine, pH 7.0, was prepared, heated at 60-65°C and 2-mercaptoethanol was added to produce a final concentration of 0.1M after the solution was filtered to sterilize it. The solution was stored at 4°C.

5.4.2. Gel electrophoresis of RNA

Total RNA was measured by spectrophotometry with a LKB Biochrom Ultrospec II (LKB, Bromma, Sweden) at 260nm with 280 nm as a reference to check the

purification quality of RNA. The loading amount of RNA for each sample for electrophoresis was 30 μg. The electrophoresis was performed with a Bio-Rad DNA-Sub-Cell in 1X MOPS (3-[N-morpholino]propanesulfonic acid), at 90 Volts for 4-5 hours until the bromophenol blue migrated over 75% of the distance down the gel. Gels were viewed and photographed along with a ruler using 31/4 x 41/4 black & white film (Polaroid Ltd, Hertfordshire, England) on a UV transilluminator (FOTODYNE Inc, New Berilin, Wisconsin), and then equilibrated in 20X SSC on an ORBIT Shaker (Lab-line Instrument Inc, Melrose Park, IL) for 1 hour before northern blotting.

Preparation of RNA for electrophoresis: The RNA was first mixed with 37% formaldehyde, 4.8 μl, 10X MOPS, 3.0 μl, deionized formamide, 15 μl, then denatured at 65°C for 10 min, and cooled on ice for 3 min. Then 4 μl of RNA tracking dye was added to the RNA preparation before loading.

Preparation of RNA gel: A solution containing 2 g of electrophoretic grade agarose (GIBCO Life Technologies Inc, Gaithersburg, MD) dissolved in 169 μl depc-H_2O, was heated in a microwave for 4-5 min until complete dissolution, then cooled to 50-55°C in a water bath, before adding 11ml of 37% formaldehyde, 20 ml of 10X MOPS, and 20 μl of ethidium bromide. This was mixed well, without generating bubbles, and the gel solution was poured into a gel tray and allowed to solidify for 30 min.

Preparation of 10X MOPS: A solution was prepared containing 0.2M MOPS, 0.05M Sodium acetate, and 0.01M EDTA, pH.7.0. This was filtered to sterilize it without heating, and stored in the dark at 4°C until use.

Preparation of DEPC-water: 1ml DEPC was added to 1000ml distilled water. This was left overnight, or stirred for 2 hours at room temperature, then autoclaved.

5.4.3. Northern blotting

Hybond-N membrane (Amersham Life Science) was used for blotting. Four pieces of 3MM chromatography paper, (Whatman, Kent, England) were placed as wicks over the glass tray in the 10X SSC reservoir, and another 4 were cut to the same size as the gel and laid on top of the gel with a stack of cut paper towels placed above them. The preparation was covered with saran wrap to prevent short-cut and evaporation. Blotting by capillary transfer was performed in 10X SSC (500ml) for 24 hours at room temperature. After blotting, the gel and membrane were viewed under a UV transluminator to check the quality of the blotting. The membranes were air-dried, and UV-irradiated for 5 min before probing.

Preparation of 20X SSC: A solution of 3 M NaCl, and 0.3 M Sodium citrate was prepared. The pH was adjusted to 7.0 with 1M HCl, the solution was autoclaved for sterilization, then stored at 4°C.

Preparation for blotting: Membranes were cut into pieces the same size as the gel, soaked in distilled water, and then in 10X SSC for 5-10 min. A piece of the membrane and 4 pieces of the 3M paper, also soaked wet in 10X SSC were laid sequentially, one by one on top the gel, carefully excluding bubbles underneath. Paper towels were placed on top of the 3M papers and an object of 1-1.5 kg in weight was placed on top.

5.4.4. Prehybridization and hybridization

The hybridization procedure was adapted from Clontech ExpressHyb protocol (Clontech Lab, Palo Alto, CA) according to the principles suggested by Sambrook et al (Sambrook J,1989). A 50ng aliquot of the cDNA probe was labelled with [α-^{32}P]-dCTP (ICN Biochemicals, Irvine, CL) to a high specific activity (approximately 1 x 10^9 dpm/μg of DNA) using the Oligolabelling kit (Pharmacia, Piscataway, NJ). The membrane was prehybridized in ExpressHyb solution in a hybridization oven (BIOCAN. SCIENTIFIC, Mississauga, Ont) at 60°C for one hour with continuous shaking. The radioactive probe was quickly mixed with 1 ml of warm hybridization solution (about 60°C) and added to the hybridization tube. This was incubated with the membrane in the hybridization oven with continuous shaking for about 14 hours at 68°C. The membrane was subsequently washed two times in 2X SSC-0.05% SDS for 30 min each time, first at room temperature and then at 50°C with continuous shaking in the hybridization oven. Finally it was washed in 0.1X SSC-0.1% SDS for 30 min at 55°C with continuous agitation. The

membrane was blotted to a semi-dry state and exposed to 8x10 Kodak autoradiography film, X-OMAT ™ (Eastman Kodak Co. Rochester, NY).

Radioactive labelling of the cDNA probe: The probe was labelled by the random priming method (Feinberg, 1983) with α-32P-dCTP(ICN) using the Oligolabelling kit (Pharmacia). A 50ng aliquot of the cDNA probe was diluted to a final volume of 34 μl with dH_2O, mixed with 10 μl Reagent Mix (provided in the kit) and denatured at 95-100°C for 10 min. It was then quickly chilled on ice for 3 min and 5 μl of Radioactive ^{32}P was added to the cDNA probe. This was mixed gently with 1 μl Klenow Fragment (provided in the kit), and incubated for one hour at 37°C. The ^{32}P labelled probe was purified by being filtered through a Sephadex G-50 (Pharmacia) soaked Microtube I (ESBE, Ville St-Laurent, Que), and denatured at 100°C for 10 min, then cooled on ice for 3 min before probing.

5.4.5. Transformation and isolation of plasmid DNA

The probe used was a 1.7 kb fragment, 229nt-1936nt of MAP_2 cDNA (Albala et al, 1993) ligated in Bluescribe® Vector provided by Dr. Shafit-Zagardo at Albert Einstein College of Medicine. The probe was first introduced into competent cells, XL1-Blue strain® (Stratagene Ltd. Cambridge, UK) to amplify it. A small-scale isolation/purification (miniprep) was carried out according to the principle of Sambrook et al (1989) with Wizard™ Minipreps DNA Purification Systems (Promega Corporation,

Madison, WI), and correct plasmids carrying the cDNA fragment were identified upon restriction mapping analysis. The 1.7kb MAP_2 cDNA fragments were released from the vectors by EcoRI (Pharmacia) digestion followed by electrophoresis, and collected by electroelution.

Transformation: The electroporation method (Stratagene Ltd.) was used to achieve optimal efficiency of transformation. The competent cells were first thawed on ice, and then gently mixed with the cDNA in a chilled 1.5 microfuge tube, using 5 μl of 10ng/μl plasmid DNA per 100ml of cells. The DNA-cell mixture was transfected into a chilled electroporation cuvette, which was electroporated at 1700V, followed by immediate suspension with 900 μl of SOC medium (Strategene Ltd). This was transferred into a 14ml Falcon 2059 polypropylene tube (Becton Dickinson Labware, Lincoln Park, NJ) for incubation with shaking at 37°C for one hour. Aliquots of 50 μl and 100 μl of the cell suspension were smeared onto Luria-Bertani (LB) agar (ICN) plates containing ampicillin (25ng/ml), and incubated at 37°C overnight.

Miniprep: Transformed cells were inoculated into 5 ml aliquots of LB culture medium, containing ampicillin 25ng/ml. These were incubated at 37°C overnight with shaking. Aliquots of 1.4ml of overnight culture were centrifuged in 1.5ml Eppendorf tubes at maximum speed for 1 min in a microcentrifuge. The pellets were resuspended in 200 μl of resuspension solution (Promega), lysed by adding 200 μl of Cell Lysis Solution (Promega), and precipitated by adding 200 μl of Neutralization solution

(Promega). The precipitants were removed by centrifugation at maximum speed in a microcentrifuge for 5 min. The supernatants were decanted to new microcentrifuge tubes, and extracted by adding 1ml of Wizard ™ Minipreps DNA Purification Resin (Promega) in a Wizard ™ Minicolumn (Promega). The plasmid DNA was finally eluted in 50 μl of TE buffer by spinning the Minicolumn at top speed in a microcentrifuge for 20 sec.

EcoR I digestion and purification: About 50 μl of plasmid DNA was digested with 3 μl of restriction enzyme, EcoRI, in the presence of 6 μl of URB buffer (Promega) at 37°C for 2 hours. Following the digestion, the 1.7kb insert was released from the vector (Fig.14) by electrophoresis with 1% agarose gel using the 1kb Ladder (GIBCO) as a molecular weight marker, in 0.5X TBE buffer at 80V for about 1hour. Bands bearing 1.7kb cDNA fragments were cut under the UV transilluminator, and were electro-eluted in an elution cell (Bio-Rad) with 0.5X TBE buffer, at 100V for about 2 hours. Equal volumes of 2-propanol were added to the elutant, which was spun at maximum speed for 10 min in a microcentrifuge. Pellets were washed with 1ml of 70% Ethanol, air-dried, and finally resuspended in millipore filtered sterile water.

5.5. ApoE GENOTYPING

5.5.1. Extraction of DNA

High molecular weight DNA was isolated from frozen tissues with a method modified from those of Davis and Goelz (Davis L et al, 1989; Goelz et al, 1986) About 3 g of frozen cortical tissue was crushed in a pestle in liquid nitrogen, and then mixed with 5ml of 1X RSB buffer (10mM Tris, pH7.4, 10mM NaCl, 25 mM EDTA), and 0.5 ml 10% SDS. The pulverized tissue mixture was incubated overnight with 10 mg proteinase K (Boehringer Mannheim), in a sterile/Gamma irradiated 14-ml round bottom polypropylene tube with cap, (Becton Dickinson Labware, Lincoln Park, NJ) at 55°C with gentle shaking. Then 0.5 ml of 5M NaCl and 5ml of salt-saturated phenol (SS-Phenol) were mixed with the proteinase K treated mixture, and it was centrifuged in a Beckman Al-20 rotor at 10,000 g, 4°C for 10 min. The supernatant was transferred into a fresh tube and extracted with an equal volume (about 2.5ml) of Phenol/Chloroform/Iso-amyl-alcohol (10:10:1) and centrifuged again under the same conditions as above. The supernatant was transferred into a fresh tube, extracted with 5ml of chloroform, and again centrifuged under the same conditions. The final supernatant was transferred into a fresh tube, and mixed with 2.5 volumes (about 20ml) of 99% cold ethanol for a few min at -20°C. The DNA was spooled out with a glass rod into an ependorff, allowed to air-dry and finally diluted in 300-500 μl of TE buffer.

Preparation of SS-Phenol: 100ml of 2M Tris, pH 7.4 and 130ml of H_2O were added to a 1-lb bottle of phenol. This was heated at 37°C until dissolved. After removing the upper aqueous phase, 100ml of 2M Tris, pH 7.4, 25ml of m-cresol (Kodak), 1ml of β-mercaptoethanol, and 500mg of 8-hydroxyquinoline were added. The solution was stored at room temperature.

Preparation of TE buffer: To make 100ml of 10mM Tris, 0.1 mM EDTA buffer, 0.5ml of 2M Tris stock, pH 7.4, and 20 μl of 0.5M EDTA stock, pH 8, were diluted with H_2O to a final volume of 100ml. The solution was stored at room temperature.

5.5.2. Polymerase chain reaction (PCR)

ApoE genotyping was performed by the allele-specific polymerase chain reaction (PCR) using the method of Main et al (1991) with some modification (Nalbantoglu, 1994). The allele-specific oligonucleotide primers labelled D,E,F,G, and H were synthesized by Genosys Biotech, The Woodlands, TX. Reactions were carried out in a volume of 50 μl containing 1 μg of DNA; deoxyadenosine triphosphate, deoxycytidine triphosphate, deoxythymidine triphosphate, and deoxyguanosine triphosphate, each at 0.2 mmol/L; 10% dimethyl sulfoxide; 12.5pmol of either primer (D,E,F,or G) or 25 pmol of primer H; and the PCR reaction buffer. The DNA in the reaction mixture was first denatured for 10min at 96°C, cooled to 4°C, mixed with one unit of Tag polymerase (Vector Biosystems, Toronto, Ont), and heated again at 96°C for 2 min and subjected

to 30 cycles in a thermal cycler. Each cycle consisted of denaturation at 96°C(10sec), annealing at 58°C (30sec), and extension at 65°C (1min). Then 10 μl of the PCR product was subjected to electrophoresis with 1% agarose gel containing Tris-phosphate EDTA (TPE) buffer (0.08 mol/L Tris-phosphate, 0.002mol/L EDTA) and ethidium bromide (0.15 μg/ml) at 67V for 1 hour. The gel was then photographed and the banding profile was determined based on a comparison with known standards. Determination of apoE genotype was performed on a total of 48 brain samples. General information on the samples is listed in table 4 in Section 6.2.3.

The primer sequences: primer D, TACTGCACCAGGCGGCCTCG; primer E, TACTGCACCAGGCGGCCTCA; primer F, GCCTGGTACACTGCCAGTCA; and primer H, AAGGAGTTGAAGGCCCCTACAAAT.

The reaction buffer: This consisted of 100 mmol/L, Tris-HCl, pH 9.0, 500 mmol/L KCl, 15mmol/L $MgCl_2$, 2mg/ml gelatin, 1% Triton X-100.(Vector Biosystems).

5.6. BIOSTATISTICAL ANALYSIS

Biostatistical analyses were performed according to standard methods (McClave & Dietrich, 1988), using the program InStat 2.01 for the MacIntosh (Graphpad). The Students' T test was used to compare the means from two sample populations, such as the means of MT or MAP_2 values from individuals with an apoE ϵ4 allele(s) versus those

without an $\epsilon 4$ allele in each age-matched group. Since the T test requires that the means compared have equal standard deviations (SDs), Bartlett's test for homogeneity of variances was used to assess the SDs of group means compared, before the T test was applied. When Bartlett's test showed a significant difference among the SDs of the group means compared, such that the T test was not applicable, the Mann-Whitney test was used instead.

One-way analysis of variance (ANOVA) was used to compare the mean values from three or more age groups to assess age-related changes, in values such as average density (no./mm^2) and size (diameter in μm) of amylosomes in the 10 consecutive age groups, and average amount of MT and MAP_2 in the four consecutive age groups. Since ANOVA requires that the means compared have approximately equal SDs, when Bartlett's test showed a significant difference among SDs of the group means compared, such that the ordinary one-way ANOVA was not applicable, the Kruskal-Wallis nonparametric ANOVA was used instead.

When comparing the age-related variables, a Post test for linear trend was performed, when ANOVA showed significant differences among the groups arranged in a natural order. The degree of linear association between the variables was determined by Runs test with $p > 0.05$ as no significant departure from linearity.

P values equal to or greater than 0.05, less than 0.05, and less than 0.01 are

interpreted as "not significant", “significant”, and “very significant” respectively.

6. RESULTS

6.1. CHARACTERISTICS OF AMYLOSOMES

6.1.1. Histological characteristics and distribution of amylosomes

The amylosomes in paraffin-embedded tissue sections were easily recognized under light microscopy with H&E stain, as bright pink homogeneous bodies, which were almost perfectly round in shape (Fig.1) and varied between 0.8 μm and 10 μm in diameter. They stained with Congo Red, without exhibiting apple-green bifringence under a polarized light, which suggested the protein content was probably not in a beta-pleated configuration. Electron microscopy revealed that amylosomes were located within dendrites. Most appeared to be homogeneous, and surrounded by a ruffled membrane, (Fig.2) while in a few amylosomes smaller substructures were visible. We confirmed the earlier reports observing these inclusions in dendrites, (Rees, 1975; Hara, 1981; 1986), and, interestingly, we also found them within neuronal cell bodies, attached to neuronal processes, close to cerebral blood vessels, on the cortical surface close to the pia, and at the boundary of the CSF (Fig.3). Their presence in these areas has not been previously reported.

The distribution of amylosomes in the human cerebral cortex was similar to that of amyloid plaques in AD. They were not present indiscriminately throughout the brain,

although they were seen in all cortical layers within the temporal, frontal, parietal and occipital lobes. They were more concentrated in specific regions of the cortex, such as the inferior temporal area, frontal lobe anterior to the precentral gyrus, and parietal lobes posterior to the post-central parietal area, as well as the hippocampus and amygdala, which are the principal areas that contain amyloid plaques in AD. They were not found in the cerebellum, nor in the deep white matter, areas where the typical amyloid plaques with a dense core are usually not found in the AD brain.

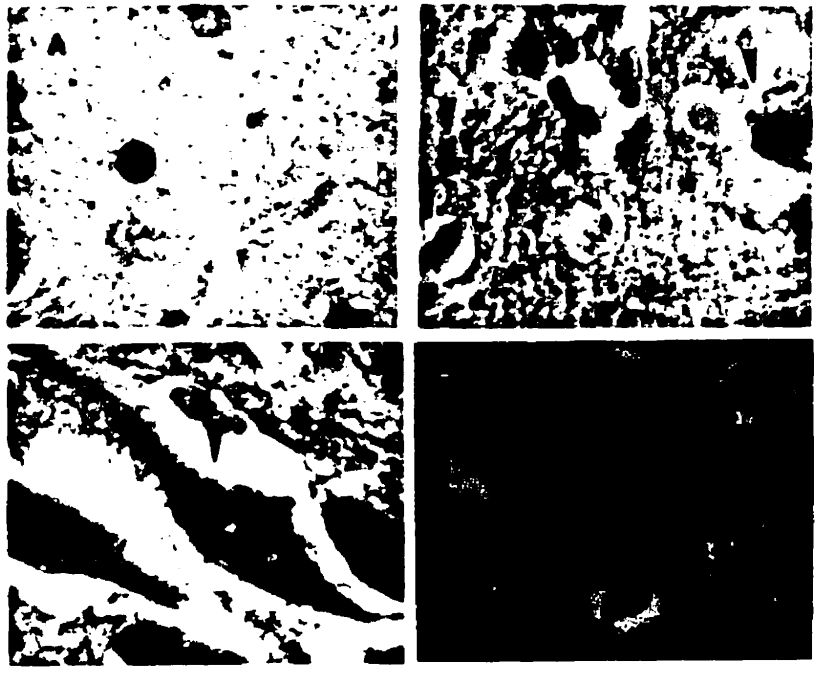


Fig. 1 Light microscopy of amylosomes with H&E stain.

A) A typical amylosome in the neuropil.

B) One intact amylosome and one that appears to have broken and could be initiating the formation of a plaque.

C) One amylosome within a neuronal cell body.

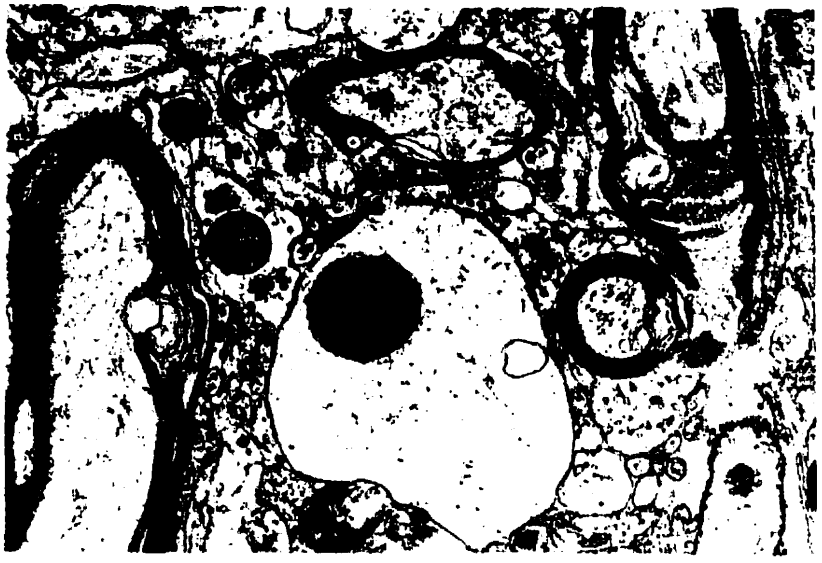
D) One amylosome attached to a dendrite.

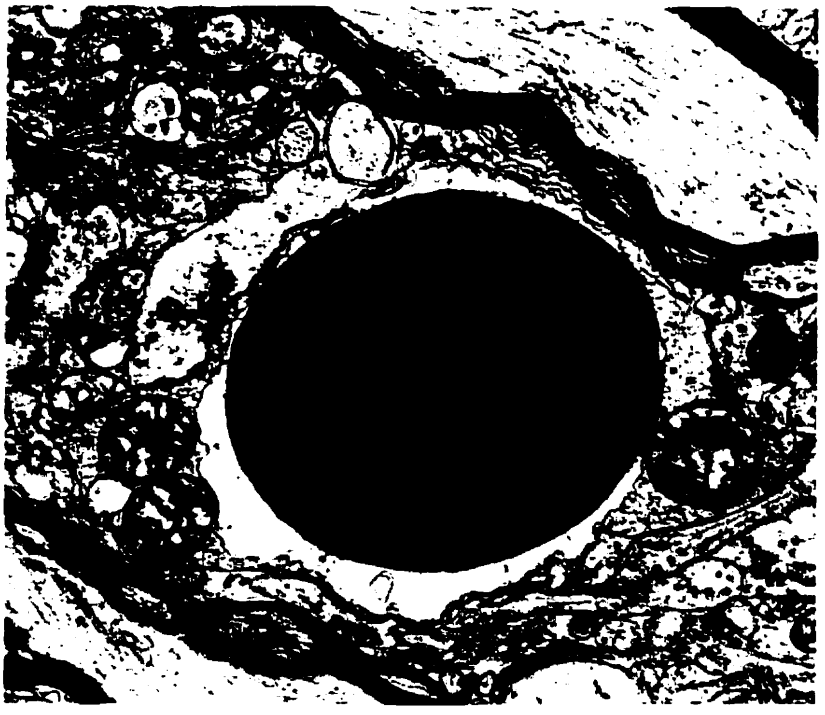
(A-D, magnification 400X).

Fig. 2 Electron micrographs of amylosomes located within dendrites.

Upper photograph, an amylosome confined by a ruffled membrane within a dendrite (approximately 35,000X),

Lower photograph, a different field, at higher magnification (50,000X) shows the homogeneous appearance of the amylosome interior, surrounded by a membrane.





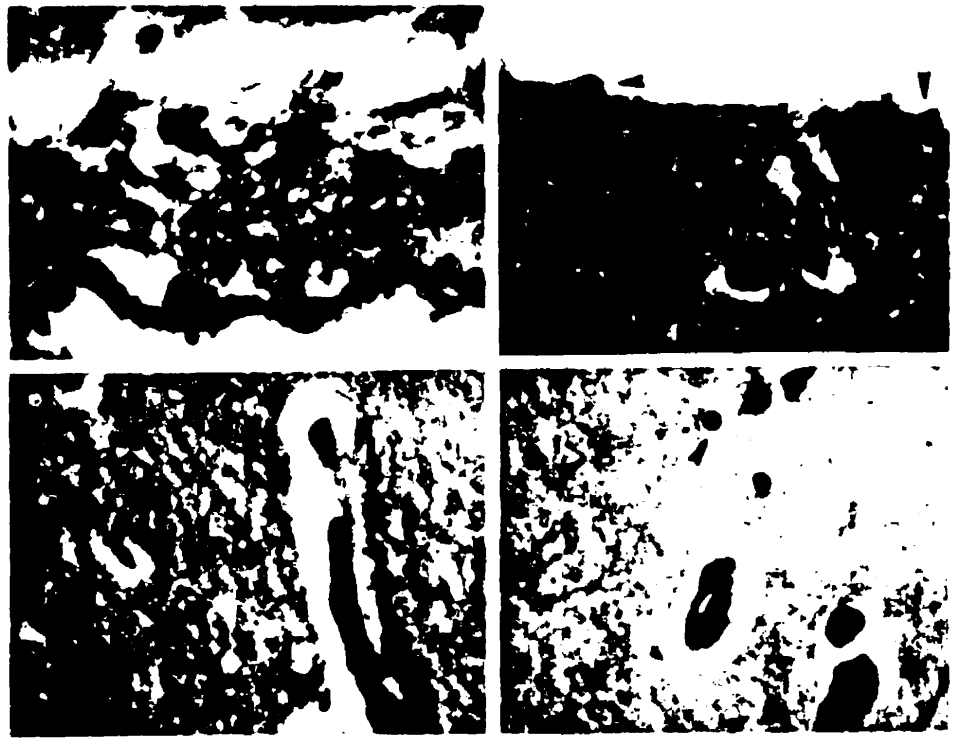


Fig. 3. Amylosomes within the human cerebral cortex.

The pictures were generated by computer from digitized data stored during image analysis. Spherical amylosomes of various size, shown by arrows, were located at the cortical surface (upper left and right), close to a blood vessel (lower left), and in the neuropil (lower right). (magnification 400X).

6.1.2. Density of amylosomes throughout the lifespan.

The average density of amylosomes in each decade of the human lifespan was expressed by calculating the number of amylosomes per millimeter squared using computer-assisted image analysis. The amylosomes were not found in any of 6 prenatal brains, examined as early as 12 weeks of gestation, nor in the newborn or infant brain. They began to appear in the brain at the age of about 1 and a half to 2 years. The average density (no./mm^2) rose quickly with age, and reached a peak at about age 10, then remained relatively stable throughout almost all of the remaining lifespan, with a decline above the age of 80 (Fig.4) The ANOVA showed no significant difference between the means for the age groups from the 20th to the 80th decade ($p=0.112$), with Bartlett's test for homogeneity of variances showing that the differences among the standard deviations were not significant.($p=0.58$). The distribution of the sample population and the values for amylosome density and size, are documented in Table 1.

6.1.3. Size of amylosomes throughout the lifespan

Under light microscopy, the amylosomes varied in size. (0.8-10 μm). In aged brains, they were relatively larger and more clearly seen, nevertheless numerous small ones still could be observed. The average diameter of amylosomes for each age group was calculated in μm using computer-assisted image analysis, which originally gave two

diameters for each amylosome targeted, the biggest and the smallest. The final results were based on the average of the two. The size of amylosomes (μm in diameter) increased in the first ten years and then did not change significantly with age, remaining relatively stable up to the age of 90 (Fig.5). The ANOVA of the means for each age group from 20 to 80 years showed no significant change ($p=0.093$), with Bartlett's test for homogeneity of variances showing that the differences among the standard deviations were not significant ($p=0.245$).

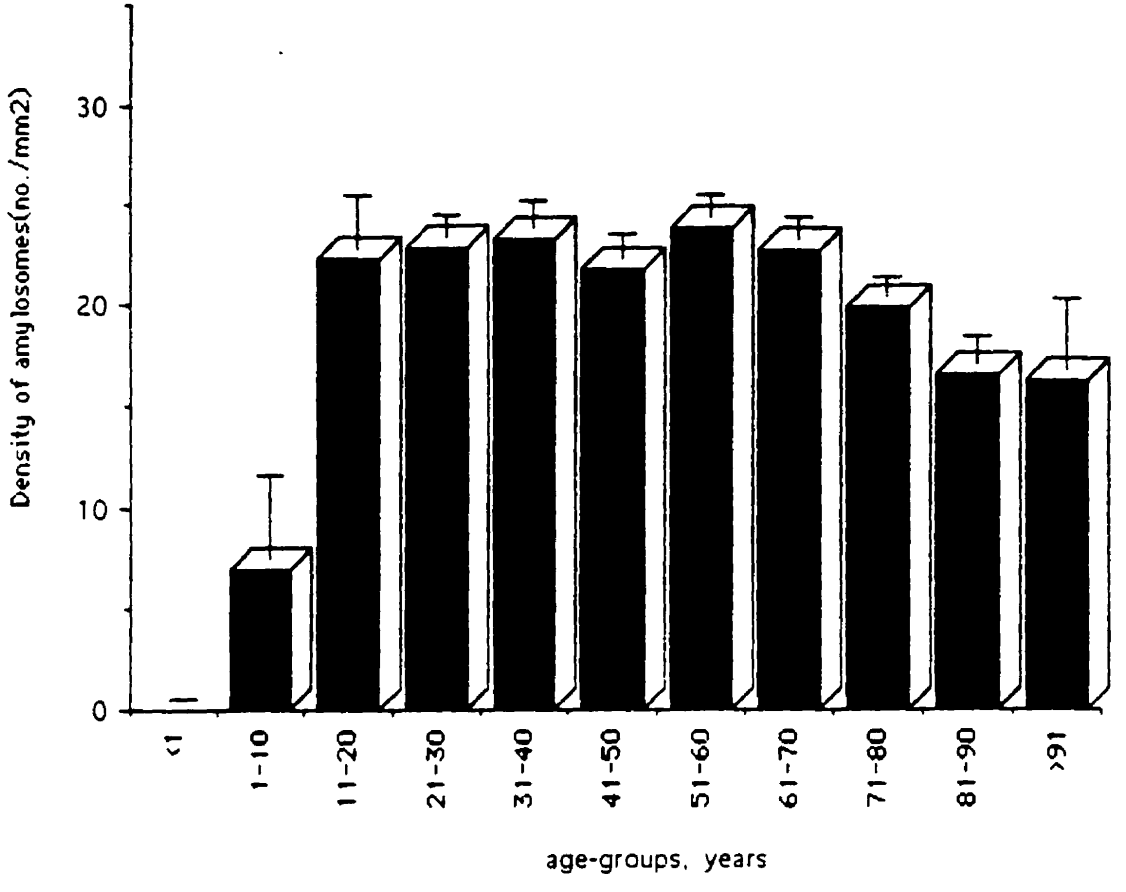


Fig. 4. Density of amylosomes in the human brain throughout life.

The density of amylosomes in each of 216 human brains, grouped by age, was expressed as the average number per millimeter squared observed during each decade of life .

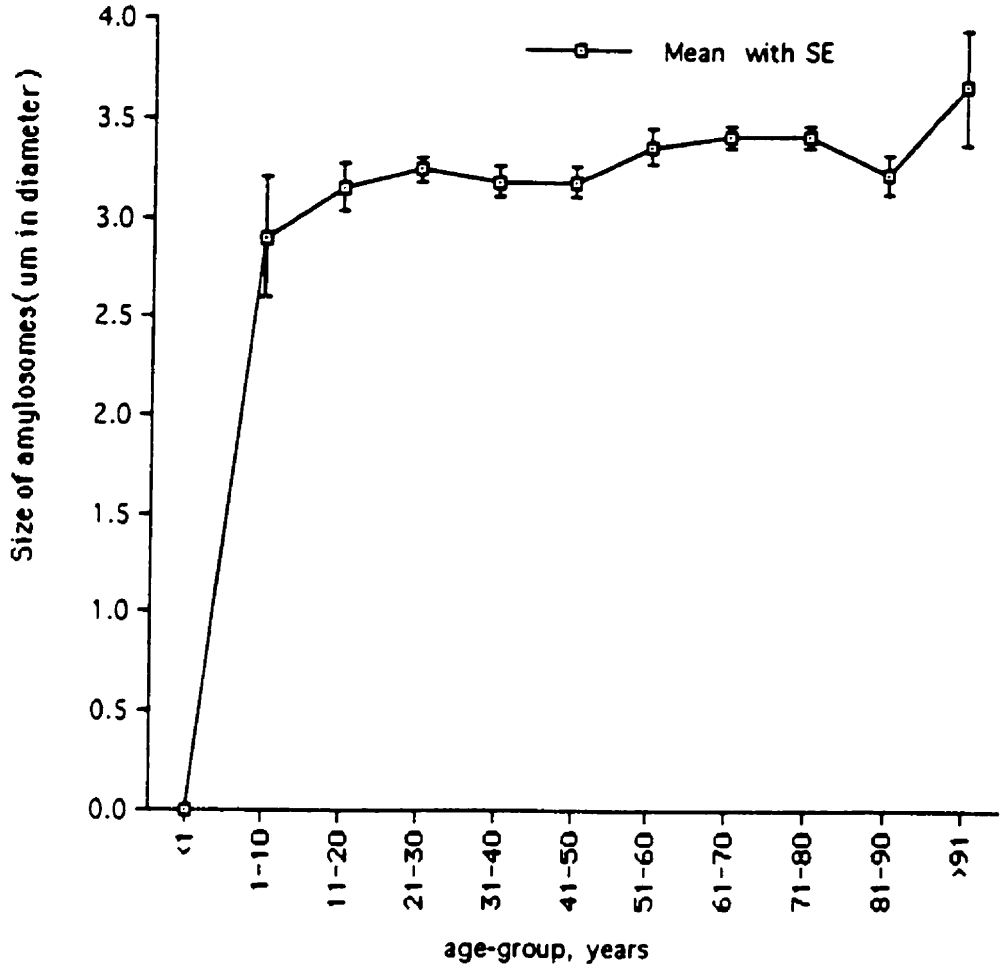


Fig. 5. Size of amylosomes in the human brain throughout life.

The size of amylosomes in each of 216 human brains, grouped by age, was expressed as the average diameter (μm) observed during each decade of life.

Table 1.

Summary of the morphometric analysis

Age group (years)	Avg.Age Years Mean ± S.D.	No.of cases T	M/F	Density (no./mm^2) Mean ± S.E.	Diameter(μm) Mean ± S.E.
<0		6	4/2	0	0
1- 10	5.4 ± 5.1	2	2/0	7.00 ± 4.0	2.90 ± 0.32
11 - 20	17.1 ± 2.0	8	4/4	22.38 ± 2.62	3.15 ± 0.12
21 - 30	24.6 ± 2.9	16	10/6	22.94 ± 1.11	3.24 ± 0.06
31 - 40	36.3 ± 2.5	22	13/9	23.36 ± 1.32	3.18 ± 0.08
41 - 50	46.4 ± 2.3	21	16/5	21.76 ± 1.24	3.18 ± 0.08
51 - 60	55.9 ± 3.1	27	17/10	23.89 ± 1.12	3.36 ± 0.09
61 - 70	65.7 ± 3.4	43	25/18	22.79 ± 1.04	3.41 ± 0.07
71 - 80	74.6 ± 2.7	40	13/17	19.95 ± 0.97	3.41 ± 0.06
81 - 90	84.7 ± 2.4	32	15/17	16.50 ± 1.37	3.22 ± 0.10
91 - 100	93.4 ± 2.3	5	1/4	16.20 ± 3.54	3.66 ± 0.29

Avg.: average. S.D: standard deviation. T: Total. M/F: Male/Female.

S.E: Standard error.

6.1.4. Immunohistochemistry of amylosomes

Antibodies directed against amyloid plaque or against synthetic Aß both consistently labelled amylosomes in histological sections of the human cerebral cortex (Fig.6) The pattern of immunostaining was similar in each of 25 normal brains, from age 14 to 85, and in 4 AD cases, the antibodies also showed positive reactions with amyloid plaques. In addition, blood vessels in the cerebral cortex were often labeled in older controls and AD brains. These results strongly suggest that amylosomes contain an amyloid peptide that is similar or identical to the constituent of amyloid plaques.

Intact, isolated amylosomes were stained by Congo Red. However, they did not demonstrate the birefringence under polarized light that is characteristic of amyloid until they were ruptured by sonication (Fig.6), suggesting that the membrane-bound peptide is maintained in a soluble, or non ß-pleated form.

6.1.5. Protein composition of amylosomes

The content of purified amylosomes was analyzed by reverse phase HPLC which yielded a pattern of peaks similar to that obtained from amyloid plaques which were isolated from the brains of AD patients (Fig.7). The HPLC peaks obtained from amylosomes were further characterized by Western blotting using antibodies against synthetic fragments of Aβ, or against amyloid plaque, and contained a 4kd band similar

to that found in amyloid plaque. Amino acid composition analysis of the amylosomes also showed a profile similar to the amino acid composition of isolated amyloid plaques (Fig.8), again suggesting that amylosomes contain a material that is similar or identical to the amyloid peptide found within the plaques in AD.

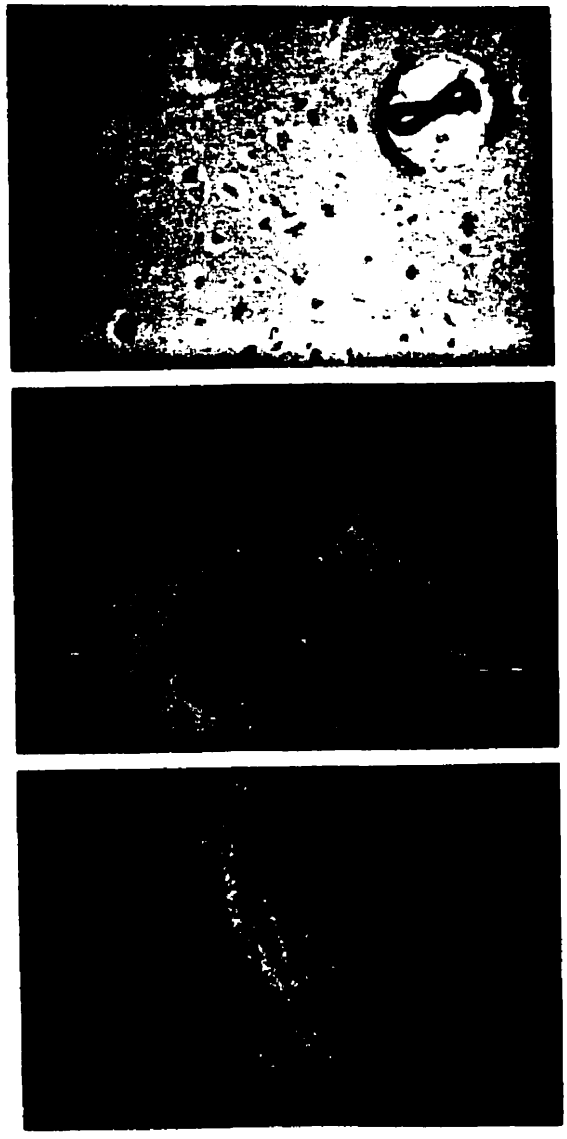


Fig. 6 Staining properties of amylosomes indicate the presence of amyloid peptide.

Upper: antibody against the synthetic peptide, Aβ17-23 (DAKO) stains both cerebral blood vessels and the amylosome.

Middle: pellets of amylosomes isolated from human cerebral cortex stain with Congo Red.

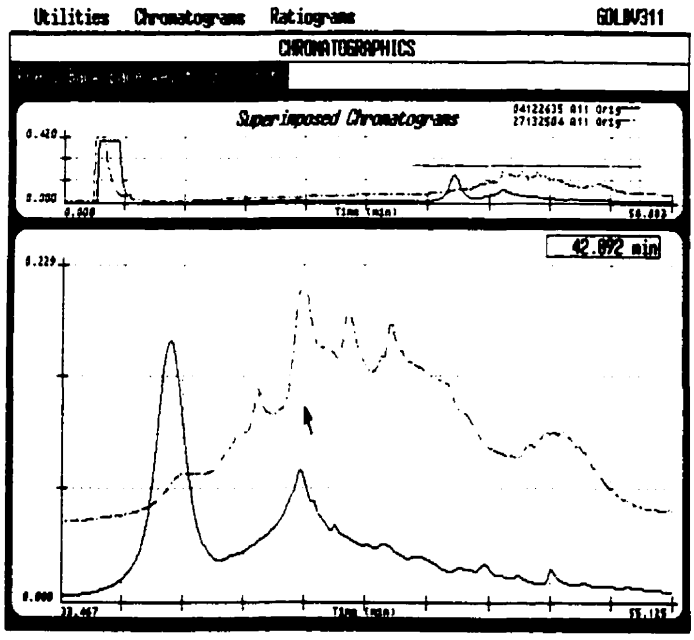
Lower: broken amylosomes stained with Congo Red show strong apple-green birefringence under polarized light.

(Magnification, upper 400X, middle 200X, bottom 200X).

Fig. 7 Protein composition of amylosomes.

Upper: HPLC profiles produced by solubilized amylosomes isolated from a human brain, age 40 years (solid line) and by solubilized amyloid plaques isolated from an 82 year old patient with AD (dashed line). There is a common peak at 42 minutes. The initial, large peak on the solid line is a formic acid, percoll artifact.

Lower: The HPLC peak collected at 42 minutes from the purified preparation of human amylosomes was immunoblotted onto an immobilon membrane. A dot blot with an antibody against the synthetic peptide Aβ16-32 showed a postive immunohistochemical reaction, with alkaline phosphatase antirabbit as the second antibody (right). A dot blot of normal rabbit serum (left) and column effluent (center) served as controls for the reaction.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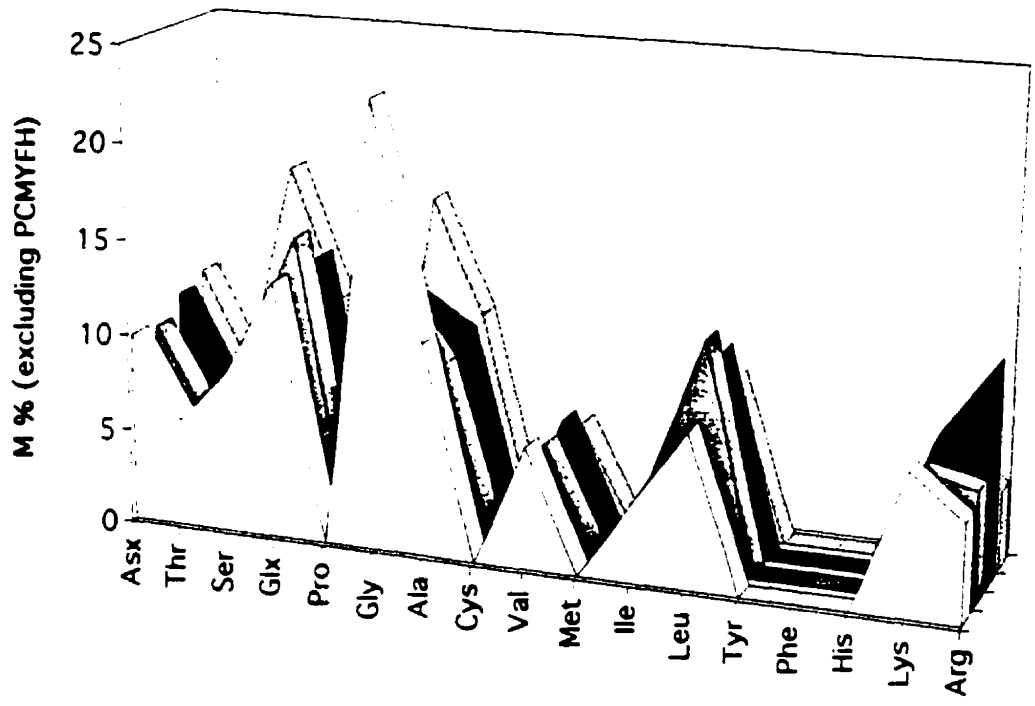


Fig. 8. Amino acid composition of amylosomes and amyloid plaques.

The amino acid composition of purified amylosomes from 2 non-demented individuals, age 43 and 40 (front two) are illustrated in comparison to the amino acid content of amyloid plaques (rear two) from 2 patients with AD, age 79 and 82.

6.2. BRAIN MICROTUBULES AND ASSOCIATED PROTEINS

6.2.1. Age-related changes in MT and associated proteins

MT and associated proteins were isolated from frozen human cortex, separated with electrophoresis, and identified on Western blots using antibodies against α-, and β-tubulin, and MAP_2 (Fig.9). The major band for MAP_2 was found at 47 kd rather than the expected 230 to 280 kd which is where MAP_2 is identified using isolates from fresh brain (Matus, 1988). The location of the major band at 47 kd was also reported by Sparkman (1992) and it probably represents a degradation product of the protein due to enzymatic activity in the postmortem interval. Two additional MAP_2 bands were located in the anticipated 230-280 kd range but they were very faint in contrast to the band at 47 kd. The proteins were also electrophoresed on gels of lower concentration to determine if this finding was consistent. In agreement with the previous observation by other investigators (Sparkman, 1992) the band remained at 47 kd. Thus, for the quantitative estimation of protein, the band at 47 kd, which reacted with the monoclonal antibody, was used in the densitometry estimations.

There was a significant decline in the quantity of both MT and MAP_2 with age in the human brain, when samples were assessed in 3 consecutive age groups. This decline followed a linear trend. (Table 2, Fig.10). The MT content in AD brains was not significantly lower than that in age-matched non-demented controls (Fig.10), while the MAP_2 content was similar in both groups.

Fig. 9. Characterization of isolated MT proteins.

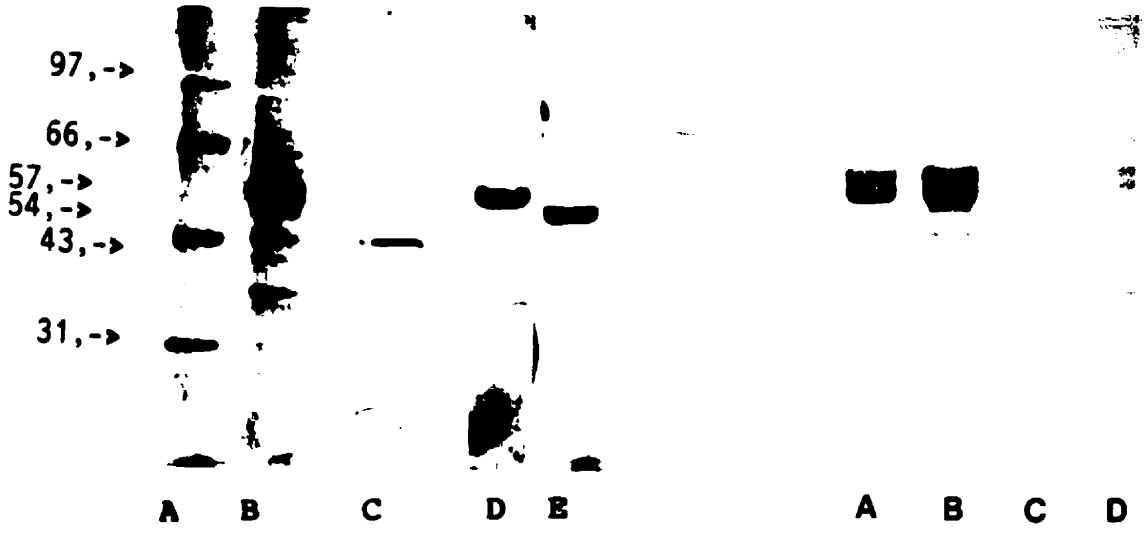
Left: Western blot analysis.

Lanes A,B: Molecular weight markers and the sample preparation from human brain, respectively, stained with Coomassie blue. Molecular weight standards, in kd, are indicated by the arrows.

Lanes C-E: MT proteins transferred to Immobilon membrane and recognized by mouse monoclonal antibodies against MAP_2 (C), α tubulin (D) and ß tubulin (E).

Alpha & beta tubulin and MAP_2 were recognized at 57, 55, and 47 kd respectively.

Right: Electrophoretic gels from which alpha and beta tubulins and MAP_2 were quantified by laser densitometry. Two samples from young brains (A 35 years, B 45 years) showed a much higher content of MT and MAP_2 compared with an older (D, 76 years), and an AD brain (C 82 years).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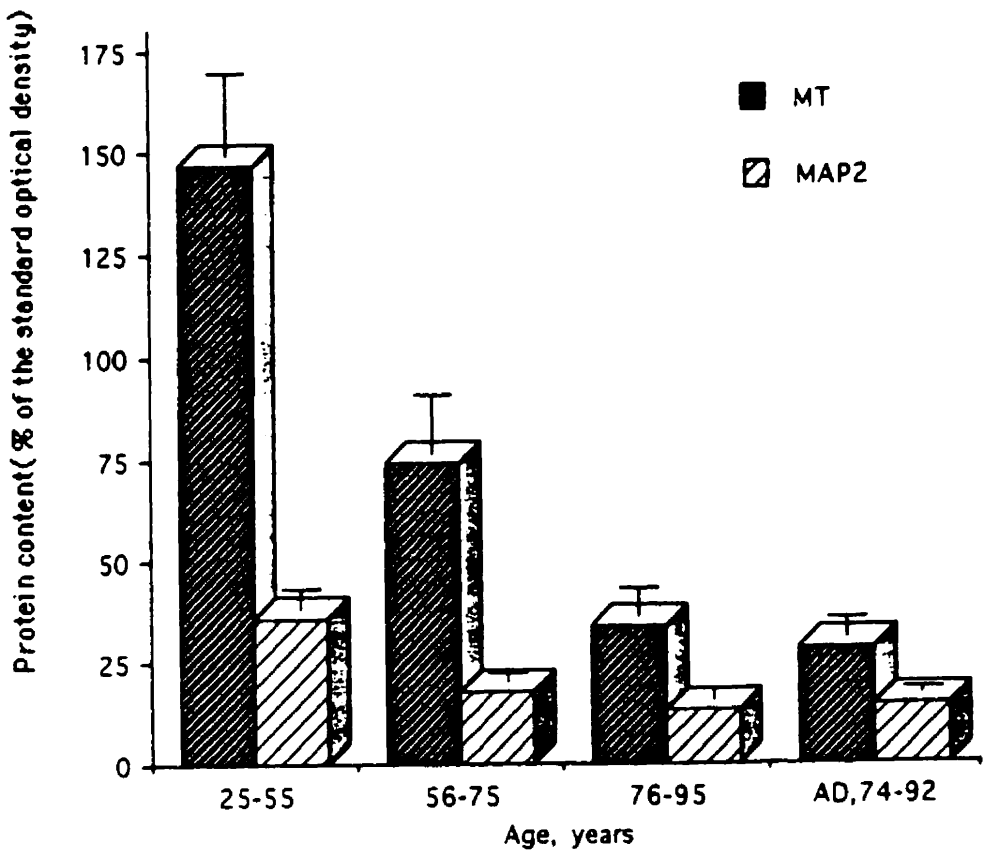


Fig. 10. Effect of age and AD on the quantity of MT proteins and MAP_2 in the human brain.

Means and standard errors are expressed as a percent of the standard. Samples were obtained from non-demented adults grouped into 3 age categories, in comparison to a group of patients with AD.

Table 2.

Summary of the data on MT and MAP_2 content in human brain

Age group(yr)	Avg. age Mean±SD	Number T (M/F)	Time hour	MT* Avg. ± SE.	MAP_2* Avg.± SE.
25-55	40.5 ± 9.4	16 (11 / 5)	9.25	146.92 ± 20.79	36.12 ± 4.62
56-75	67.3 ± 5.7	22 (18 / 4)	8.5	74.05 ± 14.80	18.70 ± 1.69
76-95	82.4 ± 5.9	9 (5 / 4)	7.8	33.97 ± 6.48	13.50 ± 2.54
ANOVA				p= 0.0011	p=0.0006
Linear Regression	CC Slope Departure			r=-0.42 p<0.0034 p>0.6	r=-0.59 P<0.0001 p>0.07
AD (74-92)	82.5 ± 8	8 (1 / 7)	8.5	28.90 ± 4.83	14.59 ± 1.42

*: Optical density, percent of the standard (2 μg BSA). Avg.: average.

SE.: standard error Time: Mean postmortem time

ANOVA: Kruskal-Wallis Nonparametric ANOVA. CC: correlation coefficient.

Slope: Slope different from 0. Departure: departure from linearity.

6.2.2. Age-related changes in MT polymerization *in vitro*

Samples of MT from 17 individuals were polymerized *in vitro* and viewed with EM. There were remarkable differences, both qualitatively and quantitatively, between MT from brains of individuals at different ages. Samples from younger brains produced well shaped, elongated MT in relatively large quantity (Fig.11), while samples from older brains yielded some MT, but these were significantly shorter and fewer in quantity. Samples from AD brains hardly polymerized into MT at all.

6.2.3. Age-related changes in MT and MAP_2 in subjects with different ApoE4 genotypes

APOE genotypes were determined from a total of 48 samples, whose MT and MAP_2 had been assessed. When the 3 age categories were further subdivided with respect to APOE genotype, decreases in MT and MAP_2 were found in groups with an APOE ϵ 4 allele(s) before the age of 75. (Table 3. Fig.12).

This difference was statistically significant in the age-group 56-75 ($p<0.01$) (Fig.13). While a difference between the samples with at least one ϵ4 allele and those without an ϵ4 allele was also observed in individuals below 55, it did not reach statistical significance. The results from individuals over 76 years of age could not be statistically assessed because of the relatively small sample size.

Interestingly, similar differences were seen in AD brains where the presence of an $\epsilon 4$ allele was also associated with a lower level of MT and MAP_2. Subdividing by apoE genotypes also revealed a difference between age-matched AD patients and normal controls (Table 3).

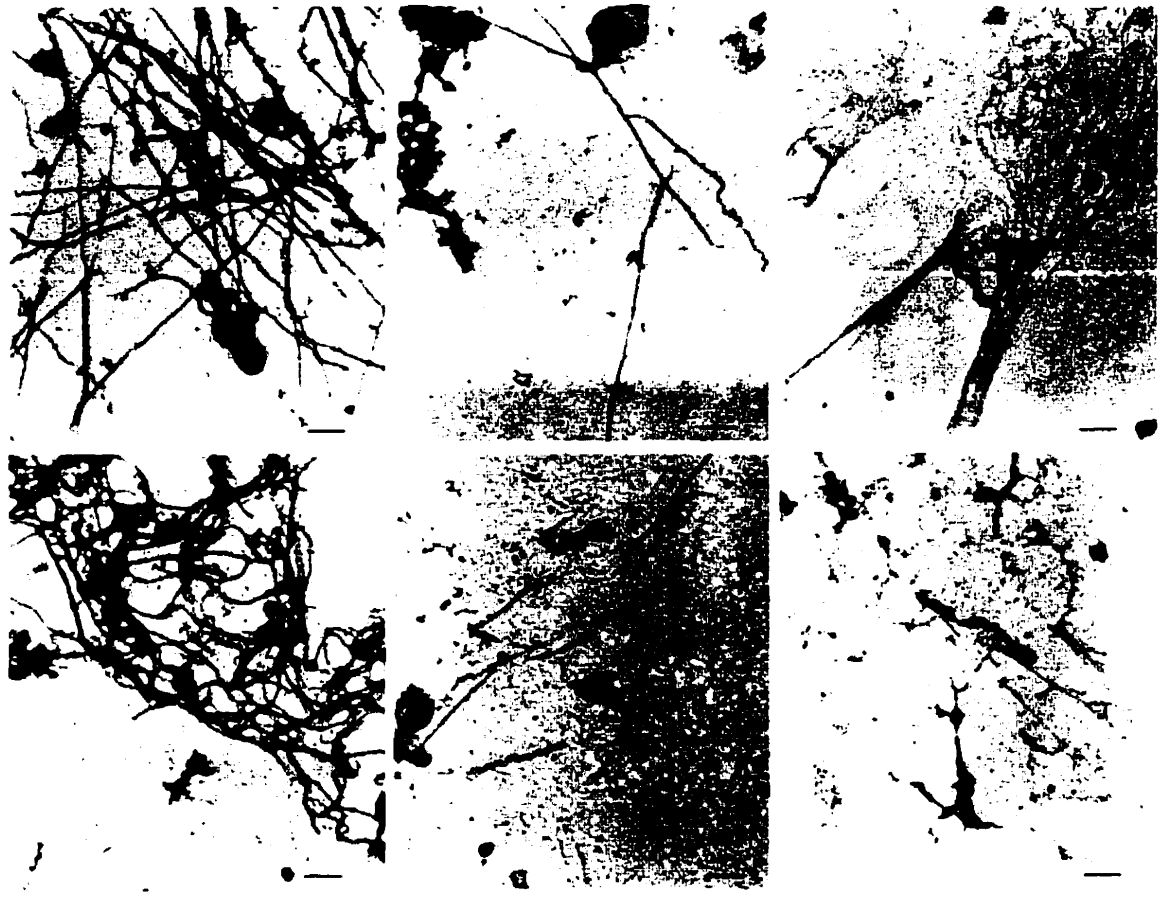


Fig. 11. Electron micrographs of MT polymerized *in vitro* from 6 samples isolated from human cerebral cortex.

Left: Tubulin proteins isolated from a 25 yr old male (upper) and a 35 yr male (lower).

Middle: Tubulin proteins isolated from a 64 yr old female (upper) and a 75 yr old male (lower).

Right: Tubulin proteins isolated from two AD brains, an 80 yr old female (upper) and an 88 yr old female (lower).

(Calibration bars, 250 nm).

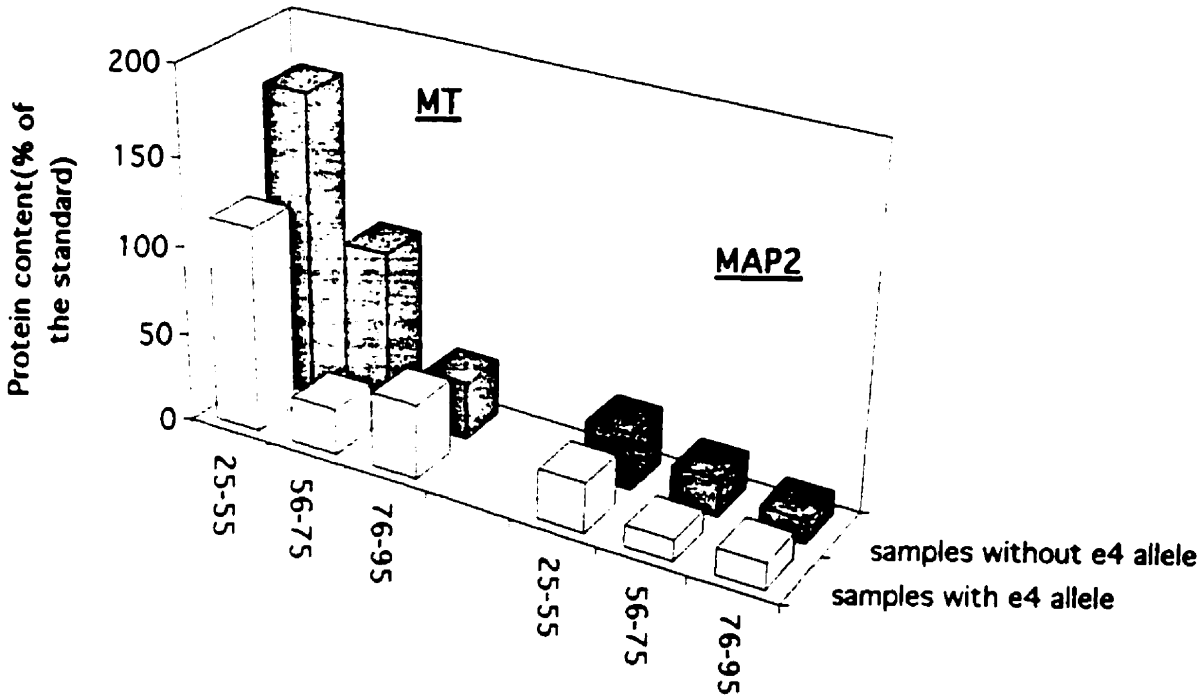


Fig. 12. Effect of age and apoE genotype on the quantity of MT proteins and MAP_2 in the human brain.

Samples were obtained from non-demented adults grouped into 3 age categories, and further subdivided into those with and without an APOE ε4 allele.

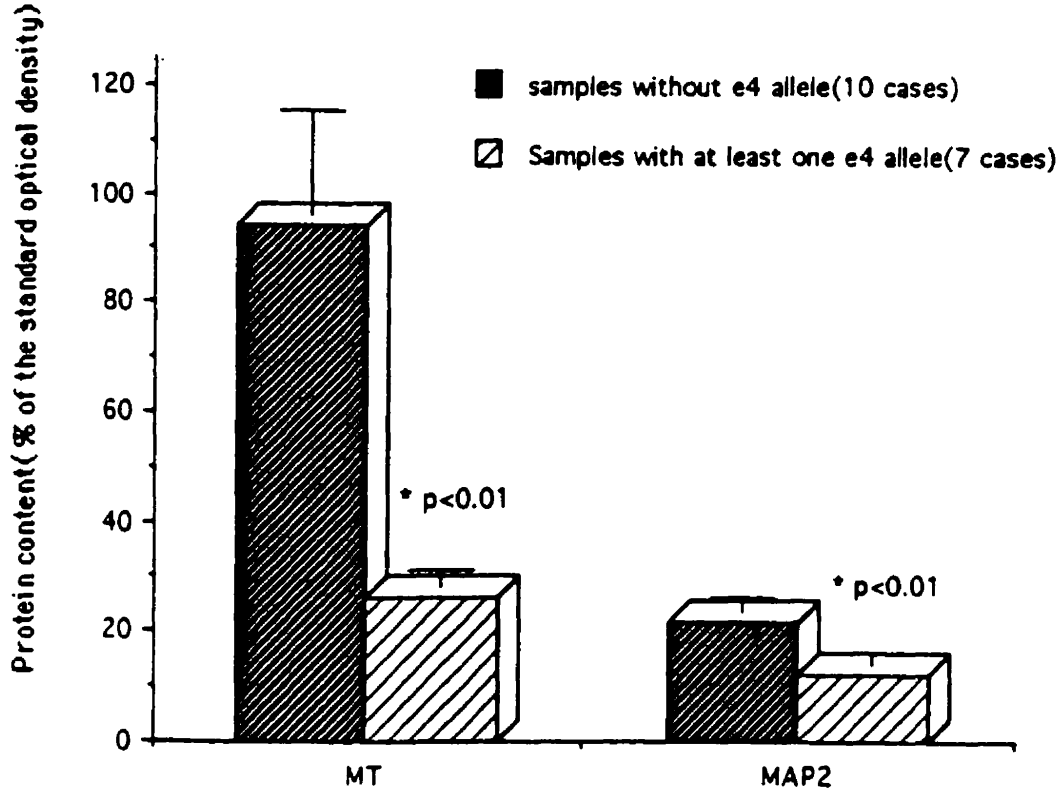


Fig. 13. MT proteins and MAP_2 quantities, for the age group 56-75 years with different apoE genotypes.

Values are expressed as a percent of the standard, for 17 cases, 7 with at least 1 apoE ε4 allele, and 10 without.

Table 3. Summary of the data on MT & MAP_2 in samples with and without an $\epsilon 4$ allele(s)

Group	Avg. age Mean±SD	Cases T (- / +)	α & β tubulin $\epsilon 4-$ / $\epsilon 4+$	MAP_2 $\epsilon 4-$ / $\epsilon 4+$
Normal 25-55	41.1 ± 9.2	14 (8 / 6)	173 ± 33 / 116 ± 32	32 ± 3 / 28 ± 3
56-75	68.2 ± 5.4	17 (10/ 7)	94 ± 20 / 26 ± 3 *$p<0.01$	21 ± 2 / 12 ± 2 *$p<0.01$
76-95	82.4 ± 5.9	9 (7 / 2)	31 ± 8 / 44 ± 3	13 ± 3 / 16 ± 2
AD 74-94	82.5 ± 8	8 (5 / 3)	31 ± 5 / 25 ± 10	17 ± 1 / 11 ± 2

T: Total, (-/+): without $\epsilon 4$ allele/ with $\epsilon 4$ allele.

*:Mann-Whitney test: very significant difference between the samples with and without apoE $\epsilon 4$.

6.3. ANALYSIS OF MAP_2 mRNA

The MAP_2 cDNA probe was first transfected into cells for amplification, and then electrophoretically released with digestion by the restriction enzyme EcoR I. The probe was labeled with P^{32} and then hybridized onto Northern blots (Fig.14). The MAP_2 mRNA bands on the northern blots, recognized with the probe, were measured by densitometry, adjusting the readings for each sample with respect to its own β-actin band (Fig.15). There was an extremely significant decrease ($p < 0.0001$) in MAP_2 mRNA with age, which showed no significant departure ($p = 0.33$) from linearity (Fig.16). There was no significant difference observed between age-matched AD and non-demented controls (Table 4).

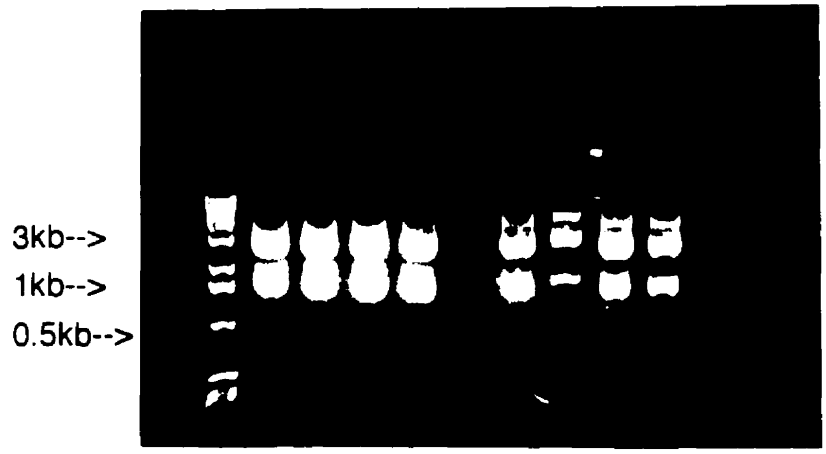
Fig. 14. Quantification of MAP_2 mRNA.

Upper: Electrophoresis with a 1% agarose gel, viewed under UV transillumination.

Left lane: Molecular weight markers, indicated by arrows.

Remaining 8 lanes show the vector (top bands) and MAP_2 cDNA probe (bottom bands). The MAP_2 probe (1.7kb) was released from plasmid DNA with EcoRI, and separated from the vector (2.9 kb) by electrophoresis.

Lower: Six examples of MAP_2 mRNA bands from brains of different ages, detected on northern blots with a P^{32} labelled probe (top). The densitometry measurement for each sample was standardized according to the corresponding β-actin band (bottom). Numbers indicate the age of each sample. All samples were from brains of non-demented individuals, with the exception of example 2, an 85-year-old AD patient.



1.7kb MAP2 cDNA prob released from vector(2.9kb) by digestion with EcoRI

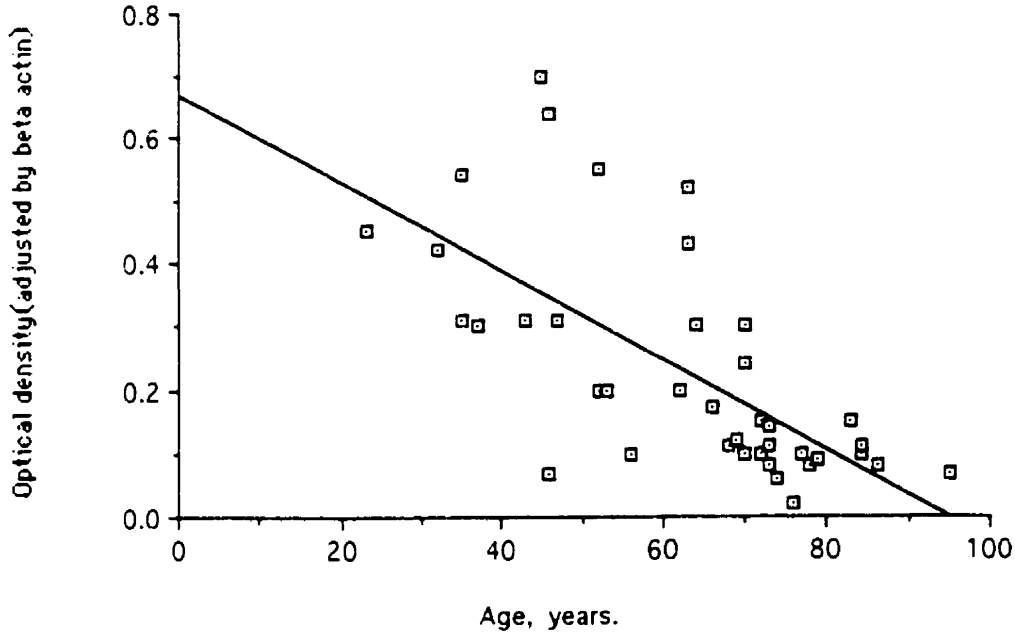


Fig. 15. Relationship between age and MAP_2 mRNA in human cerebral cortex. MAP_2 mRNA was expressed as optical density, using the corresponding ß-actin band as an internal standard for each measurement. Values are shown for each of 40 adult brains, the linear regression is also indicated.

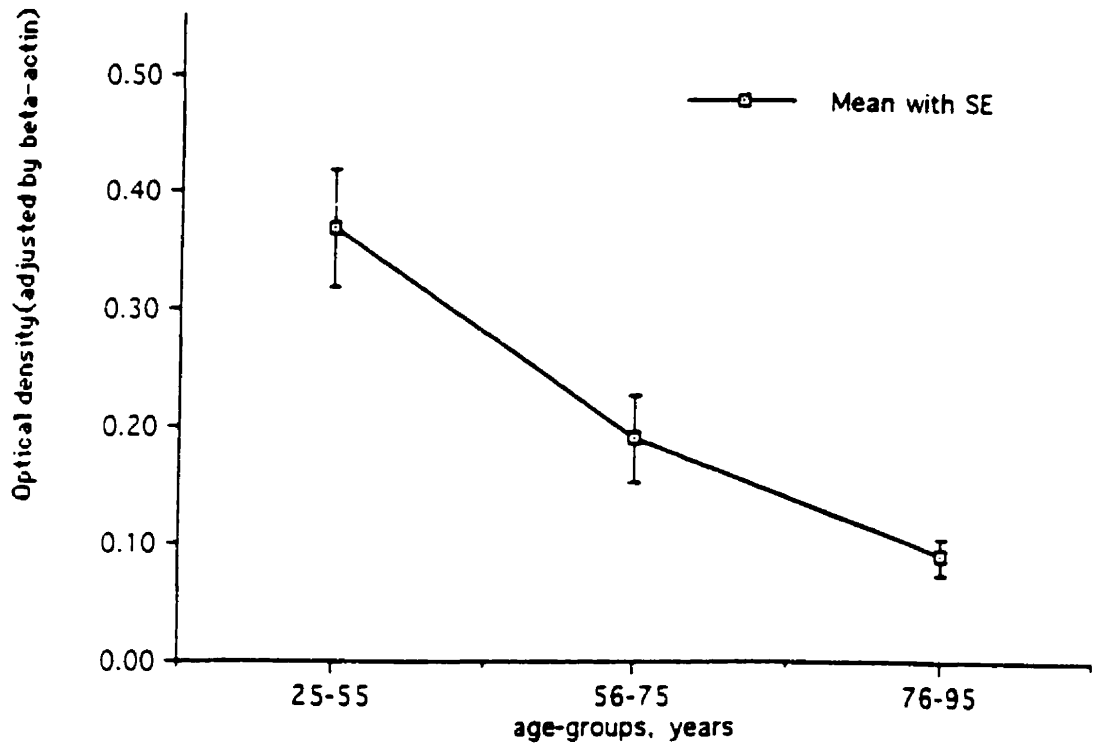


Fig. 16. Linear decrease of MAP_2 mRNA in human cerebral cortex with age. A comparison of values from adult humans grouped into 3 age categories showed no significant departure from linearity ($p=0.33$).

Table 4. Summary of the data on quantification of MAP_2 mRNA

Age groups	Avg.age	Cases T (M/F)	MAP_2 mRNA Mean ± SD
Normal 25-55 yrs	42.0 ± 9	13 (8 / 5)	0.37 ± 0.20
56-75 yrs	68.4 ± 5	18 (10 / 8)	0.19 ± 0.15
76-95 yrs	82.4 ± 5	9 (7 / 2)	0.10 ± 0.05
ANOVA(Kruska-Wallis)			P =0.0001
Linear Regression	CC Slope Departure		r=-0.61 P<0.0001 p=0.33
AD 74-94 yrs	82.5 ± 8	8 (1 / 7)	0.12 ± 0.07

ANOVA: Kruskal-Wallis Nonparametric ANOVA. CC: correlation coefficient.

Slope: Slope different from 0. Departure: departure from linearity.

7. DISCUSSION

7.1. CHARACTERISTICS OF AMYLOSOMES

7.1.1. Histological features of the amylosomes

Using the electron microscope, Rees (1975) first described these intraneuronal structures as homogeneous granular bodies. Since then there have been a few additional reports on these inclusions, mainly focusing on their histological features (Hara,1980, 1982, Averback, 1983). A relatively thorough histological description has been provided by Hara (1986). Although the histological differentiation of these intraneuronal structures from others, such as osmiophilic granular structures, Lewy bodies, corpora amylacea, and Hirano bodies, has been characterized for over a decade (Hara, 1982; 1985, Averback, 1983) these neuronal inclusions have not attracted further attention in either neuropathology or the field of research on Alzheimer Disease. The reason for this lack of interest is probably due to a number of features: a) they are more prevalent in normal brains, which are not usually scrutinized; b) they are relatively small (0.8-10 μm, in diameter), which makes them difficult to detect or easy to neglect; c) they occur in the neuropil without any particular link to glial, or other cells, and are not associated with particular morphological alterations; d) they may be misidentified as red blood cells transported out of blood vessels during processing. In reality, the histological features of these inclusions, such as the variable size (from 0.8-10 μm), the almost perfect

spherical shape, the much brighter and denser H&E staining pattern, and the location in the neuropil, together allow them to be visually differentiated from red blood cells quite easily.

Standard histological techniques reveal that amylosomes initially appear in all normal human brains during early childhood, and are constantly present after the age of one and a half years. They are not found in the fetal brain. These observations indicate a possible physiological role rather than a pathological one. In agreement with others (Hara, 1982, 1985; Averback, 1983), we found amylosomes to be invariably present in the neuropil of the grey matter in the cerebral cortex, and hippocampus, and to be absent from white matter and the cerebellum, in a pattern of distribution similar to that of the amyloid plaques in aged and AD brains. Unlike other reports, we have also detected them within neuronal cell bodies, attached to and within dendrites, in the perivascular space, and at the cortical surface. We consider this to be one of the most interesting results from the morphological studies in this thesis. Very often a histological feature that has not been described previously or been linked to a specific pathological process, will be neglected or overlooked by investigators, simply because of its "straightforward" appearance, or the lack of interest.

To our knowledge the occurrence of amylosomes in these locations has not been previously reported, nor is there any significant explanation for their presence in the brain. Amylosomes are often seen in the neuropil without any consistent relationship to

neighboring elements, such as glial cells, or blood vessels, or to pathological events such as an infarct lesion. A reasonable assumption is that the presence of amylosomes in neuronal cell bodies, within dendrites, and bordering the CSF implies a dynamic phenomenon, and they are being transported along dendrites to the perivascular space or cortical surface for subsequent removal. This is in agreement with our original prediction, that these neuronal inclusions are likely to have a physiological role. In the processes of cellular metabolism, they may provide a way for neurons to dispose of metabolic derivatives by enclosing them during transport into the CSF, where they gain access to the systemic circulation.

7.1.2. Density and size of amylosomes over the lifespan.

To further explore their dynamics in the human brain over the life span, which hopefully may shed light on their possible physiological role and any link to the pathogenesis of AD, morphometry was performed on a total of 222 brain samples. We had noted that there was some discrepancy in the previous histological and morphological descriptions of this intraneuronal structure (Hara, 1980; 1982; 1986; Averback, 1983), and we thought it possible that traditional morphometry by light microscopy was not always reliable. In order to avoid unintentional bias, and to obtain consistency and accuracy at a higher level, we adopted the computer image analysis system. Under careful scrutiny, amylosomes were not found in those brains less than 18 months old. They started to appear in brains at about age 1 and a half to 2 years, and were

consistently present in all adult brains, including those with AD, although at a lower density. It seems there were more large than small amylosomes in aged brains, however this difference was not statistically significant except at the extreme of the life span. That some of the AD brains showed a relatively lower density of amylosomes could be reasonably interpreted as the result of some amylosomes having been released into the neuropil, opened up, and formed amyloid plaques.

Adding to the impression obtained from the histological studies, the morphometric analysis also confirmed our original prediction. The constant presence, and the relatively stable density and size of the amylosomes observed in normal human brains over most of the life span support the concept that amylosomes are being continually formed and removed from neurons, otherwise, one might expect to see a change in density or size, or both, with age. Therefore it is reasonable to assume that the amylosomes have a physiological role in neuronal metabolism, and because of the striking similarity between their distribution pattern and that of amyloid plaques in AD we considered it likely that amylosomes were involved in the processing of APP/Aβ. It was therefore necessary to explore the chemical content of what these amylosomes enclose.

7.1.3. Amylosomes contain amyloid peptide

It was originally suggested (Hara, 1982; 1985; Averback, 1983) that the staining pattern of these neuronal inclusions was consistent with a protein content, while the exact

biochemical nature remained elusive. Much of our effort has since been focused on revealing the chemical constituents within the amylosomes.

All of our experiments to explore the composition of amylosomes, indicate they contain the amyloid peptide, Aβ, which is also the major constituent of the amyloid plaques in AD and in aged brains. In summary, three lines of evidence support this conclusion. Immunohistochemistry with antibodies against synthetic Aβ will label amylosomes and plaques within the AD brain and amylosomes within normal brain tissue. Congo Red staining typical of amyloid can be demonstrated when isolated pellets of amylosomes are sonicated and broken. Lastly, biochemical analysis revealed a similar profile between the content of amylosomes and that of isolated amyloid plaque, as shown by HPLC, Western blot, and by amino acid composition analysis.

Interestingly, Congo Red stained amylosomes in both histological sections and isolated pellets without the apple-green birefringence under polarized light that is characteristic of amyloid. This suggests that peptide incorporated in the amylosomes is not in a beta-pleated conformation, or is possibly bound to some molecule which prevents it from beta-pleating. In contrast Congo Red staining of broken amylosomes produced strong apple-green birefringence. This indicates that when the amylosome membrane is broken, and the Aß peptide is exposed to the surrounding environment, it may gradually beta-pleat and form the insoluble core of an amyloid plaque. This concept is in agreement with other *in vitro* studies, which showed that Aβ could assume a β-strand conformation

change under certain conditions such as a low pH environment (Burdick et al, 1992; Wisniewski et al, 1993; Lansbury, 1996; Kelly, 1996). By adopting a β-pleated conformation, the Aβ could self-assemble into amyloid fibers (Kelly, 1996; Nandi, 1996), an insoluble, pathogenic form of Aβ found in SPs and in amyloid angiopathy.

Overall, our studies on the amylosomes, particularly the histological/morphological features and the finding of Aβ in amylosomes, support our hypothesis. This hypothesis is also in agreement with many previous findings that were either interpreted differently, or left unexplained. A large amount of APP is normally produced each day, and an accordingly large amount of Aβ is continually derived (Selkoe, 1994b). One possible pathway for APP metabolism is through the endosomal/lysosomal system (Haass et al, 1992; 1993; Bernstern et al,1990; 1996), whereby APP is endocytosed into membrane-bound vesicles, most likely endosomes, to be further degraded. Amylosomes may thus be specialized endosomes. Although the physiological function of both APP and Aβ are still controversial, the beta-pleated form of Aβ is most likely to be neurotoxic and is found in amyloid plaques (Pike et al, 1993; Lorenzo & Yankner, 1994; Howlett et al, 1995), whereas soluble Aβ has been detected in culture media of neuronal cells and in the CSF of both normal and AD individuals. (Seubert et al, 1992; Shoji et al, 1992). It is therefore reasonable to assume that amylosomes containing Aß represent a normal metabolic pathway for APP, which would enable the potentially amyloidogenic and neurotoxic peptides to be sequestered intracellularly in a soluble form, while being relayed toward the systemic circulation for

further metabolism. It is then logical to assume that an alteration of this cellular pathway could enable amylosomes to accumulate, and become the source of Aß that eventually deposits as amyloid plaques in the brain.

7.2. AGE-RELATED DECLINE OF MICROTUBULE AND ASSOCIATED PROTEINS

Previous studies on brain MT have been largely done using animal brains or cultured cells, because of the difficulty in obtaining fresh samples of human brain. (Matus, 1988; Vaila, 1990). A newer method developed by Sparkman (1992), with some modification, allows isolation and reliable quantification of MT proteins from frozen human brains, and also facilitates the study of MT polymerization *in vitro*. Our results demonstrate that, through a major portion of the life span, aging is associated with a significant decline in the levels of both MT proteins and the dendritic component MAP_2 in the human cerebral cortex. The most significant rate of decline is observed between the ages of 25 and 55. There is also a progressive decrease with age in the ability of isolated MT proteins to polymerize *in vitro*. These results imply a reduction in the rate and/or capacity of the dendritic transport system with age in human cerebral cortical neurons.

Various changes related to brain MT have been reported in association with aging and/or AD. Morphological and biochemical changes in the nervous system that would probably involve MT include the loss or shortening of dendrites, the loss of dendritic

spines or synapses (Feldman et al, 1975; Coleman & Flood, 1988; Braak & Braak, 1988; Murali et al, 1990; Maslah et al, 1989; Dekosky et al, 1990), tau hyperphosphorylation and the formation of NFT (Braak & Braak, 1988; Price et al, 1991; Lee et al, 1991; 1994; Mandelkow, 1996), and the decrease in axonal transport of certain molecules (Goemaere-Vaneste et al, 1988; Raes, 1991). A lower number of MT per unit area in cortical dendrites in the AD brain, and an absence of MT polymerization in samples isolated from 2 cases of AD (Iqbal et al, 1986; Paula-Barbosa et al, 1987) have also been observed, although these results are somewhat controversial (Nieto et al, 1989).

However, a decline in the levels of MT and associated proteins in non-demented human brains as a function of aging has not previously been reported, nor have any of the related changes been integrated into a basic pathogenic process which could lead to the Alzheimer pathologies. Moreover, morphological and biochemical studies on neuronal and dendritic alterations involving MT integrity and function have shown a significant overlap, or a similar pattern (Coleman, Flood, 1987; Morris et al, 1991; 1996; Mirra, 1993; Giannakopoulos et al, 1995) between the non-demented aged human brains and the AD brains. The presence of neuritic dystrophy that by itself could account for clinical dementia, in the areas of the aged and/or AD brains devoid of NFT or SP, has also been reported. (Raes, 1991), further indicating that dendritic alteration may be an age-related basic pathological process, occurring prior to the formation of NFT and/ or amyloid plaques. These observations are particularly relevant to our findings, because they are all compatible with a reduction in the rate and/ or capacity of the dendritic

transport system with age that occurs independently and steadily prior to AD pathologies. The question is how to interpret these results as part of the basic process in the pathogenesis of AD.

Considering our histological and morphological observations on the amylosomes, and some basic cellular biology, as well as many previous reports on AD, it is reasonable to assume that normally produced Aβ is continually removed from neurons, in a membrane - confined form, to be transported along the dendrites to the perivascular space and/or cortical surface then released into the CSF (Fig.17). The decrease in MT with age would particularly impair dendritic transport and could therefore promote accumulation of amylosomes. Subsequent dissolution in the neuropil could initiate the formation of amyloid plaques.

Many additional observations are consistent with our current hypothesis, and a brief summary of the relevant information is as follows:

a) Amylosomes are topographically distributed in the cortical areas close to blood vessels and at the cortical surface, where very few neuronal cell bodies are located and dendritic processes occupy most of the parenchyma.

b) The basic function of MT is intracellular transport, and MAP_2 is predominantly located in neuronal dendrites (Matus, 1988; Vaila, 1990; Alberts et al, 1994).

c) Soluble Aβ has been found in the normal CSF (Seubert et al, 1992; Shoji et al, 1992), which assumedly prevents Aβ from aggregating and β-pleating (Wisniewski et al, 1993),

probably through binding of Aß with apoJ or other carrier molecules (Matsubara et al, 1996).

d) The soluble Aβ has been found to be quickly cleared through the CSF to the blood (Strazielle et al, 1995; Ghersi-Egen et al, 1996) in animal models.

e) There is an age-related deposition of amyloid in the choroid plexus (Eriksson & Westermark, 1986; 1990), a region of the brain where CSF volume and content are regulated. Numerous large molecules, including Aβ, are normally transported out of the CSF through the choroid plexus and Aß has also been immunoreactively detected in the plexus epithelial cells (Wen et al, 1988; Davison, 1996).

f) While Aβ deposits in the AD brain generally correlate with age and severity of the dementia (Von Dras et al, 1992; Arriagada et al, 1992; Morris et al, 1991; 1996), Aβ has been found to decrease in the CSF of AD patients in comparison to normal controls (Van Nostrand, 1992; Motter et al, 1995; Kanai et al, 1996).

g) The congophilic angiopathy is an age-related process, and predominantly involves cerebral vessels close to the cortical surface and the vessels in the leptomeninges (Hamano et al, 1997). Histological evidence (Yamaguchi et al, 1992) does not indicate that Aß deposited on vessel walls originates from circulating blood, as it is primarily deposited external to the outer basement membrane.

h) Typical SPs are always spherical, and seldom reach 100 μm in diameter, and have a dense amyloid core. Their origin is more easily explained by expansion or diffusion from a point source, rather than deposition from a circulating source such as the CSF or plasma (Selkoe, 1989; Kawai et al, 1992). Amylosomes are also a more convincing

source than migrating perivascular cells (Wisniewski & Weigel, 1993) or "primitive" ones that gradually release condensational material (Wisniewski, 1983; 1985; Bergeron, 1989). It is worth mentioning that one recent animal model did show Aβ injected into the systemic circulation leaking through the BBB into the neuropil (Pluta et al, 1996). However, this occurred under non-physiological conditions, and the brain did not develop typical amyloid plaques or other SP associated local changes. Histological studies mostly showed patchy immuno-staining, or perivascular diffusion. These observations could be interpreted to indicate that circulating Aβ is not the source of amyloid deposited as the typical AD plaque.

The observations listed above are all consistent with the hypothesis that amylosomes can suitably serve as a point source, for the Aß deposited in plaques, and an age-related decrease in dendritic transport can provide an increasingly favourable condition for them to break into the neuropil and deposit their contents.

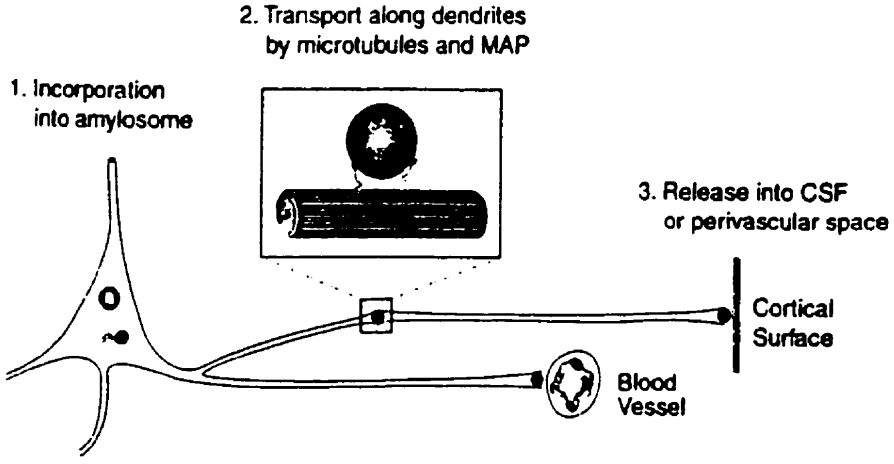


Fig. 17. Schematic representation of the hypothesis for the intraneuronal transport of Aß peptide into the CSF.

Amylosomes containing the Aß peptide are transported along dendrites by MT and MAP to the perivascular space or cortical surface, where their content is released.

7.3. RAPID DECLINE OF MICROTUBULE AND ASSOCIATED PROTEINS IN HUMANS WITH APOE4 GENOTYPE

As reviewed in the first Chapter, there is more and more supportive evidence indicating that apoE4 is a strong risk factor for AD. Some investigators even believe that apoE4 is the major, if not the only risk factor for late-onset AD, as well as for some early onset AD, and that it largely determines both the risk and the mean age of onset (Roses, 1994; Okuizumi et al, 1994; Strittmatter & Roses, 1995; 1996). This approach considers Aβ plaques to be merely a secondary consequence dependent on APOE genotype (Roses, 1994). On the other hand, the opinion that apoE4 is a risk factor, but not a causative one, is also getting more and more support, particularly since the association of apoE4 to AD as well as its dose effect is not consistent (Lannfelt et al, 1994; Sobel et al, 1995; Blacker et al, 1996). Many APOE4 carriers escape AD even at a very old age, while many AD patients do not carry the APOE4 allele (Bird, 1995; Black et al, 1996). Recently it has been confirmed (Asada et al, 1996; Sobel et al, 1996; Blacker at al, 1997) that the apoE-4 association with AD is age-dependent, with a maximum effect before age 70.

Regardless of the level of certainty in the association of apoE4 with AD, the ultimate question we need to ask is: "What is the biological mechanism by which this normal polymorphism of the apoE gene can predispose individuals to AD, particularly

by advancing the age of onset?" The functional significance of genetic variations and the rate of disease expression may be due to common variations in the gene products, the proteins and their interactions. Therefore the possible mechanism of the APOE4 effect should be examined at the level of individual proteins, and cell biology. Various mechanisms have been proposed in this regard. ApoE4 might promote NFT formation by not being able to bind tau or MAP2c as vigorously as do apoE3 or E2, and the binding may be required to prevent tau from being hyperphosphorylated (Strittmatter et al, 1994; Huang et al, 1994). ApoE4 might also promote Aβ aggregation and or beta-pleating as a chaperone (Wisniewski, 1993). ApoE4 might destabilize MT (Nathan et al, 1995) and tandem presentation of a certain apoE sequence (E141-155) may elicit neurite degeneration (Crutcher et al, 1994). Finally, apoE4 may lack the ability of apoE3 or E2 to aid in synaptic remodelling and repair, which could lead to cholinergic deficits (Poirier et al, 1995). However, a link between these observations and their associated hypotheses and an explanation for the variety of pathological and clinical features of AD, in particular the age-related amyloid plaques, is still missing.

We found a faster decrease in MT proteins and the dendritic MAP_2 in age-matched individuals with ϵ-4 allele(s) compared to individuals without an ϵ-4 allele. The most significant differences were observed in the age-group 56-75, which is the age range in which symptoms of orientation, memory and/or language deficiency start to emerge in most AD patients (Bachman et al, 1993; Becker et al, 1994; Jost & Grossberg, 1995). Similar differences also exist in the younger (25-55) and older (76-95) age-

groups, but these did not reach statistical significance, partly because of the relatively smaller sample sizes available. Interestingly, when the AD groups, which had previously shown no significant difference in the amount of MT and MAP_2 from the age-matched controls, were also divided into AD with ϵ-4 allele(s) versus AD without an ϵ-4 allele, a similar trend emerged, although this was not statistically significant because of the small sample size.

These results confirm that APOE does have an isoform-specific effect on MT integrity and function, which could account for its role in the pathogenesis of AD. We propose that normally there exists a balance between the production of APP/Aβ, and their degradation/removal, which is in large part determined by dendritic transport. Anything which can significantly alter the balance, including APP gene mutations in rare familial AD (Goate et al, 1991; Games et al, 1995) leading to overproduction of APP/Aβ, or dendritic impairment (loss, shortening, declining transport) leading to inadequate removal of Aβ, could eventually lead to a common consequence, retention of the amyloidogenic Aß in the neuropil. Of particular relevance, with aging, the intraneuronal transport at lower levels of MT might be exquisitely sensitive to variations in other components related to the cellular transport system, such as dynein or kinesin, a MT-based molecular motor (Schroer & Sheetz, 1990; Avila, 1991). Combined deficits could lead to extensive retardation or disruption of neuronal metabolism with further retention of Aß. The next consequence would logically be beta-pleating of the Aβ peptide and the formation of amyloid plaques.

Therefore the observation that APOE ϵ4 carriers lose MT function faster than non ϵ4 individuals, offers an explanation for many previous findings, including the epidemiological evidence that individuals with an ϵ4 allele(e) develop AD 10 to 15 years earlier than non ϵ4 carriers (Corder et al, 1993; Saunders et al, 1993; Roses, 1994). It can also account for the histochemical evidence, that APOE4 homozygotes have a much higher and denser Aβ load within the plaques than do APOE3 homozygotes (Schmechel et al, 1993), and the biochemical evidence (Pirttila et al, 1997), that the amounts of both acid-extractable Aβ and insoluble Aβ are higher in the frontal cortex of individuals with APOE ϵ4 allele(s) than those without it. The recent documentation that the association of apoE with AD is inconsistent, and its effect is age-dependent (Asada et al, 1996; Sobel et al, 1996; Blacker at al, 1997) also supports our conclusion that apoE is not a primary causative factor in AD. We believe its isoforms act to retard or promote the rate at which MT function declines with age toward a minimal threshold for maintenance of normal dendritic transport and removal of amylosomes from the neuron to the CSF.

The next question we need to ask is "What is the mechanism by which this normal polymorphism of the APOE gene can exercise its effect leading to a faster decline of MT and/or MAP2 with age?" Although it has not been considered to be a MAP, an apoE isoform-specific effect on MT has been observed. It would be not surprising if further studies show that apoE has a major role in maintaining and or repairing MT integrity and function, considering the critical roles already demonstrated for this

compound (Elshoubagy et al, 1985; Mahley, 1988) in both the global transport of chylomicrons and VLDL, and the local transport of lipids by redistribution among cells. It is also known to play a role in promoting neurite extension in the brain (Guillaume et al, 1995; Mahley et al, 1996), as well as in scavenging of lipids generated from axon degeneration and redistributing these lipids to spouting neurites for regeneration. The apoE isoforms differ in LDL-receptor binding which in turn affects the transport of cholesterol necessary during growth, or during repair of membranes after injury (Boyles et al, 1989; Poirier et al, 1995; Mahley et al, 1996). ApoE4 has recently been reported to block neurite extension and/or promote MT instability *in vitro* (Nathan et al, 1995; Pitas, 1996). These observations may explain, in part, the faster decline of MT in the cerebral cortex with age, in those individuals with at least one APOE $\epsilon 4$ allele.

7.4. HYPOTHESIS FOR THE PATHOGENESIS OF ALZHEIMER'S DISEASE

7.4.1. The possible source and the mechanism of the cerebral Aβ deposition

The results from the studies summarized above, particularly the histological and biochemical characteristics of amylosomes, and the age-related decrease of MT in human brains which is accelerated in APOE $\epsilon 4$ allele carriers, have led to the following hypothesis on the pathogenesis of AD (Fig.17), presented in stepwise fashion:

a) As APP is normally metabolized within the neuron, an amyloidogenic fragment, Aβ, is continually produced and incorporated or endocytosed into a membrane-bound structure, the amylosome.

b) As a part of the cellular metabolic pathway for APP, the continually formed amylosomes are normally removed from neurons along dendrites to the perivascular space or cortical surface, where their contents are released into the CSF.

c) The Aβ and possibly larger amyloidogenic APP fragments released into the CSF may be bound to other proteins, further processed extracellularly, and then transported to the systemic circulation for further metabolism or removal.

d) Any factor that significantly alters the balance between normal production of the amyloidogenic APP fragment, Aβ, and subsequent removal of Aß from neurons, in particular a gradual breakdown of the cellular transport system, would lead to the accumulation of amyloidogenic fragments in the brain, and their eventual access to the neuropil, where they beta-pleat to form amyloid plaques, and also deposit on the vessels and cortical surface.

e) The decrease in dendritic transport could be one of the major forces altering this balance. It appears to progress gradually and steadily in the normal aging population, and can therefore account for the age-related Aβ deposition as well as the

gradually increasing risk of AD.

7.4.2. The significance of this theory

This theory emphasizes that amylosomes are the source of plaque amyloid in both AD and normal aged brains, and that progressive decline of dendritic transport with age plays a key role in the pathogenesis of amyloid plaque formation. This theory offers a coherent explanation for many observations associated with AD, which have previously been either left unanswered, or difficult to interpret in a coherent way.

The following is a summary of the evidence to date:

Amylosomes are constantly present in the normal human brain from a very early stage of life and remain relatively stable in both number and size throughout the life span. This is in agreement with the large quantity of APP metabolized daily, the endo/lysosomal pathway for the APP metabolism, and many studies on the effects of APP/Aβ (Saitoh et al, 1989; Selkoe, 1994a,b; 1996; Yankner, 1996; Huber, 1997). The formation and metabolism of amylosomes in normal human brains may represent a key step in the cellular metabolic pathway for APP.

Retention of amylosomes in the brain, with release of their amyloid peptide into the neuropil, where it can beta-pleat and deposit, would account for the origin of the

amyloid deposited in plaques within both the AD and aged brains.

That the amyloid peptide deposited in the SPs could be released from the membrane-bound amylosomes offers an explanation for the constant, spherical shape of the SPs, particularly the dense core, if the consistent spherical shape (Selkoe, 1994) indicates that Aβ diffuses from a point source. In addition to its amyloidogenic content, the constant spherical shape with a diameter of about 10 μm, and the dense amyloid staining pattern make the amylosome the best candidate for a point source.

The concept that amylosomes are normally transported along dendrites to the perivascular space or cortical surface offers an explanation for the topographic distribution of the Aβ deposits in the CNS, particularly those in the cortical layer II and III, at the cortical surface, and around vessels in cortical areas and meninges.

The age-related decrease in the dendritic transport, by loss of MT integrity, can account for the age-related pattern of Aβ deposition.

Linking the pathogenesis of AD to impaired dendritic transport of amylosomes is compatible with numerous observations. Amyloid deposition could thus occur with or without APP gene and or PS1/PS2 abnormality, and tau/cytoskeleton changes are explainable, as is the role of apoE4. By considering inadequate removal of Aß as a pathogenic core of AD, it is possible to correlate the multiplicity of other factors (Hyman

& Terry, 1994; Blass, 1996; Fowler et al, 1997; Adams, 1997), that are linked to the disease.

The role of dendritic transport in APP/Aβ metabolism and in the pathogenic core process also explains why cholinomimetics do not work for the majority of AD patients. These drugs only benefit a small group, for a short period of time, mainly those who are non-APOE ϵ4 carriers (Aarsland et al, 1995; Poirier et al, 1995, CNN, 1997), because the basic pathogenic process is not targeted. It may also explain why estrogen replacement has been shown to be beneficial (Miranda et al, 1994; Fillit, 1995), if estrogen stabilizes or promotes MT function. There is some evidence to support this concept, as estrogen has a positive effect on neurite outgrowth *in vitro* (Marx, 1996). Furthermore while this thesis was being written, a positive effect of estrogen on dendritic spine density and synapses was also reported (Woolley et al, 1997; Wickelgren, 1997). Our hypothesis implies that pharmacological compounds promoting the maintenance or improvement of MT function might not only relieve symptoms, but also hopefully prevent or at least delay the onset and progression of AD.

In summary, many molecular, cellular and biochemical alterations have been associated with AD. They may occur in a sequential manner, may actually interact, or may be components of separate yet converging pathways. They may even represent separate disease processes coincidentally happening in old age. We still do not know which of them are pathogenic, and which are just consequential effects, nor do we know

the sequence of the pathogenic events. Establishing a basic pathway (Fowler et al, 1997; Adams, 1997) by which at least some verified etiological agents and many hypothetical risk factors and pathological events could be coherently linked, was the goal of this research project. Based on our results, we have proposed a hypothesis that we hope can provide some insight into the key process in the multifactorial pathogenesis of AD, and suggest some sites of possible intervention.

8. FUTURE DEVELOPMENT

To further study the pathogenic mechanism proposed in this thesis, the following topics should be investigated in the future.

a) The chemical nature of amylosomes: Since we have demonstrated that amylosomes contain amyloid peptide, by immunohistochemistry, western blot, and amino acid composition analysis, the next step would be to further confirm the structure by sequencing the amyloid peptides from the amylosomes. Modification of the isolation and purification procedures by using more efficient media may yield peptide fragments which can hopefully be sequenced. One of the choices is to use Iodixanol, a dimeric form of Nicodenz (Accurate Chemical & Scientific Co, Westbury, NY), which can produce fine resolution of membrane bound vesicles, such as endosomes and lysosomes, on the basis of their density. Iodixanal also permits high purity and yield, which is often not achieved with other gradient media (Ford, et al, 1994).

b) The dendritic transport of amylosomes: Since amylosomes have been located in dendrites by EM, the next step would be to explore their transport mechanism by MT. The confocal microscopy technique (Murray, 1992; Neri, et al, 1992) could be applied to view the physical connections between the amylosomes and the MT within dendrites. This permits three-dimensional imaging on thick tissue specimens, or on cell cultures, which cannot be achieved by immunohistochemistry, or traditional fluorescence microscopy.

c) The effect of age on MT function in other tissues: Since the age-related decreases of both MT and MAP_2 were observed in brain tissue, it would be interesting to see if these age-related changes are a general physiological phenomenon, and occur in cells of other tissues as well. If so, MT quantity, and integrity could be used as a diagnostic aid and/or criterion for monitoring treatment.

d) The correlation between MT levels and the formation of amyloid plaques or the apoE genotype: Since we have proposed that dendritic transport plays an important role in removal of the amyloid peptide, we need to explain the exceptions, primarily that low levels of MT do not invariably correlate with the disease, nor does the apoE $\epsilon 4$ genotype. Do these indicate subcategories with different properties, or some additional regulatory mechanism involved in the decline of dendritic transport, in particular, of the amylosomes? To continue the present work with a sufficient number of samples to permit reliable comparison between groups, and to correlate the amyloid load in the brains with the level of MT function would help to answer this question. Moreover, the possible effect of apoE (Masliah et al, 1995; Nathan et al, 1995) on MT could also be further pursued, by comparing MT polymerization *in vitro* in the absence of apoE, and in the presence of each of its isoforms.

e) The pharmacological alteration of MT function: The effects of MT stabilizing agents such as taxol (Schiff et al, 1973; 1979; Ruben et al, 1996) could be documented *in vitro* in comparison to the ability of putative therapeutic agents, with the intent to

develop effective drugs to promote MT integrity and function. An initial candidate would be estrogen, which has been recently (Fillit, 1986, 1995; Simpkins et al, 1997) reported to be beneficial in reducing the incidence and/or the symptoms of AD, and has also been shown to have an effect on neuronal processes and dendritic spines (Woolley et al, 1990; Miranda et al, 1994; Marx, 1996; Wickelgren, 1997). The ovariectomized rat model (Frankfurt et al, 1990; Woolley et al, 1997) could be used to study *in vivo* the estrogen effect on MT, which we suspect underlies the ability of estrogen to induce neuronal sprouting. Rat cell cultures (Maneiro et al, 1996) also offer good experimental models in which to study the mechanisms involved in neurodegenerative disorders, and to screen pharmacological compounds for neuroprotection.

9. CONCLUSIONS

We have demonstrated that membrane-bound neuronal inclusions, which we call amylosomes, contain amyloid peptide, the major constituent of the amyloid plaque, and that their presence in the human brain may represent a physiological pathway for APP/Aβ metabolism. We have also shown that there is an age-related decrease in human brain MT proteins and function, which is accelerated in individuals with APOE ϵ4, a known risk factor for AD. Therefore a novel theory about the pathogenesis of AD has been proposed, which emphasizes that amylosomes are the source of plaque amyloid, and that the age-related decrease in dendritic transport plays a key role in the pathogenesis of AD and can account for Aβ deposition in both AD brains and aged brains. We believe that this theory relates several currently different hypotheses, including cerebral amyloidosis, tau/cytoskeleton abnormalities, acceleration of aging, and apoE, by providing a central step as a pathogenic core which can be influenced by a multiplicity of other factors all acting on this primary abnormality. We believe this provides a biologically plausible and testable hypothesis for further exploration of the pathogenesis of AD. It is also our hope that this theory will indicate new and productive avenues through which to develop effective pharmacological agents for treatment and prevention of this presently incurable disease, through alteration of the pathogenesis. We hope, as predicted (Coates, 1994; Ausman, 1997), that, "25 years from now, more people in advanced countries will be living to their mid-80s while enjoying a healthier fuller life".

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