INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

UMI®

Bell & Howell Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
800-521-0600

The Effect of Age on the Accumulation of Amyloidogenic Peptides in the Human Brain and Cerebrospinal Fluid.

by

Suzanne Kunicki

Department of Pharmacology & Therapeutics

McGill University, Montreal, Canada

July, 1997

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Copyright © Suzanne Kunicki, 1997

National Library of Canada
Acquisitions and Bibliographic Services
395 Wellington Street
Ottawa ON K1A 0N4
Canada

Bibliothèque nationale du Canada
Acquisitions et services bibliographiques
395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file Votre référence

Our file Notre référence

The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

L'auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

L'auteur conserve la propriété du droit d'auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

0-612-36995-1

Canada

ABSTRACT

Amyloid deposition in cortical plaques and around cerebral vessels is a hallmark of Alzheimer's disease (AD). Amyloid also accumulates, to a lesser degree, in some nondemented elderly individuals, in the cerebral cortex and in the choroid plexus (CP), a tissue in the cerebral ventricles which produces cerebrospinal fluid (CSF). The cortical amyloid is composed mainly of aggregates of a neurotoxic peptide called amyloid-ß (Aß), of which there are two main variants, Aß42 and Aß40, but the composition of CP amyloid has not been determined, nor is it known if there is a relationship between amyloid deposition in these two locations. This thesis characterizes amyloid deposition in the CP and cortex of normal individuals, and investigates the effects of aging and AD on the levels of proteins that may modulate Aß neurotoxicity and amyloid formation in these locations. Immunohistochemistry identified Aß and its associated proteins, apolipoprotein (apo) E, apoJ and transthyretin in the CP of normal and AD subjects. However, Western blot analysis revealed that apoE, not Aß, is the major protein component of the CP amyloid. Soluble forms of Aß and apoE were identified in the CP and CSF. The concentrations of Aß42, apoE and transthyretin in the CSF remained constant with age, indicating that the reduced levels of these proteins reported in the CSF of AD patients is not age-related. In addition, we found that the levels of Aß40 declined, suggesting a change in metabolism of the Aß precursor with age. In the cerebral cortex, the ratio of Aß42- to Aß40-positive plaques remained constant with age at 2.6, but was reduced to 1.6 in AD brains. ApoE, apoJ

and transthyretin were present in a small minority of plaques in normal and AD brains, indicating that these proteins do not play a central role in modulating Aß neurotoxicity in plaques. However, apoE was strongly associated with cerebrovascular amyloid in normal individuals, while Aß42 was the predominant component in AD vessels. Together, these studies have identified changes in the levels and distribution of amyloidogenic proteins which are specific for AD and they suggest a novel role for apoE in amyloidogenesis.

RÉSUMÉ

Les dépôts d'amyloïde sous forme de plaques dans le cortex cérébral et autour des vaisseaux cérébraux constituent un trait principal de la maladie d'Alzheimer (MA). L'amyloïde s'accumule également, bien qu'en bien moindre quantité, dans les cerveaux de certains individus âgés mais non-atteints de démence. Ces accumulations se produisent dans le cortex et le plexus choroïde (PC), un tissu situé dans les ventricules cérébraux et qui sécrète la plupart du liquide céphalo-rachidien (LCR). L'amyloïde corticale se constitue principalement d'agrégats d'un peptide neurotoxique nommé amyloïde-ß (Aß), duquel il existe deux variantes, Aß42 et Aß40. Cependant, la composition de l'amyloïde dans le PC demeure inconnue. L'objectif principal de cette thèse fut d'évaluer l'accumulation d'amyloïde dans le PC et cerveau de sujets normaux et de déterminer les effet du vieillissement et de la MA sur les niveaux des protéines pouvant moduler le dépôt d'amyloïde. Une analyse immunohistochimique identifia, dans le PC de sujets normaux et d'individus atteints de MA, de l'Aß, ainsi que des protéines qui lui sont associées, soit l'apolipoprotéine (apo) E, l'apoJ et la transthyrétine. Cependant une immuno-empreinte révéla que l'amyloïde du PC se compose essentiellement d'apoE, et non de peptide Aß. De plus, de l'Aß et de l'apoE sous forme soluble furent identifiées dans le LCR. La concentration d'Aß42, d'apoE et de transthyrétine dans le LCR de sujets normaux demeura constante avec l'âge, alors que les niveaux d'Aß40 déclinèrent. Dans le cortex cérébral, le rapport entre les plaques contenant de l'Aß42 et celles contenant de l'Aß40 est de 2,6 pour les sujets normaux et

reste constant avec l'âge. Ce rapport est réduit à 1,6 pour les individus atteints de MA. L'apoE, l'apoJ et la transthyrétine sont présentes dans très peu des plaques de sujets normaux ou malades, indiquant que les protéines associées à l'Aß ne jouent pas de rôle important dans la modulation de la neurotoxicité de l'Aß. L'apoE est cependant fortement associée à l'amyloïde cérébro-vasculaire, alors que l'Aß42 est liée au dépôt d'amyloïde dans les vaisseaux de sujets atteints de MA. En resumé, ces études ont identifié des changements spécifiques à la MA dans les niveaux et la distribution des protéines amyloïdales et suggèrent un rôle - jusqu'à là méconnu - pour l'apoE dans la formation de l'amyloïde dans le cerveau.

TABLE OF CONTENTS

PREFACE

Format of the Thesis

This thesis comprises three manuscripts which are included almost entirely in the format in which they are to be submitted for publication. Connecting texts are provided in compliance with section b.2 of the "Guidelines Concerning Thesis Preparation", Faculty of Graduate Studies, and Research, McGill University. These guidelines state:

"Candidates have the option of including, as part of the thesis, the text of one or more papers submitted or to be submitted for publication, or the clearly-duplicated text of one or more published papers. These texts must be bound as an integral part of the thesis. If this option is chosen, connecting texts that provide logical bridges between the different papers are mandatory. The thesis must be written in such a way that it is more than a mere collection of manuscripts; in other words, results of a series of papers must be integrated. The thesis must still conform to all other requirements of the "Guidelines for Thesis Preparation". The thesis must include: A Table of Contents, an abstract in English and French, an introduction which clearly states the rationale and objectives of the study, a review of the literature, a final conclusion and summary, and a thorough bibliography or reference list. Additional material must be provided where appropriate (e.g. in appendices) and in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis. In the case of manuscripts co-authored by the candidate and others, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. Supervisors must attest to the accuracy of such statements at the doctoral oral defense. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of all the authors of the co-authored papers."

Chapter 1 includes a general introduction to the accumulation of proteins which may form or are associated with amyloid, with particular reference to the aging CNS, and provides the rationale for the studies presented in this thesis. Chapters 2, 3 and 4 contain the main findings of this research, presented in manuscript form. Chapter 5 includes a general discussion of the results and proposes a model for clearance of the Aß peptide from the CNS. The last section, List of Contributions, summarizes the major findings of Chapters 2 to 4. The following people have contributed to the research included in the present thesis. Dr. E. Zorychta is my thesis supervisor. Dr. J. Richardson was the resource person who made it possible to obtain tissue and cerebrospinal fluid samples and he assisted with the analysis of immunohistochemistry slides. I carried out all of the laboratory research with the exception of those activities specified in ii below.

i. **Deposition of an amyloid containing apolipoprotein E occurs in the choroid plexus with age.** S. Kunicki, J. Richardson and E. Zorychta

ii. **The effect of age, apolipoprotein E phenotype and gender on the concentration of Aß40, Aß42, apolipoprotein E and transthyretin in human cerebrospinal fluid.** S. Kunicki, J. Richardson, P.D. Mehta, K.S. Kim and E. Zorychta

P.D. Mehta developed the antibodies specific for Aß40 and Aß42. It

was his laboratory that standardized the enzyme-linked immunosorbent assays for Aß quantification. In addition, his technician analyzed 17 of the 56 samples for Aß40 and Aß42 content. K.S. Kim provided the 6E10 antibody used in the Aß ELISAs and he sequenced the commercially-obtained synthetic Aß40 and Aß42 to ensure sample purity prior to antibody development.

iii. **An immunohistochemical study of plaque composition in the brains of nondemented individuals: a comparison with Alzheimer's disease.** S. Kunicki, J. Richardson and E. Zorychta

ACKNOWLEDGEMENTS

I would like to thank the following people:

Dr. Zorychta, for showing confidence in my ideas and allowing me to be independent. I appreciate the time you spent with me, as a student, and as a person developing a philosophy of research.

Dr. Richardson, for somehow always finding the time to go over a slide (or 20!) with me, even as I dropped in unannounced.

Dr. Robaire, for guidance throughout my studies.

Hana Pelz, for showing me the basics. Your patience was much appreciated.

Michel, for helping me with the apoE ELISAs.

Larry, for calling me promptly when an autopsy was being performed.

Steve, for cutting hundreds of slides for immunohistochemistry.

Dr. Joseph, for all the direction you gave me regarding statistical analysis.

Stella, for saving my sanity by typing up those endless references.

To my family, thanks for asking how my brains were coming along. I'm sorry to report that I have been unable to create one myself. Most of all, a big thank you to my husband for translating my abstract into French and supporting me even as I underwent a Kafkaesque metamorphosis. Dziekuje habibi.

Lastly, a word of wisdom from Kahlil Gibran that helped me keep things in perspective and kept me motivated. "... But if in your fear you would seek only love's peace and love's pleasure, then it is better for you that you cover your nakedness and pass out of love's threshing-floor, into the seasonless world where you shall laugh, but not all of your laughter, and weep, but not all of your tears."

LIST OF ABBREVIATIONS

AD	Alzheimer's disease
Aß	Amyloid-ß: 39-42 amino acid peptide
apo	Apolipoprotein
APOE	The gene encoding apoE
APP	Amyloid precursor protein, 695-770 amino acids
BBB	Blood-brain barrier
CAA	Congophilic amyloid angiopathy
CNS	Central nervous system
CSF	Cerebrospinal fluid
CP	Choroid plexus
CT	Computerized tomography
FAP	Familial amyloid polyneuropathy
FAD	Familial Alzheimer's disease
IGF-II	insulin-like growth factor-II
GVD	Granulovacuolar degeneration
HCHWA	Hereditary cerebral hemorrhage with amyloidosis
HDL	High density lipoprotein
LDL	Low density lipoprotein
LRP	LDL receptor-related protein
NFT	Neurofibrillary tangles
PAS	Periodic-acid Schiff

PHF	Paired helical filaments
SSA	Senile systemic amyloidosis
TTR	Transthyretin (prealbumin)

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1.

Introduction

1.1 MOTIVATION

In this century, the average life expectancy in North America has increased from 48 years in 1900 to approximately 76 years in 1991 (66). During this time, the percent of people in the United States over the age of sixty-five has tripled from four percent to 12.5 percent. This translates into an increase from 3 million in 1900 to 31.6 million in 1990. As a result, a variety of age-associated diseases which were relatively rare even 50 years ago, are today being observed in a significant portion of our population. One of the relatively frequent disabilities seen among the elderly is dementia, an impairment of cognition and memory that can range from mild to severe.

Age is the single most important risk factor for all dementias, including Alzheimer's disease (AD), the most common cause (24,164,178). Studies conducted in North America, Europe and Asia all document an exponential increase in the prevalence of dementia with age, with a doubling approximately every five years. The incidence is around 1% at age 65 and 10% by age 80 (for a review, see ref 81).

The identification of chronological age as a risk factor for dementia initially led to the assumption that dementia was an inevitable expression of a natural aging process occurring in the central nervous system (CNS), rather than a distinct disease. Today, some 90 years after Alois Alzheimer described his now-famous case of dementia (2,3), it is recognized that dementia can usually be attributed to one of two well-documented diseases: AD or vascular

dementia (64,178).

What is intriguing about AD is that several of the characteristic histopathological changes found in the brains of demented patients can be observed, albeit to a much lesser degree, in many nondemented individuals, and the proportion of these individuals with lesions increases with age. For example, deposition of amyloid (see below) is a central feature of the family of diseases known as amyloidoses, and is a prominent characteristic of AD brains. However, some amyloid deposits can be found in the brain, and other tissues including the heart, spleen and pancreas, of many elderly individuals free of any symptoms (200).

The mechanism underlying amyloid formation in disease and in asymptomatic individuals remains elusive. A clearer understanding of amyloid formation in normal people, compared to those with AD, may provide clues to the pathogenesis of amyloidoses. The objective of this thesis is to address this question by investigating some of the factors associated with aging that may be related to amyloid formation in the human CNS. In this chapter, the somewhat generic term amyloid is defined, and some examples of the diseases in which amyloid deposits are observed are presented. This is followed by a description of three components of the CNS which are prone to amyloid deposition or which contain amyloidogenic proteins; the choroid plexus (CP), the cerebrospinal fluid (CSF) and the cerebral cortex. Finally, a hypothesis is presented concerning the relationship between the amyloid and amyloidogenic

proteins in these three areas.

1.2 AMYLOID AND AMYLOIDOGENIC PROTEINS

The term **amyloid** was originally employed by Virchow in 1853 to describe a tissue deposit of unknown composition which had the staining characteristics of starch or amylose. Following staining by iodine and sulfuric acid, the amyloid, like starch, was visualized macroscopically as blue deposits. Advances in biochemistry, together with electron microscopy, have revealed that amyloids are composed of a family of diverse proteins that have in common a specific conformation or shape, that of a **ß-pleated sheet**, illustrated in Figure 1.1 (54,88,107). The ß pleating of the peptides is responsible for the characteristic staining of amyloid deposits by Congo red dye and subsequent green coloration when viewed through a polarizing microscope (Figure 1.2), as well as for the resistance of amyloids to proteolysis and solubilization in physiological solutions (54).

Amyloids are derived from the processing of a variety of chemically unrelated precursors. One constant finding in these diverse amyloids is the presence of a serum glycoprotein which is a normal constituent of the extracellular matrix, the p-component or pentagonal substance. The presence of this protein may explain the positive staining of all amyloids by periodic-acid Schiff (54). Proteins which have the potential to ß-pleat and form amyloid are said to be **amyloidogenic**. This includes proteins that can form amyloid

spontaneously as well as those which require proteolytic processing or addition/removal of other proteins (54).

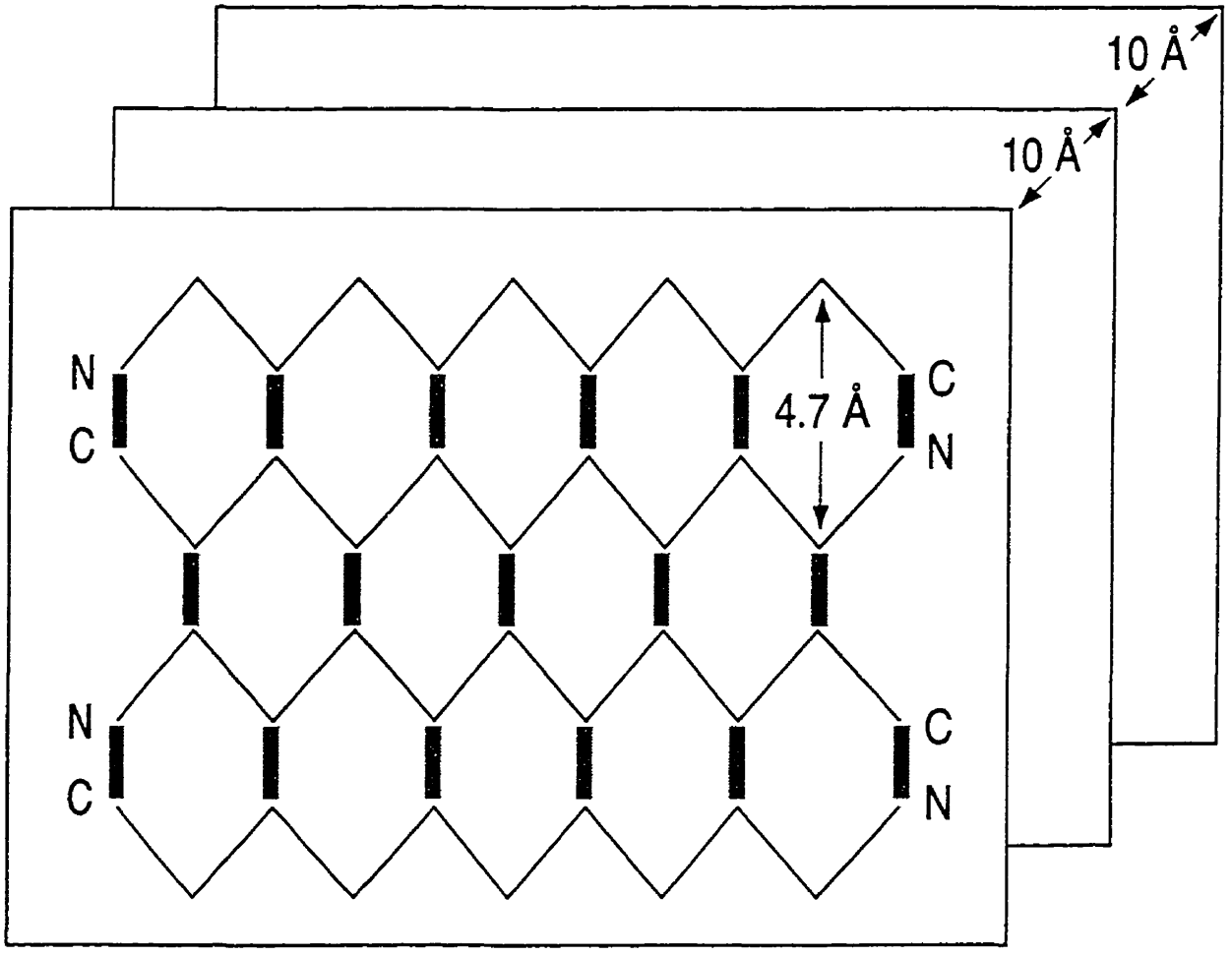
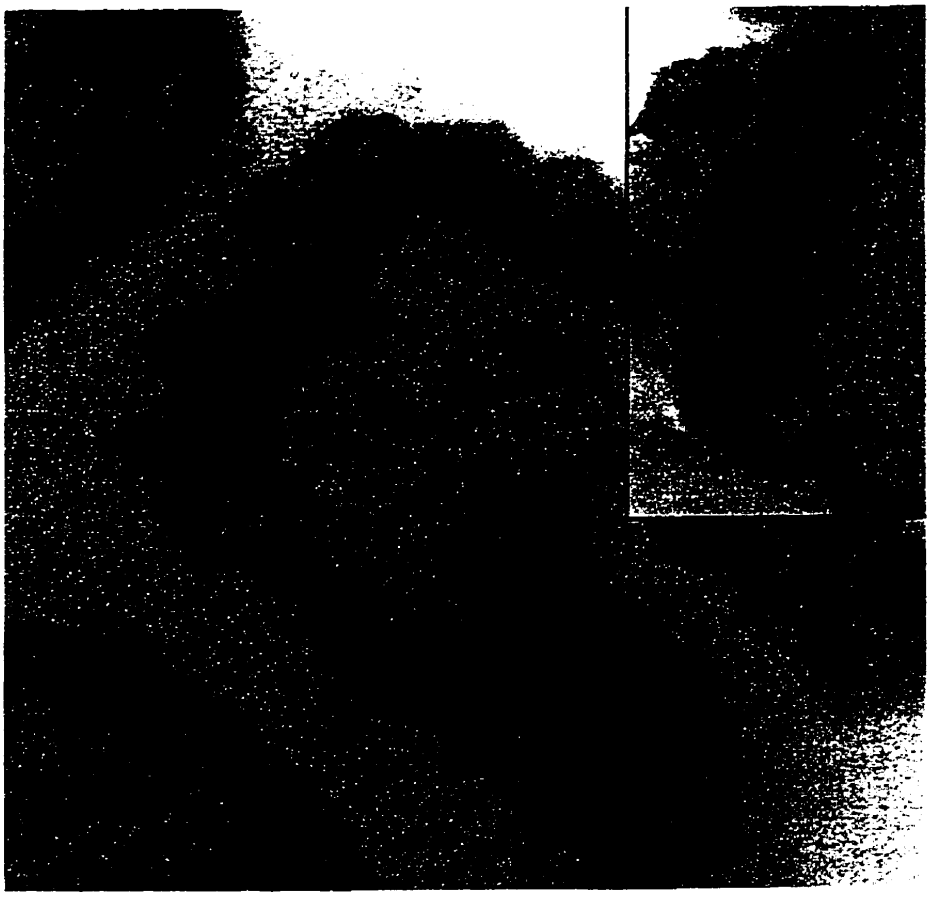
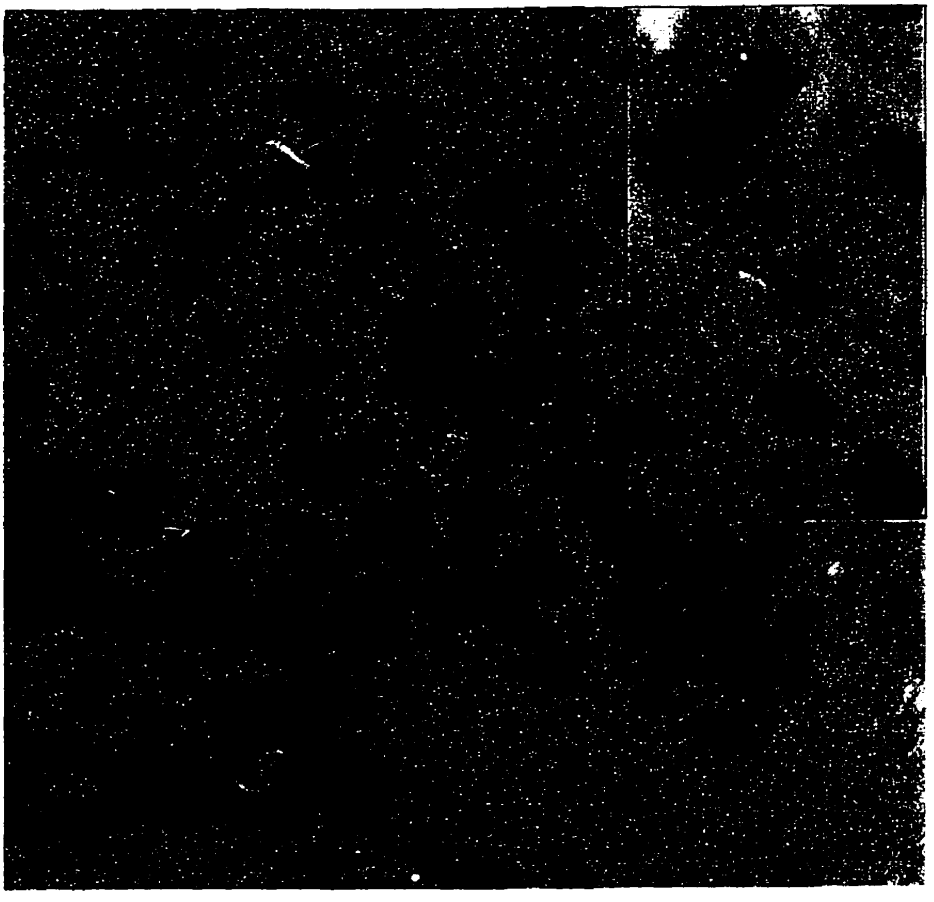


Figure 1.1 A diagrammatic representation of Pauling's model (1955) illustrating the common conformation seen in all amyloids. Each sheet contains antiparallel strands of a ß-pleated peptide (the amino terminal of one peptide subunit is joined to the carboxy terminal of another) interacting largely by hydrogen bonds (strippled bars) the length of which determines the distance between the peptide backbones (4.7 Å). The peptide backbones (four of which are illustrated here) are within the plane of the sheet, while the amino acid sidechains are perpendicular to the plane of the sheet. The more sidechains there are, the larger the distance between the sheets. For Aß amyloid, the distance is about 10 Å.

Figure 1.2 Amyloid in the CP of a normal 57 year old is visualized by staining with Congo red dye and viewing under polarized light. The amyloid deposits, commonly referred to as Biondi rings, are seen as red-green birefringent fibrils (A). These deposits can also be stained by a rabbit antiserum called AA95 (B). Original magnification is x400 (x800 for the insets).





1.2.1 Amyloidoses and their constituent proteins

Deposition of amyloid is the primary or secondary manifestation of a diverse group of systemic and cerebral disorders referred to collectively as **amyloidoses** (23). A partial list of diseases characterized by amyloid deposition is presented in Table 1.1, along with the chemical nature of the proteins which make up the amyloid. The purpose of this table is to illustrate that amyloid is formed by diverse proteins and to draw attention to some of the proteins which will be the focus of this research. For a more comprehensive study of amyloidoses and their associated amyloids, the reader is referred to a number of excellent reviews dealing with the subject (54,56,101).

In **AD**, the amyloid is deposited in roughly spherical cortical lesions known as classic neuritic plaques, as well as around cerebral vessels. It is composed mainly of aggregates of amyloid-ß (Aß), a 39-43 amino acid hydrophobic peptide that is derived from the transmembrane portion of a much larger amyloid precursor protein, APP (57,79,108,144). Several lines of evidence, including genetic predisposition associated with mutations in APP (59, 119), have implicated Aß as a key factor in the pathogenesis of AD. Aß also deposits as amyloid in the brains of patients with **Down's syndrome** (58), virtually all of whom develop a pathology identical to AD after the age of 50 (190).

Congophilic amyloid angiopathy (CAA) is characterized by amyloid deposition which is confined to the walls of the cerebral vessels. CAA is a noted cause of spontaneous cerebral hemorrhage in elderly individuals with normal blood pressure (179,184). In sporadic cases of CAA, the amyloid deposits are composed of Aß (31). In addition to sporadic cases, some individuals appear to be genetically predisposed to amyloid deposition around cerebral vessels. Amyloid angiopathy is a prominent feature of hereditary cerebral hemorrhage with amyloidosis (HCHWA) of the Dutch (96,183) and Icelandic (63) types. In HCHWA of the Dutch type, both the normal Aß and a variant form of Aß with an amino acid substitution at position 22, form amyloid deposits around cerebral grey matter vessels and in the diffuse cortical plaques which are occasionally observed in these patients (96,183). The main protein component of the amyloid in HCHWA of the Icelandic type is a mutant protease inhibitor, cystatin C, which deposits around grey matter vessels (27,51). Both types of HCHWA are associated with early-onset dementia (51,62,183), and HCHWA-I patients generally suffer a fatal cerebral hemorrhage at a strikingly young (20-40 years) age (63).

In **senile systemic amyloidosis** (SSA), a wild-type transthyretin (TTR), or fragments thereof, forms amyloid which accumulates with age, mostly in the heart (84). TTR also forms amyloid deposits in the autosomal-dominant **familial amyloid polyneuropathies** (FAP), in which over 40 TTR variants have been described. A variant of apolipoprotein A-1 and the plasma protein,

gelsolin, are also deposited in some types of FAP. This amyloid is commonly found deposited in the autonomic nervous system, peripheral nerves, heart and gastrointestinal tract (for a review see ref 141).

The mechanisms by which amyloid damages tissue are unknown. The simple fact that amyloid mechanically displaces tissue should not be overlooked as a contributing factor. In CAA for example, extensive deposition of amyloid in the media and adventitia of arterial walls destroys the normal structure of the vessels and is thought to weaken their walls (103,186). In addition, the intima is often also damaged, and this may predispose the vessels to hemorrhage (103).

In the case of Aß, more complex mechanisms may be involved. In vitro studies of primary cultures of rat hippocampus have shown that the Aß peptide is neurotoxic (204), and that the ß-pleated conformation (162) and/or aggregation of Aß (69,162) is closely linked to this toxicity. Interestingly, addition of Congo red dye to the hippocampal culture appears to attenuate this neurotoxicity (20).

DISEASE	PROTEIN	SITE
Alzheimer's Disease	Aß	Plaques, vessels
Down's Syndrome	Aß	Plaques, vessels
Sporadic CAA	Aß	Vessels
HCHWA-D	Aß and Aß variant	Vessels (plaques)
HCHWA-C	Cystatin C variant	Vessels
SSA	TTR	Heart
FAP type 1,2	TTR, apoA1 variants	ANS, Heart, GI

Table 1.1 Amyloid deposition is a prominent feature of many diseases. The proteins which form the amyloid are equally diverse. See text for details and specific references. Aß: amyloid-ß peptide, CAA: congophilic amyloid angiopathy; HCHWA: hereditary cerebral hemorrhage with amyloidosis; -D: Dutch type; -I: Icelandic type; SSA: senile systemic amyloidosis; FAP: familial amyloid polyneuropathies; ANS: autonomic nervous system; GI: gastrointestinal tract.

1.2.2 Amyloid deposition with age

Two areas known to accumulate amyloid with age are the cerebral cortex and the CP, a tissue in the cerebral ventricles. Amyloid deposits in the cortex of the elderly occur in many of the same areas prone to amyloid deposition in AD, suggesting that a common mechanism may be involved in amyloid formation. In contrast, deposition of amyloid in the CP has not been linked to any particular disorder, and so has been relatively ignored. For example, amyloid was first observed in the CP in 1918 (del Rio Hortega), a mere eleven years after Alois Alzheimer's reports, and yet the composition of this CP amyloid remains unknown. As will be shown below, there is tremendous communication between the CP and the cortex and proteins produced in one area can be transported to the other. Thus it is entirely possible that the amyloid which deposits in the brain of some normal elderly people is derived from the same source as that in the CP. If this is indeed the case, an understanding of amyloid formation in the CP could have significant implications for our comprehension of the mechanism involved in amyloid formation in the brains of AD patients.

The following sections will review what is known about the age-associated accumulation of amyloid and amyloidogenic proteins in the CP and cortex. A comparison will be made between individuals who do not present with any clinical symptoms and those with AD. We begin with an overview of the structure and function of the CP and describe its relationship with the cortex before proceeding to examine amyloid deposition in these two areas.

1.3 THE CHOROID PLEXUS AND AMYLOIDOGENIC PROTEINS

1.3.1 Morphology

The CP is a tissue found in all four ventricles of the brain (Figure 1.3). The CP of the two lateral ventricles was discovered by Herophilus (c 335-280 B.C.) (163). It was almost two thousand years later before Willis (1664) would describe the CP of the 4th ventricle and hypothesize that the CP produces the fluid found in the ventricles (191). Finally, in 1695, Ridley described the CP of the 3rd ventricle (142).

The CP is a network (plexus) of epithelium, blood vessels, connective tissue and nerves. The epithelial covering (skin or chorion) is composed of modified ependymal cells. Ependymal cells line the cerebral ventricles, aqueduct and central canal of the spinal cord, and like the CP epithelium, can pinocytose proteins and ions (123). The CP in all ventricles together is estimated to contain approximately 100 million epithelial cells, most of which are cuboidal to low columnar in shape, but which may become squamous in older individuals (187). The epithelial cells sit on a well-defined basal lamina and are sealed by apical tight junctions, forming the blood-CSF barrier (Figure 1.4) (123).

Figure 1.3 The CP is found in all four ventricles of the brain.

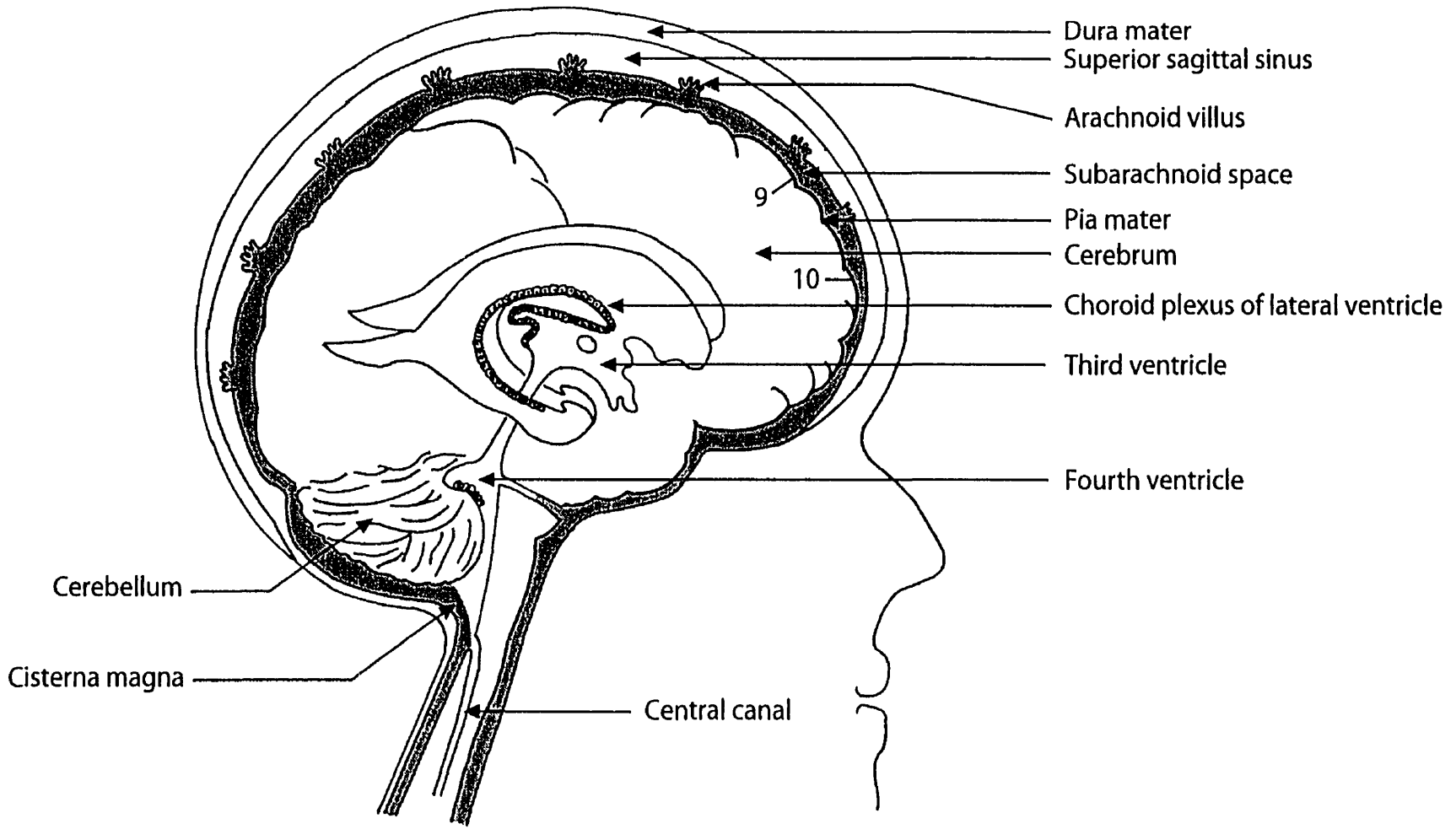
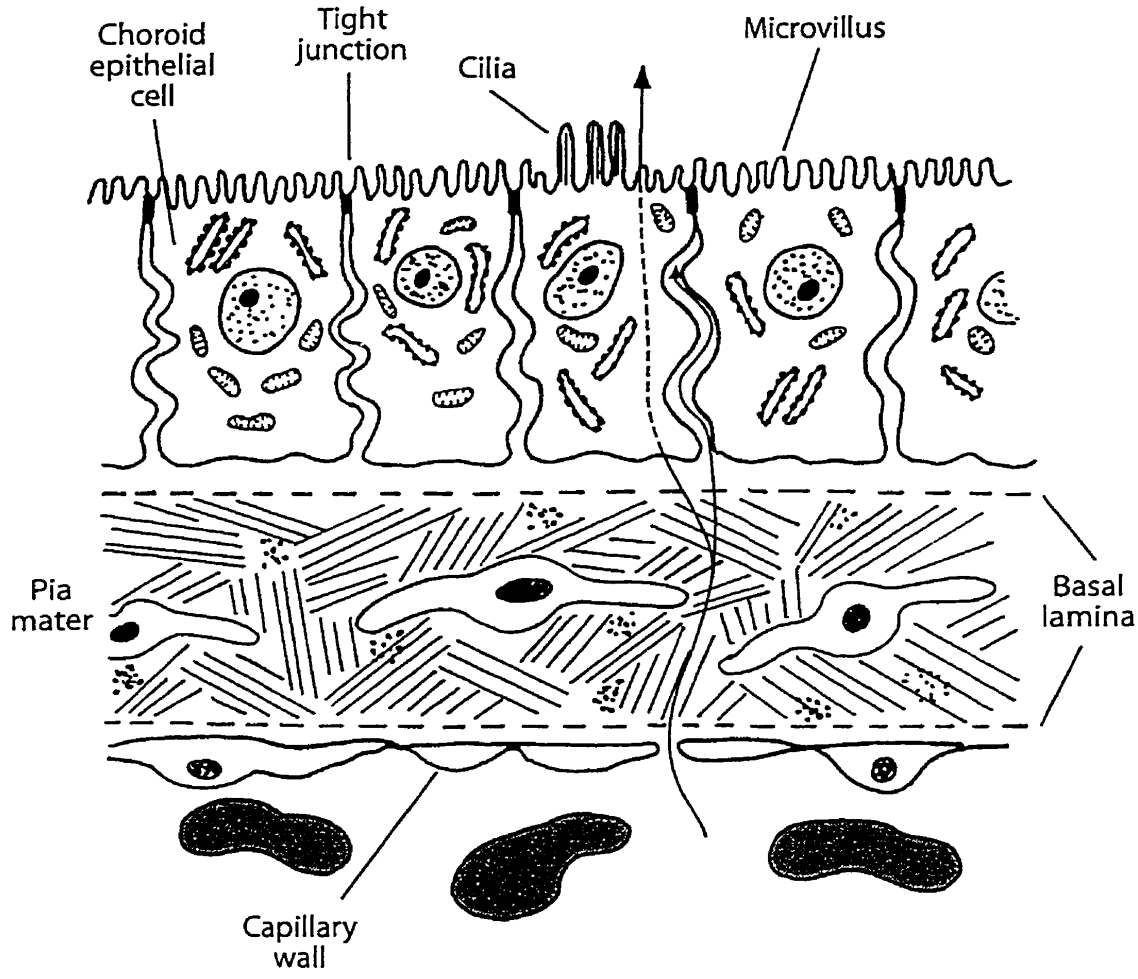

Dura mater
Superior sagittal sinus
Arachnoid villus
Subarachnoid space
9
Pia mater
Cerebrum
10
Choroid plexus of lateral ventricle
Third ventricle
Fourth ventricle
Cerebellum
Cisterna magna
Central canal

Figure 1.4 A schematic representation of the CP illustrates the blood vessels, basal lamina, numerous microvilli, tufts of cilia and tight junctions, which form the blood-CSF barrier.

CEREBROSPINAL FLUID



BLOOD

1.3.2 CP Function

The CP seems small, it weighs only about 2 grams (187), compared to the relatively large brain of approximately 1500 grams in which it resides (36). However, as will be shown below, the intimate relationship between the CP and the brain is essential for normal brain function. In particular, the CP produces most of the CSF and regulates its composition, protecting the brain from potentially toxic substances or from wild fluctuations in protein or ion concentrations. The CSF serves as a vehicle for removal of metabolic wastes and as a route of communication between various brain regions (for a review see ref 36). The many notable functions which have been ascribed to the CP depend primarily on an intact epithelium and are discussed below.

1.3.2.1 The blood-CSF barrier

The ability to regulate its chemical environment is essential for normal brain function. To achieve this, two important barriers exist which separate the blood from the brain and from the CSF which bathes the brain. The blood-brain barrier (BBB) is formed by glial processes wrapped around brain capillaries and by tight junctions between vascular endothelial cells (36). In contrast to the brain, the capillaries in the CP are fenestrated and permit the passage of macromolecules into the surrounding stroma (Figure 1.4). However, these molecules are prevented from entering the CSF by tight junctions found at the apical surface between all CP epithelial cells. These junctions effectively

regulate passage of molecules into the CSF and form the blood-CSF barrier (124). The membrane of the arachnoid villi (Figure 1.3) forms a second component of the blood-CSF barrier. It impedes passage of solutes from the relatively permeable vessels of the superior sagittal sinus into the CSF of the subarachnoid space (36).

Early studies utilized horseradish peroxidase (HRP) to characterize the blood-CSF barrier. Following intravenous injection of this tracer in rats, it entered the CP epithelium, but did not pass into the ventricles (10). In contrast, following injection of HRP into the lateral ventricles, this tracer could be found in plasma (9). These studies demonstrate that in the case of HRP, and possibly for other proteins, the epithelial cell function may be polarized.

In addition to forming a physical barrier, the CP has the capacity to take up and degrade many compounds. A number of enzymes have been identified in the CP epithelium, many of which are thought to be involved in normal cellular metabolism and others which may be important components of a chemical blood-CSF barrier. Examples of these enzymes include an endoprotease which cleaves dynorphin A, dynorphin B and α-neoendorphin (126), endopeptidase-24.11 which hydrolyses atrial natriuretic peptide and a variety of neuropeptide processing enzymes, including carboxypeptidase E (49).

The protective function of the CP is illustrated by studies performed on rats and rabbits. Following intraperitoneal administration of lead, cadmium,

mercury or arsenic, the concentration of these compounds was many fold greater in the CP of the lateral ventricles than in the CSF, brain or blood (206), indicating that the CP may sequester compounds and thereby protect the CSF and brain from influxes of toxic heavy metals from the blood.

1.3.2.2 Secretion of the CSF

The exact proportion of CSF produced by the CP is still a matter of debate, with estimates varying between 60% and 90% of the total (36, 33). The remainder of the CSF is thought to be derived from water and lipophilic substances which can directly cross the vessel walls of the brain capillaries (33,36) and from the cellular metabolites and secretions found in the interstitial fluid of the brain (154).

The average total volume of CSF in the human is about 160 ml, including roughly 25 ml in the ventricles (109). CSF is produced at between 0.2 and 0.6 ml/min (34,146), with a peak production occurring around 2 a.m. (125). The turnover rate of CSF in mammals is high, around 0.5% of the total volume per minute, that is the CSF is completely turned over 4 to 5 times per day (36). CSF production can be modulated by several factors, including sex hormones and corticosteroids (99).

The majority of CSF is contained within the ventricles and subarachnoid space, an area between the arachnoid and the pia mater of the brain and spine, the remainder is found in the extracellular fluid of the brain (Figure 1.3).

The CSF is thought to leave the subarachnoid space by means of bulk flow through the arachnoid villi of the superior sagittal sinus, under a hydrostatic pressure gradient, which is maintained by the continued secretion from the CP and arteriolar and respiratory pulsations. The CSF in the spinal cord leaves through the lymphatics (123).

The CSF provides buoyancy for the brain. A brain floating in the CSF has a virtual weight of about 50 grams, compared to approximately 1500 grams when measured in air (36). This cushions the nerve fibers, vessels and other delicate membranes from the weight of the brain and from damage during mechanical trauma (123).

The high turnover rate of CSF indicates that it serves other functions as well, including acting as a medium between brain and blood, bringing nutrients and removing waste from the brain. Molecules in the extracellular space of the brain are thought to eventually diffuse into the CSF. In fact, it is estimated that about 20% of the CSF proteins are produced by the brain (188). It has been suggested that the CSF may transport by bulk flow, substances from one part of the brain to another, as for example hypothalamic hormones (4,124,199).

1.3.2.3 The CP as part of a neuroendocrine pathway

A growing body of evidence suggests that the CP can act as a target, a source and as part of a pathway involved in neuroendocrine signalling in the brain. Autonomic nerve fibers are present in the CP. Receptors for arginine vasopressin (AVP) and atrial natriuretic peptide on the CP epithelium suggest that centrally-released transmitters liberated into the CSF can target the CP and modulate its activity (124). For instance, AVP and atrial natriuretic peptide can alter ion transport and reduce CSF production by the CP (166). The presence of prolactin receptors in the CP epithelium suggests that the CP may also be a target for pituitary hormones (19,92).

In addition to being a target, the CP can itself be a source of substances which act on the brain. For example, the CP is the main source of insulin-like growth factor-II (IGF-II) in the adult mammalian CNS (71). Receptors for IGF-II are present in many parts of the brain and IGF-II has been shown to have trophic effects on glial and neuronal cells (89,93). Similarly, during gestation, the CP of the fourth ventricle is thought to promote growth of the underlying developing cerebellum through secretion of retinoic acid (203). It is not known if the CP secretes the retinoic acid directly into the area of the cerebellum or if the retinoic acid is transported by a protein. Since TTR binds retinoic acid in plasma (61), one might speculate that TTR synthesized by the CP (see below) binds and targets the retinoic acid to TTR receptors in the cerebellum.

The CP is also part of a pathway that connects peripheral tissue to the

brain. One example is the transport of the thyroid hormone thyroxine by TTR. It has been proposed that transport of serum-derived thyroxine through the CP and into the CSF is the major route of entry for thyroxine into the brain (25).

In summary, the CP synthesizes proteins for local metabolism in the CP as well as for export to the brain. While it is evident that proteins which are unique to the CP may carry out functions which are specific to the CNS, the reason for CP synthesis of proteins which are also produced in the periphery and found in the serum is less obvious. For example, TTR is found in the serum, but it does not appear to cross the BBB at an appreciable rate and the level of TTR in the brain is determined by synthesis in the CP (40,68). Protein synthesis by the CP thus appears to be one part of a complex system by which the CP monitors and regulates the composition of the CSF as required by the brain. The importance of TTR in fundamental processes such as supplying the brain with thyroid hormones (25) may help to explain why the CP-derived TTR is regulated independently of the liver-derived serum TTR.

1.3.3 Amyloidogenic proteins in the CP

The CP synthesizes at least three proteins which are amyloidogenic: TTR, APP and cystatin C, all of which are found in the CSF (22,62,153). TTR seems to be produced solely by the CP epithelium and possibly the leptomeninges, but nowhere else in the mammalian brain (40,68). In addition to the physiological roles of TTR described above, TTR has also been

implicated in a number of amyloidoses and can be found in cortical plaques and around microvessels in AD brains (160).

The regulation of **APP** expression has been studied in numerous cell types in the human brain, but production of APP by the CP has been almost totally ignored. It has been detected in sheep and rat CP, where it appears to be synthesized early in gestation (129) and recently it was detected in the human CP, where it may be metabolized into Aß and secreted (78). Given the location and function of the CP, the metabolism of APP and the factors which regulate it in the CP warrant further study.

Cystatin C has been shown to be produced by the rat CP epithelial cells (28). In addition to its ability to form amyloid, it may promote amyloid formation by other proteins. Cystatin C inhibits cathepsin B (1), a protease which is produced in the rat CP (134) and which can cleave APP inside the Aß domain (151). Inhibition of cathepsin B by cystatin C may therefore promote accumulation and subsequent ß-pleating of the Aß peptide. Furthermore, like APP (130,133), cystatin C has been identified in the plaques of AD brains (73).

It is not known if any of these amyloidogenic proteins form amyloid in the CP epithelial cells in normal individuals. The presence of these proteins in the plaques of AD brains, together with their propensity to form amyloid elsewhere in the body, suggests the possibility that they may promote amyloid formation or form the amyloid in the CP of normal individuals. Indeed, TTR

has been shown to form amyloid in the CP of patients with systemic amyloidosis. However, this amyloid was localized to the connective tissue and arterial walls, and was not observed in the CP epithelium (74).

1.3.4 Changes with age

1.3.4.1 CSF secretion and composition

The production of CSF has been studied using magnetic resonance (MR) imaging, which is thought to provide a more accurate estimate than computerized tomography (CT) (174). However, MR imaging, like CT scanning, has yielded conflicting results. The rate of CSF secretion was shown in one study to decline from 0.41 ml/minute at age 28 to 0.19 ml/minute at age 77 years (110), and to remain constant in another study of individuals aged 22 to 76 years (53). In contrast, the CSF volume in elderly subjects has consistently been shown to be greater than in younger subjects (152,172). As the CSF volume increases while the production remains stable or declines, there appears to be a reduction in the turnover rate of CSF with age. Consistent with this possibility is the observation that the mean concentration of protein in the CSF, but not in the plasma, increases with age (110).

1.3.4.2 Amyloid deposits in the CP

Two large histological studies of CP from neonates to individuals aged 100 found age-related changes in the CP from all four ventricles (43,161). Specifically, there was an accumulation of oxidized lipid in the form of lipofuscin, and formation of numerous vacuoles in the cytoplasm. Distinctive rod and ring-like structures, which are readily identified by the PAS stain for carbohydrates, were often found in association with the cytoplasmic vacuoles.

These distinctive structures were first demonstrated in the CP epithelium by del Rio Hortega (1918), then confirmed by Gellerstedt (1932) and Biondi (1933) using silver impregnation. They have come to be known as **Biondi rings**, in reference to the fact that they are often circular in appearance. Biondi rings, which are mainly found in the perikaryon and sometimes between epithelial cells, have the staining properties of amyloid (see Figure 1.2) (41). They are seldom seen in people younger than age 50, but are almost always present in those over age 60 (161). The appearance of amyloid in the CP with age is so consistent that it has been suggested that it may be one way to verify the age of the deceased (45).

Transmission electron microscopy reveals that Biondi rings consist of a slightly irregular core 2-7 μm in diameter, of amorphous fat-like material, covered with a thin layer of amyloid fibrils (44). The appearance of these structures resembles that of a secondary lysosome or residual body. It was thus postulated that the fibrils form in conjunction with lysosomes, perhaps by

partial degradation of a larger precursor, as is the case for most amyloids (44).

Immunohistochemical studies suggest that the Biondi rings may possess Aß epitopes, but not keratin, vimentin, desmin, actin, neurofilament, glial fibrillary acidic protein, ubiquitin, tau protein or paired helical filament epitopes (189). The positive staining by PAS (123) indicates that a glycoprotein is also present in Biondi ring amyloid.

The Biondi ring amyloid has been isolated, but attempts to sequence the protein have failed (44). Nonetheless, the purified amyloid has been successfully used to develop antibodies in rabbits. This antiserum, referred to as **AA95**, stains Biondi ring amyloid in histological sections (see Figure 1.2), and recognizes a protein of about 50 kDa on immunoblots (44). The development of AA95 should facilitate efforts to identify the protein in the Biondi ring amyloid and to determine if this protein is present in the amyloid deposited in the cortex as well.

1.4 AMYLOID AND AMYLOIDOGENIC PROTEINS IN THE CEREBRAL CORTEX

The previous section showed that amyloid deposition occurs with age in the CP, a tissue which plays a major role in controlling the chemical environment of the cerebral cortex. Although the identity of the amyloid in the CP is unknown, the possibility was introduced that it may be similar to the amyloid found in the cortex in AD patients and in some normal aged

individuals. The following section will deal with amyloid deposition and associated lesions in the cortex. It will begin with a description of the lesions common in AD brains, as most of our knowledge has been derived from this source. Accumulation of amyloid and amyloidogenic proteins in the brains of normal individuals will be discussed in the subsequent section, which will include a comparison between the normal and the AD brain.

1.4.1 The Specific case of Alzheimer's disease

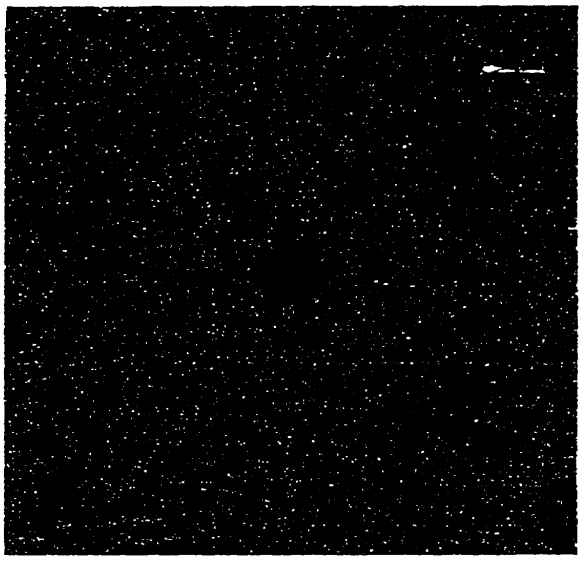
One of the best-studied cerebral amyloidoses is AD. It first came to the attention of the public in 1907, when Alois Alzheimer reported on the case of a 55 year-old women who died four years after the onset of a severe dementing illness. Postmortem examination of the brain demonstrated two principal abnormalities, as seen by Bielschowsky's silver stain. Throughout the cortex were numerous argentophilic foci, similar to those initially described in a human brain by Blocq and Marinesco (1892) and subsequently by Redlich (1898) in cases of senile atrophy. The second change consisted of widespread thickening and twisting of neurofibrils, often forming tangles, within roughly every fourth neuron. This feature has come to be known as Alzheimer's neurofibrillary tangles (NFT). Within the next couple of years, additional cases with similar histological changes had been reported, and in 1910, this dementia was given the name "Alzheimer's disease" (12).

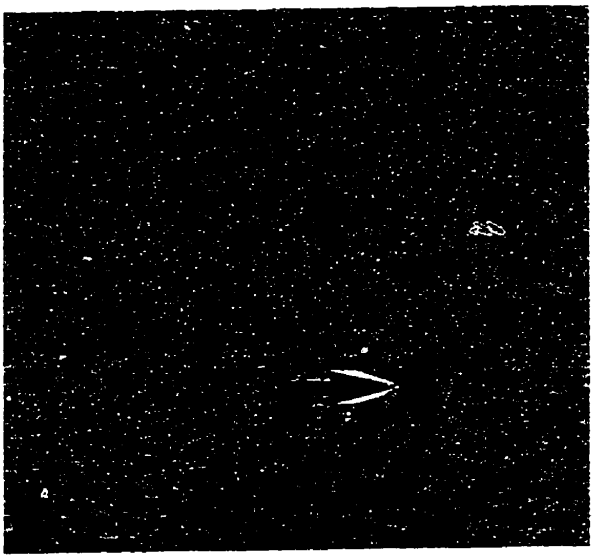
Since these initial reports, much has been learned about AD, although no cure or even reliable treatment is currently available. Modest estimates of the prevalence of AD are that it affects about 10-15% of the total population over 65 years of age, rising from 1% at age 65, to 10% at age 80 and to 20% over 80 years of age (81,176). In a Canadian study, the proportion of the population that was clinically diagnosed with AD was 1% in those 65-74 years old and 26% in individuals 85 and over (21). The estimates vary somewhat according to the sample studied, the criteria for clinical diagnosis of living patients and whether or not the diagnosis was confirmed postmortem. Nonetheless, AD is considered to be the most common cause of dementia in the Western World (24,164,178).

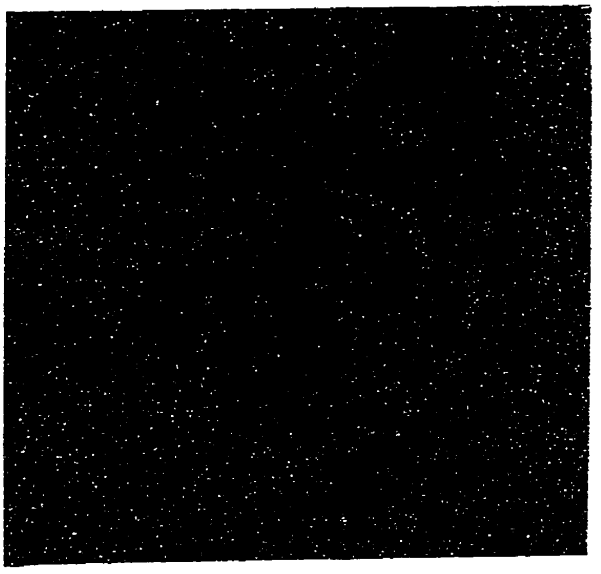
1.4.1.1 The neuropathology of AD

Although a clinical history of dementia is essential, at the present time a conclusive diagnosis of AD can only be obtained postmortem, by examining the brain for the presence of abundant plaques containing dystrophic neurites, NFTs, granulovacuolar degeneration (GVD), neuronal cell loss and amyloid deposition around cerebral vessels (for a review, see 193,194). While all of these changes are certainly important in the pathogenesis of AD, cortical plaques and NFT are considered to be the two hallmark lesions of the disease (Figure 1.5), although much debate continues as to which of these two is more closely related to the progressive and irreversible dementia in AD patients.

Figure 1.5 The hippocampus from an AD patient reveals the presence of plaques (A,C,E) and neurofibrillary tangles (B,D,F). These lesions can be visualized by silver stains, including Bielschowsky's silver stain (A) and the methenamine silver (B) as well as by antibodies to tau (C,D,F). The antibodies to tau stain dystrophic neurites (C), NFTs (arrows) in neuronal soma (D, F) as well as neuropil threads (arrowhead) (F). Reactive astrocytes seen in classic neuritic plaques can be stained by antibodies to glial fibrillary acidic protein (E). Original magnification x250.







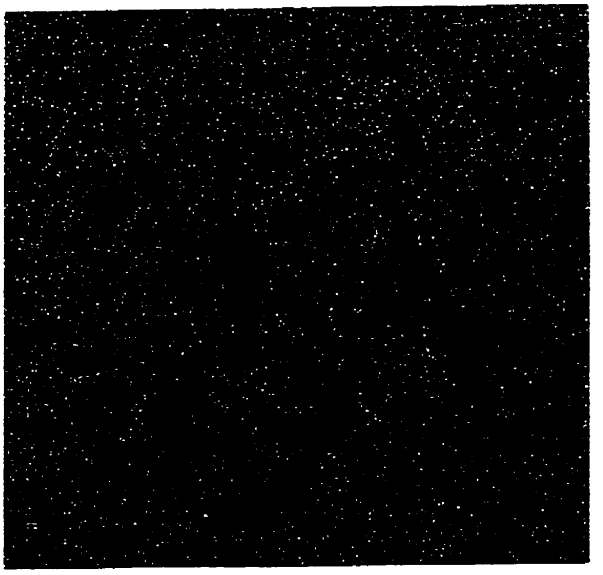
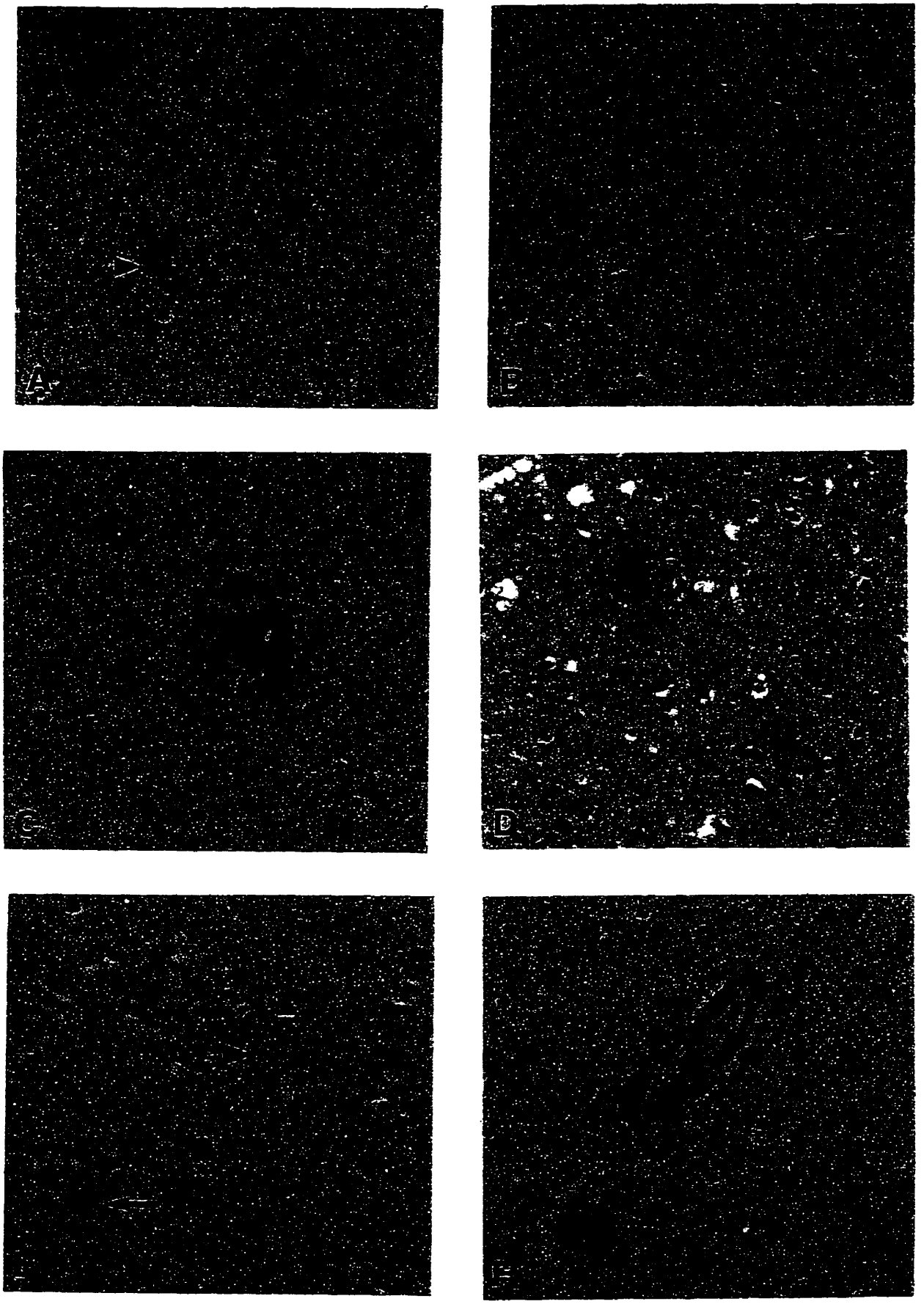


Figure 1.6 Immunohistochemical localization of proteins in the cerebral cortex of a subject with AD. The main protein component of the roughly spherical plaques is aggregated Aß (A). A number of other proteins are sometimes present in plaques. These include apoE (B), apoJ (C), cystatin C (D) and TTR (E), which can be visualized by immunohistochemistry. Both amorphous (arrow head) and cored (arrow) plaques can be seen following immunostaining. Aß immunoreactivity can also sometimes be found around cerebral vessels, extending into the neuropil (F). This is referred to as dysphoric angiopathy. Original magnification x250.


A
B
C
D
E

Plaques

At least four morphologically distinct categories of plaques have been identified in the human brain: diffuse, primitive neuritic, classic neuritic and compact. The unifying feature of these different plaque types is that they all contain the Aß peptide (57,108). Immunohistochemical studies using recently developed antibodies which can differentiate between the two main Aß variants isolated from AD brains, Aß40 and Aß42, have revealed that the Aß containing 42 amino acids is the main Aß variant in plaques. Aß40 is also present, but is found in fewer plaques than Aß42 (76).

The **diffuse** plaques are variable in shape. They do not contain amyloid and so cannot be visualized by Congo red dye, but can be detected by silver stains and antibodies against Aß (138,201,202). Diffuse plaques do not contain swollen neurites (neuronal processes) and there is virtually no detectable alteration in the surrounding neuropil (155). They can be found in most areas of the brain, commonly being numerous in regions where classical plaques are few, such as the brain stem, basal ganglia and cerebellum (192). **Primitive neuritic** plaques contain swollen neurites which are seen as enlarged silver-stained circular structures which can also be detected by antibodies against tau (192). Primitive neuritic plaques are readily visualized by anti-Aß antibodies, but do not have a central core of amyloid (112,113). **Classic neuritic** plaques contain abnormal neurites arranged around a dense central core of amyloid which is visualized by Congo red dye, and are associated with

reactive astrocytes and microglia (193). Both the primitive and classic neuritic plaques are also often referred to collectively as senile or neuritic plaques due to the presence of dystrophic neurites. **Compact** ("burned-out") plaques consist of a core of tightly packed amyloid fibers and very few dystrophic neurites (193). Table 1.2 presents a summary of the plaque types.

To confound matters, the plaque terminology is often used loosely in the literature, making clear interpretations of the various reports difficult. For example, the term amyloid plaque is often used to refer to plaques which contain Aß as shown by anti-Aß antibodies, but which have not been stained with either Congo red dye or thioiflavin S to identify amyloid. For the sake of clarity, the terminology in Table 1.2 will be used whenever possible when comparing the results obtained from various studies. Plaques which are said to contain amyloid are those which stain positively with Congo red or thioflavin S at the light microscopic level, that is classic neuritic and compact plaques. Using the above criteria, it can be said that the majority of plaques in the AD brain are primitive neuritic (201,202) and that the typical AD brain has a total of about 1400 plaques per cubic millimetre (158).

A variety of proteins have been shown to associate with cortical Aß plaques (Figure 1.6). They have been the subject of a number of excellent reviews (see especially ref 156 and 165) and so will be considered only briefly here. ApoE (121,181), apoJ (111), TTR (160) and cystatin C (73), have all been identified in plaques. ApoE accelerates the formation of amyloid by Aß

in vitro (195,197) and may itself form amyloid fibrils in the plaques (198). In contrast, apoJ and TTR inhibit amyloid formation by Aß in vitro (127, 16, 153).

The proportion of plaques containing each of these proteins is not known. Until a systematic analysis of plaque composition in both normal and AD subjects is performed, one can only speculate on the role of these proteins in amyloidogenesis in vivo.

Plaque	Aß immunoreactivity	Silver stain	Tau	Congo red
Diffuse	+	+	-	-
Primitive	+	+	+	-
Classic	+	+	+	+
Compact	+	+	+	+

Table 1.2 Plaques can be distinguished by their staining characteristics. The silver stain labels normal and dystrophic neurites, while tau immunoreactivity is restricted to plaques containing dystrophic or abnormal neurites. Congo red dye identifies amyloid. Compact plaques contain fewer dystrophic neurites than neuritic (primitive and classic) plaques and the central core of amyloid is not surrounded by a "halo" of Aß immunoreactivity as it is in classic neuritic plaques.

Neurofibrillary tangles

The NFT first described by Alzheimer are large, flame-shaped or globular masses, consisting of abnormal filaments running through the perinuclear cytoplasm and often extending into dendrites, where they are referred to as neuropil threads. They can be demonstrated by light microscopy using conventional silver staining techniques and by anti-tau immunostaining (Figure 1.5). NFT also exhibit green birefringence when stained with Congo red, and fluorescence when stained with thioflavin S. These latter staining properties are consistent with an underlying ß-pleated sheet structure of NFT (88). In addition to intracellular NFT, extracellular NFT ("ghost tangles") have been identified and are thought to be remnants of neurons which have degenerated. In AD brains, NFT occur predominantly in the pyramidal cells of the cortex, the hippocampus and the amygdala (70), as well as in the locus ceruleus and the raphe nuclei of the brainstem (75). The majority of brains from AD subjects have numerous NFT in the neocortex. However, in up to 30% of cases, NFT are restricted to the entorhinal cortex and hippocampus. These cases are classified as "AD without neocortical NFT" (175).

Ultrastructurally, NFT are composed of bundles of filaments predominantly twisted about each other in pairs (60,86,87). In addition to the **paired helical filaments** (PHF), variable amounts of straight normal neurofilaments can also be found in NFT (5). PHF are also present in the swollen neurites of neuritic plaques (60,86,87). Immunocytochemistry (37)

and protein chemistry (94) have established that the microtubule-associated protein tau forms an integral component of the PHF.

Microtubules are dynamic structures that are formed by assembly of tubulin dimers, assembly being a reversible process. In vitro, tau promotes the assembly of purified tubulin (42). The tau associated with PHF is abnormally hyperphosphorylated (13,94). This hyperphosphorylation may impair the ability of tau to bind to and stabilize microtubules, presumably preventing microtubule polymerization and allowing the microtubules to degenerate (7,122). Since microtubules are important in cell structure and transport, the accumulation of NFT in neurons may compromise axoplasmic and dendritic transport and other functions which depend on an intact cytoskeleton.

Additional cytoskeletal proteins identified in the NFT include neurofilament proteins (160 and 200 kDa) and microtubule-associated protein 2 (90). In addition, many NFT contain ubiquitin (115,132). The addition of ubiquitin to NFT is thought to follow hyperphosphorylation of tau (7,115). Ubiquitin has been implicated in a multi-enzyme pathway for metabolism of abnormal and damaged proteins (95). Despite extensive research, the precise nature of the tangle constituent which is ubiquitinated remains unresolved (for a review, see ref 100). Finally, similar to plaques, NFT contain epitopes of apoE (121) and TTR (160).

CAA

Localized deposition of amyloid in cerebral and meningeal vessels, particularly in small arteries and arterioles, occurs in almost all cases of AD (55,179,184). This is also referred to as CAA because of the affinity of the amyloid for Congo red dye. Capillaries and veins are less frequently affected, as is any type of vessel in the white matter. The deposits are generally localized to the outer media and adventitia of the vessels, and can sometimes be seen in the basal lamina of capillary endothelial cells (114). "Plaque-like angiopathy" or dysphoric angiopathy (Figure 1.6) is a less frequent form of CAA, which primarily affects cortical parenchymal capillaries and often shows infiltration of perivascular amyloid into the surrounding neuropil (145,184). Both Aß40 and Aß42 are found around the vessels in the AD brain, with much debate as to which is more prominent (106,144). As in plaques, apoE (121) and TTR (160) may sometimes also be present.

1.4.1.2 The genetics of AD

The pathogenic pathway leading to AD is not well understood. However, in a few cases a genetic etiology has been established. In these families, transmission of AD occurs through an autosomal dominant fashion, and members develop AD at a characteristically early age.

Chromosome 21

In some of these familial AD (FAD) patients, missense mutations in the APP gene on chromosome 21 appear to be causal (59,119). For example, affected members of the FAD of the Swedish type carry a double substitution at amino acids 670 and 671 of APP (119). Cells transfected with the cDNA of an APP carrying this double mutation produce 5-8 times more Aß in vitro than cells transfected with normal APP cDNA (26). A mutation at codon 717 of the APP gene that is found in some cases of AD (FAD717), is associated with an increased production of Aß42 (203). Observations such as these have led to the speculation that overproduction of Aß can cause AD. The mean age of onset for individuals in these families is 50 years (52). In addition, people with Down's syndrome, who are born with three copies of chromosome 21 instead of the normal two, exhibit an age-dependent increase in the total number of plaques and invariably develop brain lesions typical of AD in their 5th and 6th decades (105,118,190).

Chromosomes 14 and 1

Chromosome 14 has been shown to be linked to FAD by a number of earlier studies (120,149,167,182). Recently, missense mutations on a locus of chromosome 14 (designated FAD3) on a gene called S182 (65,159) have been associated with an onset of AD as early as 35 years in some families, although other mutations in this gene are associated with an age of onset of

up to 20 years later (for a review see ref 52). The autosomal dominant locus associated with AD in the Volga German kindred has been localized to chromosome 1 (97). The encoded protein of this candidate gene demonstrates significant homology to the S182 protein (98), which is predicted to have seven transmembrane domains, and is designated as STM2, for second seven transmembrane protein. The age of onset in the eight families identified to date, varies between 51 and 65 years (98). In both families, mutations in the respective genes have been linked to an increased production of Aß (203).

Chromosome 19

While cases of FAD have provided significant insight into the pathogenesis of AD, they are relatively rare. In comparison, a common risk factor for AD which affects both familial and sporadic cases has been linked to chromosome 19 (131), where the apolipoprotein (apo) E gene (APOE) is located (35). There are three common APOE alleles, $\epsilon 2$, $\epsilon 3$ and $\epsilon 4$, that encode three apoE isoforms, E2, E3 and E4 (102). The combination of these isoforms gives six phenotypes that can be identified on isoelectric focusing gels, which separate the proteins on the basis of their different net charges. The presence of the APOE $\epsilon 4$ allele is associated with an increased risk of developing AD and individuals with this allele develop AD about 15 years earlier than those without the $\epsilon 4$ allele. The APOE $\epsilon 4$ allele is found in approximately 50% of AD cases compared to only 14% of the general

population (30,135,139,148,168). On the other hand, the presence of the APOE ϵ2 allele is associated with a decrease in the risk for development of AD (29).

The exact route by which APOE ϵ4 confers increased susceptibility is not clear, but it has been linked to both plaques and NFT. AD patients with the APOE ϵ4 allele have a significantly greater number of cortical and hippocampal Aß plaques when compared to those with the ϵ3 allele. The same relationship is also seen in normal elderly subjects, even though these individuals have significantly fewer plaques than their AD counterparts (11,136,150). Both apoE3 and E4 bind Aß in vitro, although some controversy exists as to which binds Aß with greater affinity (91,168). In vitro studies suggest that binding of Aß by apoE promotes Aß amyloid formation (195,197). ApoE has also been implicated in the formation of NFT by promoting instability of microtubules (169). ApoE3, but not apoE4, binds tau in vitro (72). This binding is thought to prevent subsequent hyperphosphorylation of tau thereby promoting the stability of microtubules (122,169).

1.4.1.3 Sporadic AD

Genetic factors can predispose some individuals to AD and are even causally associated with AD in some families. However, other factors can promote or even cause AD independently of the known genetic components.

A study of the incidence of AD in identical twins found only a 50% concordance, pointing to the importance of non-genetic factors in AD as well (77). Similarly, autopsy-confirmed AD in two identical twins with an age of onset that differed by 15 years has been reported (205). Environmental factors such as head injury (117,143) and exposure to estrogen therapy in postmenopausal women (67,173) can respectively facilitate and retard the development of AD.

1.4.1.4 The possible role of Aß in AD

Although still a matter of much debate, several lines of evidence now support the hypothesis that Aß plays a central role in the pathogenesis of AD. The mutations in the genes for APP, S182 and STM2 found in some FAD, are associated with an increase in the production of Aß, especially Aß42, and the apoE ϵ4 allele appears to promote formation of Aß cortical plaques (see above). Thus the inherited mutations and the presence of the apoE ϵ4 allele provide support for the idea that increased Aß generation or aggregation may lead to the development of AD. Recently transgenic mice have been produced which overexpress the APP gene or carry the APP717 mutation (208,209). These mice are characterized by an increase in the production of Aß and by the appearance with age of AD pathology and behavioral deficits. Such models further underline the central role of Aß in the pathogenesis of AD (208,209). In addition, Aß can initiate apoptosis in primary cultures of

neurons and it may promote formation of NFT by stimulating an increase in the intracellular levels of calcium (211). Aß has also been shown to be cytotoxic to vascular endothelial cells (210). The evidence linking Aß, and in particular the formation of Aß fibrils, to the pathogenesis of AD has prompted a search for therapeutic agents which can inhibit or even reverse formation of Aß fibrils (207).

1.4.2 The aging human brain

1.4.2.1 Chronological age as a risk factor

One of the most important, yet often overlooked aspects of the etiology and pathogenesis of AD is that the disease is an age-related condition (80). Even in individuals with a genetic predisposition, AD rarely develops before age 55 (52,82). In patients with Down's syndrome, NFT, plaques and amyloid deposits around vessels are absent before age 20, but numerous after age 50 (see ref 104 for a review). Furthermore, these lesions are virtually absent in young healthy individuals in the general population, but can be observed, albeit to a much lesser degree than in AD, in elderly people who are cognitively intact (see below). This suggests that there may be factors inherent to the aging process that modulate the ability of the brain to maintain homeostasis or to respond to injuries, so that in those individuals who are predisposed by genetic and/or environmental factors, NFT, plaques and amyloid deposits may

accumulate with age. For this reason many investigators have begun to characterize the changes which occur in the brain in normal aging and to compare these changes to those observed in disease.

1.4.2.2 The age-associated accumulation of cortical lesions.

Cortical Plaques

Several studies have examined the brains of nondemented individuals and found an increase with age in the proportion of subjects in which plaques could be detected. The plaques were identified by a variety of techniques, including the von Braunmühl silver stain (177) and later, using antibodies against the Aß peptide (128). The focus of earlier studies was to identify quantitative differences between normal and AD brains. These studies suggest that some plaque formation is inherent to aging and unassociated with dementia (32,171). Indeed, the adjustment with age of the number of neuritic plaques required for the diagnosis of AD (85,112,113) is a reflection of such a belief. That is to say that the number of neuritic plaques per unit area required for the diagnosis of AD increases with age.

Recent studies showing AD pathology in some clinically normal individuals have begun to blur the line between AD and normal brains. While the brains of most nondemented individuals contain few or no neuritic plaques, numerous neuritic plaques can be observed in a few people with mild or no dementia. In one investigation of nondemented individuals, large numbers of

plaques and NFT had already accumulated in a group of patients with only very mild, barely detectable dementia, compared to the brains of the elderly nondemented individuals who demonstrated few or no plaques (137). In another study of patients who were clinically assessed six months prior to death (48), it was observed that some nondemented subjects fulfilled the neuropathological criteria of AD (85). Morris and co-workers (1996) performed a prospective study of individuals over many years. These patients were all found to be clinically normal at the last exam, about one year before death. About half of these subjects (mean age 83 years) demonstrated very sparse numbers of plaques, roughly 0.3% of which were classic neuritic plaques (thioflavin S-positive), the remaining plaques were either diffuse or primitive neuritic. In contrast, the other half of the subjects (mean age 87 years) had enough neuritic plaques to fulfil the histopathological criteria of AD (85), and about 15% of plaques were classic neuritic.

There are three possible explanations for these observations. The first is that the presence of abundant neuritic plaques in clinically normal individuals represents preclinical or incipient AD. Since a significant proportion of people, including those into their ninth and tenth decades of life, are free of any plaques (18,180), it may be argued that the individuals with numerous neuritic plaques are not aging "normally" and that they have presymptomatic AD (48,116).

A second possibility is provided by Katzman and co-workers who found

a neuropathology consistent with AD in a group of patients (mean age 87 years) with preserved mental status (83). This group also had a significantly greater brain weight and a greater number of large neurons (> 90 μm) than either their age-matched nondemented controls or AD patients. It was speculated that these individuals either had less brain atrophy than is normally found by this age or that these subjects started with more neurons and a larger brain and thus had a greater reserve.

An alternative explanation may be that although the numbers of plaques are comparable, the composition may differ in normal and demented individuals and that consequently, the degree of neuronal damage may be less in nondemented individuals. Thus, for example, while the classic neuritic plaque is relatively rare in most nondemented subjects, it is commonly observed in AD brains. Indeed, it appears that one of the differences between normal and AD brains is that the neuritic plaques in AD are associated with a greater degree of neuronal degeneration, as is evidenced by the presence of tau immunoreactivity in paired helical filaments (8,47). In this regard, studies performed by Iwatsubo and colleagues are particularly relevant. They showed that in AD brains, virtually all plaques are immunoreactive for Aß42, but only a subset contain Aß40 (76). Importantly, they noticed that the proportion of plaques with Aß40 immunoreactivity was significantly greater in AD brains than in those from nondemented individuals (48). Thus in addition to the number of plaques, the composition of plaques may differ in AD brains.

To summarize, it may be said that our limited knowledge suggests that at least some clinically normal individuals develop plaques while others do not. The ability of the brain to compensate may vary and is perhaps reflected in the age-adjusted criteria for the diagnosis of AD. Some differences in the composition of these plaques between normal and AD subjects have been identified and merit further study.

NFT

There is considerably less controversy surrounding the presence of NFT in nondemented individuals. The ultrastructure of NFT is identical in aging and AD (194), however, the NFT do not appear in the same areas. So for example, while abundant NFT are common in certain neurons of the neocortex in AD patients, neocortical NFT are either rare or absent in nondemented aged individuals, even in those cases in which abundant neocortical neuritic plaques are present (48,178). When present in normal people, NFT are generally confined to pyramidal cells in CA1 of the hippocampus and to the entorhinal cortex (17,116,137). Thus while the formation of NFT appears to be part of the aging process, large numbers of NFT are present only in demented individuals, including those with AD or supranuclear palsy (170).

CAA

The incidence of CAA increases with age (46,179,185). In normal

elderly people, amyloid deposition is limited to the vessels of the cerebral cortex with deposits only rarely identified in other parts of the brain. Most investigators have found little evidence for amyloid in those under age 60, however CAA can be observed in about 30% of individuals over 60 years old (23,185). The main protein in CAA in normal individuals is Aß, but it is not known if Aß40 or Aß42 is the more common variant. While numerous proteins, like apoE, TTR and cystatin have been identified around vessels in AD brains, it remains to be established whether they are also present in normal individuals.

1.5 THE AGING BRAIN AS A MODEL

The preceding sections described some of the changes observed in normal individuals with age, with particular reference to the accumulation of amyloid in the CNS. The main observations were that amyloid deposition is a constant feature of the aged CP, where it is known as Biondi rings, and is frequently present in the cortex, mainly around vessels and in cortical plaques. Together these observations suggests that there are factors inherent to aging which promote amyloid formation. The identification of aging as a risk factor for AD suggests that AD may result from an augmentation of a process that invariably accompanies brain aging (157). If this is so, then the aging human brain may provide us with a model to identify the temporal sequence of events leading to AD, in the much the same way that the aging Down's syndrome

brain has contributed to our understanding of AD.

One of the potential advantages of studying normal people instead of Down's patients is that the normals are free of any known underlying disorder which may directly affect plaque formation. For example, the concentration of the APP in the serum and brain of Down's patients is increased about 1.5-fold compared to controls and AD patients, possibly as a result of the extra copy of the APP gene (147). Thus while increased APP levels may be important in Down's syndrome, additional factors may be involved in sporadic AD and the underlying pathological mechanism in operation in Down's syndrome may not be present in most AD brains. Similarly, studies of familial forms of AD like those involving the Swedish mutations have yielded some important clues about the pathogenesis of AD, but they, like the Down's syndrome cases, may represent only subsets of the AD population. A study of nondemented aging brains could therefore provide information of particular relevance to the majority of AD patients, for whom environmental factors may be more significant.

A fundamental contribution of the aging brain model would be to identify some of the changes associated with amyloid accumulation in normal individuals. An understanding of some of the factors which promote amyloid deposition may provide us with possible therapeutic targets. For example, an age-related decline in the concentration of a protein such as TTR, which is thought to keep Aß soluble, may be one factor which would foster amyloid

formation by Aß.

1.6 THESIS HYPOTHESIS AND OBJECTIVES

The main goal of this project is to identify changes that occur in the human CNS with age that could foster or possibly retard amyloid deposition. The focus will be on the production and clearance of amyloidogenic proteins and amyloid-associated proteins. Specifically, it will examine the age-associated accumulation of amyloid and its constituent proteins, in the CP, CSF and cerebral cortex, and it will attempt to determine how they may be related. Based on the observation that there is an active exchange between the brain, CSF and CP (see section 1.3.2), together with the age-associated presence of amyloid in the cortex and CP, we hypothesize that the protein composition of the amyloid in the CP is similar to that found in the amyloid plaques of the brain and that the protein components of this amyloid can also be detected in the CSF.

The first objective of this thesis is thus to characterize the amyloid in the CP. In Chapter 2, we test the hypothesis that the CP amyloid contains Aß, apoE, apoJ, TTR and cystatin C. In Chapter 3, we examine the quantitative changes in Aß40, Aß42, apoE and TTR in the CSF of nondemented patients, aged 19 to 82 years, in an attempt to identify age-related changes which may be associated with amyloid deposition in the

cortex and/or CP. In Chapter 4, we characterize some of the changes in amyloid-associated proteins in the cortex with age and address the question of whether these changes are qualitatively, as well as quantitatively, different from those seen in AD patients. Finally, chapter 5 integrates these results in the form of a model.

1.7 REFERENCES

1. Abrahamson M, Barrett AJ, Salvesen G and Grubb A (1986) Isolation of six proteinase inhibitors from human urine. *J Biol Chem* 261: 11282-11289.

2. Alzheimer A (1907a) Ueber eine eigenartige Erkrankung der Hirnrinde. *Allgemeine Zeitschrift für Psychiatrie und Psychiatrie-Gerichtliche Medizin* 64: 146-148.

3. Alzheimer A (1907b) Ueber eine eigenartige Erkrankung der Hirnrinde. *Zentralblatt für die gesamte Neurologie und Psychiatrie* 18: 177-179. The original reports became available in English in 1987 (see reference 12).

4. Anand Kumar TL and Thomas GH (1968) Metabolites of ^{3}H-oestradiol-17b in the cerebrospinal fluid of the rhesus monkey. *Science* 219: 628-629.

5. Anderton BH, Breinburg D, Downes MJ, Green PJ, Tomlinson BE, Ulrich J, Wood JN and Kahn J (1982) Monoclonal antibodies show that neurofibrillary tangles and neurofilaments share antigenic determinants. *Nature* 298: 84-86.

6. Arnold SE, Hyman BT, Flory J, Damasio AR and Van Hoesen GW (1991) The topographical and neuroanatomical distribution of neurofibrillary tangles and neuritic plaques in cerebral cortex of patients with Alzheimer's disease. *Cerebral Cortex* 1: 103-116.

7. Bancher C, Grundke-Iqbal I, Iqbal K, Fried VA, Smith HT and Wisniewski HM (1991) Abnormal phosphorylation of tau precedes ubiquitination in neurofibrillary pathology of Alzheimer's disease. *Brain Res* 539: 11-18.

8. Barcikowska M, Wisniewski HM, Bancher C and Grundke-Iqbal I (1989) About the presence of paired helical filaments in dystrophic neurites participating in the plaque formation. *Acta Neuropathol* 78: 225-231.

9. Becker NH and Almazon R (1968) Evidence for the functional polarization of micropinocytotic vesicles in the rat choroid plexus. *J Histochem Cytochem* 16: 278-279.

10. Becker NH, Novikoff AB and Zimmerman HM (1967) Fine structure observations of the uptake of intravenously injected peroxidase by the rat choroid plexus. *J Histochem Cytochem* 15: 160-165.

11. Berr C, Hauw J-J, Delaère P, Duyckaerts C and Amouyel P (1994) Apolipoprotein E allele $\epsilon 4$ is linked to increased deposition of the amyloid ß-peptide (A-ß) in cases with or without Alzheimer's disease. *Neurosci Lett* 178: 221-224.

12. Bick K, Amaducci L and Pepeu G, eds. *The Early Story of Alzheimer's disease*. Padova, Italy: Liviana Press, 1987.

13. Biernat J, Mandelkow EM, Schroter C, Lichtenberg-Kraag B, Steiner B, Berling B, Meyer H, Mercken M, Vandermeeren A, Goedert M et al (1982) The switch of tau protein to an Alzheimer-like state includes the phosphorylation of two serine-proline motifs upstream of the microtubule binding region. *EMBO J* 11: 1593-1597.

14. Biondi G (1933) Ein neuer histologischer befund am epithel des plexus chorioideum. Ztschr ges Neurol 114:161-165.

15. Blocq P and Marinesco G (1892) Sur les lesions et la pathogénie de l'épilepsie die essentielle. *Semaine Médicale* 12: 445-446.

16. Boggs LN, Fuson KS, Baez M, Churgay L, McClure D, Becker G and May PC (1996) Clusterin (Apo J) protects against amyloid-beta (1-40) neurotoxicity. *J Neurochem* 67: 1324-1327.

17. Braak H and Braak E (1990) Neurofibrillary changes confined to the entorhinal region and an abundance of cortical amyloid in cases of presenile and senile dementia. *Acta Neuropathol* 80: 479-486.

18. Braak H, Braak E, Bohl J and Reintjes R (1996) Age, neurofibrillary changes, A-beta-amyloid and the onset of Alzheimer's disease. *Neurosci Lett* 210: 87-90.

19. Brooks PJ, Funabashi T, Kleopoulos SP, Mobbs CV and Pfaff DW (1992) Prolactin receptor messenger RNA is synthesized by the epithelial cells of the choroid plexus. *Brain Res. Mol Brain Res* 16: 163-167.

20. Burgevin M-C, Passat M, Daniel N, Capet M and Doble A (1994) Congo red protects against toxicity of ß-amyloid peptides on rat hippocampal neurones. *NeuroReport* 5: 2429-2432.

21. Canadian Study of Health and Aging Working Group. Canadian Study of Health and Aging: study methods and prevalence of dementia (1994) *Can Med Assoc J* 150: 899-913.

22. Carroll RT, Lust MR, Kim KS, Doyle PD and Emmerling MR (1995) An age-related correlation between levels of ß-amyloid precursor protein and ß-amyloid in human cerebrospinal fluid. *Biochem Biophys Res Comm* 210: 345-349.

23. Castaño EM and Frangione B (1988) Human amyloidosis, Alzheimer's disease and related disorders. *Lab Invest* 58: 122-132.

24. Castaño EM and Frangione B (1991) Alzheimer's disease from the perspective of the systemic and localized forms of amyloidosis. *Brain Pathol* 1: 263-271.

25. Chanoine JP, Alex S, Fang SL, Stone S, Leonard JL, Korhle J and Braverman LE (1992) Role of transthyretin in the transport of thyroxine from the blood to the choroid plexus, the cerebrospinal fluid, and the brain. *Endocrinol* 130: 933-938.

26. Citron M, Oltersdorf T, Haas C, McConlogue L, Humg AY, Seubert P, Vigo-Pelfrey C, Lieberburg I and Selkoe DJ (1992) Mutation of the ß-amyloid precursor protein in familial Alzheimer's disease increases ß-protein production. *Nature* 360: 672-674.

27. Cohen DH, Feiner H, Jensson O and Frangione B (1983) Amyloid fibril in hereditary cerebral hemorrhage with amyloidosis (HCHWA) is related to the gastroentero-pancreatic neuroendocrine protein , gamma trace. *J Exp Med* 158: 623-628.

28. Cole T, Dickson PW, Esnard F, Averill S, Risbridger G, Gauthier F and Schreiber G (1989) The cDNA structure and expression analysis of the genes for the cysteine protease inhibitor cystatin C and for ß(2)-microglobulin in rat brain. *Eur J Biochem* 186: 35-42.

29. Corder EH, Saunders AM, Risch NJ, Strittmatter WJ, Schmechel DE, Gaskell PC Jr, Rimmler JB, Locke PA, Conneally PM, Schmader KE and Tanzi RE (1994) Apolipoprotein E type 2 allele decreases the risk of late-onset Alzheimer disease. *Nature Genet* 7: 180-184.

30. Corder EH, Saunders AM, Strittmatter WJ, Schmechel DE, Gaskell PC, Small GW, Roses AD, Haines JL and Pericak-Vance MA (1993) Gene dosage of apolipoprotein E type 4 allele and the risk of Alzheimer's disease in late onset families. *Science* 261: 921-923.

31. Coria F, Castaño EM and Fragione B (1987) Brain amyloid in normal aging and cerebral amyloid angiopathy is antigenically related to Alzheimer's disease ß-protein. *Am J Pathol* 129: 422-428.

32. Crystal H, Dickson D, Fuld P, Masur D, Scott R, Mehler M, Masdeu J, Kawas C, Aronson M and Wolfson L (1988) Clinico-pathologic studies in dementia: nondemented subjects with pathologically confirmed Alzheimer's disease. *Neurology* 38: 1682-1687.

33. Cserr HF and Patlak CS. Regulation of brain volume under isosmotic and anisosmotic conditions. In R Gilles et al (Eds.), *Advances in Comparative and Environmental Physiology*, Vol 9, Springer, Berlin/Heidelberg, 1991, pp 61-80.

34. Cutler RWP, Page L, Galicich J and Watters GV (1968) Formation and absorption of cerebrospinal fluid in man. *Brain* 91: 707-720.

35. Das HK, McPherson J, Bruns GA, Karathanasis SK and Breslow JL (1985) Isolation, characterization, and mapping to chromosome 19 of the human apolipoprotein E gene. *J Biol Chem* 260: 6240-6247.

36. Davson H, Welch K and Segal MB *The Physiology and Pathophysiology of the Cerebrospinal Fluid*, Churchill Livingstone, Edinburgh, 1987.

37. Delacourte A and Defossez A (1986) Alzheimer's disease: tau proteins, the promoting factors of microtubule assembly, are major components of paired helical filaments. *J Neurol Sci* 76: 173-186.

38. Delaère P, Duyckaerts C, Masters C, Beyreuther K, Piette F and Hauw J-J (1990) Large amounts of neocortical ßA4 deposits without neuritic plaques or tangles in a psychometrically assessed, non-demented person. *Neurosci Lett* 116: 87-93.

39. del Rio Hortega P (1918) Noticia de un nuevo y facil metodo para la coloracion de la neuroglia y del tejido conjuntivo. *Trab Lab Invest Biol Univ Madrid* 15: 367-378.

40. Dickson PW and Schreiber G (1986) High levels of mRNA for transthyretin (prealbumin) in human choroid plexus. *Neurosci Lett* 66: 311-315.

41. Divry P (1955) De la nature des formations argentophiles des plexus choroideus. *Acta Neurol Psychiatr Belg* 55:282-283

42. Drechsel DN, Hyman AA, Cobb MH and Kirschner MW (1992) Modulation of the dynamic instability of tubulin assembly by the microtubule-associated protein tau. *Mol Biol Cell* 3: 1141-1154.

43. Dunn J Jr. and Kernohan JW (1955). Histologic changes within the choroid plexus of the lateral ventricle: their relation to age. *Proc Mayo Clin* 30:607-615.

44. Eriksson L and Westermark P (1990a) Characterization of intracellular amyloid fibrils in the human choroid plexus epithelial cells. *Acta Neuropathol* 80: 597-603.

45. Eriksson L and Westermark P (1990b) Amyloid inclusions in choroid plexus epithelial cells. A simple autopsy method to rapidly obtain information on the age of an unknown dead person. *Forensic Science Internatl* 48: 97-102.

46. Esiri MM and Wilcock GK (1986) Cerebral amyloid angiopathy in dementia and old age. *J Neurol Neurosurg Psychiatry* 49: 1221-1226.

47. Flament S, Delacourte A, Delaère P, Duyckaerts C and Hauw J-J (1990) Correlation between microscopical changes and tau 64 and 69 biochemical detection in senile dementia of the Alzheimer type. *Acta Neuropathol* 80: 212-215.

48. Fukumoto H, Asami-Odaka A, Suzuki N, Shimada H, Ihara Y and Iwatsubo T (1996) Amyloid ß protein deposition in normal aging has the same characteristics as that in Alzheimer's disease. Predominance of Aß42(43) and association of Aß40 with cored plaques. *Am J Pathol* 148: 259-265.

49. Gee P, Rhodes CH, Fricker LD and Angeletti RH (1993) Expression of neuropeptide processing enzymes and neurosecretory proteins in ependyma and choroid plexus epithelium. *Brain Res* 617: 238-248.

50. Gellerstedt N (1932) Histologiska iakttagelser oever funktionen hos plexus chorioideus. *Svenska Läkartidningen* 29: 1169-1173.

51. Ghiso J, Jensson O and Frangione B (1986) Amyloid fibril in hereditary cerebral hemorrhage with amyloidosis of Icelandic type is a variant of τ-trace basic protein (cystatin C). *Proc Natl Acad Sci USA* 83: 2974-2978.

52. Giannakopoulous P, Hof PR, Savioz A, Guimon J, Antonarkis SE and Bouras C (1996) Early-onset dementias: clinical, neuropathological and genetic characteristics. *Acta Neuropathol* 91: 451-465.

53. Gideon P., Ståhlberg, T.C., Henriksen, O. (1994) Cerebrospinal fluid production and dynamics in normal aging: a MRI phase-mapping study. *Acta Neurol Scand* 89: 362-366.

54. Glenner GG (1980). Amyloid deposits and amyloidosis. *New Engl J Med* 302: 1283-1292.

55. Glenner GG, Henry JH and Fujihara S (1981) Congophilic angiopathy in the pathogenesis of Alzheimer's degeneration. *Ann Pathol* 1: 120-129.

56. Glenner GG and Murphy M.A. (1989) Amyloidosis of the nervous system. *J Neurol Sci* 94: 1-28.

57. Glenner GG and Wong CW (1984a) Alzheimer's disease: initial report of the purification and characterization of a novel cerebrovascular amyloid protein. *Biochem Biophys Res Comm* 120: 885-890.

58. Glenner GG and Wong CW (1984b) Alzheimer's disease and Down's syndrome: sharing of a unique cerebrovascular amyloid fibril protein. *Biochem Biophys Res Commun* 122: 1131-1135.

59. Goate A, Chartier-Harlin M-C, Mullan M, Brown J, Crawford F, Fidani L, Giuffra L, Haynes A, Irving N, James L, Mant R, Newton P, Rooke K, Roques P, Talbot C, Pericak-Vance M, Roses A, Williamson R, Rossor M, Owen M and Hardy J (1991) Segregation of a missense mutation in the amyloid precursor protein gene with familial Alzheimer's disease. *Nature* 349:704-706.

60. Gonatas NK, Anderson W and Evangelista I (1967) The contribution of altered synapses in the senile plaques: An electron microscopic study in Alzheimer's dementia. *J Neuropathol Exp Neurol* 26: 25-39.

61. Goodman DS (1976) Retinol-binding protein, prealbumin and vitamin A transport. *Progr Clin Biol Res* 5: 313-330.

62. Grubb A, Jensson O, Gudmundsson G, Arnason A, Lofberg H and Malm J (1984) Abnormal metabolism of gamma trace alkaline microprotein. The basic defect in hereditary cerebral hemorrhage with amyloidosis. *New Engl J Med* 311: 1547-1549.

63. Gudmundson G, Hallgrímsson J, Jónasson TA and Bjarnason O (1972) Hereditary cerebral hemorrhage with amyloidosis. *Brain* 59: 387-404.

64. Haines A and Katona C (1992) Dementia in old age. *Occasional Paper-Royal College of Gen Practitioners* 58: 62-66.

65. Haltia M, Vitanen M, Sulkava R, Ala-Hurula V, Poyhonen M, Goldfarb L, Brown P, Levy E, Houlden H, Crook R, Goate A, Clark R, Korenblat K, Pandit S, Keller HD, Lilius L, Liu L, Axelman K, Forsell L, Winblad B, Lannfelt L and Hardy J (1994) Chromosome 14-encoded Alzheimer's disease: Genetic and clinicopathological description. *Ann Neurol* 36: 362-367.

66. Hayflick L How and why we age. Ballantine Books, New York, USA, 1st edition, 1994, pp 53-62.

67. Henderson VW, Paganini-Hill A, Emanuel CK, Dunn ME and Buckwalter JG (1994) Estrogen replacement therapy in older women. Comparisons between Alzheimer's disease cases and nondemented control subjects. *Arch Neurol* 51: 896-900.

68. Herbert J, Wilcox JN, Pham KTC, Fremeau RT Jr., Zeviani M, Dwork A, Soprano DR, Makover A, Goodman DS, Zimmerman EA, Roberts JL and Schon EA (1986) Transthyretin: A choroid plexus-specific transport protein in human brain. *Neurol* 36: 900-911.

69. Hilbich C, Kisters-Woike B, Reed J, Masters CL and Beyreuther K (1991) Aggregation and secondary structure of synthetic amyloid ßA4 peptides of Alzheimer's disease. *J Mol Biol* 218:149-163.

70. Hirano A and Zimmerman HM (1962) Alzheimer's neurofibrillary changes. *Arch Neurol* 7: 227-242.

71. Holm NR, Hansen LB, Nilsson C and Gammeltoft S (1994) Gene expression and secretion of insulin-like growth factor-II and insulin-like growth factor binding protein-2 from cultured sheep choroid plexus epithelial cells. *Brain Res. Molecular Brain Res* 21: 67-74.

72. Huang DY, Goedert M, Jakes R, Weisgraber KH, Garner CC, Saunders AM, Pericak-Vance MA, Schmechel DE, Roses AD and Strittmatter WJ (1994) Isoform-specific interactions of apolipoprotein E with the microtubule-associated protein MAP2c: implications for Alzheimer's disease. *Neurosci Lett* 182: 55-58.

73. Ii K, Ito K, Kominami E and Hirano A (1993) Abnormal distribution of cathepsin proteases and endogenous inhibitors (cystatins) in the hippocampus of patients with Alzheimer's disease, parkinsonism-dementia complex in Guam, and senile dementia in the aged. *Virchows Arch A Pathol Anat* 423: 185-194.

74. Ishihara T, Nagasawa T, Yokota T, Gondo T, Takahashi M and Uchino F (1989) Amyloid protein of vessels in leptomeninges, cortices, choroid plexuses, and pituitary glands from patients with systemic amyloidosis. *Hum Pathol* 20: 891-895.

75. Ishii T (1966) Distribution of Alzheimer's neurofibrillary changes in brainstem and hypothalamus of senile dementia. *Acta Neuropathol* 6: 181-187.

76. Iwatsubo T, Odaka A, Suzuki N, Mizusawa H, Nukina N and Ihara Y (1994) Visualization of Aß42(43) and Aß40 in senile plaques with end-specific Aß monoclonals: Evidence that an initially deposited species is Aß42(43). *Neuron* 13: 45-53.

77. Jarvik LF, Ruth V and Matsuyama SS (1980) Organic brain syndrome and aging. A six year follow-up of surviving twins. *Arch Gen Psychiatry* 37: 280-286.

78. Kalaria RN, Premkumar DRD, Pax AB, Cohen DL and Lieberburg I (1996) Production and increased detection of amyloid ß protein and amyloidogenic fragments in brain microvessels, meningeal vessels and choroid plexus in Alzheimer's disease. *Mol Brain Res* 35: 58-68.

79. Kang J, Lemaire H-G, Unterbeck A, Salbaum JM, Masters CL, Grzeschik K-H, Multhaup G, Beyreuther K, MÜller-Hill B (1987) The precursor of Alzheimer's disease amyloid A4 protein resembles a cell-surface receptor. *Nature* 325: 733-736.

80. Katzman R (1986) Alzheimer's disease. *New Engl J Med* 314: 964-973.

81. Katzman R and Kawas C. The epidemiology of dementia and Alzheimer disease. In: Terry RD, Katzman R and Bick KL, eds. *Alzheimer disease*. New York: Raven Press, 1994, pp 105-122.

82. Katzman R and Saitoh T (1991) Advances in Alzheimer's disease. *FASEB J* 5: 278-286.

83. Katzman R, Terry R, DeTeresa R, Brown T, Davies P, Fuld P, Renbing X, Peck A (1988) Clinical, pathological, and neurochemical changes in dementia: a subgroup with preserved mental status and numerous neocortical plaques. *Ann Neurol* 23: 138-144.

84. Kelly JW and Lansbury PT Jr (1994) A chemical approach to elucidate the mechanism of transthyretin and ß-protein amyloid fibril formation. *Amyloid: Int J Exp Clin Invest* 1: 186-205.

85. Khachaturian ZS (1985) Diagnosis of Alzheimer's disease. *Arch Neurol* 42: 1097-1105.

86. Kidd M (1963) Paired helical filaments in electron microscopy of Alzheimer's disease. *Nature* 197: 192-193.

87. Kidd M (1964) Alzheimer's disease - an electron microscopic study. *Brain* 87: 307-321.

88. Kishner DA, Abraham C and Selkoe DJ (1986) X-ray diffraction from intraneuronal paired helical filaments and extraneuronal amyloid fibers in Alzheimer disease indicates cross-ß conformation. *Proc Natl Acad Sci USA* 83: 503-507.

89. Knusel B, Michel PP, Schwaber JS and Hefti F (1990) Selective and nonselective stimulation of central cholinergic and dopaminergic development in vitro by nerve growth factor, epidermal growth factor, insulin and the insulin-like growth factors I and II. *J Neurosci* 10: 558-570.

90. Kosik KS, Duffy LK, Dowling MM, Abraham C, McCluskey A and Selkoe DJ (1984) Microtubule-associated protein 2: Monoclonal antibodies demonstrate the selective incorporation of certain epitopes into Alzheimer neurofibrillary tangles. *Proc Natl Acad Sci USA* 81: 7941-7945.

91. LaDu MJ, Pederson TM, Frail DE, Reardon CA, Getz GS and Falduto MT (1995) Purification of apolipoprotein E attenuates isoform-specific binding to beta-amyloid. *J Biol Chem* 270: 9039-9042.

92. Lai Z, Roos P, Olsson Y, Larsson C and Nyberg F (1992) Characterization of prolactin receptors in human choroid plexus. *Neuroendocrinol* 1992 56: 225-233.

93. Lauterio TJ, Marson L, Daughaday WH and Baile CA (1987) Evidence for the role of insulin-like growth factor II (IGF-II) in the control of food intake. *Physiol Behavior* 40: 755-758.

94. Lee VM-Y, Balin BJ, Otvos L Jr and Trojanowski JQ (1991) A68: a major subunit of paired helical filaments and derivatized forms of normal tau. *Science* 251: 675-678.

95. Leigh PN, Dodson A, Swash M, Brion JP and Anderton BH (1989) Cytoskeletal abnormalities in motor neuron disease. An immunocytochemical study. *Brain* 112: 521-535.

96. Levy E, Carmen MD, Fernandez-Madrid IJ, Power MD, Lieberburg I, Van Duinen SG, Bots GTAM, Luyendijkj W and Frangione B (1990) Mutation of the Alzheimer's disease amyloid gene in hereditary cerebral hemorrhage Dutch type. *Science* 284: 1124-1126.

97. Levy-Lehad E, Wasco W, Poorkaj P, Romano DM, Oshima J, Pettingell WH, Yu C-E, Jondro PD, Schmidt SD, Wang K, Crowley AC, Fu Y-H, Guenette SY, Galas D, Nemens E, Wijsman EM, Bird TD, Schellenberg GD and Tanzi RE (1995a) Candidate gene for the chromosome 1 familial Alzheimer's disease locus. *Science* 269: 973-977.

98. Levy-Lehad E, Wijsman EM, Nemens E, Anderson L, Goddard KAD, Weber JL, Bird TD and Schellenberg GD (1995b) A familial Alzheimer's disease locus on chromosome 1. *Science* 269: 970-973.

99. Lindvall-Axelsson M and Owman C (1990) Actions of sex steroids and corticosteroids on rabbit choroid plexus as shown by changes in transport capacity and rate of cerebrospinal fluid formation. *Neurological Res* 12: 181-186.

100. Lowe J, Mayer RJ and Landon M (1993) Ubiquitin in neurodegenerative diseases. *Brain Pathol* 3: 55-65.

101. Maggio JE and Mantyh PW (1996) Brain amyloid- A physiochemical perspective. *Brain Pathol* 6: 147-162.

102. Mahley RW (1988) Apolipoprotein E: Cholesterol transport protein with expanding role in cell biology. *Science* 240: 622-630.

103. Mandybur TI (1986) Cerebral amyloid angiopathy: The vascular pathology and complications. *J Neuropathol Exp Neurol* 45: 79-90.

104. Mann DMA (1989) Cerebral amyloidosis, aging and Alzheimer's disease: a contribution from studies on Down's syndrome. *Neurobiol Aging* 10: 397-399.

105. Mann DMA and Esiri MM (1988) The site of the earliest lesion of Alzheimer's disease. *New Engl J Med* 318:789-790.

106. Mann DMA, Iwatsubo T, Ihara Y, Cairns NJ, Lantos PL, Bogdanovic N, Lannfelt L, Winblad B, Maat-Schieman MLC and Rossor MN (1996) Predominant deposition of amyloid-$\beta_{42(43)}$ in plaques in cases of Alzheimer's disease and hereditary cerebral hemorrhage associated with mutations in the amyloid precursor protein gene. *Am J Pathol* 148: 1257-1266.

107. Marsh RE, Corey RB and Pauling L (1955) An investigation of the structure of silk fibroin. *Biochem Biophys Acta* 16: 1171-1174.

108. Masters CL, Simms G, Weinman NA, Multhaup G, McDonald BL, Beyreuther K (1985) Amyloid plaque core protein in Alzheimer's disease and Down Syndrome. *Proc Natl Acad Sci USA* 82:4245-4249.

109. Matsumae M, Kikinis R, Mórocz IA, Lorenzo AV, Sándor T, Albert MS, Black PM and Jolesz FA (1996) Age-related changes in intracranial compartment volumes in normal adults assessed by magnetic resonance imaging. *J Neurosurg* 84: 982-991.

110. May C, Kaye JA, Atack JR, Schapiro MB, Friedland RP and Rapoport SI (1990) Cerebrospinal fluid production is reduced in healthy aging. *Neurology* 40: 500-503, 1990.

111. McGeer PL, Kawamata T and Walker DG (1992) Distribution of clusterin in Alzheimer brain tissue. *Brain Res* 579: 337-341.

112. Mirra SS, Hart MN, Terry RD (1993). Making the diagnosis of Alzheimer's disease. A primer for practicing pathologists. *Arch Pathol Lab Med* 117:132-144.

113. Mirra SS, Heyman A, McKeel D, Sumi SM, Crain BJ, Brownlee LM, Vogel SF, Hughes JP, van Belle G, Berg L, and participating CERAD neuropathologists (1991) The consortium to establish a registry for Alzheimer's disease (CERAD). II. Standardization of the neuropathologic assessment of Alzheimer's disease. *Neurol* 41: 470-486.

114. Miyakawa T, Sumiyoshi S, Murayama E and Deshimaru M (1974) Ultrastructure of capillary-like degeneration in senile dementia. *Acta Neuropathol* 29: 229-236.

115. Mori H, Kondo J and Ihara Y (1987) Ubiquitin is a component of paired helical filaments in Alzheimer's disease. *Science* 325: 1641-1644.

116. Morris JC, Storandt M, McKeel DW Jr., Rubin EH, Price JL, Grant EA and Berg L (1996) Cerebral amyloid deposition and diffuse plaques in "normal" aging: Evidence for presymptomatic and very mild Alzheimer's disease. *Neurology* 46: 707-719.

117. Mortimer JA (1990) Epidemiology of dementia: cross-cultural comparisons. *Adv Neurol* 51: 27-33.

118. Motte J and Williams RS (1989) Age-related changes in the density and morphology of plaques and neurofibrillary tangles in Down syndrome brain. *Acta Neuropathol* 77: 535-546.

119. Mullan M, Crawford F, Axelman K, Houlden H, Lilius L, Winblad B and Lannfelt L (1992a). A pathogenic mutation for probable Alzheimer's disease in the APP gene at the N-terminus of beta-amyloid. *Nature Genet* 1: 345-347.

120. Mullan M, Houlden H, Windelspecht M, Fidani L, Lombardi C, Diaz P, Rossor M, Crook R, Hardy J, Duff K and Crawford F (1992b). A locus for early onset Alzheimer's disease on the long arm of chromosome 14 proximal to the alpha 1 antichymotrypsin gene. *Nature Genet* 2: 340-343.

121. Namba Y, Tomonaga M, Kawasaki H, Otomo E, Ikeda K (1991) Apolipoprotein E immunoreactivity in cerebral amyloid deposits and neurofibrillary tangles in Alzheimer's disease and Kuru plaque amyloid in Creutzfeldt-Jakob disease. *Brain Res* 541: 163-166.

122. Nathan BP, Chang K-C, Bellosta S, Brisch E, Ge N, Mahley RW and Pitas RE (1995) The inhibitory effect of apolipoprotein E4 on neurite outgrowth is associated with microtubule depolymerization. *J Biol Chem* 270: 19791-19799.

123. Netsky MG and S Shuangshoti In *The Choroid Plexus in Health and Disease*, University Press of Virginia, 1975, Great Britain, 351 pages.

124. Nilsson C, Lindvall-Axelsson M and Owman C (1992a) Neuroendocrine regulatory mechanisms in the choroid plexus-cerebrospinal fluid system. *Brain Res Reviews* 17: 109-138.

125. Nilsson C, Ståhlberg F, Thomsen C, Henriksen O, Herning M and Owman C (1992b). Circadian variation in human cerebrospinal fluid production measured by magnetic resonance imaging. *Am J Physiol* 262: R20-R24.

126. Nyberg F, Kankanranta S, Brostedt P, Silberring J (1991) Purification and characterization of endoproteases from human choroid plexus cleaving pro-dynorphin-derived opioid peptides. *Brain Res* 552: 129-135.

127. Oda T, Pasinetti GM, Osterburg HH, Anderson C, Johnson SA and Finch CE (1994) Purification and characterization of brain clusterin. *Biochem Biophys Res Commun* 204: 1131-1136.

128. Ogomori K, Kitamoto T, Tateishi J, Sato Y, Suetsugu M and Abe M (1989) ß-protein amyloid is widely distributed in the central nervous system of patients with Alzheimer's disease. *Am J Pathol* 134: 243-251.

129. Ohta M, Kitamoto T, Iwaki T, Ohgamai T, Fukui M and Tateshi J (1993) Immunohistochemical distribution of amyloid precursor protein during normal rat development. *Devel Brain Res* 75: 151-161.

130. Palmert MR, Podlisny MB, Witker DS, Oltersdorf T, Younkin LH, Selkoe DJ, Younkin SG (1988) Antisera to an amino-terminal peptide detect the amyloid protein precursor of Alzheimer's disease and recognize senile plaques. *Biochem Biophys Res Commun* 156:432-437.

131. Pericak-Vance MA, Bebout JL, Gaskell PC Jr, Yamaoka LH, Hung WY, Alberts MJ, Walker AP, Bartlett RJ, Haynes CA and Welsh KA (1991) Linkage studies in familial Alzheimer's disease: evidence for chromosome 19 linkage. *Am J Hum Genet* 48: 1034-1050.

132. Perry G, Friedman R, Shaw G and Chau V (1987) Ubiquitin is detected in neurofibrillary tangles and senile plaque neurites of Alzheimer's disease brains. *Proc Natl Acad Sci USA* 84: 3033-3036.

133. Perry G, Lipphardt S, Mulvihill P, Kancherla M, Mijares M, Gambetti P, Sharma S, Maggiora L, Cornette J, Lobi T and Greenberg B (1988) Amyloid precursor protein is in senile plaques of Alzheimer's disease. *Lancet* ii: 746.

134. Petanceska S, Burke S, Watson SJ and Devi L (1994) Differential distribution of messenger RNAs for cathepsins B, L and S in adult rat brain: an in situ hybridization study. *Neurosci* 59: 729-738.

135. Poirier J, Davignon J, Bouthillier D, S Kogan, Bertrand P and Gauthier S (1993) Apolipoprotein E polymorphism and Alzheimer's disease. *Lancet* 342: 697-699.

136. Polvikoski T, Sulkava R, Haltua M, Kainulainen K, Vuorio A, Verkkoniemi A, Niinistö L, Halonen P and Kontula K (1995) Apolipoprotein E, dementia, and cortical deposition of ß-amyloid protein. *N Engl J Med* 333: 1242-1247.

137. Price JL, Davis PB, Morris JC and White DL (1991) The distribution of tangles, plaques and related immunohistochemical markers in healthy aging and Alzheimer's disease. *Neurobiol Aging* 12: 295-312.

138. Probst A, Brunnschweiler H, Lautenschlager C and Ulrich J (1987) A special type of senile plaque, possibly an initial stage. *Acta Neuropathol* (Berl) 74: 133-141.

139. Rebeck GW, Reiter JS, Strickland DK and Hyman BT (1993) Apolipoprotein E in sporadic Alzheimer's disease: allelic variation and receptor interactions. *Neuron* 11: 575-580.

140. Redlich E (1898) Über miliäre Sklerose der Hirnrinde bei seniler Atrophie. *Jahrbücher für Psychologie und Neurologie* 17: 208-216.

141. Reilly MM and Staunton H (1996) Peripheral nerve amyloidosis. *Brain Pathol* 6: 163-177.

142. Ridley *The Anatomy of the Brain*, London, 1695

143. Roberts GW, Gentleman SM, Lynch A, Murray L, Landon M and Graham DI (1994) ß amyloid protein deposition in the brain after severe head injury: implications for the pathogenesis of Alzheimer's disease. *J Neurol Neurosurg Psychiatry* 57: 419-425.

144. Roher A, Wolfe D, Palutke M and KuKuruga D (1986) Purification, ultrastructure, and chemical analysis of Alzheimer's disease amyloid plaque core protein. *Proc Natl Acad Sci USA* 83: 2662-2666.

145. Rosemuller JM, Eikelenboom P, Stam FC, Beyreuther K and Masters CL (1989) A4 protein in Alzheimer's disease: Primary and secondary cellular events in extracellular amyloid deposition. *J Neuropathol Exp Neurol* 48: 674-691.

146. Rubin RC, Henderson ES, Ommaya AK, Walker MD and Rall DP (1966) The production of cerebrospinal fluid in man and its modification by acetazolamide. *J Neurosurg* 25: 430-436.

147. Rumble B, Retallack R, Hilbich C, Simms G, Multhaupt G, Martins R, Hockey A, Montgomery P, Beyreuther K and Masters CL (1989) Amyloid A4 protein and its precursor in Down's syndrome and Alzheimer disease. *N Engl J Med* 320: 1446-1452.

148. Saunders AM, Strittmatter WJ, Schmechel D, St.George-Hyslop PH, Pericak-Vance MA, Joo SH, Rosi BL, Gusella JF, Crapper-MacLachlan DR, Alberts MJ, Hulette C, Crain B, Goldgaber D and Roses AD (1993) Association of apolipoprotein E allele $\epsilon 4$ with late-onset familial and sporadic Alzheimer's disease. *Neurology* 43: 1467-1472.

149. Schellenberg GD, Bird T, Wijsman E, Orr HT, Anderson L, Nemens E, White JA, Bonnycastle L, Weber JL, Alonso ME, Potter H, Heston LL and Martin GM (1992) Genetic linkage evidence for a familial Alzheimer's disease locus on chromosome 14. *Science* 258: 668-671.

150. Schmechel DE, Saunders AM, Strittmatter WJ, Crain BJ, Hulette CM, Joo SH, Pericak-Vance MA, Goldgaber D and Roses AD (1993) Increased amyloid ß-peptide deposition in cerebral cortex as a consequence of apolipoprotein E genotype in late-onset Alzheimer disease. *Proc Natl Acad Sci* 90: 9649-9653.

151. Schönlein C, Probst A and Huber G (1993) Characterization of protease with the specificity to cleave the secretase-site of ß-APP. *Neurosci Lett* 161: 33-36.

152. Schwartz M, Creasey H, Grady CL, DeLeo JM, Frederickson HA, Cutler NR and Rapoport SI (1985) Computed tomographic analysis of brain morphometrics in 30 healthy men, aged 21 to 81 years. *Ann Neurol* 17: 146-157.

153. Schwarzman AL, Gregori L, Vitek MP, Lyubski S, Strittmatter WJ, Enghilde JJ, Bhasin R, Silverman J, Weisgraber KH, Coyle PK, Zagorski MG, Talafous J, Eisenberg M, Saunders AM, Roses AD, Goldgaber D (1994) Transthyretin sequesters amyloid ß protein and prevents amyloid formation. *Proc Natl Acad Sci* 91: 8368-8372.

154. Segal MB (1993) Extracellular and cerebrospinal fluids. *J Inherit Metab Dis* 16: 617-638.

155. Selkoe DJ (1986) Altered structural proteins in plaques and tangles: What do they tell us about the biology of Alzheimer's disease ? *Neurobiol Aging* 7: 425-432.

156. Selkoe DJ (1989) Biochemistry of altered brain proteins in Alzheimer's disease. *Ann Rev Neurosci* 12:463-490.

157. Selkoe DJ (1993) Physiological production of the beta-amyloid protein and the mechanism of Alzheimer's disease [Review]. *Trends Neurosci* 16: 403-409.

158. Selkoe DJ, Abraham CR, Podlisny MB and Duffy LK (1986) Isolation of low-molecular-weight proteins from amyloid plaque fibers in Alzheimer's disease *J Neurochem* 146: 1820-1834.

159. Sherrington R, Rogaev EI, Liang Y, Rogaeva EA, Levesque G, Ikeda M, Chi H, Lin C, Li G, Holman K, Tsuda T, Mar L, Foncin J-F, Bruni AC, Montes MP, Sorbi S, Rainero I, Pinessi L, Nee L, Chumakov I, Pollen D, Brookes A, Sanseau P, Polinsky RJ, Wasco W, Da Silva HAR, Haines JL, Pericak-Vance MA, Tanzi RE, Roses AD, Fraser PE, Rommens JM and St George-Hyslop PH (1995) Cloning of a gene bearing missense mutations in early-onset familial Alzheimer's disease. *Nature* 375: 754-760.

160. Shirahama T, Skinner M, Westermark P, Rubinow A, Cohen AS, Brun A and Kemper TL (1982) Senile cerebral amyloid. Prealbumin as a common constituent in the neuritic plaque, in the neurofibrillary tangle, and in the microangiopathic lesion. Am J Pathol 1982, 107:41-50.

161. Shuangshoti S and Netsky MG (1970) Human choroid plexus: morphologic and histochemical alterations with age. *Am J Anat* 128:73-96.

162. Simmons LK, May PC, Tomaselli KJ, Rydel RE, Fuson KS, Brigham EF, Wright S, Lieberburg I, Becker GW, Brems DN and LI WY (1994) Secondary structure of amyloid ß peptide correlates with neurotoxic activity *in vitro*. *Mol Pharmacol* 45 373-379.

163. Singer C, *Galen on Anatomical Procedures*, Oxford University Press, New York, 1956, 289 pages.

164. Smith JS and Kiloh LG (1981) The investigation of dementia: results in 200 consecutive admissions. *Lancet* 1: 824-827.

165. Snow AD and Wight TN (1989) Proteoglycans in the pathogenesis of Alzheimer's disease and other amyloidoses. *Neurobiol Aging* 10: 481-497.

166. Steardo L and Nathanson JA (1987) Brain barrier tissues: End organs for atriopeptins. *Science* 235: 470-473.

167. St George-Hyslop PH, Haines J, Rogaev EI, Mortilla M, Vaula G, Pericak-Vance M, Focin JF, Montesi M, Bruni A, Sorbi S, Rainero I, Pinessi L, Pollen D, Polinsky R, Nee L, Kennedey J, Macciardi F, Rogaeva E, Liang Y, Alexandrova N, Luqiw W, Schlumpf K, Tanzi R, Tsuda T, Farrer L, Kantu J-M, Duara R, Amaducci L, Bergamini L, Gusella J, Roses A and Crapper-Mclachlan D (1992) Genetic evidence for a novel familial Alzheimer's disease locus on chromosome 14. *Nature Genet* 2: 330-334.

168. Strittmatter WJ, Saunders AM, Schmechel D, Pericak-Vance M, Enghild J, Salvesen GS and Roses AD (1993) Apolipoprotein E: high-avidity binding to beta-amyloid and increased frequency of type 4 allele in late-onset familial Alzheimer disease. *Proc Natl Acad Sci USA* 90: 1977-1981.

169. Strittmatter WJ, Weisgraber KH, Goedert, Saunders AM, Huang D, Corder EH, Dong LM, Jakes R, Alberts MJ, Gilbert JR, Han S-H, Hulette C, Einstein G, Schmechel DE, Pericak-Vance MA and Roses AD (1994) Microtubule instability and paired helical filament formation in the Alzheimer disease brain as a function of apolipoprotein E genotype. *Exp Neurol* 125: 163-171.

170. Tabaton M, Mandybur TI, Perry G, Onorato M, Autilio-Gambetti L and Gambetti P (1989) The widespread alteration of neurites in Alzheimer's disease may be unrelated to amyloid deposition. *Ann Neurol* 26:771-778.

171. Tagliavini F, Giaccone G, Frangione B, Bugiani O (1988) Preamyloid deposits in the cerebral cortex of patients with Alzheimer's disease and nondemented individuals. Neurosci Letters 93:191-196.

172. Takeda S and Matsuzawa T (1984) Brain atrophy during aging: a quantitative study using computed tomography. *J Am Geriatr Soc* 32: 520-524.

173. Tang M-X, Jacobs D, Stern Y, Marder K, Schofield P, Gurland B, Andrews H and Mayeux R (1996) Effect of oestrogen during menopause on risk and age at onset of Alzheimer's disease. *Lancet* 348: 429-32.

174. Tanna NK, Kohn MI, Horwich BA, Jolles PR, Zimmerman RA, Alves WM and Abass A (1991) Analysis of brain and cerebrospinal fluid volumes with MR imaging: Impact on PET data correction for atrophy. Part II. Aging and Alzheimer dementia. *Radiol* 178: 123-130.

175. Terry RD, Hansen LA, DeTeresa R, Davies P, Tobias H, Katzman R (1987) Senile dementia of Alzheimer type without neocortical neurofibrillary tangles. *J Neuropathol Exp Neurol* 46: 262-268.

176. Terry RD and Katzman R (1983) Senile dementia of the Alzheimer type. *Ann Neurol* 14: 497-506.

177. Tomlinson BE, Blessed G and Roth M (1968) Observations on the brains of non-demented old people. *J Neurol Sci* 7:331-356

178. Tomlinson BE, Blessed G and Roth M (1970) Observations on the brains of non-demented old people. *J Neurol Sci* 7: 331-356.

179. Tomonaga M (1981) Cerebral amyloid angiopathy in the elderly. *J Am Geriatr Soc* 29: 151-157.

180. Troncoso JC, Martin LJ, Dalforno G and Kawas CH (1996) Neuropathology in controls and demented subjects from the Baltimore longitudinal study of aging. *Neurobiol Aging* 17: 365-371.

181. Uchihara T, Duyckaerts C, Lazarini F, Mohtari K, Seilhean D, Amouyel P, Hauw J-J (1996) Inconstant apolipoprotein E (ApoE)-like immunoreactivity in amyloid ß protein deposits: relationship with APOE genotype in aging brain and Alzheimer's disease. *Acta Neuropathol* 92: 180-185.

182. Van Broeckhoven C, Backhovens H, Cruts M, De Winter G, Bruyland M, Cras P and Martin JJ (1992) Mapping of a gene predisposing to early-onset Alzheimer's disease to chromosome 14q24.3. *Nature Genet* 2: 335-339.

183. Van Duinen SG, Castaño EM, Prelli F, Bots GTAM, Luyendijik W and Frangione B (1987) Hereditary cerebral hemorrhage with amyloidosis in patients of Dutch origin is related to Alzheimer disease. *Proc Natl Acad Sci USA* 84: 5991-5994.

184. Vinters HV (1987) Cerebral amyloid angiopathy: a critical review. *Stroke* 18: 311-324.

185. Vinters HV and Gilbert JJ (1983) Cerebral amyloid angiopathy: incidence and distribution in the aging brain. II. The distribution of vascular changes. *Stroke* 14: 924-928.

186. Vinters HV, Wang ZZ and Secor DL (1996) Brain parenchymal and microvascular amyloid in Alzheimer's disease. *Brain Pathol* 6: 179-195.

187. Voetmann E (1949) On the structure and surface area of the human choroid plexuses: a quantitative anatomical study. *Acta Anat* 8: (Suppl 10): 1-116.

188. Watson MA and Scott MG (1995) Clinical utility of biochemical analysis of cerebrospinal fluid. *Clin Chem* 41: 343-360.

189. Wen GY, Rudelli RD, Kim KS and Wisniewski HM (1988) Tangles of ependyma-choroid plexus contain ß-amyloid protein epitopes and represent a new form of amyloid fiber. *Arch Neurol* 45: 1298-1299.

190. Whalley LJ (1987) The dementia of Down's syndrome and its relevance to etiological studies of Alzheimer's disease. *Ann NY Acad Sci* 396: 39-53.

191. Willis T, *Cerebri Anatome*, Flésher, London, 1664.

192. Wisniewski HM, Iqbal K, Bancher C, Miller D and Currie J (1989) Cytoskeletal protein pathology and the formation of beta-amyloid fibers in Alzheimer's disease. *Neurobiol Aging* 10: 409-412.

193. Wisniewski HM and Terry RD (1973) Reexamination of the pathogenesis of the senile plaque. *Prog Neuropathol* 2: 1-26.

194. Wisniewski HM and Terry RD (1976) Neurobiology of the ageing brain. *In* Terry RD, Gershon S (eds): Neurobiology of Ageing. New York, Raven Press, 1976, pp 256-280.

195. Wisniewski T, Castaño EM, Golabek A, Vogel T and Frangione B (1994) Acceleration of Alzheimer's fibril formation by apolipoprotein E in vitro. *Am J Pathol* 145: 1030-1035.

196. Wisniewski T, Ghiso J and Frangione B (1994) Alzheimer's disease and soluble Aß. *Neurobiol Aging* 15: 143-152.

197. Wisniewski T, Golabek A, Matsubara E, Ghiso J and Frangione B (1993) Apolipoprotein E: binding to soluble Alzheimer's beta-amyloid. *Biochem Biophys Res Commun* 192: 359-365.

198. Wisniewski T, Lalowski M, Golabek A, Vogel T and Frangione B (1995) Is Alzheimer's disease an apolipoprotein E amyloidosis? *Lancet* 345: 956-958.

199. Wood JG (1982) Neuroendocrinology of cerebrospinal fluid: peptides, steroids and other hormones. *Neurosurgery* 11: 293-305.

200. Wright JR, Calkins E, Breen WJ, Stolte G and Schultz RT (1969) Relationship of amyloid to aging, review of the literature and systematic study of 83 patients derived from a general hospital population. *Medicine* 48: 39-60.

201. Yamaguchi H, Hirai S, Morimatsu M, Shoji M and Harigaya Y (1988a) Diffuse type of senile plaques in the brains of Alzheimer-type dementia. *Acta Neuropathol* 77: 113-119.

202. Yamaguchi H, Hirai S, Morimatsu M, Shoji M and Ihara Y (1988b) A variety of cerebral amyloid deposits in the brains of the Alzheimer-type dementia demonstrated by ß protein immunostaining. *Acta Neuropathol* 76: 541-549.

203. Yamamoto M, McCaffery P and Drager UC (1996) Influence of the choroid plexus on cerebellar development- analysis of retinoic acid synthesis. *Brain Res* 93: 182-190.

204. Yankner BA, Duffy LK and Krischner DA (1990) Neurotrophic and neurotoxic effects of amyloid ß protein: Reversal by tachykinin neuropeptides. *Science* 250: 279-282.

205. Zhang M, Katzman R, Jin H, Cai G, Wang Z, Qu G, Grant I, Yu E, Levy P and Liu WT (1990) The prevalence of dementia and Alzheimer's disease (AD) in Shanghai, China: impact of age, gender and education. *Ann Neurol* 27: 428-437.

206. Zheng W, Perry DF, Nelson DL and Aposhian HV (1991) Choroid plexus protects cerebrospinal fluid against toxic metals. *FASEB J* 5: 2188-2193.

207. Esler WP, Stimson ER, Ghilardi JR, Felix AM, Lu Y-A, Vinters HV, Mantyh PW and Maggio JE (1997) Aß deposition inhibitor screen using synthetic amyloid. *Nature Biotech* 15: 258-263.

208. Games D, Adams C, Alessandrini R, Barbour R, Berthelette P, Blackwell C, Carr T, Clemens J, Donaldson T, Gillespie F, Guido T, Hagopian S, Johnson-Wood K, Kahn K, Lee M, Leibowitz P, Leiberburg I, Little S, Masliah E, McConlogue L, Montaya-Zavala M, Mucke L, Paganini L, Pinniman E, Power M, Schenk D, Seubert P, Snyder B, Soriano F, Tan H, Vitale J, Wadsworth S, Wolozin B and Zhao J (1995) Alzheimer-type neuropathology in transgenic mice overexpressing V717F ß-amyloid precursor protein. *Nature* 373: 523-527.

209. Nalbantoglu J, Trado-Santiago G, Lahsaini A, Poirier J, Goncalves O, Verge G, Momoli F, Welner SA, Massicotte G, Julien J-P and Shapiro ML (1997) Impaired learning and LTP in mice expressing the carboxy terminus of the Alzheimer amyloid precursor protein. *Nature* 387: 500-505.

210 Thomas T, Thomas G, McLendon C, Sutton T and Mullan M (1996) ß-amyloid-mediated vasoactivity and vascular endothelial damage. *Nature* 380: 168-171.

211. Yankner BA (1996) Mechanisms of neuronal degeneration in Alzheimer's disease. *Neuron* 16 921-932.

PREFACE TO CHAPTER 2

In this chapter, two main questions are addressed. First, can the amyloid-ß (Aß) protein be found in the choroid plexus of normal and AD subjects, and does it form amyloid in this tissue ? Second, can Aß be found in the CSF of these individuals ? We address these issues using two complimentary approaches. First, immunohistochemistry is used to characterize the CP amyloid. This is followed by biochemical analysis of the amyloid, using reverse-phase high-performance liquid chromatography and Western blot analysis.

CHAPTER 2.

Deposition of an amyloid containing apolipoprotein E occurs in the choroid plexus of the human brain with age.

S. Kunicki, J. Richardson and E. Zorychta

2.1 ABSTRACT

Amyloid accumulates with age in the cerebral cortex and in the choroid plexus, a tissue in the cerebral ventricles which regulates the composition of cerebrospinal fluid. Cortical amyloid deposits, which are more numerous in Alzheimer's disease, are composed mainly of amyloid-ß, and sometimes also contain apolipoproteins E and J, and transthyretin. These four proteins are also present in cerebrospinal fluid. To investigate a possible relationship between cortical and choroid plexus amyloid, known as Biondi rings, we analyzed choroid plexuses obtained at autopsy from 37 non-demented subjects aged 27-95 years, and from seven Alzheimer's disease patients, aged 62-94 years. Ring-like structures were identified with increasing frequency with age by a monoclonal antibody against apolipoprotein E and by the polyclonal serum AA95, which labels Biondi ring amyloid. Such structures were occasionally labelled by antibodies against apolipoprotein J and amyloid-ß. Western blot analysis revealed that AA95 and anti-apolipoprotein E antibodies labelled recombinant apolipoprotein E, as well as 34 kD and 45-80 kD proteins from the choroid plexus and cerebrospinal fluid. These results demonstrate that the choroid plexus contains proteins found in cortical amyloid and that with age, one of these proteins, apolipoprotein E, may by itself, or in association with another protein, form Biondi ring amyloid.

2.2 INTRODUCTION

As humans age, localized depositions of amyloid occur within the brain, both in the cerebral cortex (1) and in the choroid plexus (CP) (2,3), a tissue in the cerebral ventricles which produces most of the cerebrospinal fluid (CSF) and regulates its composition (4). Cortical amyloid accumulates in plaques and around vessels, and individuals with Alzheimer's disease (AD) are characterized by larger quantities of cerebral amyloid than their age-matched controls (5). This amyloid is composed mainly of a ß-pleated amyloid ß (Aß) peptide (6,7), but a number of associated proteins, including apolipoprotein (apo) E (8), apo J (9) and transthyretin (TTR) (10), can often be found in the amyloid deposits. These amyloid-associated proteins are also present in the CSF (11,12,13) and blood (14,15,16) where they are thought to bind soluble Aß. The contribution of these amyloid-associated proteins to the formation of amyloid is unclear. It has been proposed that apoE may be a pathological chaperone protein which binds to Aß and promotes ß-pleating of soluble Aß peptide into insoluble amyloid (17). The role of apo J (9) and TTR (10) in Aß amyloid plaques is not known, but both of these proteins have been shown to inhibit amyloid formation by Aß in vitro (13,18).

Much less is known about the amyloid in the CP. Rod and ring-like inclusions, which were first observed in the cytoplasm of the CP epithelial cells of aged humans using silver staining (19,20), were later found to have the staining properties of amyloid (2). These sites of amyloid deposition, known

as Biondi rings, are named after one of the first investigators to study their histological features (20). Ultrastructurally, Biondi rings consist of an amorphous core of lipid-like appearance 2-7 μm in diameter covered with a thin layer of amyloid fibrils (21). It has been proposed that the amyloid fibrils are bound to either residual bodies (22) or cytoplasmic vacuoles (3), which may explain the circular appearance of many of the amyloid inclusions. Immunohistochemical studies suggest that Biondi rings may contain Aß epitopes, but not tau, paired helical filaments, keratin, vimentin, desmin, actin, glial fibrillary acidic protein or ubiquitin epitopes (21,23). The amyloid has been isolated from human CP, but the protein sequence could not be determined (21). Characterization of the amyloid in the CP would be an important first step toward understanding how amyloid accumulates in the aging CP, and whether its accumulation is related to age-associated changes in CP function.

The well-documented exchange of proteins between the cortex, CSF and CP (4,24), together with the age-associated accumulation of amyloid in the cortex and CP (1-3), led us to postulate a relationship between the amyloid in these two locations. We have previously shown that Aß can be isolated from the CP (25). In the present report, immunohistochemical techniques and Western blot analysis were used to investigate the possibility that Biondi ring amyloid may be composed of the same proteins found in cortical amyloid deposits, including Aß. In addition, we tested the possibility that the CP-

derived cystatin C (26), which forms amyloid in hereditary cerebral hemorrhage with amyloidosis of the Icelandic type (27), may form amyloid in the CP. In this paper we present evidence that Biondi rings contain apoE, and that apo J and Aß, but not TTR or cystatin C, may sometimes associate with these structures.

2.3 MATERIALS AND METHODS

2.3.1 Tissue

CP and CSF from the lateral ventricles of 37 normal subjects and CP from seven AD patients were obtained at autopsy performed within a mean postmortem period of 12 (5-25) hours. Cortical sections were obtained from 13 normal subjects and from five of the AD cases. Control subjects had no clinical history of neurological disorders and postmortem examination of the brains by a neuropathologist revealed no anatomical abnormalities or atypical histology. Diagnosis of AD was made by clinical history and confirmed by postmortem quantification of cortical plaques and tangles using the CERAD criteria (5). Although tissue was obtained from a random sampling of autopsies performed within a limited time postmortem, most control subjects were male and most AD subjects were female. It was not possible to reduce the number of male control or female AD subjects as this would have resulted in a sample size that was too small for analysis. A summary of the cases is provided in Table 2.1.

2.3.2 Histochemistry

Tissues were fixed in 10% formalin and embedded in paraffin. Nine serial sections of each CP were stained with Congo red to identify amyloid, or incubated with an antibody and processed for immunohistochemistry. The eight antibodies used were AA95, a polyclonal antibody against CP amyloid (1:370), rabbit polyclonal antibodies against TTR (Boehringer Mannheim, 1:200) and cystatin C (Dako, 1:200) and monoclonal antibodies against human apoE (Boehringer Mannheim, 14 μg/ml), apo J (Quidel, 1:32), and three different regions of Aß. The AA95 serum, a gift from Dr. P. Westermark, was obtained from rabbits injected with amyloid pooled from four human CP (21). The anti-Aß antibodies were against amino acids 1-15 (6E10, 1:50), 17-24 (4G8, 1:250) and 8-17 (Dako m872, 1:50) of the Aß peptide. Antibodies 6E10 and 4G8 were gifts from Dr. K.S. Kim. Serial sections of frontal or temporal cortexes were incubated with AA95 or anti-apoE antibodies in order to ascertain if the protein labelled by AA95 is present in the cortex and to compare its distribution to that of apoE.

To enhance immunoreactivity, tissues were immersed in 90% formic acid for three to six hours before exposure to anti-Aß antibodies, and for three hours prior to staining with all other antibodies. Immunohistochemical staining was performed as per manufacturers instructions using an Omnitags avidin/biotin kit, with 3-amino-9-ethylcarbazole as the chromogen (Immunon, Fisher), which produces a red reaction product. Sections were counterstained

with hematoxylin. As a negative control, all antibodies, with the exception of AA95, were preabsorbed with their respective antigens. In addition, control sections, which were prepared for each antibody, were incubated with the secondary but not the primary, antibody. Each slide was examined without prior knowledge of case history, by two of the investigators.

For immunoabsorption analysis, 70 μl (50 μg) of recombinant apoE, isoform 3, was added to 300 μl AA95 solution (1:370) and left at 4°C for 48 hours. Immunohistochemistry of the CP was then carried out using this suspension as the primary antibody.

2.3.3 Biochemical Analysis

The protocol of Glenner and Wong (6), which was developed to isolate amyloid from cerebral vessels, was used as the initial step in isolation of protein from the CP. The resulting protein pellet was solubilized in 90% formic acid then an equal volume of 0.1M tris-buffered saline was added to the solution. Samples were purified on a superose 12 (prep grade) column (Pharmacia) using a buffer of 70% formic acid, 1% acetonitrile and a flow rate of 0.5 ml/minute. The superose column peaks were each run on a Vydac C4 reverse-phase high performance liquid chromatography (RP-HPLC) column, using buffers previously described (28) at a flow rate of 1 ml/minute. Samples of CSF were also chromatographed. The 3 to 4 major peaks which eluted from the RP-HPLC column between 30 and 40 minutes, were each lyophilized,

run on a 15% SDS-polyacrylamide gel (29), then electrophoretically transferred to a polyvinylidene fluoride membrane (Immobilon-P^{SQ}, Millipore Corp.) using standard protocol (30). Membranes were blocked in 5% skimmed milk in Tris-buffered saline, incubated in primary antibody overnight at 4°C, washed, then incubated in secondary antibodies for 1 hour at room temperature. The primary antibodies were mouse monoclonals m872 (1:100), anti-apoE (2 μg/ml) and anti-apo J (1:100) and rabbit polyclonal AA95 (1:100). Alkaline phosphatase labelled goat anti-mouse and anti-rabbit IgG (Sigma) at a dilution of 1:4000 were the secondary antibodies and immunoblots were developed using 5-bromo-4-chloro-3-indoyl-phosphate and nitroblue tetrazolium tablets (Sigma). Synthetic Aβ_{1-40} (Research Biochemicals Incorporated), recombinant human apoE (Calbiochem), apo J (Quidel Corporation), TTR (Sigma), cystatin (Sigma) and tubulin (ICN) were purchased commercially and used as standards in Western blot analysis.

2.3.4 Statistics

To analyze changes in the CP with age, logistic regression analysis was performed using SAS version 6.07, with age entered as a continuous variable. A two-tailed Fisher's Exact Test was used to test for correlations between the presence of amyloid, AA95, apoE, apo J and Aß immunoreactivities. A p value of 0.05 or less was considered significant.

2.4 RESULTS

2.4.1 Congo Red Staining and Immunoreactivity in Control CP

The presence of amyloid, as detected by Congo red staining, was first observed in a 49-year-old and increased significantly with age ($p = 0.003$). Rod- and ring-like amyloid deposits were observed in the CP epithelium (Figure 2.1) from all subjects 64 years and older, with the exception of one 73-year-old. In total, amyloid was detected in 19 of the 37 cases. Vessels of the CP were never found to have amyloid angiopathy.

The polyclonal serum AA95, which was raised against amyloid purified from human CP and has been shown to label rod- and ring-like amyloid inclusions (21), identified such structures in 30 CP. The remaining seven CP did not demonstrate any form of AA95 immunoreactivity.

Epitopes of apoE and apoJ were frequently observed in the CP epithelial cells, either diffusely throughout the cell, or in association with structures resembling Biondi rings. However, antibodies against apoE and apoJ did not label ring-like structures as consistently as AA95 did (Figure 2.2) and in contrast to AA95, they sometimes produced only diffuse cytoplasmic staining. Structures resembling Biondi rings were labelled in 26 of the 29 CP that were apoE-positive, and in only 13 of the 28 CP that were apoJ-positive.

Similar to the results with Congo red staining, the percent of individuals with Biondi rings detected by AA95-immunoreactivity in the CP increased significantly with age ($p = 0.008$). However, AA95 reactivity was detected

earlier and more frequently than Congo red-positive staining (Figure 2.3), being first detected at age 45. CP from subjects 49 years and older, with the exception of one 54 and one 62 year-old, all demonstrated AA95 immunoreactivity. Interestingly, AA95 immunoreactivity was observed not only in all CP which contained amyloid, but was also seen in 61% of CP which did not yet contain amyloid. The presence of AA95 immunoreactivity in the absence of detectable amyloid was more frequently observed in young subjects (Figure 2.3).

The percent of CP with anti-apoE immunoreactivity increased slightly with age ($p = 0.06$), while anti-apoJ immunoreactivity remained unchanged (Figure 2.3). A positive correlation was observed between the presence of amyloid and immunoreactivity for AA95 ($p = 0.003$) and apoE ($p = 0.019$), but not for apoJ. The ability of AA95 to identify Biondi rings was lost when AA95 was incubated with recombinant apoE prior to immunostaining (Figure 2.2). In comparison, preabsorption of AA95 with apoJ did not eliminate staining of Biondi rings by AA95.

In only two cases was Aß immunoreactivity found to associate with ring-like structures. In these cases, Biondi rings could be identified by Congo red, AA95, and antibodies against apoE and apoJ. Aß has recently been shown to be produced by human CP (31), but it was observed in only 16 of the 37 CP examined, despite the use of three different anti-Aß antibodies to maximize the probability of detection. The ability to detect Aß did not vary

between the antibodies since Aß was identified in the same 16 CP by all of them. The percent of CP with Aß immunoreactivity did not change with age (Figure 2.3). In comparison, immunoreactivity for TTR and cystatin, which are also produced by the CP epithelium (26,32), was present in every case examined and in the majority of epithelial cells in each section. Antibodies against TTR and cystatin never stained any identifiable structures, but instead produced diffuse staining throughout the cytoplasm (Figure 2.2).

2.4.2 Congo Red Staining and Immunoreactivity in CP from AD subjects

The CP from the seven AD cases all contained amyloid and exhibited AA95, apoE, cystatin C and TTR immunoreactivity. Aß and apoJ immunoreactivity was observed in three and six of these cases respectively. As in normal subjects, only anti-apoE antibodies stained the vessels of the CP and reactivity was confined to the tunica adventitia. The three cases which demonstrated apoE epitopes around blood vessels also possessed immunoreactivity in the epithelium. The Congo red and immunoreactivity profile of AD cases (mean age of 81.7 years) was comparable to that seen in the group of normal individuals over age 75 (mean age 85.8 years).

2.4.3 Biochemical Analysis

Aß and apoE, but not apoJ, were isolated from the CP by the techniques employed. In most normal cases tested, only a faint band of 4 kD Aß-

immunopositive protein was visible, indicating only a negligible amount of Aß was isolated. However, in some cases a much darker 4 kD immunopositive band was observed (Figure 2.4a). ApoE, which could be consistently isolated from the CP, was present in both a 34 kD and 45-80 kD protein bands (Figure 2.4b).

AA95 labelled apoE-immunopositive protein bands of approximately 45 to 80 kD from the CP and CSF of normal subjects. In addition, it labelled protein of 34 kD from the CSF of normal patients and the 34 kD recombinant human apoE (Figure 2.4c). It did not react with the commercially-obtained synthetic $Aß_{1-40}$, apoJ, TTR, cystatin or tubulin.

2.4.4 Immunoreactivity in the Cortex

The study of cortices from normal subjects aged 14 to 72 years, revealed that both AA95 and anti-apoE immunoreactivity could be detected in astrocytes, neurons, corpora amylacea and around vessels, particularly in older individuals (Figure 2.5). As in the CP experiments, some differences in immunoreactivity between AA95 and apoE were observed, with anti-apoE antibodies demonstrating greater reactivity (Table 2.2).

Although statistical analysis was not possible due to the small number of AD cases, both apoE and AA95 immunoreactivity were more prevalent in AD than in control brains.

Figure 2.1 Amyloid and AA95 immunoreactivity in the CP from a normal 57 year old stained with Congo red dye to identify amyloid (A), and with the AA95 serum, which was raised against CP amyloid and labels Biondi rings (B). Congo red-positive amyloid inclusions are indicated by arrows. Immunopositive protein is visualized as red 3-amino-9-ethylcarbazole reaction products in the section counterstained with hematoxylin. Original magnification is x400 and x800 for the inserts.

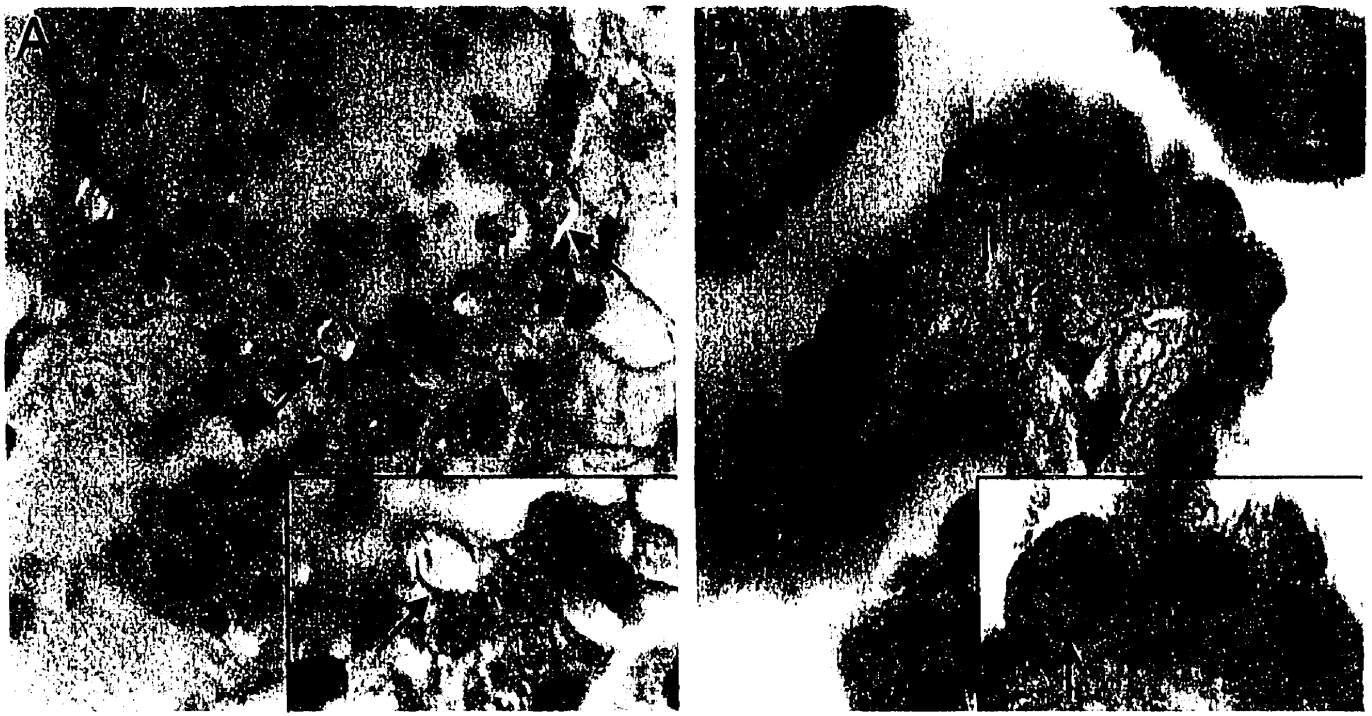

A

Figure 2.2 Immunoreactivity in the CP of a normal 69 year old stained with antibodies against apoE (A,B) and apoJ (C). ApoE immunoreactivity around vessels was confined to the adventitia in all cases. Diffuse staining was observed with antibodies against transthyretin (D) and amino acids 8-17 of the Aß peptide (E) in a 53 year old subject. AA95 adsorbed with apoE results in a loss of staining of ring-like structures (F). Immunopositive protein is visualized as red reaction products. Sections were counterstained with hematoxylin. Original magnification is x800.

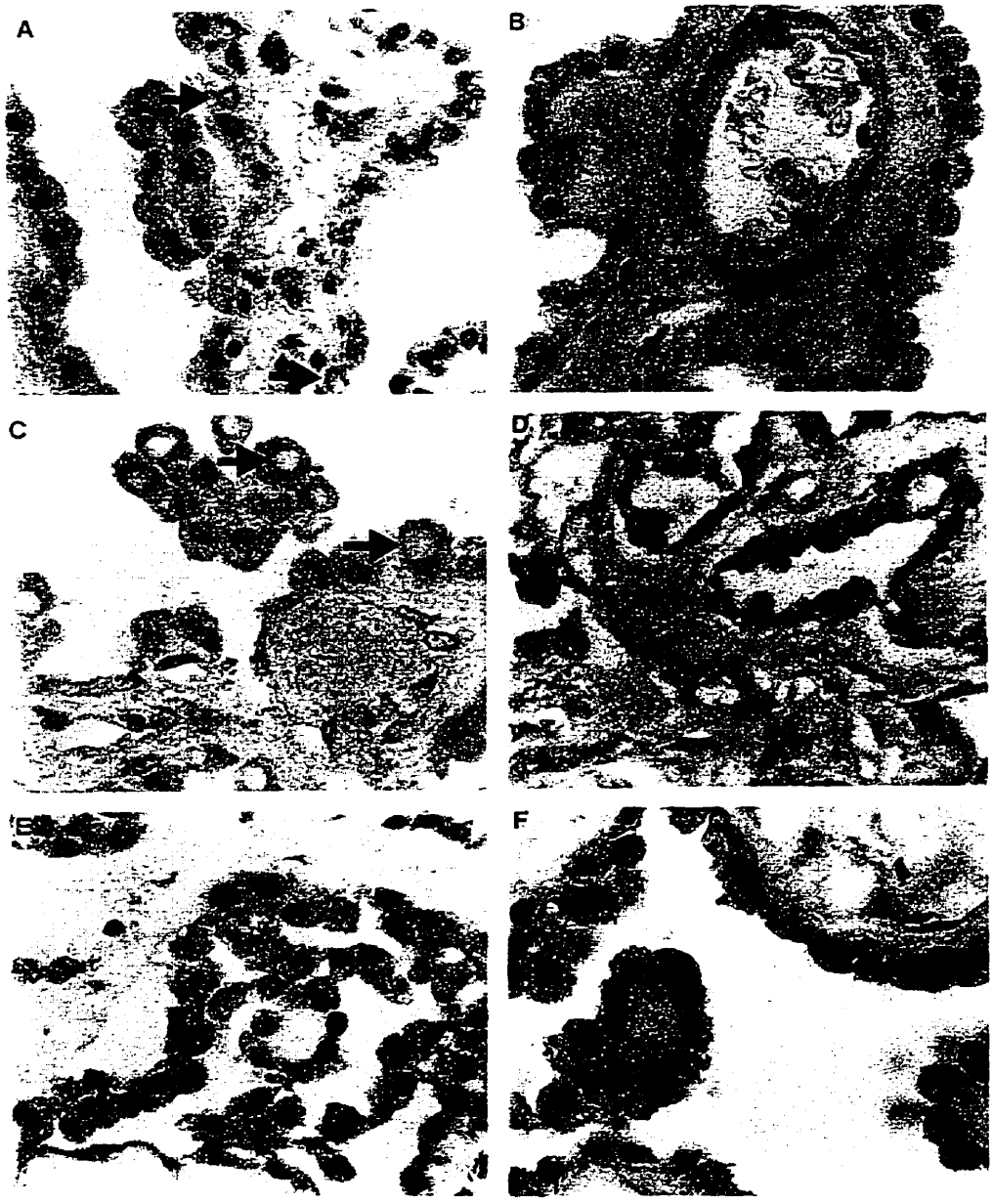

A
B
C
D
E
F

Figure 2.3 Changes in the choroid plexus with age. The accumulation of amyloid, as detected by Congo red staining, is compared with the presence of AA95, anti-apoE, anti-apoJ and anti-Aß immunoreactivity in serial sections of 37 CP from non-demented subjects. Logistic regression analysis revealed a significant increase with age in the presence of amyloid ($p = 0.003$) and AA95 ($p = 0.008$) immunoreactivity and a slight increase in apoE ($p = 0.060$) immunoreactivity that did not reach significance.

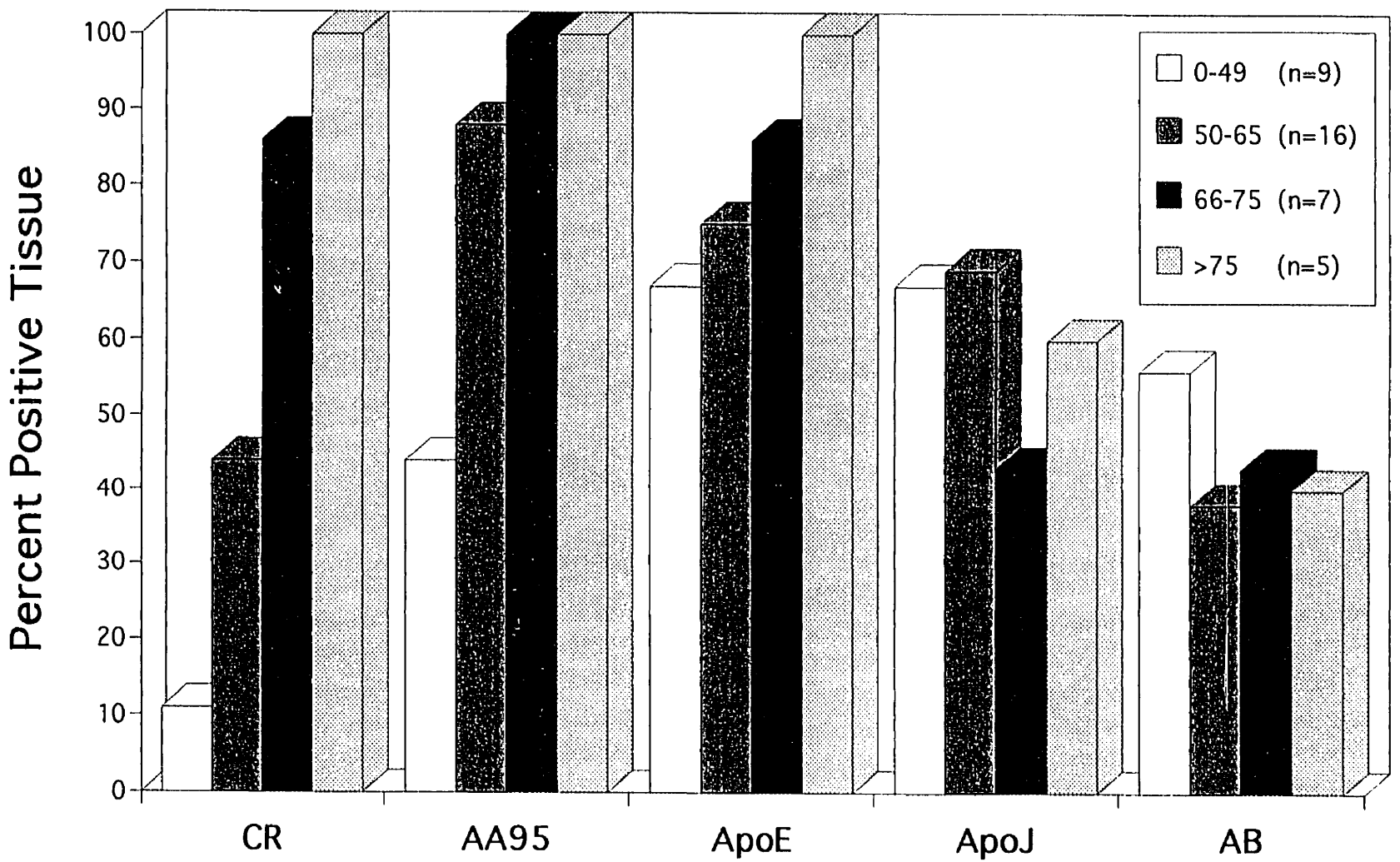
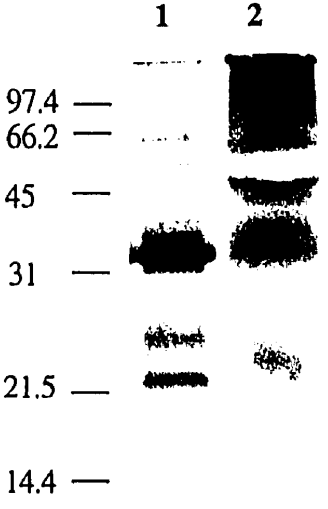

Percent Positive Tissue
100
90
80
70
60
50
40
30
20
10
0
0-49 (n=9)
50-65 (n=16)
66-75 (n=7)
>75 (n=5)
CR
AA95
ApoE
ApoJ
AB

Figure 2.4 Isolation and Western blot analysis of Aß, apoE and Biondi ring protein. A: immunoblot of five micrograms of synthetic $Aß_{1-40}$ (*lane 1*) and protein isolated from the CP of a normal 45 year old (*lane 2*) reacted with an antibody against amino acids 8-17 of Aß. B: five micrograms of recombinant apoE (*lane 1*) and protein from the CP of a normal 66 year old (*lane 2*) reacted with anti-apoE antibody. The low molecular weight bands represent either incompletely synthesized fragments of recombinant apoE or a degradation product of apoE (47). C: samples probed with polyclonal antibody AA95. Five micrograms of recombinant apoE (*lane 1*), protein from the CP of a normal 46 year old (*lane 2*) and CSF from a normal 35 year old (*lane 3*). The migration positions of the molecular weight markers are indicated on the left in kilodaltons.

A

B



C

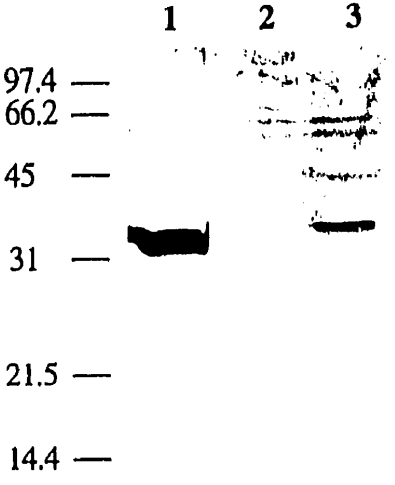
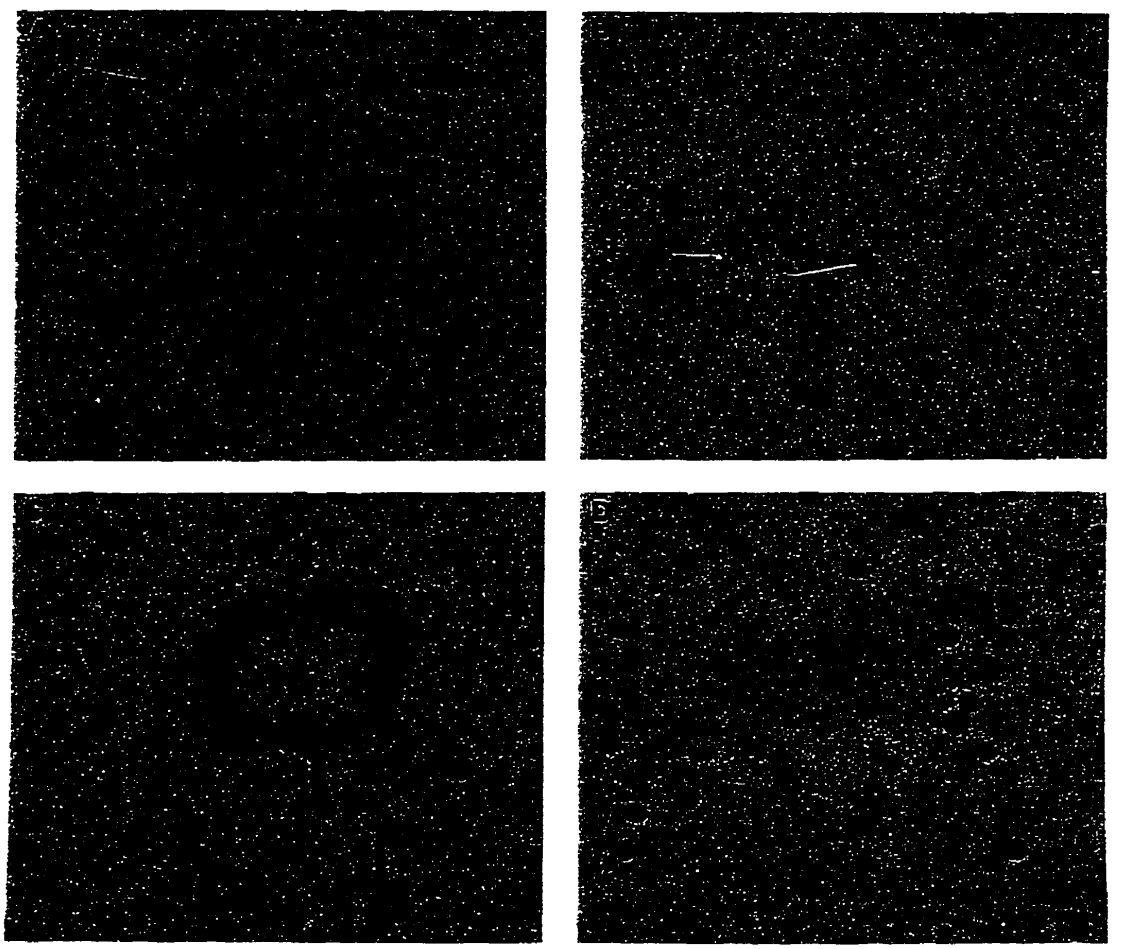


Figure 2.5 AA95 immunoreactivity in the temporal cortex. AA95 immunoreactivity was frequently observed in nondemented aged subjects and also in AD patients. AA95 immunoreactivity in astrocytes (A) and around vessels (C) in the cortex of a normal 61 year old. Diffuse cytoplasmic staining in neurons (B) and in corpora amylacea (D) in the cortex from an 89 year old AD subject. Immunopositive protein is visualized as red reaction products. Sections were counterstained with hematoxylin. Original magnification is x800.



	Mean Age	Females (age)	Males (age)
Control (years)			
0-49	38.7 ± 8.9	3 (27-46)	6 (25-49)
50-65	57.4 ± 4.7	3 (50-65)	13 (52-64)
66-75	70.1 ± 3.3	1 (75)	6 (66-73)
>75	85.8 ± 6.7	2 (89,95)	3 (77-84)
Alzheimer's disease	81.7 ± 10.2	6 (62-94)	1 (83)

Table 2.1 Summary of cases. The mean age (± SD), gender distribution and age range studied for each age group is indicated. Patients with Alzheimer's disease have been grouped together.

	Percent Immunopositive Tissue	
	AA95	Anti-ApoE
Normal		
Astrocytes	46	54
Neurons	38	69
Vessels	31	54
Plaques	0	67
Alzheimer's disease		
Astrocytes	100	60
Neurons	100	60
Vessels	80	80
Plaques	80	80

Table 2.2 Immunoreactivity in the cortex of normal and AD subjects. Frontal or temporal cortical sections from normal and Alzheimer disease subjects were stained with AA95, a serum raised against choroid plexus amyloid, or with an antibody against apoE. The mean age was 55.4 (14-72 years) for control subjects (n = 13) and 88.2 (80-94 years) for the group with Alzheimer's disease (n = 5). Plaques were present in only three of the normal cases examined. The percent immunopositive tissue signifies the percent of brains in which immunoreactivity could be observed.

2.5 DISCUSSION

We have demonstrated that proteins found in cortical amyloid, which accumulates with age (1) and is a hallmark of AD (5,6,7), are present in the CP of both normal individuals and AD subjects. Moreover, we have shown that at least one of these proteins, apoE, is a component of amyloid-laden Biondi rings, lipid-like structures in the CP (21) which accumulate with age (2,3).

Western blot analysis demonstrated that the polyclonal antibody AA95, which was raised against CP amyloid and labels all Congo red-positive Biondi rings in histological sections (21), reacts with recombinant human apoE. In addition, protein between 45 and 80 kD from the CP and CSF as well as a 34 kD CSF protein were labelled by both AA95 and anti-apoE antibodies. These results, combined with the observation that preabsorption of AA95 with recombinant apoE results in a loss of staining of Biondi rings, strongly suggest that at least one of the epitopes in CP amyloid is apoE.

The precise nature of the high molecular weight AA95- and apoE-immunopositive protein remains unclear. It may simply be a heavily glycosylated apoE (33), or a dimer of apoE. A recent study has demonstrated that high molecular weight apoE-immunoreactive proteins of 50 and 66 kD can be isolated from the CSF of non-demented and AD patients (34). Although the identity of the 50 kD protein was undetermined, it was suggested that the 66 kD protein was apoE covalently crosslinked by malondialdehyde and 4-

hydroxy-2-nonenal, two reactive byproducts of lipid peroxidation. This cross-linked apoE may be transported into the CP. In addition, such a conjugate could form in the CP epithelium, which accumulates oxidized lipids with age (3).

Alternatively, Biondi rings may be formed by apoE complexed to a second, unidentified protein or compound. Indeed, electrophoresis of CP amyloid revealed that a protein of approximately 6 kD co-purifies with the approximately 50 kD AA95-immunoreactive Biondi ring protein, but is not recognized by AA95 (21). Furthermore, in a brief report, Aß epitopes were identified in amyloid fibrils in the CP of one individual (23). Similarly, in our studies AA95 did not react with $Aß_{1-40}$, apoJ, TTR, cystatin or tubulin on immunoblots, but antibodies against Aß and apoJ, in addition to apoE, sometimes stained ring-like structures within the epithelium. Together, these results suggest the possibility that Biondi rings may be formed by a complex of proteins, including apoE, and occasionally Aß and apoJ. We are currently attempting to generate such complexes in vitro to determine if other proteins associate with apoE to form Biondi rings and to identify the nature of this association.

The high molecular weight protein appears to be the predominant form of apoE detected by AA95 in the CP, in contrast to the CSF which demonstrated more robust labelling of the 34 kD apoE. The inability of AA95 to detect the 34 kD apoE in the CP as compared to the CSF may reflect a

quantitative difference. In addition, it is highly likely that the apoE epitope(s) of the polyclonal AA95 serum differ from that of the monoclonal anti-apoE antibody as some differences in immunoreactivity between AA95 and anti-apoE antibodies were observed in the cortex as well. It is possible that these antibodies differ in their ability to detect apoE in diverse states, such as glycosylated, dimerized or crosslinked, as discussed above. The production of antibodies which recognize oxidatively modified, but not normal, neurofilaments is consistent with such a possibility (35).

Both AA95 and anti-apoE antibodies could identify ring-like structures in CP from individuals in which amyloid was not yet present, demonstrating for the first time that in young individuals, some protein(s) accumulates in Biondi rings prior to the deposition of amyloid. This was not apparent from previous studies which have focused on amyloid deposition in the elderly.

The origin of apoE in the CP is unknown, and to our knowledge, it has not been shown to be produced by the human CP. Two possible sources are the CSF and the blood. The apoE in the CSF is derived from the brain (36), where it is produced by astrocytes (37) and taken up by neurons (14). In addition, we detected apoE and AA95 immunoreactivity in circular structures called corpora amylacea, which accumulate with age (38,39) and are believed to be extruded from neurons, possibly into the CSF (40). The presence of apoE immunoreactivity around vessels in some CP indicates that the serum may provide an additional source of apo E.

The mechanism by which apoE may enter the CP from the CSF has yet to be identified. A novel human apoE receptor resembling the LDL receptor, has recently been identified and is postulated to play a role in the uptake of apoE-enriched HDL, including that found in the CSF (41). In support of this concept, Northern blot analysis demonstrated abundant expression of the mRNA for this receptor in the rat CP and ependyma of the ventricles (41). The LDL receptor related protein (LRP), which is found in the CP and ependyma of mice (42) is a second possible mechanism of entry for apoE.

We have shown that proteins found in cortical amyloid are present in the CP, yet unlike in the cortex, Aß does not appear to be a major component of CP amyloid. This indicates that either the concentration of Aß in CP epithelial cells does not reach a level at which Aß can form amyloid, or that a mechanism exists in the CP to prevent the formation of amyloid by Aß. In this respect, it is noteworthy that the CP-derived TTR (32), which has been shown to inhibit the ability of Aß to form amyloid fibrils in vitro (13), was not found to associate with the Biondi ring amyloid. It may be that TTR acts as a physiological chaperone to prevent amyloid formation by Aß in the CP. A study of the metabolism of Aß and its precursor protein in the CP may help to clarify why Aß does not seem to readily form amyloid in this tissue.

Our results demonstrate that proteins associated with Biondi rings exist in a non-ß-pleated state in young individuals and may evolve with time into the amyloid characteristically observed in many older individuals and in all AD

subjects. Although our study did not specifically address the mechanism of amyloid deposition in the CP, our results lead us to speculate that there are two possible processes by which preamyloid Biondi rings may accumulate amyloid. The apoE of Biondi rings may be present initially in a form which is not ß-pleated, and therefore identified by AA95 but not by Congo red staining, and it may ß-pleat only later in life. In this respect it may resemble Aß, which can be found in a soluble form in the CSF (43,44) and in Congo-red negative diffuse cortical plaques (45) but which deposits as insoluble amyloid in the plaques and around vessels of the cortex with age (1) and in AD (5,6,7). Support for this possibility comes from a report in which a 10 kD carboxy-terminal fragment of apoE was isolated from cortical amyloid plaques, and a recombinant apoE of this fragment was generated. This fragment was shown to form amyloid fibrils in vitro (46). Alternatively, it may be that apoE never ß-pleats but instead with age becomes associated with another protein(s) which does ß-pleat. One candidate protein is Aß, which has been found in amyloid in the CP of one individual (23), but other proteins may also contribute to this amyloid, and further studies are required to clarify the exact composition of Biondi rings at different stages of life. Identification of the protein(s) in CP amyloid is an important step towards defining the mechanism of amyloid formation in this tissue, and it may contribute significantly to our understanding of the age-associated changes in CP function and the pathogenesis of AD.

2.6 ACKNOWLEDGEMENTS

The authors gratefully acknowledge the generous gifts of antibodies from Dr K.S. Kim of *The Institute for Basic Research in Developmental Disabilities*, New York, USA and from Dr P. Westermark of the *Hälsouniversitetet Institute för Pathologi*, Linköping, Sweden.

2.7 REFERENCES

1. Tomlinson BE, Blessed G and Roth M (1968) Observations on the brains of non-demented old people. *J Neurol Sci* 7: 331-56.
2. Divry P (1955) De la nature des formations argentophiles des plexus choroideus. *Acta Neurol Psychiatr Belg* 55:282-83.
3. Shuangshoti S and Netsky MG (1970) Human choroid plexus: morphologic and histochemical alterations with age. Am J Anat 1970; 28:73-96.
4. Davson H, Welch K and Segal MB (eds). The secretion of the cerebrospinal fluid. In: Physiology and Pathophysiology of the Cerebrospinal Fluid, Edinburgh: Churchill Livingstone, 1987: 189-246.
5. Mirra SS, Heyman A, McKeel D, Sumi SM, Crain BJ, Brownlee LM, Vogel SF, Hughes JP, van Belle G, Berg L, and participating CERAD neuropathologists (1991) The consortium to establish a registry for Alzheimer's disease (CERAD). II. Standardization of the neuropathologic assessment of Alzheimer's disease. *Neurol* 41: 470-486.
6. Glenner GG and Wong CW (1984) Alzheimer's disease: initial report of the purification and characterization of a novel cerebrovascular amyloid protein. *Biochem Biophys Res Comm* 1984; 120:885-90.
7. Masters CL, Simms G, Weinman NA, Multhaup G, McDonald BL and Beyreuther K (1985) Amyloid plaque core protein in Alzheimer's disease and Down Syndrome. *Proc Natl Acad Sci USA* 82: 4245-4249.

8. Namba Y, Tomonaga M, Kawasaki H, Otomo E and Ikeda K (1991) Apolipoprotein E immunoreactivity in cerebral amyloid deposits and neurofibrillary tangles in Alzheimer's disease and Kuru plaque amyloid in Creutzfeldt-Jakob disease. *Brain Res* 541: 163-166.

9. McGeer PL, Kawamata T and Walker DG (1992) Distribution of clusterin in Alzheimer brain tissue. *Brain Res* 579:337-41.

10. Shirahama T, Skinner M, Westermark P, Rubinow A, Cohen AS, Brun A and Kemper TL (1982) Senile cerebral amyloid. Prealbumin as a common constituent in the neuritic plaque, in the neurofibrillary tangle, and in the microangiopathic lesion. *Am J Pathol* 107:41-50

11. Pittas RE, Boyles JK, Lee SH, Hui D and Weisgraber KH (1987) Lipoproteins and their receptors in the central nervous system. *J Biol Chem* 262:14352-143560.

12. James RW, Hochstrasser A-C, Borghini I, Martin B, Pometta D and Hochstrasser D (1991) Characterization of a human high density lipoprotein-associated protein, NA1/NA2. *Arteriosclerosis Thrombosis* 11: 645-652.

13. Schwarzman AL, Gregori L, Vitek MP, Lyubski S, Strittmatter WJ, Enghilde JJ, Bhasin R, Silverman J, Weisgraber KH, Coyle PK, Zagorski MG, Talafous J, Eisenberg M, Saunders AM, Roses AD and Goldgaber D (1994) Transthyretin sequesters amyloid ß protein and prevents amyloid formation. *Proc Natl Acad Sci* 91: 8368-8372.

14. Mahley RW (1988) Apolipoprotein E Cholesterol transport protein with expanding role in cell biology. *Science* 240:622-630.

15. Kirszbaum L, Sharpe JA, Murphy B, d'Apice AJF, Classon B, Hudson P and Walker D (1989) Molecular cloning and characterization of the novel, human complement-associated protein SP-40,40: A link between the complement and the reproductive system. *EMBO J* 8:711-718.

16. Blake CCF, Geisow MJ, Swan IDA, Rerat C and Rerat B (1979) Structure of human plasma prealbumin at 2.5 Å resolution. *J Mol Biol* 88:1-12.

17. Wisniewski T and Frangione B (1992) Apolipoprotein E: a pathological chaperone protein in patients with cerebral and systemic amyloid. *Neurosci Lett* 135:235-238.

18. Oda T, Pasinetti GM, Osterburg HH, Anderson C, Johnson SA and Finch CE (1994) Purification and characterization of brain clusterin. *Biochem Biophys Res Commun* 204: 1131-1136.

19. del Rio Hortega P (1918) Noticia de un nuevo y facil metodo para la coloracion de la neuroglia y del tejido conjuntivo. *Trab Lab Invest Biol Univ Madrid* 15: 367-378.

20. Biondi G (1933) Ein neuer histologischer Befund am Epithel des Plexus Chorioideum. *Ztschr Ges Neurol* 114: 161-165.

21. Eriksson L and Westermark P (1990) Characterization of intracellular amyloid fibrils in the human choroid plexus epithelial cells. *Acta Neuropathologica* 80: 597-603.

22. Eriksson L and Westermark P (1986) Intracellular neurofibrillary tangle-like aggregations. A constantly present amyloid alteration in the aging choroid plexus. *Am J Pathol* 125: 124-129.

23. Wen GY, Rudelli RD, Kim KS and Wisniewski HM (1988) Tangles of ependyma-choroid plexus contain ß-amyloid protein epitopes and represent a new form of amyloid fiber. *Arch Neurol* 45: 1298-1299.

24. Nilsson C, Lindvall-Axelsson M and Owman C (1992) Neuroendocrine regulatory mechanisms in the choroid plexus-cerebrospinal fluid system. *Brain Res Reviews* 17: 109-138.

25. Kunicki S, Zorychta E and Richardson J (1994) Amyloid in the human brain: relation to age and disease. (Abstract) *Brain Pathol* 4: 553.

26. Benditt EP, Erikssen N, Hermodson NA and Ericcson LH (1971) The major proteins of human and monkey amyloid substance: Common properties including unusual N-terminal amino acid sequences. *FEBS Lett* 19: 169-173.

27. Gudmundsson G, Hallgrimsson J, Jónasson TA and Bjarnason Ó (1972) Hereditary cerebral hemorrhage with amyloidosis. *Brain* 95: 387-404.

28. Mori H, Takio K, Ogawara M and Selkoe DJ (1992) Mass spectrometry of purified amyloid ß protein in Alzheimer's disease. *J Biol Chem* 267: 17082-17086.

29. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680-685.

30. Towbin H, Stoehelin T and Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* 76: 4350-4354.

31. Kalaria RN, Premkumar DRD, Pax AB, Cohen DL and Lieberburg I (1996) Production and increased detection of amyloid ß protein and amyloidogenic fragments in brain microvessels, meningeal vessels and choroid plexus in Alzheimer's disease. *Mol Brain Res* 35: 58-68.

32. Herbert J, Wilcox JN, Pham KTC, Fremeau RT Jr., Zeviani M, Dwork A, Soprano DR, Makover A, Goodman DS, Zimmerman EA, Roberts JL and Schon EA (1986) Transthyretin: A choroid plexus-specific transport protein in human brain. *Neurol* 36: 900-911.

33. Weisgraber KH (1994) Apolipoprotein E: Structure-function relationships. *Adv Protein Chem* 45: 249-302.

34. Montine TJ, Huang DY, Valentine WM, Amarnath V, Saunders A, Weisgraber KH, Graham DG and Strittmatter WJ (1996) Crosslinking of apolipoprotein E by products of lipid peroxidation. *J Neuropathol Exp Neurol* 55: 202-210.

35. Smith MA, Rudnicka-Nawrot M, Richey PL, Praprotnik D, Mulvihill P, Miller CA, Sayre LM and Perry G (1995) Carbonyl-related posttranslational modification of neurofilament protein in neurofibrillary pathology of Alzheimer's disease. *J Neurochem* 64: 2660-2666.

36. Carlsson J, Armstrong VW, Reiber H, Felgenhauer K and Seidel D (1991) Clinical relevance of the quantification of apolipoprotein E in cerebrospinal fluid. *Clin Chim Acta* 196: 167-176.

37. Boyles JK, Pitas RE, Wilson E, Mahley RW and Taylor JM (1985) Apolipoprotein E associated with astrocytic glia of the central nervous system and with nonmyelinating glia of the peripheral nervous system. *J Clin Invest* 76: 1501-1513.

38. Ramsey HJ (1965) Ultrastructure of corpora amylacea. *J Neuropathol Exp Neurol* 24:29-39.

39. Sakai M, Austin J, Witmer F and Trueb L (1969) Studies of corpora amylacea 1: isolation and preliminary characterization by chemical and histochemical techniques. *Arch Neurol* 21: 526-544.

40. Singhrao SK, Neal JW and Newman GR (1993) Corpora amylacea could be an indicator of neurodegeneration. *Neuropathol Appl Neurobiol* 19: 269-276.

41. Kim D-H, Iijima H, Goto K, Sakai J, Ishii H, Kim H-J, Suzuki H, Kondo H, Saeki S and Yamamoto T (1996) Human apolipoprotein E receptor 2. *J Biol Chem* 271: 8373-8380.

42. Kounnas MZ, Haudenschild CC, Strickland DK and Argraves WS (1994) Immunological localization of glycoprotein 330, low density lipoprotein receptor related protein and 39 kDa receptor associated protein in embryonic mouse tissues. *In Vivo* 8: 343-351.

43. Seubert P, Vigo-Pelfrey C, Esch F, Lee M, Dovey H, Davis D, Sinha S, Schlossmacher M, Whaley J, Swindlehurst C, McCormack R, Wolfert R, Selkoe D, Lieberburg I and Schenk D (1992) Isolation and quantification of soluble Alzheimer's ß-peptide from biological fluid. *Nature* 359: 325-327.

44. Shoji M, Golde TE, Ghiso J, Cheung TT, Estus S, Shaffer LM, Cai X-D, McKay DM, Tintner R, Frangione B and Younkin SG (1992) Production of the Alzheimer amyloid ß protein by normal proteolytic processing. *Science* 258: 126-129.

45. Tagliavini F, Giaccone G, Fragione B and Bugiani O (1988) Preamyloid deposits in the cerebral cortex of patients with Alzheimer's disease and nondemented individuals. *Neurosci Letters* 93: 191-196.

46. Wisniewski T, Lalowski M, Golabek A, Vogel T and Fragione B (1995) Is Alzheimer's disease an apolipoprotein E amyloidosis ? *Lancet* 345: 956-958.

47. Wernette-Hammond ME, Lauer S, Corsini A, Walker D, Taylor JM and Rall SC (1989) Glycosylation of human apolipoprotein E. The carbohydrate attatchment site is threonine 194. *J Biol Chem* 264: 9094-9101.

PREFACE TO CHAPTER 3

The previous chapter demonstrated three important points. The first is that although a number of amyloidogenic proteins can be identified and isolated from the CP epithelium, it is apoE which constitutes the most prominent component of the CP amyloid. Secondly, Aß is present in the CP epithelium, and it may sometimes associate with Biondi rings. The third important finding was the detection of Aß in the CSF of normal and AD patients, indicating that Aß is not a product of aberrant metabolism, and that it can be found in a soluble form.

The next chapter examines the concentrations of Aß40, Aß42, apoE and TTR in the human CSF at different ages in order to determine if there are progressive changes in the amounts of any of these proteins with age and also whether a relationship can be discerned between the levels of these proteins. ApoJ and cystatin levels were not determined as the volume of CSF available for analysis limited the number of proteins we could test for and we did not have access to techniques which would allow accurate determination of their levels. Thus the focus will be on the main protein component of plaques, Aß, with particular reference to the two major forms identified in plaques, Aß42 and Aß40.

CHAPTER 3

The effect of age, apolipoprotein E phenotype and gender on the concentration of Aß40, Aß42, apolipoprotein E and transthyretin in human cerebrospinal fluid.

S. Kunicki, J. Richardson, P.D. Mehta, K.S. Kim and E. Zorychta

3.1 ABSTRACT

The two major isoforms of the amyloid-ß (Aß) peptide, Aß40 and Aß42, are normal constituents of the cerebrospinal fluid (CSF). Both Aß40 and Aß42 can ß-pleat and form amyloid deposits in the brains of Alzheimer's disease (AD) patients, and to a lesser extent in the brains of some nondemented aged individuals, but the mechanisms regulating this process are not understood. Factors which promote amyloidogenesis in vitro are an elevated concentration of Aß, the two carboxy-terminal amino acids of Aß42 and the presence of apoE. In contrast, transthyretin (TTR) inhibits formation of amyloid by Aß. We therefore tested whether there is a correlation between three well-documented risk factors for AD, chronological age, gender and the presence of the apoE4 allele, and the concentrations of Aß40, Aß42, apoE and TTR in the CSF of normal individuals, age 19 to 82 years. The CSF levels of all these proteins except Aß40 are reduced in AD. In contrast, we found that the concentration of Aß40 declined with age while the concentration of Aß42, apoE and TTR remained constant. There was no relationship between gender or apoE phenotype and the level of any of these proteins. These results indicate that the changes observed in the CSF of AD patients are specific to AD and are not modulated by age, gender or apoE phenotype.

3.2 INTRODUCTION

Chronological age, gender and the inheritance of the apolipoprotein (apo) E ϵ4 allele are well-established factors affecting the individual risk of Alzheimer's disease (AD) (3,38,41,47,48) . However, the mechanisms linking these risk factors to the pathogenesis of the disease are not understood. What is known is that the incidence of AD is higher in women (3) and that some of the neuropathological hallmarks of AD, including deposition of amyloid in classic neuritic plaques and around cerebral vessels, can be observed in many nondemented older individuals (48), although to a much lesser degree than in AD subjects of a similar age (26). The number of neuritic plaques in the brains of both normal (1) and AD patients (1,35,41) is influenced by the APOE genotype, being greater in people with an APOE ϵ4 allele compared to those with either APOE ϵ2 or APOE ϵ3 alleles.

The amyloid deposits are formed primarily of aggregates of a ß-pleated peptide of 39 to 42(43) amino acids, called amyloid-ß (Aß) (11,24), which is derived from a much larger amyloid precursor protein, APP (18). However, the Aß peptide can also be found in a non-ß-pleated conformation in diffuse plaques as well as around vessels in the brain (15,37,54). Soluble Aß has also been identified in plasma (33,43) and in the cerebrospinal fluid (CSF) (31,43,45). It is unclear what causes soluble Aß to ß-pleat and form insoluble amyloid deposits.

Three factors considered to influence the propensity of Aß to form amyloid are the concentration of Aß, the length of the peptide and its interaction with diverse proteins. Elevated concentrations of Aß have been shown to promote Aß aggregation and formation of amyloid fibrils in vitro (17) and may do so in vivo (6). Similarly, the additional two C-terminal amino acids of Aß42 confer an increased tendency towards aggregation into amyloid in comparison to Aß40 (17). The ability of numerous proteins to modulate Aß amyloidogenesis in vitro has also been recognized. Special attention has been given to proteins which occasionally co-localize with Aß amyloid deposits and which are also found along with Aß in the CSF, including apolipoprotein (apo) E (29), apo J (9,25) and transthyretin (TTR) (44). These proteins all bind Aß (9,42,47,53), but with very different results. Apo E promotes formation of amyloid by Aß in vitro (52,53), while apo J (30) and TTR (42) inhibit it. It has been suggested that TTR is the major protein which sequesters Aß in the CSF (42). The mechanisms by which these proteins influence ß-pleating of Aß is not known.

The concentrations of Aß42 (27) and apo E (2,22) are significantly reduced in the CSF of AD patients compared to age-matched controls. However, it is not known if the decline in these proteins occurs throughout life and is simply accelerated in AD or if these changes are specific to the disease process of AD. Since aging is associated with an increase in the incidence of AD and an increase in the prevalence of amyloid deposition in the brains of

normal individuals, we hypothesized that the concentration of Aß and its associated proteins may gradually change throughout life. A systematic evaluation of the effect of age on these proteins is lacking as studies of nondemented individuals have been primarily on subjects over the age of 60, included as age-matched controls for AD patients. We therefore measured the concentration of Aß40, Aß42, apo E and TTR in human CSF throughout adulthood, in males and females, from age 19 to 82 years. We also determined the APOE phenotype in order to assess the impact of APOE $\epsilon 4$ on the CSF concentration of the proteins under investigation.

3.3 MATERIALS AND METHODS

3.3.1 CSF

CSF samples were obtained from lumbar punctures performed for diagnostic purposes using routine protocol at the Montreal Neurological Institute. Informed consent was obtained for the use of any remaining fluid for research purposes. The CSF was spun at 1000 RPM and 0.5 to 1.0 ml aliquots were stored at -80°C until analysis. The sample population was restricted to individuals with no case histories of dementia, stroke or recent head trauma. In addition, only CSF which had cell counts, total protein and glucose concentrations that fell within the normal range were included in this study. The most common clinical reasons for obtaining CSF samples were to rule out disorders such as peripheral neuropathy (n = 11), a central nervous

system tumor (n=9), multiple sclerosis (n=8), meningitis (n=3) or vasculitis (n=3) and as part of a follow up after the diagnosis of a tumor in the periphery, to rule out metastasis to the central nervous system (n=6). In total, CSF from 39 female and 25 male subjects with a mean age of 51.2 ± 17.0 years was studied (Figure 3.1). Both sexes were equally represented among the different ages, as demonstrated by linear regression analysis and by a two-sample t-test comparing the age distributions of males and females.

3.3.2 APOE phenotyping.

A CSF volume of 50 to 80 μl was prepared for isoelectric focusing as described previously (13). In brief, the neuraminidase-treated CSF was run on an acrylamide gel for 900 volt-hours, then electrophoretically transferred to a polyvinylidene fluoride membrane (Immobilon-P^{SQ}, Millipore Corp.) using standard protocol (49). Membranes were blocked in 5% skim milk in Tris-buffered saline (TBS), incubated in anti-apo E antibody (2 μg/ml, Boehringer Mannheim) overnight at 4°C, washed, then incubated in alkaline phosphatase-labelled goat anti-mouse antibody (Sigma) at a dilution of 1:4000, for one hour at room temperature (RT). Antibodies were diluted in 0.5% skim milk in TBS. Immunoblots were developed using 5-bromo-4-chloro-3-indoyl-phosphate and nitroblue tetrazolium tablets (Sigma). Recombinant human apo E 3 and apo E 4 (Calbiochem), along with plasma from subjects of known genotypes (gift from Dr. Gilfix), were used as standards. In one of the 64 cases, the CSF

volume remaining following protein concentration analysis was insufficient to obtain an accurate phenotype.

3.3.3 Description of antibodies R162 and R164.

The polyclonal antibodies R162 and R164 have been characterized elsewhere (36, submitted for publication). Briefly, rabbit antisera were raised against synthetic peptides (AnaSpec, San Jose, CA) that included Aß residues 28 to 40, and 33-42 respectively. Western blot analysis demonstrated that 25 ng of Aß40 can be detected by R162 (1:1000) but not R164 (1:1000) and that 25 ng of Aß42 is detected by R164 but not R162. In addition, enzyme linked immunosorbant assays (ELISAs) demonstrated that the dilution of R162 required to detect 100 μg of Aß was 1:15,000 for Aß40 compared to <1:20 for Aß42. The dilution of R164 required to detect Aß42 was 1:8,000 compared to <1:50 for Aß40. Thus although there is some cross-reactivity as shown by the ELISAs, R162 was much more specific for Aß40 and R164 was much more specific for Aß42. The sensitivity of these antibodies as demonstrated by sandwich ELISAs is shown in Figure 3.2.

3.3.4 Quantitative analysis of CSF protein concentrations.

The amounts of Aß and apo E were determined using a sandwich ELISA as previously described (4,32). For Aß ELISAs, 96-well NUNC U96 Maxisorb Immuno plates were incubated overnight at 4°C with the monoclonal capturing

antibody 6E10 (2.5 μg/ml), against amino acids 1-15 of Aß (20). The plates were washed using a microplate autowasher EL404 (Bio-Tek Instruments), blocked with 10% normal sheep serum (Gibco BRL) for one hour at RT then washed. CSF or Aß standards (Aß40, Aß42 Research Biochemicals International) were subsequently added and left to incubate for two hours at RT. Following a wash cycle, biotinylated detecting antibody was added and left at RT for 75 minutes. The rabbit polyclonal antibodies R162 (1:200) and R164 (1:100) were the detecting antibodies for Aß40 and Aß42 respectively. Following a wash cycle, the wells were incubated with an avidin-biotin solution prepared from a Vectastain ABC kit (Vector Laboratories, USA) for 45 minutes at RT. The wells were washed, a substrate buffer containing o-phenylenediamine dihydrochloride (Sigma, USA) was added for 30 minutes and the reaction was stopped using sulfuric acid. The color reaction was read by a Bio-Tek Instruments ELISA Reader EL311 at a wavelength of 490 nm. Nonlinear curve fitting was performed using Bio-Tek Kineticalc EIA Application software version 2.03 to convert optical densities to estimates of the protein concentrations in the CSF. The linear assay range for Aß40 and Aß42 was 0.39 to 10 ng/ml (Figure 3.2).

The amount of apo E was determined in a similar manner, with a few modifications. An affinity-purified goat anti-apo E antibody (Genzyme) was used as both the capturing and detecting antibody. The wells were incubated with capturing antibody for 2 hours at 37°C, blocked for one hour at room

temperature, then incubated with either CSF or apo E3 standard (Calbiochem) for two hours at 37 °C. The capturing antibody was conjugated with peroxidase. The substrate reaction was carried out as above, and the colored reaction products were measured using a Bio-Tek Instruments ELISA Reader EL310 at a wavelength of 490 nm. The linear assay range was 0.5 to 10 μg/ml. The protein concentrations were estimated from the absorbance values using Sigma Plot.

TTR levels were quantified using a radial immunodiffusion kit from Behring Diagnostic Reagents, which includes TTR standards and anti-TTR antibodies. To ensure that the protein concentrations fell within the assay range of 33 to 467 μg/ml, the CSF was concentrated approximately five-fold using a Speedvac.

For all analyses, samples were run in duplicate on the same day, which allowed for calculation of the within-assay variability. The mean of the two values was recorded as the concentration for each case.

3.3.5 Statistical analysis.

Chi square analysis was used to determine if the distribution of APOE phenotypes in our sample set is comparable to those reported in four separate Canadian studies, which included a total of 811 normal subjects (13,28,34,40,46). The distribution of the six APOE isoforms (ε 2.2, 2.3, 2.4, 3.3, 3.4 and 4.4) was compared between males and females using the

Fisher's Exact test.

Linear regression analysis was used to determine if the concentrations of the proteins varied with age or APOE phenotype. Following this, the concentrations of Aß40, Aß42 and apo E were standardized as a ratio of TTR, which has been shown to remain constant with age (8), in order to correct for variability between individuals in protein concentration. The linear regression analysis was then repeated. Multivariate regression models were developed to test for possible interaction between age, gender and APOE phenotype. A Bayes factor (BF) was generated for each model (19). Best models were selected using the Bayesian information criterion (BIC), which has been shown to produce more robust final models compared to the usual backwards or forwards model selection techniques. Standard F statistics and two-tailed p values were also calculated. Finally, Pearson correlation coefficients were calculated to determine if a relationship between the concentrations of any of the proteins could be identified.

3.4 RESULTS

3.4.1 APOE phenotype distribution.

Chi square analysis did not reveal any significant differences between the distribution of phenotypes within our sample and within a larger population of subjects studied previously (Table 3.1). Regression analysis did not detect any age-associated trend in the distribution, such as preferential survival of

individuals carrying certain APOE phenotypes. This was also true when subjects were grouped into one of two categories: those with at least one APOE ϵ4 allele (2.4, 3.4, 4.4) and those without any ϵ4 alleles (2.2, 2.3, 3.3), thus the APOE ϵ4 allele does not appear to have a negative impact on survival.

The distribution of the six APOE isoforms was not found by the Fisher's Exact test to be different between females and males, although the less common phenotypes ϵ 2/4 and ϵ 4/4 were absent from the female and male sets respectively. Similarly, the distribution of APOE phenotypes was not found to be different between females and males when subjects were grouped into those with at least one APOE ϵ4 allele and those without any.

3.4.2 APOE phenotype and CSF protein concentration.

The mean CSF concentrations of each protein along with the intra-assay and inter-assay variability are presented in Table 3.2. The CSF levels of Aß42 are significantly lower than the concentration of Aß40. As a result, the Aß42 assay originally was not sensitive enough to detect Aß42 in any of the initial 23 CSF samples tested. Following optimization of the Aß42 assay (see materials and methods for current sensitivity), this peptide was measured in the remaining 33 CSF samples, 25 of which contained measurable amounts of Aß42. The age range of these 25 subjects was 21 to 77 years.

Linear regression analysis showed that the concentrations of Aß40, Aß42, apoE and TTR did not appear to be modulated by the APOE phenotype.

In each case, the BIC favored the null model compared to any other. These results are presented in Figure 3.3. To address the possibility that there is an interaction between age, APOE phenotype and gender which is masking the effect of APOE phenotype on protein concentration, further modelling was performed. As with APOE phenotype alone, the BIC favored the null model over those containing age and/or gender and/or APOE phenotype as parameters. These data suggest that the APOE phenotype does not modulate protein concentration, regardless of subject age or gender.

3.4.3 Age, gender and CSF protein concentration.

After eliminating APOE phenotype as a potential confounding or interactive variable, we proceeded to test for an effect of age on the CSF concentrations using linear regression analysis with age as a continuous variable. A significant decrease in Aß40 occurred with age ($p=0.025$). On average, the concentration of Aß40 declined by 0.04 ng/ml each year (Figure 3.4). There was a similar trend observed for Aß42, however, it was not found to be significant ($p=0.093$). Regression analysis failed to demonstrate any significant changes with age in any of the other proteins or in the ratio of Aß40 to Aß42. Comparable results were obtained when CSF protein concentrations were standardized as a ratio of TTR concentration. A positive correlation between the concentrations of Aß42 and Aß40 throughout life ($r=0.619$, $p=0.0010$) was observed (Figure 3.5). No other correlations

between the various proteins were detected. Gender had no significant effect on protein concentration, although the ratio of Aß40 to Aß42 was greater ($p = 0.0519$) in males (8.06) than in females (5.57).

Figure 3.1 Distribution of age and gender of subjects tested. The mean age of each group is indicated by a horizontal bar.

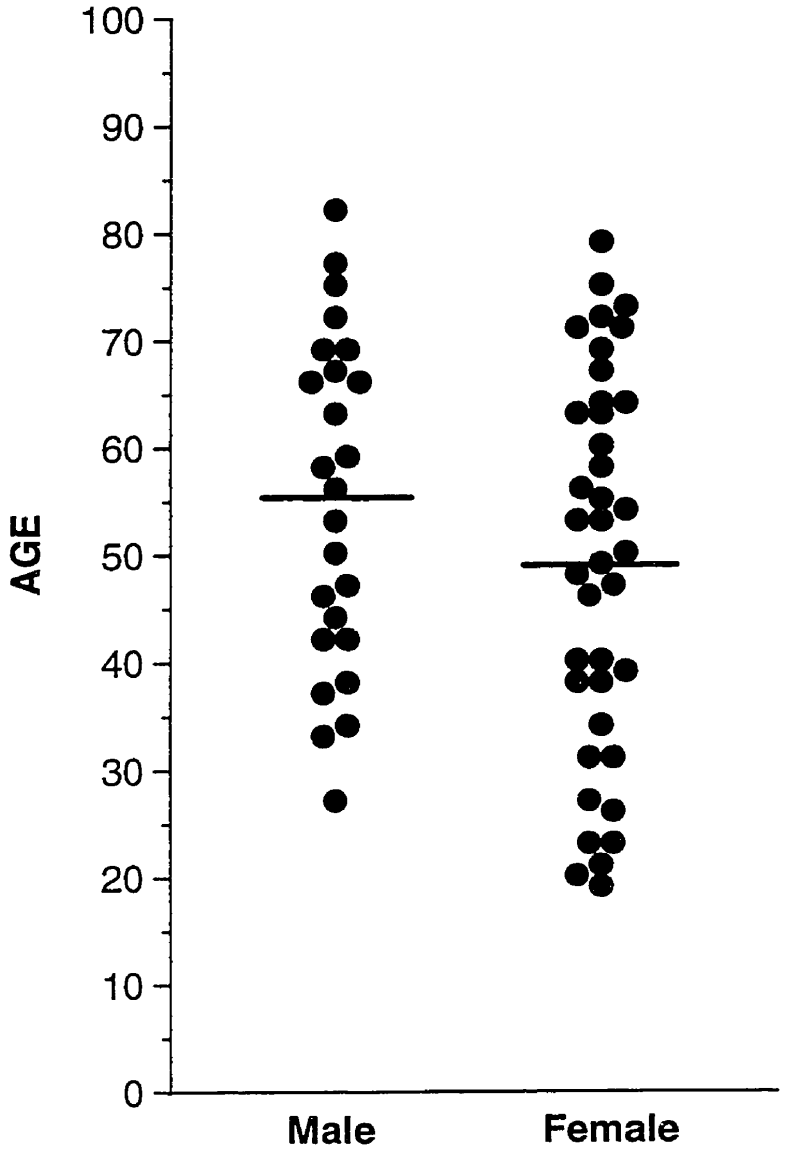
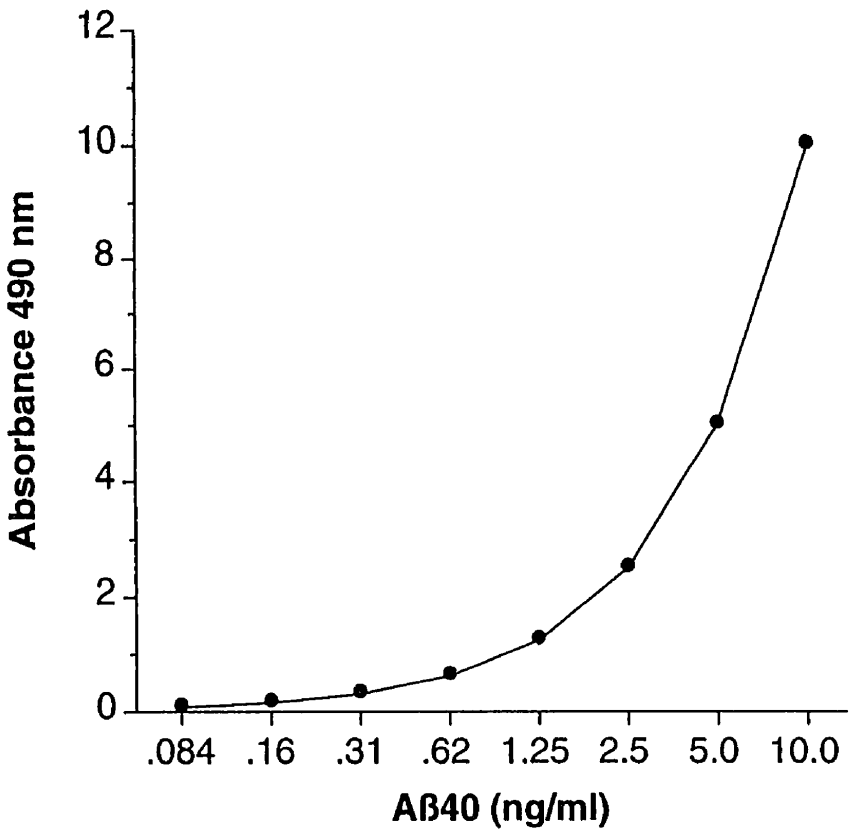

100
90
80
70
60
50
40
30
20
10
0
AGE
Male
Female

Figure 3.2 Aß sandwich ELISAs. Synthetic Aß40 or Aß42 was added to 6E10-coated wells. Bound antigen was detected by biotinylated R162 (anti-Aß40, dilution 1:200) or R164 (anti-Aß42, dilution 1:100).

R162 ELISA



R164 ELISA

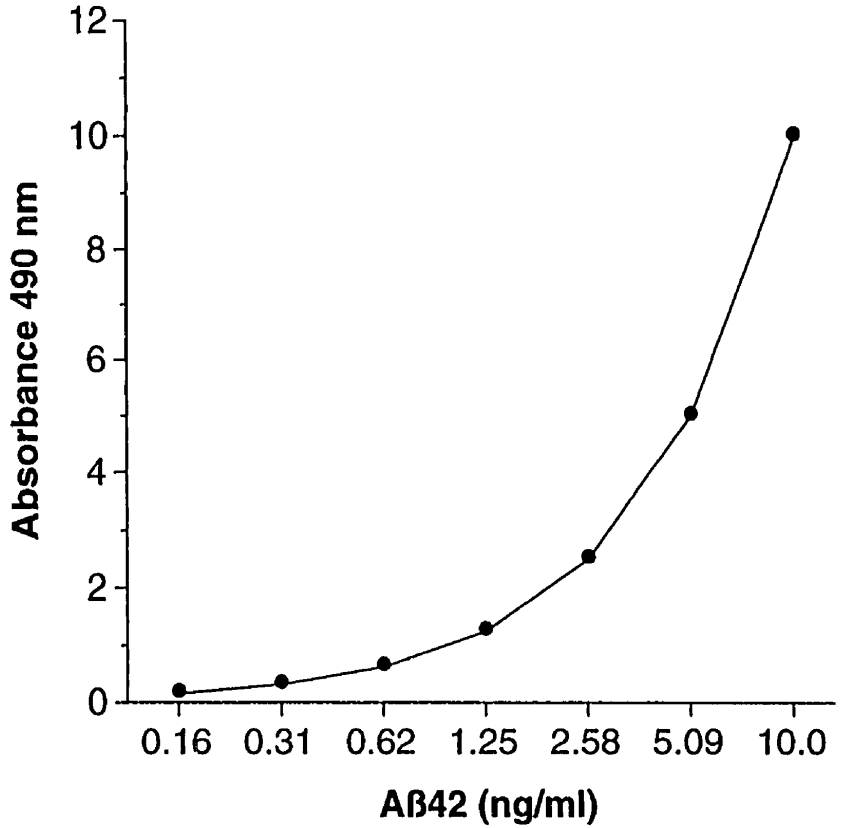


Figure 3.3 CSF concentrations of Aß (ng/ml), apo E (μg/ml) and TTR (μg/ml) grouped by APOE phenotype. The mean protein concentrations and standard deviation are indicated for subjects with at least one apo ϵ4 allele (●) and those without any ϵ4 alleles (○). There was no observable effect of APOE phenotype on CSF proteins.

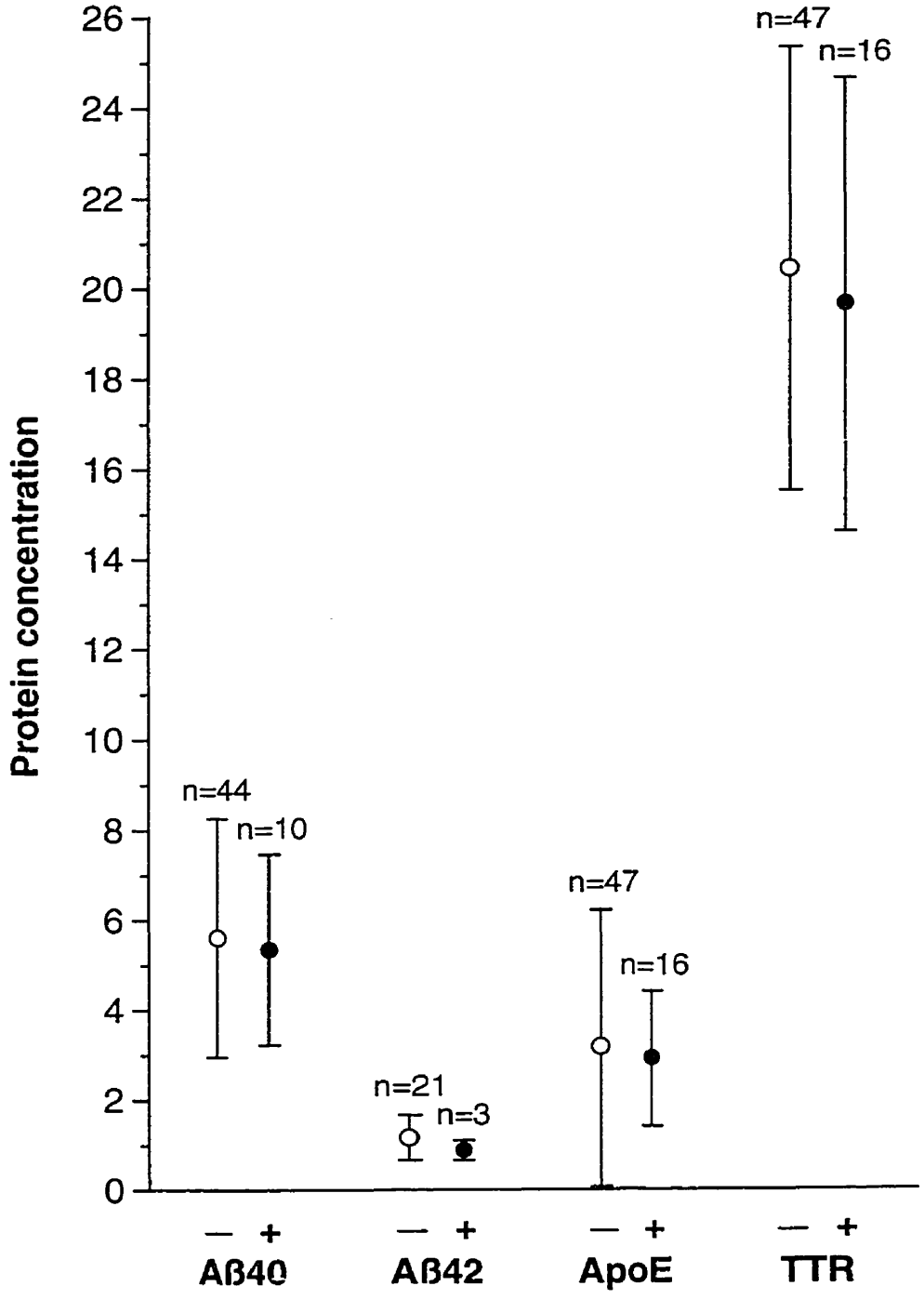
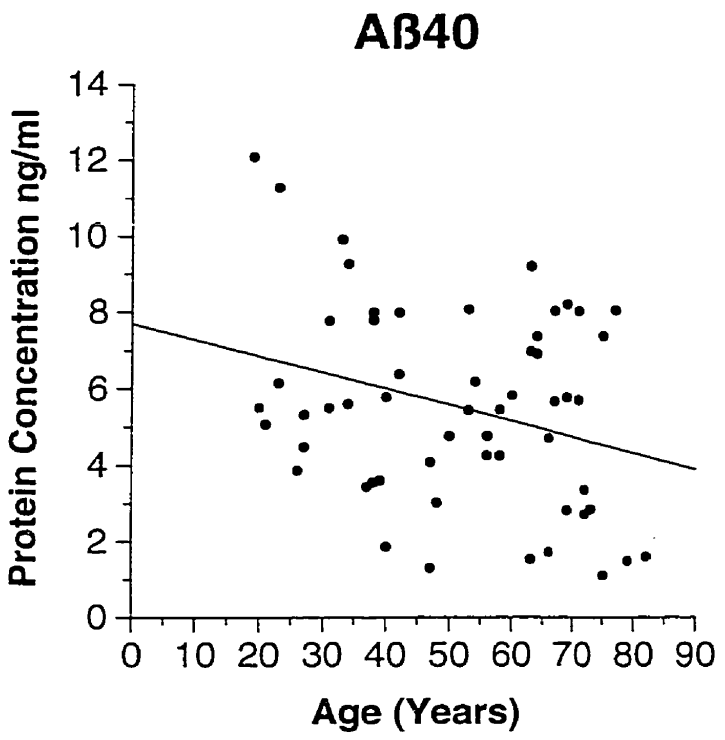
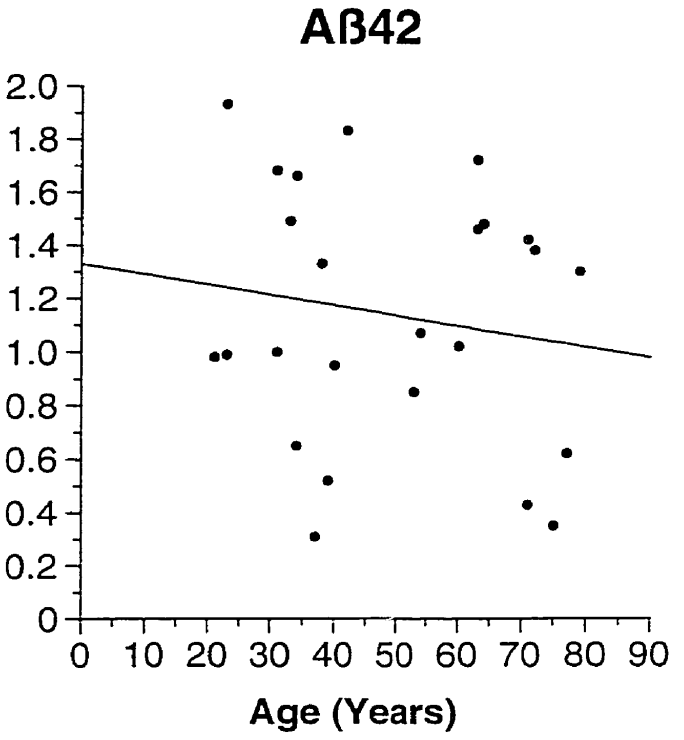
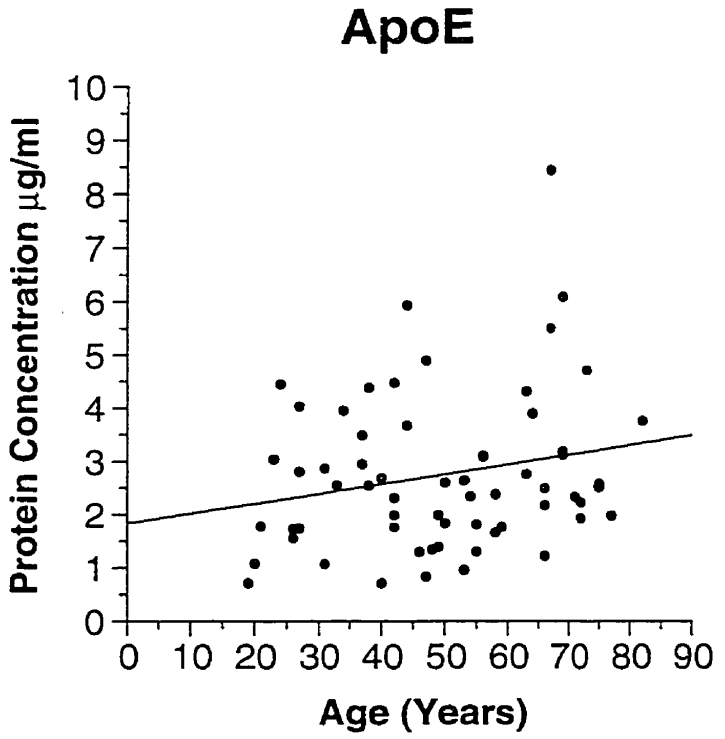

Protein concentration
26
24
22
20
18
16
14
12
10
8
6
4
2
0
n=44
n=10
n=21
n=3
n=47
n=16
n=47
n=16
— +
Aß40
— +
Aß42
— +
ApoE
— +
TTR

Figure 3.4 The effect of age on the concentration of Aß40, Aß42, apoE and TTR as determined by regression analysis. Only Aß40 demonstrated a significant change with age.


Aß40
Protein Concentration ng/ml
0
2
4
6
8
10
12
14
0 10 20 30 40 50 60 70 80 90
Age (Years)


Aß42
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
0 10 20 30 40 50 60 70 80 90
Age (Years)


ApoE
Protein Concentration µg/ml
0
1
2
3
4
5
6
7
8
9
10
0 10 20 30 40 50 60 70 80 90
Age (Years)

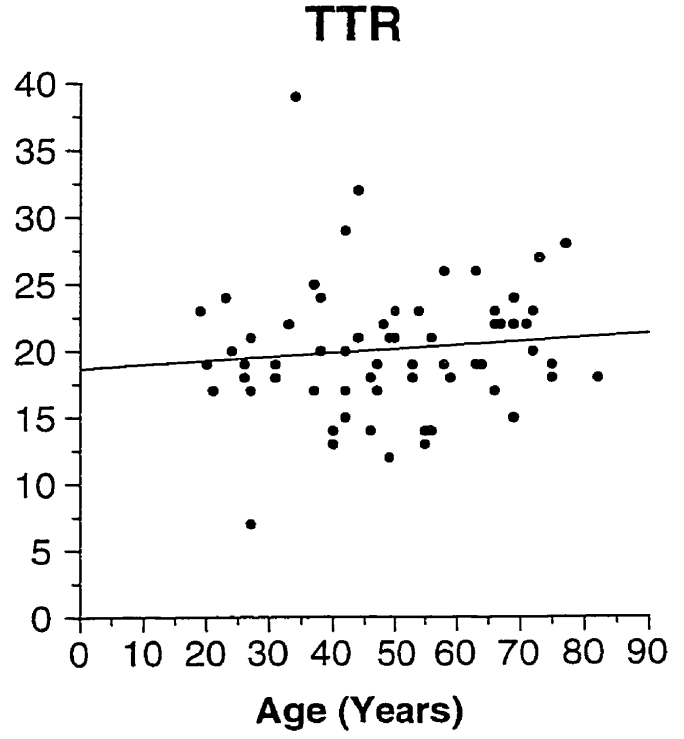
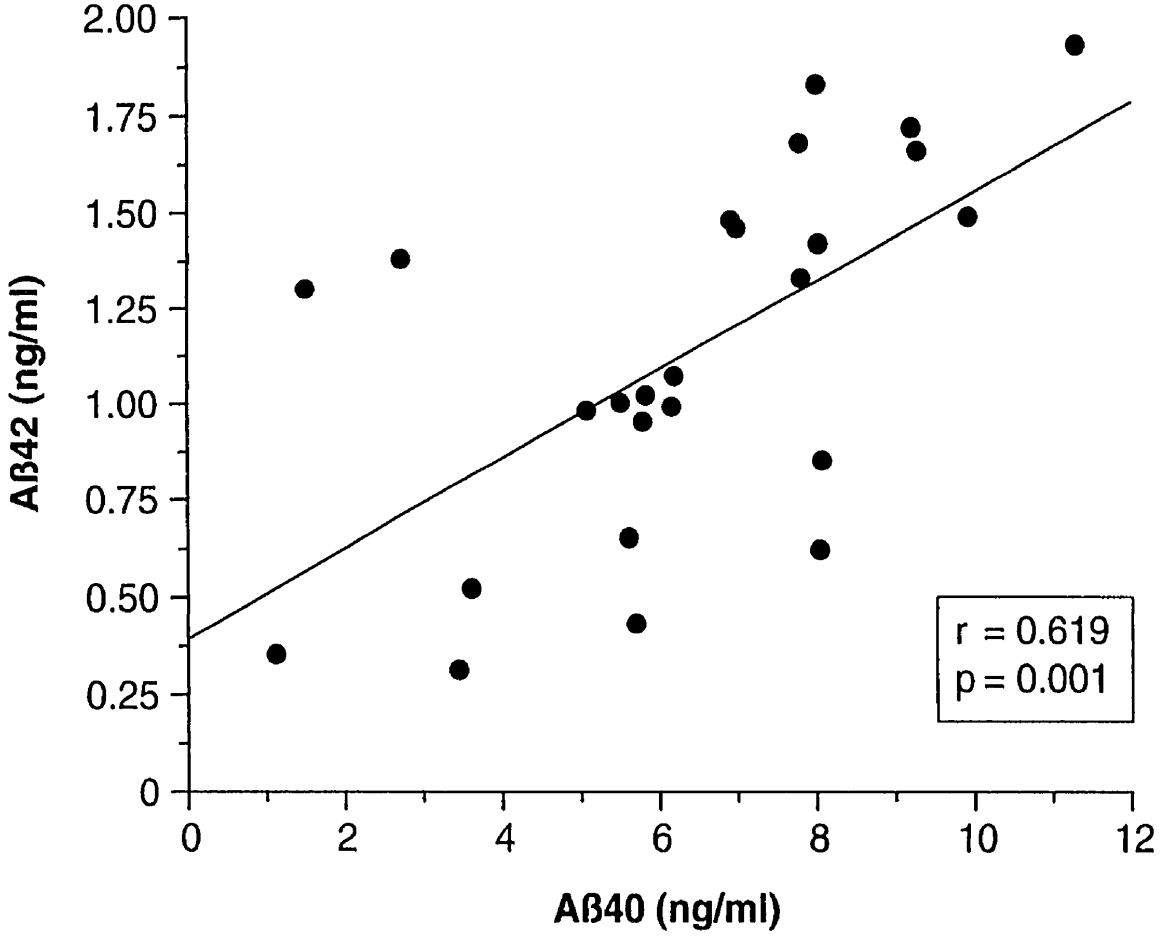

TTR
0
5
10
15
20
25
30
35
40
0 10 20 30 40 50 60 70 80 90
Age (Years)

Figure 3.5 Correlation analysis of Aß40 and Aß42 concentration in the CSF.


2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0
Aß42 (ng/ml)
0
2
4
6
8
10
12
Aß40 (ng/ml)
r = 0.619
p = 0.001

APOE Isoform	This Population		Reported Prevalence
	F:M	% Total	Percent
ϵ 2/2	1:2	4.7	0-2
ϵ 2/3	7:4	17.2	10-31
ϵ 2/4	0:1	1.6	0-3
ϵ 3/3	24:11	54.7	58-80
ϵ 3/4	5:7	18.8	19-27
ϵ 4/4	2:0	3.1	1-4

Table 3.1 Distribution of apoE phenotypes. CSF samples were phenotyped by isoelectric focusing. The number of females (F) and males (M) with each phenotype is indicated. The distribution of APOE isoforms in this study was found by Chi square analysis not to differ significantly from that reported in four other North American populations, which when combined, included 811 individuals (13,28,34,40,46).

	n	Mean ± S.D.	Assay Variability (%)	
			Intra	Inter
Aß40	56	5.52 ± 2.56 ng/ml	4.9	8.6
Aß42	25	1.14 ± 0.48 ng/ml	3.5	11.0
Aß40:Aß42	25	6.27 ± 2.91	NA	NA
Apo E	64	3.04 ± 2.75 µg/ml	4.0	6.6
TTR	64	20.13 ± 4.94 µg/ml	7.0	15.0

Table 3.2 Average CSF protein concentrations in adult humans aged 19 to 82 years. Assay variability values are given for the intra-assay and inter-assay coefficients of variation. The levels of Aß42 and apo E were below the limit of detection in eight and one case respectively. NA, not applicable.

3.5 DISCUSSION

The Aß peptide is produced throughout life and can be found circulating in the CSF (5), yet Aß rarely forms amyloid deposits in the brains of nondemented people under the age of 60 (48). AD, which is characterized by significant Aß amyloid deposition in the brain, is also uncommon before the age of 60 (10). It is unclear why the aged brain is more prone to forming these Aß amyloid deposits. One possibility is that the capacity of the brain to excrete the proteins involved in amyloidogenesis may decline with age, resulting in their eventual accumulation.

The amyloid deposits are formed primarily of aggregates of the Aß42 variant and include Aß40 to a lesser extent (7,11,16,24). Additional proteins are also present, including apo E (50) and TTR (44). These proteins are all found in the CSF of normal individuals and, with the exception of Aß40 (14,27), are significantly reduced in the CSF of AD patients (2,14,22,27,39). Since age is a risk factor for AD, we wanted to determine if there is a gradual decline in these proteins in the CSF with age or if the reduction in their concentration is specific for the disease process.

The major findings of this work are that the proteins which are reduced in AD are maintained at a constant level in nondemented individuals throughout life, while Aß40 declines significantly with age. The gradual decline in Aß40 was shown using regression analysis on our data and is supported by the observation that the average concentration of Aß40 in the

CSF of our subjects, who had a mean age of 50 years, was 5.52 ng/ml, while it was 3.04 ng/ml in a group of older individuals with an average age of 67 years (14). Although a negative trend in the concentration of Aß42 with age was also observed, it was not found to be significant. The mean concentration of Aß42 is comparable to that reported in older subjects (14,27), further suggesting that the concentration of Aß42 in the CSF is maintained in a more consistent manner throughout life.

The concentrations of both the brain-derived apoE (4,23), and the choroid plexus-derived TTR (12), two proteins thought to bind Aß in the CSF (42,47,53), also remained constant with age in our subjects. The mean CSF concentration of apo E in our population is comparable to the mean concentration reported for older normal subjects (2,21), further supporting the conclusion that the CSF concentration of apoE remains stable throughout life. Thus, the secretion of apo E and TTR into the CSF appears to be unaffected by age, but is specifically disrupted in AD.

In addition to age, we examined the possible effect of a second risk factor for AD, the APOE phenotype (38,41,47). Individuals with the ϵ4 allele are at an increased risk of developing AD although the mechanism conferring this increased susceptibility is unknown. Aß deposition in control and AD brains has been reported to increase as a function of APOE genotype, from APOE ϵ2/3 to APOE ϵ3/3 to APOE ϵ3/4 (35,38,41). However, we failed to detect any modulating effect of APOE phenotype on the CSF concentration of

any of the proteins examined, regardless of the age of the individual. Evidence supporting this conclusion includes a study of normal older individuals in which the APOE phenotype was found not to modulate the CSF apo E concentrations (21), as well as from a study of AD patients in which the APOE genotype had no effect on the CSF concentration of total Aß or Aß42 (51). Taken together, these results suggest that the mechanism by which the APOE $\epsilon 4$ allele promotes Aß deposition in the brain of normal individuals and AD patients is not through a direct regulation of the secretion and/or removal of specific proteins from the CSF.

The uniform distribution of males and females among the different ages in our population allowed us to evaluate whether protein concentrations varied as a function of gender. Although the incidence of AD is higher in women (3), we did not detect any appreciable effect of gender on the concentration of any of the CSF proteins examined.

In conclusion, these data demonstrate that three of the risk factors for AD, namely age, APOE isoform and gender, do not have a demonstrable effect on the concentrations of Aß42, apo E or TTR in the CSF of nondemented individuals. These proteins appear to be cleared from the brain or secreted into the CSF at a uniform rate throughout adult life. Their reduced level in the CSF of AD patients is a reflection of the disease itself, rather than an accelerated form of the normal aging process. In contrast, the concentration of Aß40 gradually declines with age, and the implications of this observation

remain to be explained.

3.6 ACKNOWLEDGEMENTS

The authors would like to thank Dr. B. Gilfix, Department of Medicine, Royal Victoria Hospital, Montreal, for the serum samples used as isoelectric focusing standards. We also gratefully acknowledge Dr. L. Joseph, Associate Professor in the Department of Epidemiology and Biostatistics, McGill University, for the helpful advice regarding statistical analysis of our data.

3.7 REFERENCES

1. Berr C, Hauw J-J, Delaère P, Duyckaerts C, Amouyel P (1994) Apolipoprotein E allele $\epsilon 4$ is linked to increased deposition of the amyloid ß-peptide (Aß) in cases with or without Alzheimer's disease. *Neurosci Lett* 178: 221-224.

2. Blennow K, Hesse C and Fredman P (1994) Cerebrospinal fluid apolipoprotein E is reduced in Alzheimer's disease. *NeuroReport* 5: 2534-2536.

3. Bretler MB, Claus JL, van Duijn CM, Launer LJ and Hofman A (1992) Epidemiology of Alheimer disease. *Epidemiol Rev* 14: 59-82.

4. Carlsson J, Armstrong VW, Reiber H, Felgenhauer K and Seidel D (1991) Clinical relevance of the quantification of apolipoprotein E in cerebrospinal fluid. *Clin Chim Acta* 196: 167-176.

5. Carroll RT, Lust MR, Kim KS, Doyle PD and Emmerling MR (1995) An age-related correlation between levels of ß-amyloid precursor protein and ß-amyloid in human cerebrospinal fluid. *Biochem Biophys Res Comm* 210: 345-349.

6. Citron M, Vigo-Pelfrey C, Teplow DB, Miller C, Schenk D, Johnston J, Winblad B, Venizelos N, Lannfelt L and Selkoe DJ (1994) Excessive production of amyloid beta-protein by peripheral cells of symptomatic and presymptomatic patients carrying the Swedish familial Alzheimer disease mutation. *Proc Natl Acad Sci USA* 91: 11993-11997.

7. Fukumoto H, Asami-Odaka A, Suzuki N, Shimada H, Ihara Y and Iwatsubo T (1996) Amyloid ß protein deposition in normal aging has the same characteristics as that in Alzheimer's disease. Predominance of Aß42(43) and association of Aß40 with cored plaques. *Am J Pathol* 148: 259-265.

8. Garton MJ, Keir G, Lakshmi MV and Thompson EJ (1991) Age-related changes in cerebrospinal fluid protein concentrations. *J Neurolog Sci* 104: 74-80.

9. Ghiso J, Matsubara E, Koudinov A, Choi-Miura NH, Tomita M, Wisniewski T and Frangione B (1993) The cerebrospinal-fluid soluble form of Alzheimer's amyloid beta is complexed to SP-40,40 (apolipoprotein J), an inhibitor of the complement membrane-attack complex. *Biochem J* 293:P 27-30.

10. Giannakopoulous P, Hof PR, Savioz A, Guimon J, Antonarkis SE and Bouras C (1996) Early-onset dementias: clinical, neuropathological and genetic characteristics. *Acta Neuropathol* 91: 451-465.

11. Glenner GG and Wong CW (1984) Alzheimer's disease: initial report of the purification and characterization of a novel cerebrovascular amyloid protein. *Biochem Biophys Res Comm* 1984, 120: 885-890.

12. Herbert J, Wilcox JN, Pham KTC, Fremeau RT Jr., Zeviani M, Dwork A, Soprano DR, Makover A, Goodman DS, Zimmerman EA, Roberts JL and Schon EA (1986) Transthyretin: A choroid plexus-specific transport protein in human brain. *Neurology* 36: 900-911.

13. Hill JS and Pritchard PH (1990) Improved phenotyping of apolipoprotein E: Application to population frequency distribution. *Clin Chem* 36: 1871-1874.

14. Ida N, Hartmann T, Pantel J, Schröder J, Zerfass R, Förstl H, Sandbrink R, Masters CL and Beyreuther K (1996) Analysis of heterogeneous ßA4 peptides in human cerebrospinal fluid and blood by a newly developed sensitive western blot assay. *J Biol Chem* 271: 22908-22914.

15. Iwatsubo T, Mann DMA, Odaka A, Suzuki N and Ihara Y (1995) Amyloid ß-protein (Aß) deposition: Aß42(43) precedes Aß40 in Down's syndrome. *Ann Neurol* 37: 294-299.

16. Iwatsubo T, Odaka A, Suzuki N, Mizusawa H, Nukina N and Ihara Y (1994) Visualization of Aß42(43) and Aß40 in senile plaques with end-specific Aß monoclonals: Evidence that an initially deposited species is Aß42(43). *Neuron* 13: 45-53.

17. Jarrett JT, Berger EP and Lansbury PT Jr. (1993) The carboxyl terminus of the ß amyloid protein is critical for the seeding of amyloid formation: implications for the pathogenesis of Alzheimer's disease. *Biochemistry* 32: 4693-4697.

18. Kang J, Lemaire H-G, Unterbeck A, Salbaum JM, Masters CL, Grzeschik K-H, Multhaup G, Beyreuther K and MÜller-Hill B (1987) The precursor of Alzheimer's disease amyloid A4 protein resembles a cell-surface receptor. *Nature* 325:733-736.

19. Kass RE and Raftery AE (1995) Bayes Factors. *J Amer Statisical Assoc* 90: 773-795.

20. Kim KS, Miller DL, Sapienza VJ, Chen CM, Bai C, Grundke-Iqbal I, Currie JR and Wisniewski HM (1988) Production and characterization of monoclonal antibodies reactive to synthetic cerebrovascular amyloid peptide. *Neurosci Res Commun* 2: 121-130.

21. Lefranc D, Vermersch P, Dallongeville J, Daems-Monpeurt C, Petit H and Delacourte A (1996) Relevance of the quantification of apolipoprotein E in the cerebrospinal fluid in Alzheimer's disease. Neurosci Lett 212: 91-94.

22. Lehtimäki T, Pirttilä T, Mehta PD, Wisniewski HM, Frey H and Nikkari T (1995) Apolipoprotein E (apoE) polymorphism and its influence on apoE concentrations in the cerebrospinal fluid in Finnish patients with Alzheimer's disease. *Hum Genet* 95: 39-42.

23. Linton MF, Gish R, Hubl ST, Butler E, Esquivel C, Bry WI, Boyles JK, Wardell MR and Young SG (1991) Phenotypes of apolipoprotein B and apolipoprotein E after liver transplantation. *J Clin Invest* 88: 270-281.

24. Masters CL, Simms G, Weinman NA, Multhaup G, McDonald BL and Beyreuther K (1985) Amyloid plaque core protein in Alzheimer's disease and Down Syndrome. *Proc Natl Acad Sci USA* **82**, 4245-4249.

25. McGeer PL, Kawamata T and Walker DG (1992) Distribution of clusterin in Alzheimer brain tissue. *Brain Res* **579**, 337-341.

26. Mirra SS, Heyman A, McKeel D, Sumi SM, Crain BJ, Brownlee LM, Vogel SF, Hughes JP, van Belle G, Berg L and participating CERAD neuropathologists (1991) The consortium to establish a registry for Alzheimer's disease (CERAD). II. Standardization of the neuropathologic assessment of Alzheimer's disease. *Neurol* 41, 479-486.

27. Motter R, Vigo-Pelfrey C, Kholodenko D, Barbour R, Johnson-Wood K, Galasko D, Chang L, Miller B, Clark C, Green R, Olson D, Southwick P, Wolfert R, Munroe B, Lieberburg I, Seubert P and Schenk D (1995) Reduction of ß-amyloid peptide$_{42}$ in the cerebrospinal fluid of patients with Alzheimer's disease. *Ann Neurol* 38: 643-648.

28. Myers RH, Schaefer EJ, Wilson PW, D'Agostino R, Ordovas R, Knoefel JE, Cobb JI, McNulty KA, Beiser A and Wolf PA (1996) Apolipoprotein E epsilon4 association with dementia in a population-based study: The Framingham study. *Neurology* 46: 673-677.

29. Namba Y, Tomonaga M, Kawasaki H, Otomo E and Ikeda K (1991) Distribution of clusterin in Alzheimer brain tissue. *Brain Res* 541: 163-166.

30. Oda T, Pasinetti GM, Osterburg HH, Anderson C, Johnson SA and Finch CE (1994) Purification and characterization of brain clusterin. *Biochem Biophys Res Commun* 204: 1131-1136.

31. Palmert MR, Podlisny MB, Witker DS, Oltersdorf T, Younkin LH, Selkoe DJ and Younkin SG (1989) The ß-amyloid protein precursor of Alzheimer disease has soluble derivatives found in human brain and cerebrospinal fluid. *Proc Natl Acad Sci USA* 86: 6338-6342.

32. Pirttilä T, Kim KS, Mehta PD, Frey H and Wisniewski HM (1994) Soluble amyloid ß-protein in the cerebrospinal fluid from patients with Alzheimer's disease, vascular dementia and controls. *J Neurol Sci* 127: 90-95.

33. Podlisny MB, Mammen AL, Schlossmacher MG, Palmert MR, Younkin SG and Selkoe DJ (1990) Detection of soluble forms of the ß-amyloid precursor protein in human plasma. *Biochem Biophys Res Commun* 167: 1094-1101.

34. Poirier J, Davignon J, Bouthillier D, Kogan S, Bertrand P and Gauthier S (1993) Apolipoprotein E polymorphism and Alzheimer's disease. *Lancet* 342: 697-699.

35. Polvikoski T, Sulkava R, Haltua M, Kainulainen K, Vuorio A, Verkkoniemi A, Niinistö L, Halonen P and Kontula K (1995) Apolipoprotein E, dementia, and cortical deposition of ß-amyloid protein. *N Engl J Med* 333: 1242-1247.

36. Potempska A, Mack K, Mehta P, Kim KS and Miller DL (1997) Detection of subfentomolar amounts of Alzheimer amyloid ß peptides. *Analytical Biochem* (submitted).

37. Probst A, Brunnschweiler H, Lautenschlager C and Ulrich J (1987) A special type of senile plaque, possibly an initial stage. *Acta Neuropathol* (Berl) 74: 133-141.

38. Rebeck GW, Reiter JS, Strickland DK and Hyman BT (1993) Apolipoprotein E in sporadic Alzheimer's disease: allelic variation and receptor interactions. *Neuron* 11: 575-580.

39. Rissøen H (1988) Reduced prealbumin (transthyretin) in CSF of severely demented patients with Alzheimer's disease. *Acta Neurol* 78: 455-459.

40. Robitaille N, Cormier G, Couture R, Bouthillier D, Davignon J and Pérusse L (1996) Apolipoprotein E polymorphism in a French Canadian population of Northeastern Quebec: Allele frequencies and effects on blood lipid and lipoprotein levels. *Hum Biol* 68: 357-370.

41. Schmechel D, Saunders AM, Strittmatter WJ, Crain BJ, Hulette CM, Joo SJ, Pericak-Vance MA, Goldgaber D and Roses AD (1993) Increased amyloid-ß peptide deposition in cerebral cortex as a consequence of apolipoprotein E genotype in late-onset Alzheimer disease. *Proc Natl Acad Sci* 90: 9649-9653.

42. Schwarzman AL, Gregori L, Vitek MP, Lyubski S, Strittmatter WJ, Enghilde JJ, Bhasin R, Silverman J, Weisgraber KH, Coyle PK, Zagorski MG, Talafous J, Eisenberg M, Saunders AM, Roses AD and Goldgaber D (1994) Transthyretin sequesters amyloid ß protein and prevents amyloid formation. *Proc Natl Acad Sci* 91: 8368-8372.

43. Seubert P, Vigo-Pelfrey C, Esch F, Lee M, Dovey H, Davis D, Sinha S, Schlossmacher M, Whaley J, Swindlehurst C, McCormack R, Wolfert R, Selkoe D, Lieberburg I and Schenk D (1992) Isolation and quantification of soluble Alzheimer's ß-peptide from biological fluid. *Nature* 359:325-327.

44. Shirahama T, Skinner M, Westermark P, Rubinow A, Cohen AS, Brun A and Kemper TL (1982) Senile cerebral amyloid. Prealbumin as a common constituent in the neuritic plaque, in the neurofibrillary tangle, and in the microangiopathic lesion. *Am J Pathol* 107:41-50

45. Shoji M, Golde TE, Ghiso J, Cheung TT, Estus S, Shaffer LM, Cai X-D, McKay DM, Tintner R, Frangione B and Younkin SG (1992) Production of the Alzheimer amyloid ß protein by normal proteolytic processing. *Science* 258:126-129.

46. Sing CF and Davignon J (1985) Role of the apolipoprotein E polymorphism in determining normal plasma lipid and lipoprotein variation. Am J Hum Genet 37:268-285.

47. Strittmatter WJ, Saunders AM, Schmechel D, Pericak-Vance M, Enghild J, Salvesen GS and Roses AD (1993) Apolipoprotein E: high-avidity binding to beta-amyloid and increased frequency of type 4 allele in late-onset familial Alzheimer disease. *Proc Natl Acad Sci USA* 90: 1977-1981.

48. Tomlinson BE, Blessed G and Roth M (1968) Observations on the brains of non-demented old people. *J Neurol Sci* 7:331-356.

49. Towbin H, Stoehelin T and Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* 76:4350-4354.

50. Uchihara T, Duyckaerts C, Lazarini F, Mohtari K, Seilhean D, Amouyel P and Hauw J-J (1996) Inconstant apolipoprotein E (ApoE)-like immunoreactivity in amyloid ß protein deposits: relationship with APOE genotype in aging brain and Alzheimer's disease. *Acta Neuropathol* 92: 180-185.

51. Vigo-Pelfrey C, Lee D, Keim P, Lieberburg I and Schenk DB (1994) Characterization of ß-amyloid peptides from normal cerebral spinal fluid. *J Neurochem* 61: 1965-1968.

52. Wisniewski T, Ghiso J and Frangione B (1994) Alzheimer's disease and soluble Aß. *Neurobiol Aging* 15: 143-152.

53. Wisniewski T, Golabek A, Matsubara E, Ghiso J and Frangione B (1993) Apolipoprotein E: binding to soluble Alzheimer's beta-amyloid. *Biochem Biophys Res Commun* 192: 359-365.

54. Yamaguchi H, Hirai S, Morimatsu M, Shoji M and Ihara Y (1988) A variety of cerebral amyloid deposits in the brains of the Alzheimer-type dementia demonstrated by ß protein immunostaining. *Acta Neuropathol* 76: 541-549.

PREFACE TO CHAPTER 4

In the previous chapter we measured the CSF concentrations of proteins which can deposit as amyloid in the CP and in the cerebral cortex. We found that with the exception of a decline in Aß40 with age, the concentration of these proteins was relatively unaffected by three of the most widely acknowledged risk factors associated with AD: age, gender and apoE genotype. Thus, we concluded that the reduction of Aß42, apoE and TTR observed in the CSF of AD patients is specific to AD, and is not part of an accelerated aging process.

We hypothesized that the differences in the composition of the CSF between normal and AD patients may be reflected by differences in the composition of plaques. More specifically, we speculated that there may be detectable differences in the plaque composition which might account for the greater degree of neuronal degeneration associated with the plaques of AD subjects. The next chapter addresses this possibility by comparing the plaque composition between normal and AD brains.

CHAPTER 4.

An immunohistochemical study of plaque composition in the brains of nondemented individuals: a comparison with Alzheimer's disease.

S Kunicki, J Richardson and E Zorychta

4.1 ABSTRACT

The brain of a patient with Alzheimer's disease (AD) is characterized by the presence of numerous cortical plaques containing amyloid-ß (Aß) and dystrophic neurites, and by amyloid deposition around vessels. A smaller number of cortical Aß plaques can be observed in some nondemented elderly individuals, but they are not usually associated with neuronal degeneration. Apolipoprotein (apo) E promotes Aß neurotoxicity in vitro while apoJ and transthyretin inhibit it. We used immunohistochemical techniques to determine the distribution of the Aß variants Aß40 and Aß42 as well as apoE, apoJ and transthyretin in the plaques and vessels of normal and AD brains and to investigate whether there is a gradual change with age. We found that apoE immunoreactivity was more common in AD plaques but, as for apoJ and transthyretin, it was restricted to a small proportion of plaques in both groups of subjects. Thus, none of these proteins appear to play a central role in modulating Aß neurotoxicity in plaques. The ratio of plaques containing Aß40 to Aß42 remained constant with age, but was significantly increased in AD brains, suggesting that the greater presence of Aß40 in AD plaques is not part of an aging phenomenon. Finally, we found that cerebrovascular amyloid deposition in normal subjects was closely associated with apoE immunoreactivity, which increased with age, and not with Aß immunoreactivity, as in the AD brains.

4.2 INTRODUCTION

Numerous studies have shown that deposition of amyloid-ß (Aß) in cortical plaques and around cerebral vessels is a characteristic histopathological feature of Alzheimer's disease (AD) (44). Cortical plaques identified by immunostaining with antibodies against Aß, can be defined morphologically as either amorphous with irregular boundaries, or cored with a well-circumscribed centre that is usually, but not always, surrounded by a halo (26). Aß is a 39 to 42(43) amino acid peptide, derived from the membrane-spanning amyloid precursor protein, APP (14,28). Immunohistochemical studies have shown that the main Aß variant in plaques is Aß42, while a small proportion of plaques contain Aß40 (11,17). Both of these Aß variants have been shown to be neurotoxic in vitro (4,29,47,57).

Cortical plaques are also found in some intellectually intact elderly humans, particularly those over the age of 65, but they are fewer in number (27,31,49). Remarkably, although the plaques in nondemented individuals contain Aß40 and Aß42 (11), most plaques do not demonstrate any detectable degree of neuronal degeneration (43,55). This is in contrast to AD plaques, in which swollen neurites are a common trait (20,31). In fact, one of the distinguishing features of AD brains is that the dystrophic neurites in plaques are readily visualized by antibodies to tau, while plaques in normal brains rarely contain tau immunoreactivity (2,9). Abnormal hyperphosphorylation of tau in degenerating neurites may explain the selective

immunostaining of AD plaques (18).

The greater degree of neuronal injury associated with AD plaques has not been explained. A number of proteins can modulate Aß neurotoxicity in vitro, including apoE, apoJ and TTR, all of which have been identified in the plaques of AD brains (30,34,46,51). ApoE enhances Aß neurotoxicity in vitro by accelerating amyloid fibril formation (23,53). In contrast, apoJ attenuates the neurotoxicity of Aß40 (3) and both apoJ and TTR inhibit amyloid formation by Aß (35,42). Cystatin C, an inhibitor of the cysteine protease cathepsin B (1), has also been localized, along with APP in AD plaques (5,15). Cathepsin B may cleave APP inside the Aß domain (41), and inhibiting this enzyme could therefore increase the level of intact Aß.

It has yet to be resolved whether or not these Aß-associated proteins are a constant feature of plaques and thus their role in neurodegeneration within the plaques remains to be established. We therefore wanted to determine whether proteins which promote Aß toxicity are more common in AD plaques and whether proteins which attenuate this toxicity are more common in the plaques of normal brains. To investigate this hypothesis, the density of plaques containing apoE, apoJ, TTR and cystatin C was determined in the frontal cortex and hippocampus from nondemented persons, aged 14 to 90 years, and in AD patients, aged 76 to 94 years. A wide age range of nondemented individuals was included in order to determine the earliest age at which plaques could be detected using newly-developed, highly sensitive

anti-Aß40 and anti-Aß42 antibodies, and to determine if there is an evolution from Aß42 to Aß40 in plaques with age, as there may be in Down's Syndrome (16,17). Based on their morphology following immunostaining, plaques were classified as either amorphous or cored. Since it has been suggested that the cored plaques represent a more mature plaque form (24), we wanted to test whether the composition of the amorphous plaques differs from that of the cored plaques, and whether or not their relative progression changes with age.

Cerebral blood vessels were also examined since Aß is often deposited both as amyloid and as soluble Aß in the vasculature of AD brains and, to a lesser extent, in normal individuals (50,52). Both Aß40 and Aß42 have been identified around cerebral vessels in AD, although it has not yet been established which of these two Aß variants is the major component (26,40). The Aß variant which deposits around cerebral vessels in normal aged individuals is unknown.

4.3 MATERIALS AND METHODS

4.3.1 Tissue

Cortical blocks from 27 nondemented individuals and 7 AD patients were obtained from the Montreal General Hospital and the Montreal Neurological Institute. The mean age (and standard deviation) of control and AD subjects was 63 ± 19 and 85.6 ± 6.6 years respectively, with a mean postmortem delay of 16 ± 7 hours. Controls were free of any reported

dementia while AD cases had a clinical history of dementia and a diagnosis of AD was confirmed postmortem (31). Although tissue was obtained from a random sampling of autopsies, most control subjects were male and all AD subjects were female. Cases with a history of cerebral hemorrhage, stroke or other illness related to the central nervous system (CNS) were not included in the study, nor were those involving head injuries, which have been associated with diffuse Aß cerebral deposits at postmortem examination (39). The most common immediate causes of death were cardiac failure (n=14) and carcinoma, without CNS involvement (n=6). A summary of the age and gender distribution is provided in Figure 4.1.

4.3.2 Histochemistry

For histochemical analysis, portions of the frontal cortex (Brodmann areas 9/10) and the hippocampus (transverse section through the body of the hippocampus) from all subjects were fixed in 10% formalin at room temperature, embedded in paraffin and serially sectioned at 5 μm. Tissue sections were stained with alkaline Congo red dye to identify amyloid, with a modified methenamine silver technique, and with a variety of antibodies described below. The modified methenamine silver technique has been shown to be an effective method to identify plaques and neurofibrillary tangles (10).

4.3.3 Immunohistochemistry

Frontal cortical and hippocampal sections from all cases were reacted with a monoclonal antibody against amino acids 8-17 of Aß (Dako m872, 1:50) and with rabbit polyclonal antibodies against Aß40 (R162) and Aß42 (R164), at a dilution of 1:100. R162 and R164 were gifts from Dr. P. Mehta and are characterized elsewhere (38). Monoclonal antibodies against apoE (Boehringer Mannheim, 14 μg/ml), apoJ (Quidel, 1:35) and tau (Sigma, 1:50) as well as rabbit polyclonal antibodies against TTR and cystatin C (Dako, 1:375) were employed.

To enhance immunostaining, tissues were immersed in 90% formic acid for 30 to 60 minutes before exposure to all antibodies except anti-tau (21). They were subsequently washed in 0.1 M tris-buffered saline (pH 7.2) for three hours and exposed to the primary antibodies overnight at 4°C. Immunohistochemical staining was performed following manufacturers instructions using an Omnitags avidin/biotin kit, with 3-amino-9-ethylcarbazole as the chromogen (Immunon, Fisher), which produces a red reaction product. Sections were lightly counterstained with hematoxylin. Control sections were incubated with the secondary but not the primary antibody. In addition, preabsorption of R164 with Aß42, but not Aß40, resulted in a loss of staining as did preabsorption of R162 with Aß40, but not with Aß42. Each slide was examined without prior knowledge of case history.

The density of both amorphous and cored plaques detected by each

method was determined by counting plaques at a magnification of x125 using an ocular with a ruled graticule of one square millimetre. For each slide, plaques were counted in ten consecutive fields of one square millimetre and the average count obtained was reported as the plaque count for that slide. Each of the ten fields selected contained only grey matter. For hippocampal sections, plaques were counted in Ammon's horn, the dentate gyrus, areas CA1 and CA2 and the subiculum. At the same time, the mean area of plaques was determined using the image analysis package NIH Image 1.44 for the Macintosh. The area of each plaque was calculated for the first 20 consecutive plaques. The mean value was recorded for each tissue section.

4.3.4 Statistics

To determine if there were significant differences between the numbers of plaques stained by each of the techniques, a one-way analysis of variance (ANOVA) was performed for both control and AD groups. This was followed by paired t-tests, with two-tailed p values reported. To determine whether the incidence of plaques (i.e. the likelihood of finding a plaque) increased with age in normal cases, linear regression analysis was performed, with age entered as a continuous variable. Paired t-tests were used to compare frontal and hippocampal regions and to compare the number of cored and amorphous plaques. Two-sample t-tests were used to compare plaque composition between control and AD brains. A p value of 0.05 or less was considered

significant. In those cases where the number of observations was small, nonparametric Wilcoxon and Mann-Whitney tests were employed in addition to the paired and two-sample t-tests. Comparable results were obtained with parametric and nonparametric analysis and so only the results from parametric analysis are presented. All analyses were performed using Systat version 6.0.1.

4.4 RESULTS

4.4.1 Characterization of plaques in nondemented controls

4.4.1.1 General Features

Aß-immunoreactive plaques were identified in nine of the 27 nondemented subjects in both the frontal and hippocampal cortical sections (Figure 4.1). The remaining 18 cases were free of any plaques. In the nine cases, silver-stained plaques were detected in all sections except in the frontal cortex of a 75 year old, which demonstrated only Aß-positive plaques. The anti-tau antibody stained plaques in the hippocampus of two of the nine cases. In the first case (80 year old), six tau-immunopositive plaques clustered in an area of about two square millimetres and in the second case (89 year old), one tau-immunoreactive plaque was detected in the entire hippocampal section of over two square centimetres. Congo red staining detected sparse amyloid deposits in plaques in one of the nine cases, the 80 year old described above.

The R164 antiserum consistently labelled a greater number of plaques

than either of the two other antibodies against Aß, indicating that Aß42 is the predominant Aß variant (Figure 4.2). The ratio of R164-positive to R162-positive plaques was 2.2 in the frontal cortex and 3.0 in the hippocampus. Epitopes of apoJ, TTR and cystatin C were present in plaques in a minority of the brains, in contrast to apoE, which was found in most cases (Table 4.1). However, in all cases, the antibodies to these proteins detected significantly fewer plaques than did the antibodies against Aß, and the staining was usually not as robust (Figure 4.3).

All anti-Aß antibodies identified cored plaques, but these plaques represented only a small fraction of the total number of plaques seen in each section, the vast majority of plaques were amorphous. The number of cored plaques detected by R164, R162 or m872 was not significantly different, in either the frontal cortex or hippocampus, implying that plaque cores contain both Aß42 and Aß40. This is in contrast to amorphous plaques in which Aß42 is the predominant Aß variant (Figure 4.2). Aß antibodies labelled about ten times more cored plaques than the anti-apoE antibody, indicating that apoE is not a significant component of plaque cores. The proportion of cored apoE plaques was 10% in the frontal cortex and 33% in the hippocampus.

The only notable differences observed between the frontal cortex and hippocampus were the greater number of R164 ($p=0.055$) and R162 ($p=0.044$) cored plaques in the frontal cortex (Figure 4.2) and the greater prevalence of epitopes for apoJ, TTR and cystatin C in the hippocampus (Table

4.1). The density of plaques detected by antibodies to Aß or apoE, or by the silver stain was comparable in the frontal cortex and hippocampus as was the average size of R164-positive and of R162-positive plaques (Table 4.2).

4.4.1.2 Age-Related Changes

The youngest individual to demonstrate plaques was a 53 year old, with numerous (12/mm^2) Aß-positive plaques. The mean age of subjects with plaques (70 years), was significantly greater ($p = 0.016$) than the mean age of subjects without plaques (55 years). However, the actual number of plaques detected by the three anti-Aß antibodies or by silver staining did not increase with age in the nine subjects (53 to 90 years) and the composition of plaques, with respect to the Aß variants, also remained constant with age. Since the plaque density and the ratio of R164-positive to R162-positive plaques did not vary with age, it appears that Aß42 plaques do not evolve with time into Aß40 plaques. In addition, the size of R164 and R162 plaques was comparable, and it also remained constant with age.

In contrast to plaque composition, the morphology of the plaques did appear to change with age. Although the amorphous plaque was found to be the most common at all ages, the number of R164 plaques that were amorphous decreased with age while the number of cored plaques increased in both the frontal cortex ($p = 0.025$) and hippocampus ($p = 0.014$). No such trend was observed with R162 or m872 antibodies.

4.4.2 Plaques in AD brains

4.4.2.1 General Features

The R164 antiserum identified a greater number of plaques than either the R162 antiserum or m872 (Figure 4.2). The ratio of R164 to R162 plaques was 1.5 in the frontal cortex and 1.6 in the hippocampus. ApoE-positive plaques were found in all AD sections, but like apoJ, TTR and cystatin C, apoE was found in only a small number of plaques (Table 4.1).

In the frontal cortex, Aß42 was more prominent in both cored and amorphous plaques than Aß40 as R164 detected greater numbers of both types of plaques than did R162. In contrast, in the hippocampus the two Aß variants appeared in an equal number of cored plaques, but antibodies against Aß40 detected fewer amorphous plaques (Figure 4.2). The fraction of apoE plaques that were cored was 34% and 9% for the frontal cortex and hippocampus respectively.

4.4.3 A comparison of plaques in normal and AD brains

In addition to differences in the density of plaques (Figure 4.2), qualitative differences in plaque composition and morphology were observed between nondemented controls and AD subjects. The most obvious difference was that tau-immunoreactive plaques were identified in every AD section examined as were numerous plaques containing amyloid deposits, in contrast to brains from nondemented subjects, in whom these plaques were rare.

Moreover, plaques immunoreactive for apoE, apoJ, TTR and cystatin C were more commonly found in AD brains (Table 4.2). In addition, the proportion of (R164) plaques which had a central core was greater in both the frontal ($p = 0.028$) and hippocampal ($p = 0.012$) sections of AD brains than in normal brains. The plaque size (R164) in AD brains was greater than in normal brains, in both the frontal cortex ($p = 0.034$) and hippocampus ($p = 0.033$) (Table 4.2).

4.4.4 Vessels.

In normal individuals, the protein most consistently observed around cerebral vessels was apoE, which was present in eight of the nine normal cases with plaques and in 11 of the 18 cases without plaques (Figure 4.4). In contrast to all other proteins, apoE immunoreactivity increased with age ($p = 0.036$), although this increase was restricted to the hippocampus ($n = 13/27$). R164 immunoreactivity, which was more prominent around vessels than R162 immunoreactivity, was present in five of the nine cases with cortical plaques and in six of the 18 cases which were free of any plaques.

Congo red-positive amyloid appeared first around vessels in the hippocampus of a 53 year old and later in the frontal cortex of a 61 year old. In total, amyloid angiopathy was present in the frontal cortex of five and in the hippocampus of six normal subjects. Surprisingly, in cases with amyloid angiopathy, Aß immunoreactivity was frequently absent, while apoE immunoreactivity was always present (Figure 4.5). Amyloid deposition around

vessels in the frontal cortex was found only in those individuals with cortical plaques, but was present in the hippocampus of two individuals that were free of plaques.

A different pattern emerged in the cortex of AD patients. Amyloid deposition was observed around blood vessels in the majority of AD cases (Figure 4.4), and when present, was always accompanied by Aß immunoreactivity (Figure 4.5). In contrast, apoE immunoreactivity was absent from two cases in which amyloid angiopathy was observed.

As in nondemented individuals, the predominant Aß species around vessels in AD subjects was Aß42. However, infiltration of Aß immunoreactivity into the surrounding neuropil (dysphoric angiopathy) was present in some AD cases, but never in normal subjects. These Aß immunoreactive areas were not counted as plaques.

Figure 4.1 The age and gender distribution is indicated for nondemented subjects, including those with (closed circle) and those without (open circle) cortical plaques, and for AD subjects (triangles).

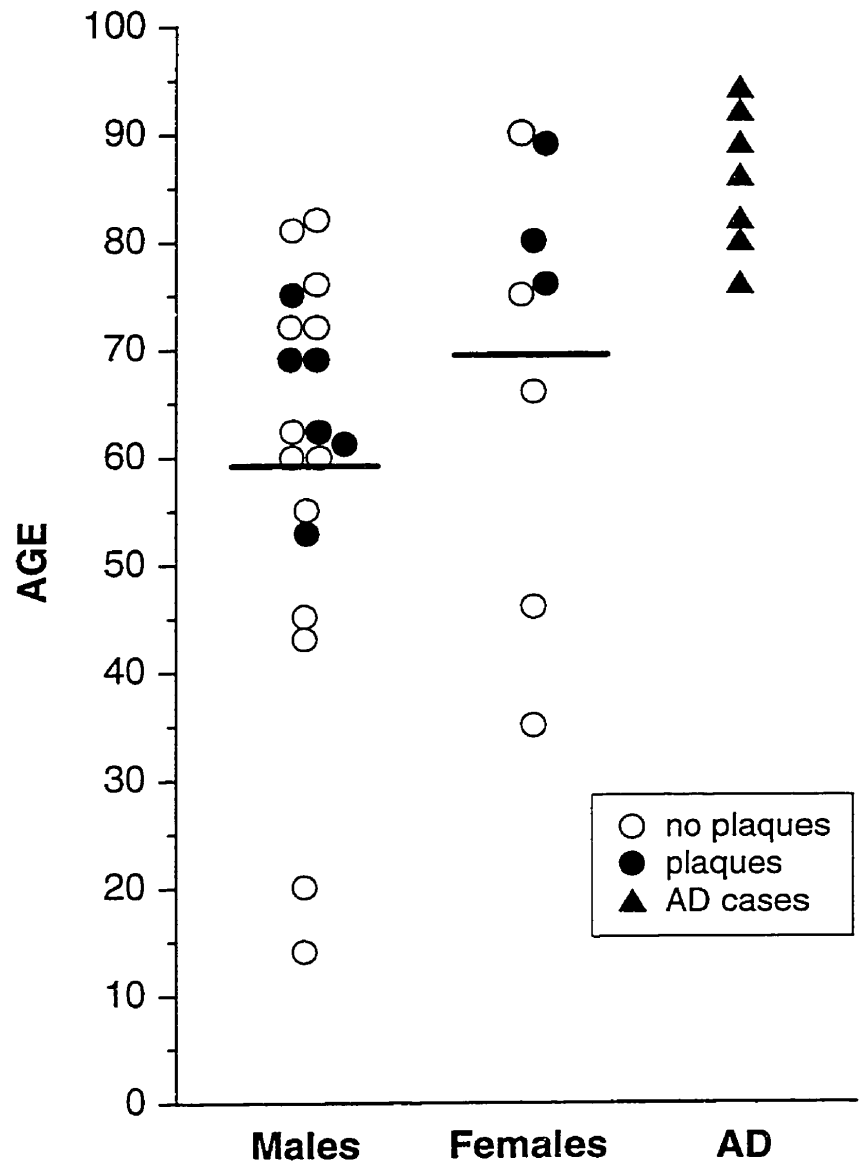
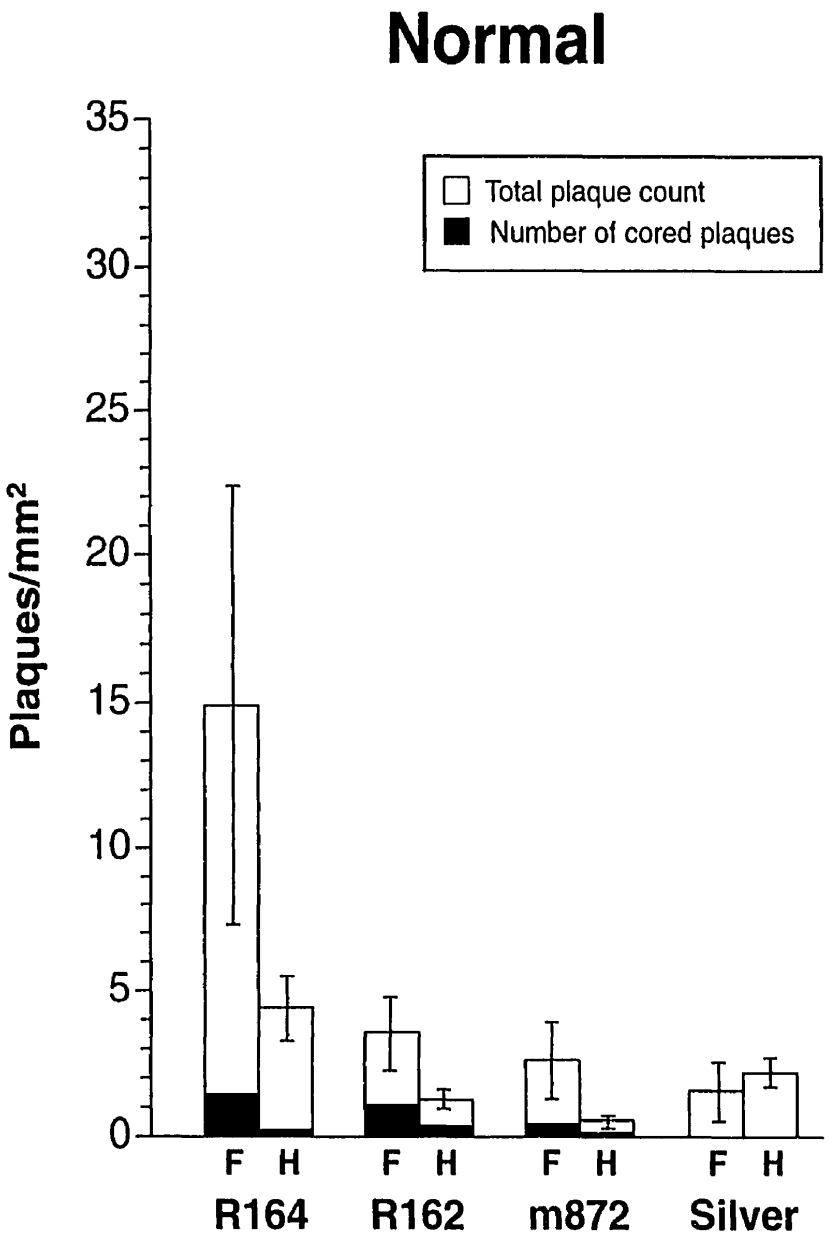

100
90
80
70
60
50
40
30
20
10
0
AGE
Males
Females
AD
no plaques
plaques
AD cases

Figure 4.2 Comparison between the number of plaques per square millimetre in the frontal cortex (F) and hippocampus (H) of nine normal and seven AD brains. Serial sections were reacted with rabbit antisera against Aß42 (R164) and Aß40 (R162) and with a monoclonal antibody against amino acids 8-17 of Aß (m872) and by a modification of the methenamine silver technique (Silver). The number of Aß-positive plaques in which a central core could be identified is indicated by solid boxes. The mean number of plaques detected in both the frontal and hippocampal sections was significantly greater in the AD sections ($n=7$) than in cortical and hippocampal sections from nondemented individuals ($n=9$) for R162 ($p<0.005$), R164 ($p<0.005$) and m872 ($p<0.01$). Bars indicate standard deviation.


Normal
Total plaque count
Number of cored plaques
Plaques/mm²
35
30
25
20
15
10
5
0
F H
R164
F H
R162
F H
m872
F H
Silver

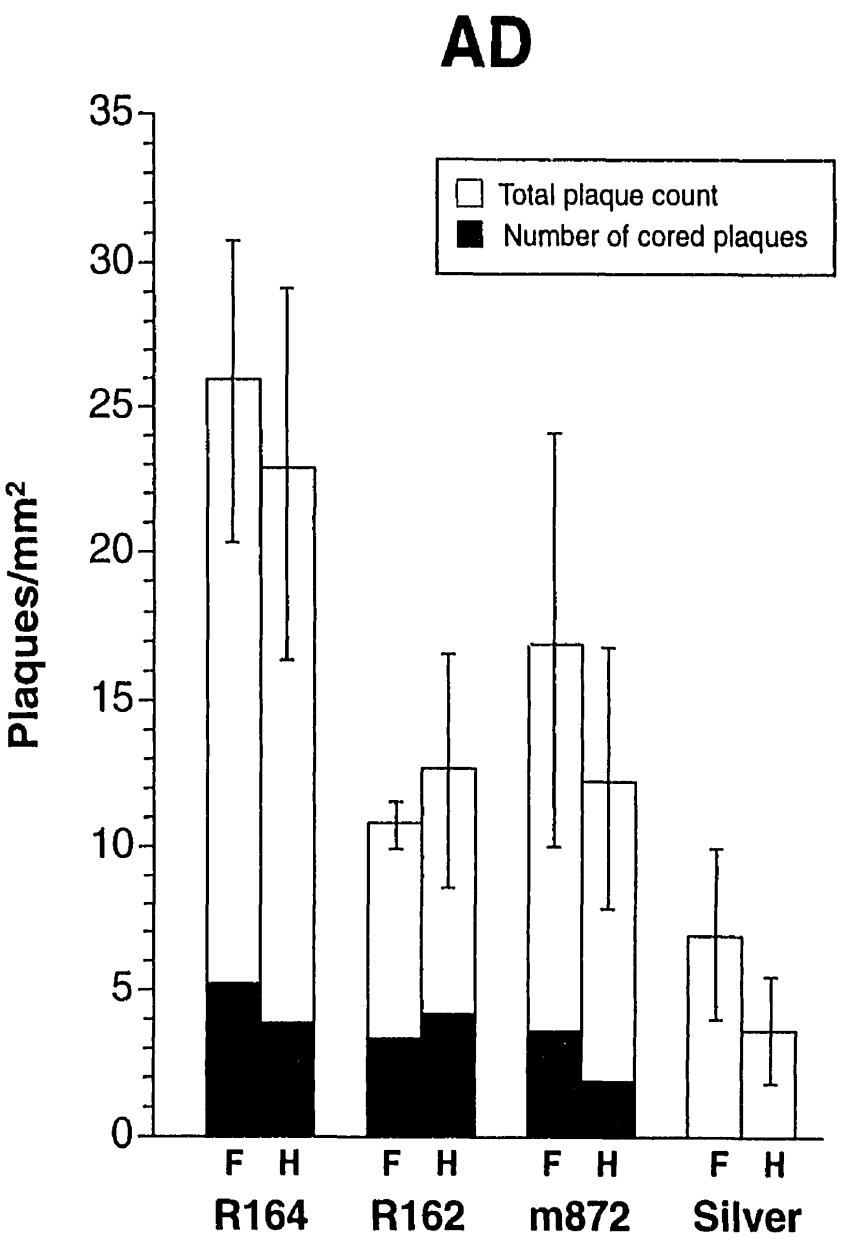

AD
Total plaque count
Number of cored plaques
Plaques/mm²
35
30
25
20
15
10
5
0
F H
R164
F H
R162
F H
m872
F H
Silver

Figure 4.3 Immunohistochemical staining characteristics of cortical plaques in the hippocampus of a normal 69 year old. The rabbit sera R164 (A), R162 (B) stained numerous plaques, compared to antibodies against apoE (C) and cystatin (D). Both cored plaques (arrows) and amorphous plaques (arrow heads) can be seen. Immunopositive protein is visualized as red 3-amino-9-ethylcarbazole reaction products. All sections were counterstained with hematoxylin. Original magnification is x 250.

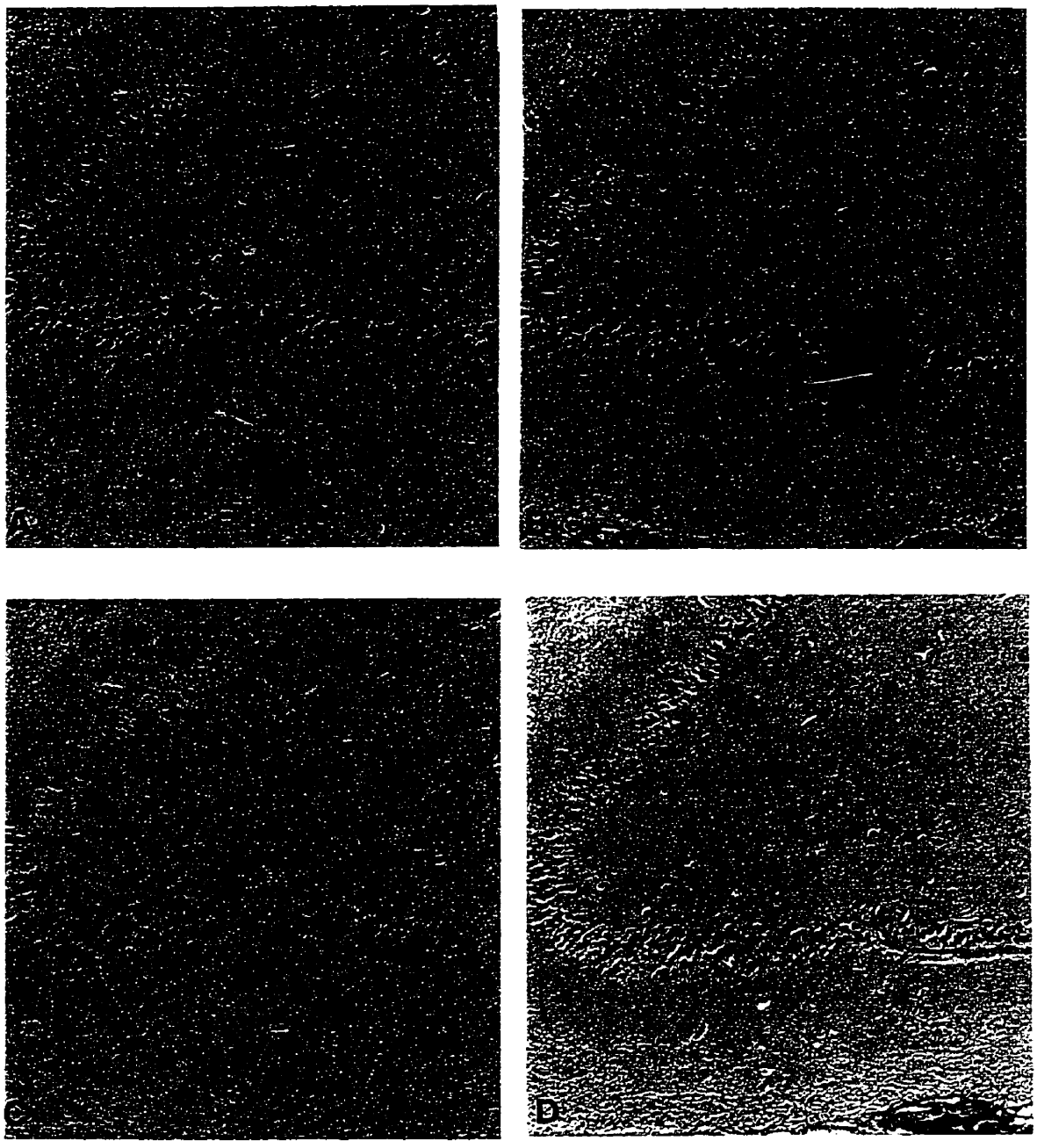

A
B
C
D

Figure 4.4 The presence of Congo red (CR)-positive deposits and immunoreactivity around cerebral vessels in nondemented controls (n = 27) and AD subjects (n = 9). The percentage of positive cases refers to the percent of sections in which amyloid deposition or immunoreactivity was observed around vessels. Aß immunoreactivity is reported for rabbit polyclonal antibodies specific for Aß42 (R164) and Aß40 (R162).

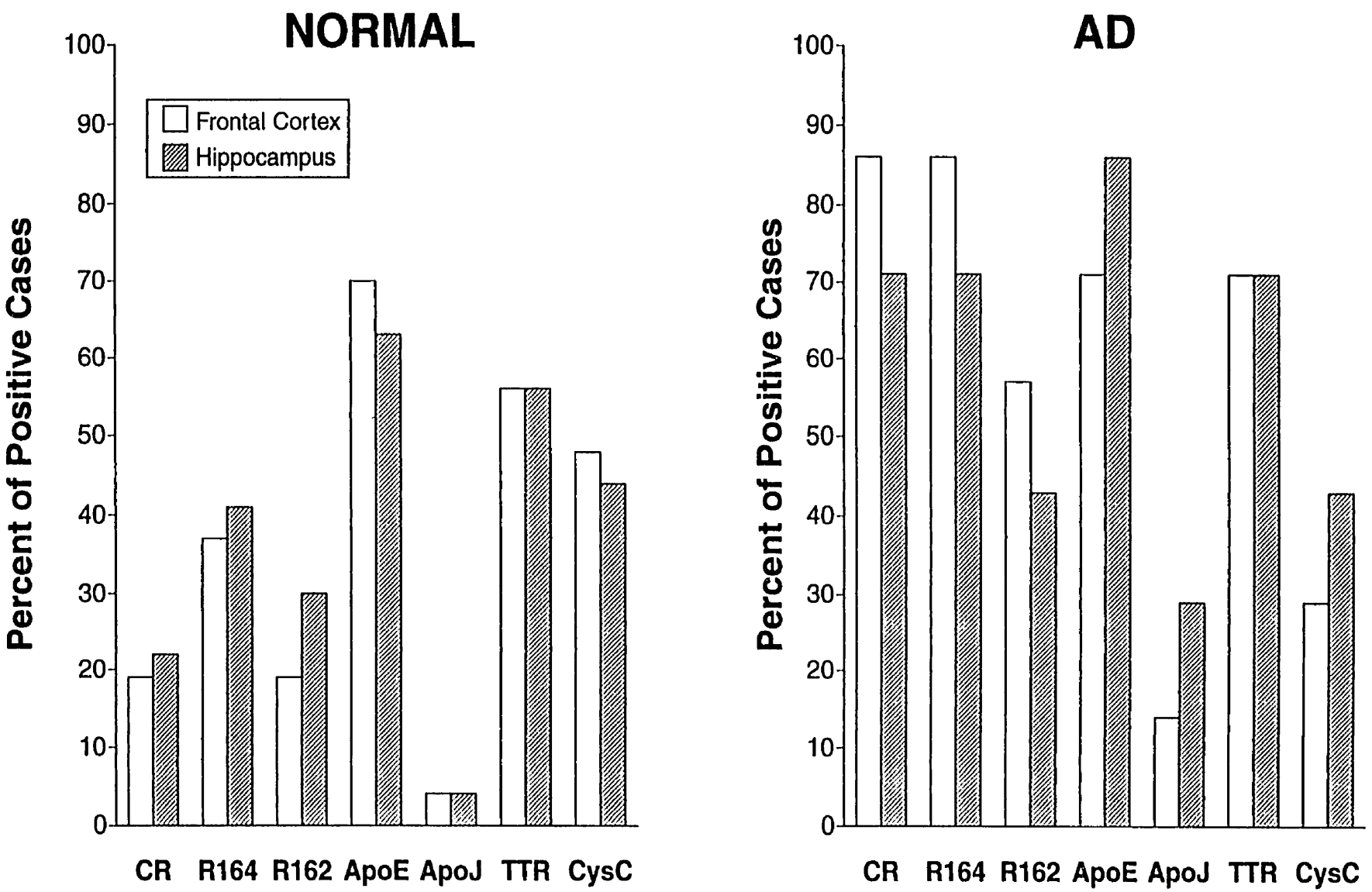
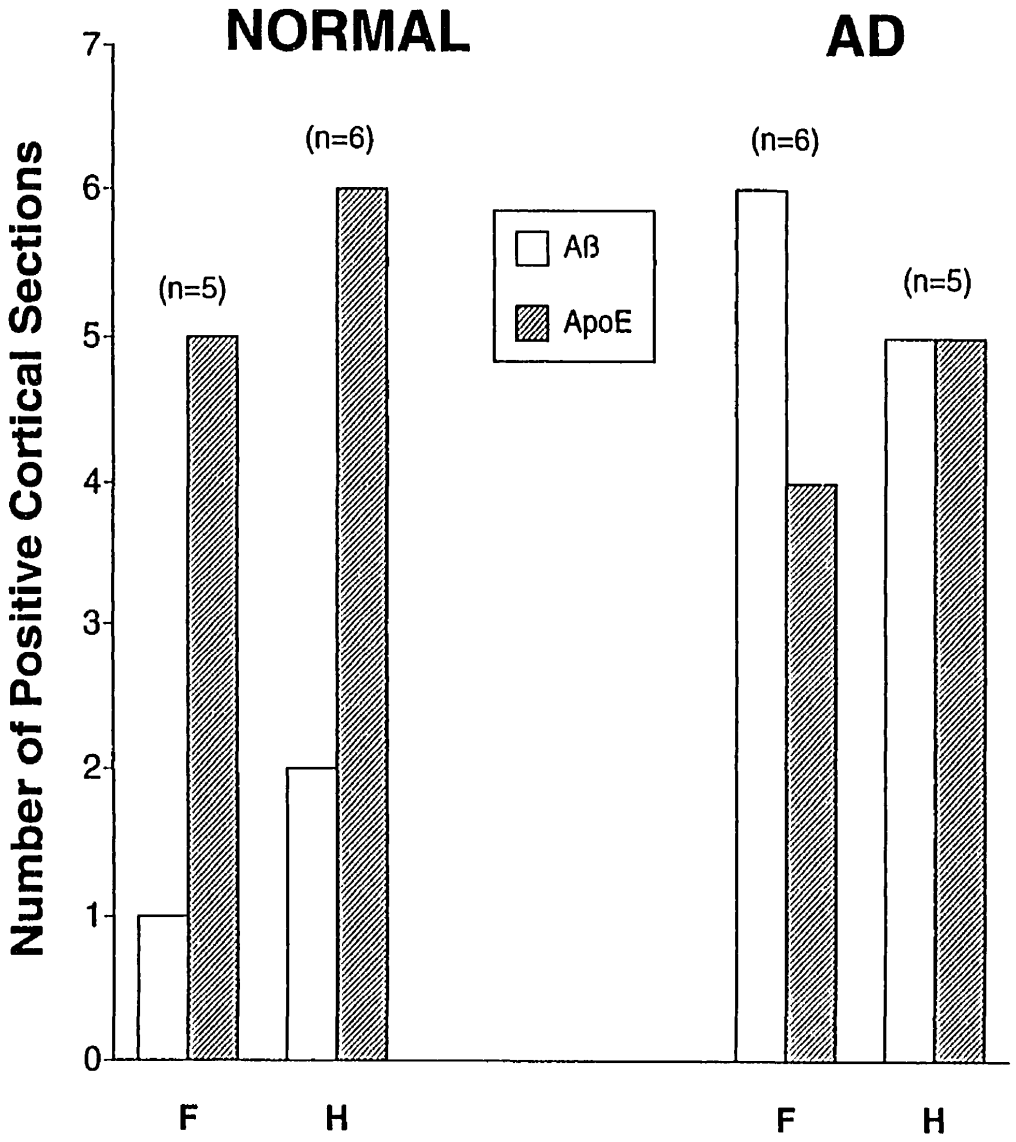

NORMAL
AD
Frontal Cortex
Hippocampus
Percent of Positive Cases
100
90
80
70
60
50
40
30
20
10
0
CR
R164
R162
ApoE
ApoJ
TTR
CysC

Figure 4.5 A comparison between amyloid-ß (Aß) and apolipoprotein E (apoE) immunoreactivity in sections demonstrating congophilic angiopathy. The presence of apoE immunoreactivity is linked to amyloid deposition in nondemented individuals, while Aß (either Aß42 or Aß40) immunoreactivity is strongly associated with vascular amyloid deposition in AD patients. The number of frontal (F) and hippocampal (H) sections in which vascular amyloid deposition was noted is indicated in parentheses above the vertical bars.


NORMAL
AD
Number of Positive Cortical Sections
7
6
5
4
3
2
1
0
(n=5)
(n=6)
(n=6)
(n=5)
Aß
ApoE
F
H
F
H

Case	Antibody			
	ApoE	ApoJ	TTR	Cystatin C
Normal				
Frontal cortex	0.48 ± 0.39 (7)	0.08 ± 0.02 (3)	0.09 (1)	0.11 ± 0.04 (3)
Hippocampus	0.18 ± 0.04 (8)	0.04 ± 0.02 (4)	0.02 ± 0.002 (4)	0.05 ± 0.02 (5)
AD				
Frontal cortex	0.64 ± 0.17 (7)	0.62 ± 0.54 (4)	0.36 ± 0.27 (2)	0.17 ± 0.04 (6)
Hippocampus	0.93 ± 0.37 (7)	0.04 ± 0.02 (5)	0.10 ± 0.05 (4)	0.34 ± 0.16 (4)

Table 4.1 The number of plaques per square millimetre identified in frontal and hippocampal sections by antibodies against apolipoprotein E (apoE), apoJ, transthyretin (TTR) and cystatin C in nondemented individuals (n = 9) and in AD subjects (n = 7). The mean and standard deviations are reported. Parentheses indicate the number of cases in which the respective immunoreactivity was observed.

	Plaque Size (μm^2)	
	R164	R162
Normal Frontal Cortex	163 ± 70	347 ± 464
Normal Hippocampus	198 ± 62	187 ± 44
AD Frontal Cortex	288 ± 138*	380 ± 224
AD Hippocampus	364 ± 274**	316 ± 244

Table 4.2 Comparison of plaque sizes (± S.D) detected by antibodies against Aß42 (R164) and Aß40 (R162). The average sizes of R164 plaques in both the frontal cortex and hippocampus are significantly greater in AD patients than in normal subjects (*p=0.034; **p=0.033).

4.5 DISCUSSION

The histological characteristics and chemical composition of plaques have been studied extensively in the AD brain which provides an abundant source, having typically more than 1400 plaques per cubic millimetre (11,17,45). In comparison, the composition of plaques in the brains of nondemented individuals has been less well documented. We speculated that the increased neuronal damage observed in AD plaques compared to plaques control brains may be associated with differences in plaque composition between these two populations. The aim of the present study was to determine if such differences could be detected and whether changes in plaque composition also occur with age in our group of nondemented individuals, as it may in Down's syndrome (16,17).

We found that most plaques in both AD and nondemented brains are amorphous and that the principal Aß peptide species in these plaques is Aß42. However, the ratio of Aß40 to Aß42 plaques was greater in AD brains. Our results confirm earlier reports on plaques in normal (11) and AD brains (16,17). In addition, we found that both plaque density and the ratio of Aß40- to Aß42-immunopositive plaques remained constant with age, indicating that a gradual accumulation of Aß40 with time does not occur in nondemented individuals.

A small proportion of plaques in both normal and AD brains had a core of Aß immunoreactive material at their centre. In nondemented subjects these plaques were labelled equally well by the R164 and R162 antisera, indicating

that both Aß42 and Aß40 are present in the plaque core. The number of Aß42-positive plaques which had a central core increased with age both in the frontal and hippocampal sections, suggesting that there is a progressive change in the morphology of plaques with age. Although the significance of this change is unclear, it is interesting that the cored plaque is thought to be a more mature plaque form (24). Moreover, the proportion of (Aß42) plaques which had a central core was more than doubled in AD brains compared to normal brains. This suggests that a greater proportion of AD plaques have "matured". Together these results indicate that a gradual change in plaque morphology may be a natural component of aging, in contrast to the accumulation of Aß40 in plaques, which may be more specific to AD.

Both Aß40 and Aß42 are neurotoxic (4,29,47,57), yet plaques in normal brains generally do not contain the dystrophic neurites which are typical of AD plaques (2,9,48,55). We therefore examined plaques for the presence of proteins which can modulate Aß toxicity in vitro. Immunoreactivity for apoE, apoJ, TTR and cystatin C was observed in brain sections from both AD and normal individuals, but appeared to be confined to only a small fraction of plaques.

While some researchers have indicated that apoE immunoreactivity is common in AD plaques (56), others have shown that apoE immunoreactivity is present in only a small number of AD plaques (12,51). In the present study, apoE immunoreactivity in plaques was observed in most control brains and in

all AD brains. However, in both groups, the number of plaques labelled by the anti-apoE antibody was only a small fraction of the total number of Aß plaques. In addition, only about 20% of apoE plaques were cored in either normal or AD brains. These findings suggest that Aß can accumulate in plaques, including those with cores, in the absence of apoE and that apoE does not contribute significantly to neurodegeneration by promoting Aß neurotoxicity in plaques.

Both apoJ and TTR have previously been identified in the AD brain (30,46), but it is not clear from these studies how often they present in AD plaques and whether they are also present in cortical plaques of normal individuals. By quantifying the plaque densities, we were able to directly demonstrate that both apoJ and TTR are present in normal and AD brains, but they are restricted to a very small subset of cortical plaques in both groups. The significance of apoJ and TTR in these plaques is unclear. However, since both proteins bind Aß in the CSF and inhibit aggregation of Aß in vitro (3,42), their presence in Aß plaques may have more to do with a failed attempt to remove Aß from the brain than a direct role in plaque formation.

Cystatin C was found in the plaques of brains from normals and AD subjects, although again, only in a small number of plaques. Cystatin C could theoretically promote Aß accumulation by blocking enzymatic cleavage of APP within the Aß domain (41), through inhibition of cathepsin B (1). However, although APP is found in plaques (36,37), the infrequent occurrence of

cystatin C indicates that this mechanism does not contribute significantly to the accumulation of Aß within the plaques.

The densities of Aß plaques in the subjects with AD and those without dementia showed considerable overlap in this study, suggesting the possibility that some normal subjects with plaques had incipient AD. However, while plaques containing amyloid and tau-immunoreactive neurites were present in every AD section, these plaques were rarely found in the normal brains. In addition, a number of postmortem studies conducted on nondemented individuals who were being clinically monitored as part of a prospective study of aging, have reported plaque densities well in excess of what was observed in our group of nondemented individuals, in the absence of any cognitive impairment (6,7,8,11,19,33). Thus numerous plaques, particularly those unassociated with tau immunoreactive neurites, can be observed in clinically normal individuals.

Both Aß40 and Aß42 have been found in the cerebral vessels of AD patients, but some controversy exists as to which variant is more common (32,40). Our study found that, as in cortical plaques, Aß42 is the predominant form around vessels in both nondemented and AD subjects. This prompted us to test whether or not other plaque associated proteins are also present around vessels.

ApoE immunoreactivity was very common around vessels in normal and AD brains. However, in contrast to AD brains, apoE immunoreactivity was

more prominent than Aß immunoreactivity in control brains and was more closely linked to amyloid deposition than was Aß immunoreactivity (see Figure 4.5). Furthermore, we found apoE immunoreactivity became increasingly frequent with age in nondemented individuals. Since the prevalence of amyloid angiopathy also increases with age (50,52), it may be that apoE contributes to amyloid formation around cerebral vessels. We have previously shown that apoE is present in the amyloid deposits in the choroid plexus (22) and a recombinant apoE fragment has been found to form amyloid fibrils in vitro (54). Together, these results indicate that apoE may be another source of amyloid in the brain.

TTR (46) and cystatin C (13) have been identified in cerebral vessels of patients with AD and with hereditary cerebral hemorrhage of the Icelandic type respectively, and we show here that they are also found around vessels of normal individuals. The observation that many of the proteins found in plaques are also present around cerebral vessels, suggests a common pathway leading to amyloid deposition at both sites.

The recent development of sensitive antibodies specific for different Aß species has provided a powerful tool to help define plaque composition, particularly in non-AD cases in which the number of plaques, and consequently the amount of protein deposited in the brain, may be insufficient for biochemical analysis. Our study provides no evidence to suggest that in nondemented individuals Aß42 plaques evolve into Aß40 plaques. In addition, despite the reported in vitro effects of apoE on Aß, apoE does not appear to

play a direct role in plaque formation in either AD or nondemented individuals.

4.6 ACKNOWLEDGEMENTS

The authors greatfully acknowledge the gift of antibodies from Dr. P.D. Mehta of *The Institute for Basic Research in Developmental Disabilities*, New York, NY, USA.

4.7 REFERENCES

1. Abrahamson M, Barrett AJ, Salvesen G and Grubb A (1986) Isolation of six proteinase inhibitors from human urine. *J Biol Chem* 261: 11282-11289.

2. Barcikowska M, Wisniewski HM, Bancher C and Grundke-Iqbal I (1989) About the presence of paired helical filaments in dystrophic neurites participating in the plaque formation. *Acta Neuropathol* 78: 225-231.

3. Boggs LN, Fuson KS, Baez M, Churgay L, McClure D, Becker G and May PC (1996) Clusterin (Apo J) protects against amyloid-beta (1-40) neurotoxicity. *J Neurochem* 67: 1324-1327.

4. Busciglio J, Gabuzda DH, Matsudaira P and Yankner BA (1993) Generation of ß-amyloid in the secretory pathway in neuronal and nonneuronal cells. *Proc Natl Acad Sci USA* 90: 2092-2096.

5. Cole GM, Masliah E, Shelton ER, Chan HW, Terry RD and Saitoh T (1990) Accumulation of amyloid precursor fragment in Alzheimer plaques. *Neurobiol Aging* 12: 85-91.

6. Crystal H, Dickson D, Fuld P, Masur D, Scott R, Mehler M, Masdeu J, Kawas C, Aronson M and Wolfson L (1988) Clinico-pathologic studies in dementia: nondemented subjects with pathologically confirmed Alzheimer's disease. *Neurology* 38: 1682-1687.

7. Delaère P, Duyckaerts C, Masters C, Beyreuther K, Piette F and Hauw J-J (1990) Large amounts of neocortical ßA4 deposits without neuritic plaques or tangles in a psychometrically assessed, non-demented person. *Neurosci Lett* 116: 87-93.

8. Dickson DW, Crystal HA, Mattiace LA, Masur DM, Blau AD, Davies P, Yen S-H and Aronson MK (1991) Identification of normal and pathological aging in prospectively studied nondemented elderly humans. *Neurobiol Aging* 13: 179-189.

9. Flament S, Delacourte A, Delaère P, Duyckaerts C and Hauw J-J (1990) Correlation between microscopical changes and tau 64 and 69 biochemical detection in senile dementia of the Alzheimer type. *Acta Neuropathol* 80: 212-215.

10. Flowers D, Harasty J, Halliday G and Kril J (1996) Microwave modification of the methenamine silver technique for demonstration of Alzheimer-type pathology. *J Histotechnol* 19: 33-38.

11. Fukumoto H, Asami-Odaka A, Suzuki N, Shimada H, Ihara Y and Iwatsubo T (1996) Amyloid ß protein deposition in normal aging has the same characteristics as that in Alzheimer's disease. Predominance of Aß42(43) and association of Aß40 with cored plaques. *Am J Pathol* 148: 259-265.

12. Gearing M, Schneider JA, Robbins RS, Hollister RD, Mori H, Games D, Hyman BT and Mirra SS (1995) Regional variation in the distribution of apolipoprotein E and Aß in Alzheimer's disease. *J Neuropathol Exp Neurol* 54: 833-841

13. Ghiso J, Jensson O and Frangione B (1986) Amyloid fibril in hereditary cerebral hemorrhage with amyloidosis of Icelandic type is a variant of τ-trace basic protein (cystatin C). *Proc Natl Acad Sci* USA 83: 2974-2978.

14. Glenner GG and Wong CW (1984) Alzheimer's disease: initial report of the purification and characterization of a novel cerebrovascular amyloid protein. *Biochem Biophys Res Comm* 120: 885-890.

15. Ii K, Ito K, Kominami E and Hirano A (1993) Abnormal distribution of cathepsin proteases and endogenous inhibitors (cystatins) in the hippocampus of patients with Alzheimer's disease, parkinsonism-dementia complex in Guam, and senile dementia in the aged. *Virchows Arch Pathol Anat* 423: 185-194.

16. Iwatsubo T, Mann DMA, Odaka A, Suzuki N and Ihara Y (1995) Amyloid ß-protein (Aß) deposition: Aß42(43) precedes Aß40 in Down's syndrome. *Ann Neurol* 37: 294-299.

17. Iwatsubo T, Odaka A, Suzuki N, Mizusawa H, Nukina N and Ihara Y (1994) Visualization of Aß42(43) and Aß40 in senile plaques with end-specific Aß monoclonals: Evidence that an initially deposited species is Aß42(43). *Neuron* 13: 45-53.

18. Joachim CL, Morrid JH, Selkoe DJ and Kosik KS (1987) Tau epitopes are incorporated into a range of lesions in Alzheimer's disease. *J Neuropathol Exp Neurol* 46: 611-622.

19. Katzman R, Terry R, DeTeresa R, Brown T, Davies P, Fuld P, Renbing X and Peck A (1988) Clinical, pathological, and neurochemical changes in dementia: a subgroup with preserved mental status and numerous neocortical plaques. *Ann Neurol* 23: 138-144.

20. Khachaturian ZS (1985) Diagnosis of Alzheimer's disease. *Arch Neurol* 42: 1097-1105.

21. Kitamoto T, Ogomori K, Tateishi J and Prusiner SB (1987) Formic acid pretreatment enhances immunostaining of cerebral and systemic amyloids. *Lab Invest* 57: 230-236.

22. Kunicki S, Zorychta E and Richardson J (1995) Biondi ring protein is found in the human cortex and cerebrospinal fluid. *Mol Biol Cell* 6: 94a.

23. Ma J, Brewer HB Jr. and Potter H (1996) Alzheimer Aß neurotoxicity: Promotion by antichymotrypsin, ApoE4; Inhibition by Aß-related peptides. *Neurobiol Aging* 17: 773-780.

24. Mann DMA (1989) Cerebral amyloidosis, aging and Alzheimer's disease: a contribution from studies on Down's syndrome. *Neurobiol Aging* 10: 397-399.

25. Mann DMA and Esiri MM (1988) The site of the earliest lesion of Alzheimer's disease. *New Engl J Med* 318: 789-790.

26. Mann DMA, Iwatsubo T, Ihara Y, Cairns NJ, Lantos PL, Bogdanovic N, Lannfelt L, Winblad B, Maat-Schieman MLC and Rossor MN (1996) Predominant deposition of amyloid-$ß_{42(43)}$ in plaques in cases of Alzheimer's disease and hereditary cerebral hemorrhage associated with mutations in the amyloid precursor protein gene. *Am J Pathol* 148: 1257-1266.

27. Mann DMA, Tucker CM and Yates PO (1987) The topographic distribution of senile plaques and neurofibrillary tangles in the brains of non-demented persons of different ages. *Neuropathol Appl Neurobiol* 13: 123-139.

28. Masters CL, Simms G, Weinman NA, Multhaup G, McDonald BL and Beyreuther K (1985) Amyloid plaque core protein in Alzheimer's disease and Down Syndrome. *Proc Natl Acad Sci USA* 82: 4245-4249

29. May PC, Gitter BD, Waters DC, Simmons IK, Becker GW, Small JS and Robinson PM (1992) ß-Amyloid in vitro toxicity: lot-to-lot variability. *Neurobiol Aging* 13: 605-607.

30. McGeer PL, Kawamata T and Walker DG (1992) Distribution of clusterin in Alzheimer brain tissue. *Brain Res* 579: 337-341.

31. Mirra SS, Heyman A, McKeel D, Sumi SM, Crain BJ, Brownlee LM, Vogel SF, Hughes JP, van Belle G, Berg L, and participating CERAD neuropathologists (1991) The consortium to establish a registry for Alzheimer's disease (CERAD). II. Standardization of the neuropathologic assessment of Alzheimer's disease. *Neurol* 41: 470-486.

32. Mori H, Takio K, Ogawara M and Selkoe DJ (1992) Mass spectrometry of purified amyloid ß protein in Alzheimer's disease. *J Biol Chem* 267: 17082-17086.

33. Morris JC, Storandt M, McKeel DW Jr., Rubin EH, Price JL, Grant EA and Berg L (1996) Cerebral amyloid deposition and diffuse plaques in "normal" aging: Evidence for presymptomatic and very mild Alzheimer's disease. *Neurology* 46: 707-719.

34. Namba Y, Tomonaga M, Kawasaki H, Otomo E and Ikeda K (1991) Apolipoprotein E immunoreactivity in cerebral amyloid deposits and neurofibrillary tangles in Alzheimer's disease and Kuru plaque amyloid in Creutzfeldt-Jakob disease. *Brain Res* 541: 163-166.

35. Oda T, Pasinetti GM, Osterburg HH, Anderson C, Johnson SA and Finch CE (1994) Purification and characterization of brain clusterin. *Biochem Biophys Res Commun* 204: 1131-1136.

36. Palmert MR, Podlisny MB, Witker DS, Oltersdorf T, Younkin LH, Selkoe DJ and Younkin SG (1988) Antisera to an amino-terminal peptide detect the amyloid protein precursor of Alzheimer's disease and recognize senile plaques. *Biochem Biophys Res Commun* 156: 432-437.

37. Perry G, Lipphardt S, Mulvihill P, Kancherla M, Mijares M, Gambetti P, Sharma S, Maggiora L, Cornette J, Lobi T and Greenberg B (1988) Amyloid precursor protein is in senile plaques of Alzheimer's disease. *Lancet* ii: 746.

38. Potemska A, Mack K, Mehta, Kim KS and Miller DL (1997) Detection of subfemtomolar amounts of Alzheimer amyloid ß peptides. *Analytical Biochemistry* (submitted).

39. Roberts GW, Gentleman SM, Lynch A, Murray L, Landon M and Graham DI (1994) ß amyloid protein deposition in the brain after severe head injury: implications for the pathogenesis of Alzheimer's disease. *J Neurol Neurosurg Psychiatry* 57: 419-425.

40. Roher AE, Lowenson JD, Clarke S, Woods AS, Cotter RJ, Gowing E and Ball MJ (1993) ß-amyloid-(1-42) is a major component of cerebrovascular amyloid deposits: implications for the pathology of Alzheimer's disease. *Proc Natl Acad Sci USA* 90: 10836-10840.

41. Schönlein C, Probst A and Huber G (1993) Characterization of protease with the specificity to cleave the secretase-site of ß-APP. *Neurosci Lett* 161: 33-36.

42. Schwarzman AL, Gregori L, Vitek MP, Lyubski S, Strittmatter WJ, Enghilde JJ, Bhasin R, Silverman J, Weisgraber KH, Coyle PK, Zagorski MG, Talafous J, Eisenberg M, Saunders AM, Roses AD and Goldgaber D (1994) Transthyretin sequesters amyloid ß protein and prevents amyloid formation. *Proc Natl Acad Sci* 91: 8368-8372.

43. Selkoe DJ (1994) Cell biology of the amyloid ß-protein precursor and the mechanism of Alzheimer's disease. *Annu Rev Cell Biol* 10: 373-403.

44. Selkoe DJ (1994) Normal and abnormal biology of the ß amyloid precursor protein. *Annu Rev Neurosci* 17: 489-517.

45. Selkoe DJ, Abraham CR, Podlisny MB and Duffy LK (1986) Isolation of low-molecular-weight proteins from amyloid plaque fibers in Alzheimer's disease. *J Neurochem* 146: 1820-1834.

46. Shirahama T, Skinner M, Westermark P, Rubinow A, Cohen AS, Brun A, Kemper TL (1982) Senile cerebral amyloid. Prealbumin as a common constituent in the neuritic plaque, in the neurofibrillary tangle, and in the microangiopathic lesion. *Am J Pathol* 107: 41-50.

47. Simmons LK, May PC, Tomaselli KJ, Rydel RE, Fuson KS, Brigham EF, Wright S, Lieberburg I, Becker GW, Brems DN and LI WY (1994) Secondary structure of amyloid ß peptide correlates with neurotoxic activity *in vitro*. *Mol Pharmacol* 45: 373-379.

48. Tagliavini F, Giaccone G, Fragione B and Bugiani O (1988) Preamyloid deposits in the cerebral cortex of patients with Alzheimer's disease and nondemented individuals. *Neurosci Letters* 93: 191-196.

49. Tomlinson BE, Blessed G and Roth M (1968) Observations on the brains of non-demented old people. *J Neurol Sci* 7:331-356

50. Tomonaga M (1981) Cerebral amyloid angiopathy in the elderly. *J Am Geriatr Soc* 29: 151-157.

51. Uchihara T, Duyckaerts C, Lazarini F, Mohtari K, Seilhean D, Amouyel P and Hauw J-J (1996) Inconstant apolipoprotein E (ApoE)-like immunoreactivity in amyloid ß protein deposits: relationship with APOE genotype in aging brain and Alzheimer's disease. *Acta Neuropathol* 92: 180-185.

52. Vinters HV and Gilbert JJ (1983) Cerebral amyloid angiopathy: incidence and distribution in the aging brain. II. The distribution of vascular changes. *Stroke* 14: 924-928.

53. Wisniewski T, Castaño EM, Golabek A, Vogel T and Frangione B (1994) Acceleration of Alzheimer's fibril formation by apolipoprotein E in vitro. *Am J Pathol* 145: 1030-1035.

54. Wisniewski T, Lalowski M, Golabek A, Vogel T and Frangione B (1995) Is Alzheimer's disease an apolipoprotein E amyloidosis ? *Lancet* 345: 956-958.

55. Yamaguchi H, Hirai S, Morimatsu M, Shoji M and Harigaya Y (1988) Diffuse type of senile plaques in the brains of Alzheimer-type dementia. *Acta Neuropathol* 77: 113-119.

56. Yamaguchi H, Ishiguro K, Sugihara S, Nakazato Y, Kawarabayashi T, Sun X and Hirai S (1994) Presence of apolipoprotein E on extracellular neurofibrillary tangles and on meningeal blood vessels precedes the Alzheimer beta-amyloid deposition. *Acta Neuropathologica* 88: 413-419.

57. Yankner BA, Duffy LK and Krischner DA (1990) Neurotrophic and neurotoxic effects of amyloid ß protein: Reversal by tachykinin neuropeptides. *Science* 250: 279-282.

CHAPTER 5

DISCUSSION

Amyloid deposition is a central feature of many diseases, including AD, the most common cause of dementia in the Western world (5,41). The factors which promote amyloid formation in the brains of patients with AD are just beginning to be defined. Since advanced age is recognized as an epidemiological risk factor for AD and is associated with an increase in the prevalence of amyloid deposition in nondemented persons, it follows that there may be changes which occur with age that promote amyloid deposition in the CNS. Indeed, it has been suggested that AD is an accelerated form of aging (37). Moreover, it is the greater density, not merely the presence, of the neuropathological hallmarks of AD (classic neuritic plaques and NFT) that is currently used to differentiate between AD and non-AD brains (20,28).

The experiments in this thesis were designed to further our understanding of the age-associated accumulation of specific amyloidogenic proteins and amyloid in the human CNS. This work focused on the cerebral cortex and CP, as both accumulate amyloid deposits with age and they are intimately connected to each other. In particular, the CP produces most of the CSF in which the brain is suspended, and there is an active exchange of proteins between these two areas.

This chapter will be divided into three sections. In the first part, the data presented in this thesis are summarized and the contribution to our understanding of amyloidogenesis is discussed. The data are then integrated with the work of others into a model describing amyloid accumulation in the

cortex and in the CP. The last section concludes by suggesting further studies which could directly test this model.

5.1 AMYLOID AND AMYLOIDOGENIC PROTEINS IN THE CP, CSF AND CEREBRAL CORTEX: IMPLICATIONS OF THE PRESENT FINDINGS

In order to understand how and why amyloid forms, a critical first step is to identify the protein components of the amyloid. The main protein component of cortical amyloid is an aggregate of Aß, which in some familial cases of AD is derived from a mutant APP (10,31). The Biondi ring amyloid of the CP, like all amyloids, contains a glycosylated protein (9,33) and Aß epitopes have previously been demonstrated in ring-like structures in the CP of one individual (45). Little else was known about this amyloid until Eriksson and Westermark (see Chapter 2) developed an antiserum (AA95) which stains the amyloid-laden Biondi rings.

The AA95 antiserum was used in this thesis research to test the possibility that the CP amyloid in Biondi rings contains the same proteins as the amyloid found in the plaques of AD brains. As it turns out, although the proteins found in the cortical plaques are present in the CP epithelium, the composition of CP amyloid differs somewhat from that in the cortex (Chapter 2). Nonetheless, while the original hypothesis failed to be confirmed, the testing of this hypothesis lead to several novel discoveries.

Biochemical analysis revealed that the main protein component of Biondi rings is apoE, although additional proteins like apoJ and Aß may associate with this amyloid. These results suggest the possibility that apoE, in addition to Aß, may form amyloid in the cortex. Support for this speculation comes from three separate studies. Wisniewski and colleagues (1995) isolated an apoE fragment from the cortical plaques from AD subjects and showed that a recombinant apoE of this fragment forms amyloid in vitro. In addition, Chapter 4 provided evidence that apoE forms amyloid around cerebral vessels in normal individuals. Furthermore, a variant of another apolipoprotein, apoA1, forms amyloid in some cases of FAP (see ref 19 for a review). Together, these data suggest a novel role for apoE in amyloidogenesis.

Importantly, this analysis also revealed that Aß is present in both the CP and CSF (Chapter 2). This demonstrated two important points. First, that Aß production is not a result of aberrant metabolism of its precursor APP. Secondly, that Aß can be produced and maintained in a soluble form, as is evidenced by its presence in the CSF. To further substantiate these results, the protein was submitted for sequencing. However, the unique chemical properties of Aß present many technical difficulties which could not be resolved by our collaborators. Fortunately, at this time, two independent laboratories (38,40) were able to sequence the protein from the CSF and neuronal cultures and so were able to confirm these rather controversial observations. With the publication of these findings came a revolution in our

understanding of AD. The mere production of Aß could no longer be held accountable for AD.

Perhaps of equal importance, our results (Chapter 2) demonstrated that although amyloid deposition occurs only later in life, all the ingredients necessary for amyloid formation are present in the human CP throughout life. In addition, by testing the AA95 serum and antibodies to apoE on sections of CP from young subjects, it could be shown that Biondi rings appear first as pre-amyloid Congo red-negative structures. The pre-amyloid Biondi ring is an intriguing analogue to the diffuse plaques which predominate in the brains of young Down syndrome patients and in some older nondemented individuals. Taken together, these results indicate that, as in the cortex, there is some mechanism which prevents Aß and apoE from forming amyloid in young individuals, and that this mechanism may break down in some older people.

Thus, in both the CP and cortex, the presence of these amyloidogenic proteins is, by itself, insufficient for amyloid formation to occur, at least in young individuals. One reason may be that these proteins do not reach a critical concentration necessary to form amyloid. To explore the possibility that the concentrations of Aß, apoE and TTR increase with age, these proteins were measured in the CSF.

The concentrations of apoE and TTR in the CSF remained constant with age, suggesting that synthesis and/or secretion in comparison to removal of these proteins, remains stable throughout life. The apoE in the CSF is derived

from the brain (4,25,35) while TTR is derived from the CP (12). The concentration of Aß40 in the CSF was shown to decline with age, while Aß42 concentrations remained stable throughout life (Chapter 3). The preferential decline of Aß40 in the CSF could result from a selective deposition of Aß40 in tissue. However, Aß42, not Aß40, is the predominant Aß variant deposited in the brain of nondemented individuals (Chapter 4). Alternatively, the decline in Aß40 may indicate that less Aß40 is produced in the CNS with age, reflecting a shift towards production of the more amyloidogenic Aß42 with time. Such a shift in APP metabolism, if indeed it occurs, could promote the deposition of Aß42 and the formation of amyloid. For example, a mutation in the APP gene observed in some familial cases of AD (FAD717), has been shown *in vitro* to cause a preferential increase in the production and secretion of Aß42 as compared to Aß40 (44).

In direct contrast to normal individuals, the regulation of the concentration of these proteins is notably impaired in AD. The CSF concentration of Aß42, apoE and TTR (1,30), but not Aß40 (14,30), is significantly reduced in AD patients compared to age-matched controls. Since these proteins do not decline progressively with age (Chapter 3), it can be concluded that these changes are specific to AD and that they may be linked to the pathogenesis of AD.

The reason Aß42 levels are reduced in the CSF of AD patients is unknown, but several lines of evidence suggest that it is likely a consequence

of deposition in the brain, rather than as a result of a decline in Aß42 synthesis. First, Aß42 is the predominant Aß variant deposited in plaques (17, Chapter 4). Second, production of Aß42 in particular is increased relative to Aß40 in some cases of FAD (3). Third, a decline in the CSF concentrations of another protein, cystatin C, is observed in patients with hereditary amyloidoses of the Icelandic type, who are characterized by deposition of cystatin C amyloid around cerebral vessels (11).

The reduction of TTR in the CSF indicates that the CP is also affected in AD. The concentration of TTR may decline as a result of either a decrease in synthesis and/or a decrease in its secretion into the CSF. The CP has been found to undergo some changes in AD, including an accumulation of antibodies to the basement membrane of epithelial cells (39), but the effect that such changes may have on protein synthesis by the CP has not been directly assessed. Since TTR may act as an anti-amyloidogenic agent, an analysis of protein synthesis by the CP in AD cases is warranted.

ApoE, apoJ and TTR have all been identified in cortical and hippocampal plaques, but unlike Aß, it is not clear how common they are in the plaques and thus their role in amyloidogenesis and neurodegeneration in plaques remains unknown. To address this question, the composition of plaques in normal and AD brains was compared and we reported several unexpected findings (Chapter 4). First of all, although apoE was present in plaques in every AD brain examined, it was confined to a small minority of the plaques, suggesting

that Aß accumulation can occur independently of apoE. In addition, since apoE was present in some nondemented individuals, most of whom did not have any classic (amyloid) neuritic plaques, it appears that the presence of apoE in vivo is not sufficient to cause Aß to form amyloid. Thus although apoE can accelerate amyloid formation in vitro (23,47), it does not necessarily play this role in vivo within the plaques.

The second observation which was also surprising was that apoE was found to be strongly correlated with amyloid deposition in the vessels of normal individuals, much more so than Aß (see Figure 4.5). Together with the results from Chapter 2 and from studies which showed apoE can form amyloid in vitro (48), these results support the hypothesis that apoE, independently of Aß, can form amyloid in the brain and CP.

ApoJ and TTR were observed in cortical plaques in both normal and AD brains. However, they were present in only a few brains and in a small number of plaques. Given that in vitro studies have demonstrated an anti-amyloidogenic effect of these proteins (2,34,36), it was hypothesized that they would be common in the diffuse plaques of normal individuals, but the data of Chapter 4 suggest that this is not the case.

Finally, one of the most pronounced differences between plaques in normal and AD brains was the greater ratio of Aß40 plaques to Aß42 plaques in AD brains. Since Aß42 has been shown in vitro to form amyloid more readily than Aß40 (18,42), it is difficult to interpret these findings. One

possibility is raised from studies of aging Down's syndrome brains. In young Down's syndrome brains, plaques contain almost entirely Aß42, deposition of Aß40 begins only about ten years later (16,17). Since individuals with Down's syndrome invariably develop the lesions typical of AD by the time they reach their fifties (27,29,46), it may be that plaques evolve with time in AD as well. In normal individuals however, plaque composition (Aß variants) does not appear to evolve with age (Chapter 4). This suggests that the changes observed in plaques of Down's patients, and possibly of AD subjects, are specific for the disease.

This leaves unanswered the question of why aging is associated with an increase in the prevalence of AD. With respect to plaque formation, one possibility is that there is a **gradual increase in Aß production** and/or a gradual **decline in transport** of Aß out of the brain with age (see Figure 5.1). Overproduction of Aß is associated with AD in at least some patients (FAD Swedish), and in an animal model of AD (6), but it is unknown whether or not such an increase is common to all AD cases or whether it may occur gradually with age.

There is some evidence to suggest that reduced transport may be associated with plaque formation in AD brains. For example, there are NFT in neurons of layers II and IV of the entorhinal cortex which project to regions of the hippocampus that are rich in classic neuritic plaques (13). In addition, the ability of microtubules to polymerize, a process that is essential for normal

microtubule function, is significantly reduced in AD brains (15), perhaps in an apoE ϵ4 dose-dependent manner (32,43). Interestingly, a decline with age in the amount of microtubules in the human brain and in the degree of microtubule polymerization has been observed (49). The decline in microtubules and their ability to polymerize may result in a decreased transport of Aß and other proteins out of the neurons, into the CSF. It is not yet known if this decline is generalized or if, like the appearance of NFT, it is restricted to specific neurons, perhaps those in areas prone to plaque formation.

A gradual decline with age in the ability of the brain to transport substances, including metabolic waste and proteins such as apoE or Aß might facilitate the changes specific to the pathogenesis of AD. This possibility is discussed in greater detail in the following section.

5.2 REDUCED TRANSPORT IN THE CNS PROMOTES AMYLOID FORMATION

In order to test the possibility that a reduced ability to clear Aß from the brain contributes to the accumulation of Aß, it is paramount to identify the system by which this peptide is removed. I propose that one route of transport involves secretion of Aß into the CSF, into the ventricles directly, or into the subarachnoid space, at the surface of the brain or via the perivascular space, also known as the Virchow-Robin space (see Figure 5.1). The perivascular space is continuous with the subarachnoid space and could

receive both brain-derived and serum-derived Aß. From the CSF, Aß may enter the CP or leave the CNS by either the superior sagittal sinus or by the lymphatics of the spinal column. A recent study in rats supports this hypothesis. Injection of labelled Aß into the rat ventricles was followed by rapid uptake by the CP (7). The labelled Aß was later found in the arteries, but none of it entered the brain.

The Aß may travel on its own, but the observation that numerous proteins in the CSF can bind Aß suggests that it may be transported by a carrier protein, which perhaps maintains it in a soluble state and targets it to specific receptors for eventual removal. It is possible that Aß binds to these proteins only after it reaches the CSF, however, the large volume of CSF would make such a chance meeting unlikely.

One candidate transporter is TTR, since it is found in the cortex, binds Aß in the CSF and inhibits the ability of Aß to form amyloid (36). The observation that the TTR level in the CSF remains constant throughout life (Chapter 3), but is specifically reduced in AD, makes it tempting to speculate that this may be one factor leading to the accumulation of Aß in the AD brain.

ApoJ is a second candidate transporter molecule which also binds Aß in the CSF (8). The presence of a receptor for this lipoprotein on the epithelial cells of the CP (23) indicates that apoJ and possibly an apoJ-Aß complex may be taken up by this tissue. The observation that apoJ binds Aß in the serum and may promote Aß sequestration by cerebral capillaries (50), suggests the

possibility that it may also promote Aß sequestration in the CP.

Since apoE binds Aß in the CSF in normal and AD subjects (43) and the apoE in the CSF is derived from the brain (4,25), apoE must also be considered as a potential transporter of Aß. There are at least two distinct receptors for apoE found on the CP epithelial cells (21,23). One of these, the LDL receptor related protein (LRP), triggers endocytosis of the secreted form of APP (APP_s) in mouse embryonic fibroblasts (24). However, only APP_s derived from APP751 and APP770, isoforms which contain the Kunitz-type protease inhibitor domain, are endocytosed by the LRP pathway (24). The endocytosed complex is then directed to lysosomes where the apoE and or APP/APP_s is metabolized (24). Importantly, APP751 and APP770 isoforms are elevated in the aging brain (22). Thus, it is conceivable that with age, the lysosomes in the CP epithelium accumulate apoE and possibly its ligands, such as APP or Aß, to form Biondi rings.

Taken together, these studies suggest some possible causes of Aß accumulation in the aging brain and in AD, and they suggest one possible mechanism for apoE accumulation in the CP Biondi rings. Some possible experiments to test this model are discussed below.

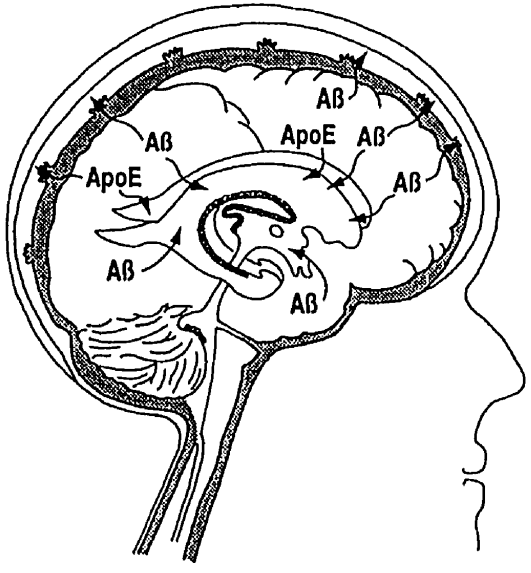
5.3 FUTURE DIRECTIONS

To address the possibility that the production or concentration of Aß and apoE in the brain increase with age, these proteins could be measured in the brains of humans of different ages. An immunohistochemical evaluation of the brains would be necessary to exclude those which have cortical plaques, as these brains would presumably have higher levels of Aß and other plaque-associated proteins. Ideally, CSF from these subjects would also be analyzed in order to assess the relationship between cortical protein production and secretion into the CSF.

Transport from the CSF into the CP could be examined in two ways. First, labelled apoE, APP_s, Aß and complexes thereof could be injected into the ventricles of an appropriate animal model and the uptake by the CP could be monitored. To explore the possibility that these proteins enter the CP epithelium by the apoE receptor and are subsequently targeted to lysosomes, these same proteins could be added to culture media in the presence of apoE receptor antagonists like the 39 kDa receptor-associated protein (26) or inhibitors of lysosome enzymes, respectively. In addition, it would be interesting to test whether formation of Biondi rings (preamyloid and amyloid) could be promoted or accelerated by exposing the epithelial cells to elevated concentrations of apoE or Aß variants.

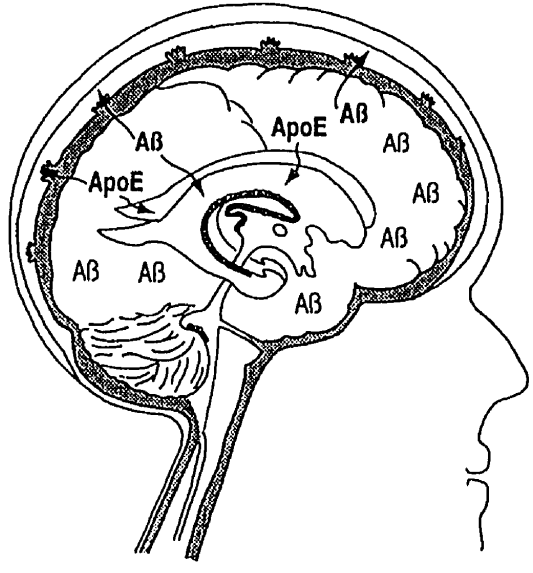
Finally, the ability of the human CP to suppress amyloid formation by Aß, despite the continual presence of Aß in the CSF is intriguing. Tissue cultures of the CP could be used to explore whether proteins such as the CP-derived TTR act as physiological inhibitors of amyloidosis in this tissue.

Figure 5.1 A model proposing a possible route of Aß transport out of the cerebral cortex. Aß and other proteins are continuously secreted into the CSF, either into the ventricles for removal by the CP, or into the subarachnoid space. An overproduction of these proteins and/or a decline in the transport system could promote accumulation of Aß and other proteins in the cerebral cortex.



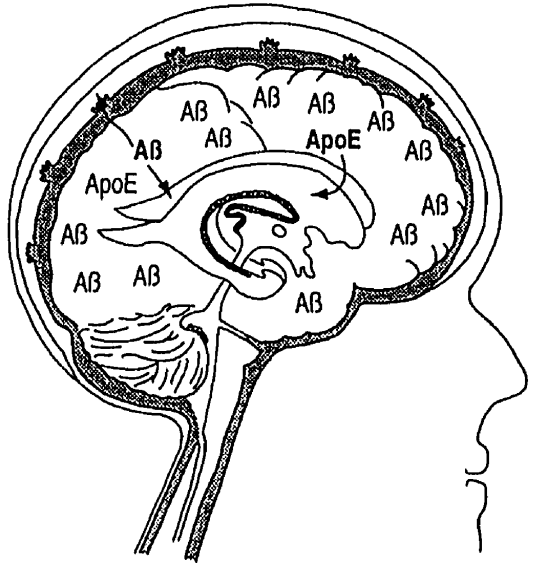
Young Normal

Proteins normally produced by the brain, including Aß and ApoE, are excreted into the CSF (ventricles, subarachnoid space, shown here in grey). The proteins are either removed from the CSF by the CP or enter the superior sagittal sinus.



Aged "Normal"

Protein production is increased and/or transport of proteins out of the brain is slightly decreased. Some protein is therefore left to accumulate in the brain while most Aß, ApoE and other proteins still reach the CP for removal. Residual amounts of protein gradually accumulate to form Biondi rings in the CP.



AD Patient

Protein production is significantly increased and/or protein transport is severely impaired. Consequently, a greater amount of protein is left to accumulate in the cortex. A reduced amount of Aß and ApoE reach the CSF and subsequently the CP.

5.4 REFERENCES

1. Blennow K, Hesse C and Fredman P (1994) Cerebrospinal fluid apolipoprotein E is reduced in Alzheimer's disease. *NeuroReport* 5: 2534-2536.
2. Boggs LN, Fuson KS, Baez M, Churgay L, McClure D, Becker G and May PC (1996) Clusterin (Apo J) protects against amyloid-beta (1-40) neurotoxicity. *J Neurochem* 67: 1324-1327.
3. Cai XD, Golde T and Younkin SG (1993) Release of excess amyloid ß protein from a mutant amyloid ß protein precursor. *Science* 259: 514-516.
4. Carlsson J, Armstrong VW, Reiber H, Felgenhauer K and Seidel D (1991) Clinical relevance of the quantification of apolipoprotein E in cerebrospinal fluid. *Clin Chim Acta* 196: 167-176.
5. Castaño EM and Fragione B (1991) Alzheimer's disease from the perspective of the systemic and localized forms of amyloidosis. *Brain Pathol* 1: 263-271.
6. Games D, Adams C, Alessandrini R, Barbour R, Berthelette P, Blackwell C, Carr T, Clemens J, Donaldson T, Gillespie F, Guido T, Hagopian S, Johnson-Wood K, Kahn K, Lee M, Leibowitz P, Leiberburg I, Little S, Masliah E, McConlogue L, Montaya-Zavala M, Mucke L, Paganini L, Pinniman E, Power M, Schenk D, Seubert P, Snyder B, Soriano F, Tan H, Vitale J, Wadsworth S, Wolozin B and Zhao J (1995) Alzheimer-type neuropathology in transgenic mice overexpressing V717F ß-amyloid precursor protein. *Nature* 373: 523-527.

7. Ghersi-Egea J-F, Gorevic PD, Ghiso J, Frangione B, Patlak CS and Fenstermacher JD (1996) Fate of cerebrospinal fluid-borne amyloid ß-peptide: Rapid clearance into blood and appreciable accumulation by cerebral arteries. *J Neurochem* 67: 880-883.

8. Ghiso J, Matsubara E, Koudinov A, Choi-Miura NH, Tomita M, Wisniewski T and Frangione B (1993) The cerebrospinal-fluid soluble form of Alzheimer's amyloid beta is complexed to SP-40,40 (apolipoprotein J), an inhibitor of the complement membrane-attack complex. *Biochem J* 293: 27-30.

9. Glenner GG (1980) Amyloid deposits and amyloidosis. *New Engl J Med* 302: 1283-1292.

10. Goate A, Chartier-Harlin M-C, Mullan M, Brown J, Crawford F, Fidani L, Giuffra L, Haynes A, Irving N, James L, Mant R, Newton P, Rooke K, Roques P, Talbot C, Pericak-Vance M, Roses A, Williamson R, Rossor M, Owen M and Hardy J (1991) Segregation of a missense mutation in the amyloid precursor protein gene with familial Alheimer's disease. *Nature* 349: 704-706.

11. Grubb A, Jensson O, Gudmundsson G, Arnason A, Lofberg H and Malm J (1984) Abnormal metabolism of gamma trace alkaline microprotein. The basic defect in hereditary cerebral hemorrhage with amyloidosis. *New Engl J Med* 311: 1547-1549.

12. Herbert J, Wilcox JN, Pham KTC, Fremeau RT Jr., Zeviani M, Dwork A, Soprano DR, Makover A, Goodman DS, Zimmerman EA, Roberts JL and Schon EA (1986) Transthyretin: A choroid plexus-specific transport protein in human brain. *Neurol* 36: 900-911.

13. Hyman BT, Van Hoesen GW, Damasio AR and Barnes CL (1984) Alzheimer's disease: cell-specific pathology isolates the hippocampal formation. *Science* 225: 1168-1170.

14. Ida N, Hartmann T, Pantel J, Schröder J, Zerfass R, Förstl H, Sandbrink R, Masters CL and Beyreuther K (1996) Analysis of heterogeneous ßA4 peptides in human cerebrospinal fluid and blood by a newly developed sensitive western blot assay. *J Biol Chem* 271:22908-22914.

15. Iqbal K, Grundke-Iqbal I, Zaidi T, Merz PA, Wen GY, Shaikh SS and Wisniewski HM (1989) Defective brain microtubule assembly in Alzheimer's disease. *Lancet* ii: 421-426.

16. Iwatsubo T, Mann DMA, Odaka A, Suzuki N, Ihara Y (1995) Amyloid ß-protein (Aß) deposition: Aß42(43) precedes Aß40 in Down's syndrome. *Ann Neurol* 37: 294-299.

17. Iwatsubo T, Odaka A, Suzuki N, Mizusawa H, Nukina N, Ihara Y (1994) Visualization of Aß42(43) and Aß40 in senile plaques with end-specific Aß monoclonals: Evidence that an initially deposited species is Aß42(43). *Neuron* 13: 45-53.

18. Jarrett JT, Berger EP and Lansbury PT (1993) The carboxy terminus of the ß amyloid protein is critical for the seeding of amyloid formation: implication for the pathogenesis of Alzheimer's disease. *Biochemistry* 32: 4693-4697.

19. Julien J (1993) Les neuropathies amyloides familiales. *Revue Neurologique* 149: 517-523.

20. Khachaturian ZS (1985) Diagnosis of Alzheimer's disease. *Arch Neurol* 42: 1097-1105.

21. Kim D-H, Iijima H, Goto K, Sakai J, Ishii H, Kim H-J, Suzuki H, Kondo H, Saeki S and Yamamoto T (1996) Human apolipoprotein E receptor 2. *J Biol Chem* **271**, 8373-8380.

22. Koo EH, Sisodia SS, Archer DR, Martin LJ, Weidemann A, Beyreuther K, Fischer P, Masters CL and Price DL (1990) Precursor of amyloid protein in Alzheimer disease undergoes fast anterograde axonal transport. *Proc Natl Acad Sci USA* 87: 1561-1565.

23. Kounnas MZ, Haudenschild CC, Strickland DK and Argraves WS (1994) Immunological localization of glycoprotein 330, low density lipoprotein receptor related protein and 39 kDa receptor associated protein in embryonic mouse tissue. *In vivo* 8: 343-351.

24. Kounnas MZ, Moir RD, Rebeck GW, Bush AI, Argraves WS, Tanzi RE, Hyman BT and Strickland DK (1995) LDL receptor-related protein, a multifunctional ApoE receptor, binds secreted ß-amyloid precursor protein and mediates its degredation. *Cell* **82**, 331-340.

25. Linton MF, Gish R, Hubl ST, Butler E, Esquivel C, Bry WI, Boyles JK, Wardell MR and Young SG (1991) Phenotypes of apolipoprotein B and apolipoprotein E after liver transplantation. *J Clin Invest* 88: 270-281.

26. Mahley RW (1988) Apolipoprotein E: Cholesterol transport protein with expanding role in cell biology. *Science* 240, 622-630.

27. Mann DMA and Esiri MM (1988) The site of the earliest lesion of Alzheimer's disease. *New Engl J Med* 318: 789-790.

28. Mirra SS, Heyman A, McKeel D, Sumi SM, Crain BJ, Brownlee LM, Vogel SF, Hughes JP, van Belle G, Berg L, and participating CERAD neuropathologists (1991) The consortium to establish a registry for Alzheimer's disease (CERAD). II. Standardization of the neuropathologic assessment of Alzheimer's disease. *Neurol* 41: 470-486.

29. Motte J and Williams RS (1989) Age-related changes in the density and morphology of plaques and neurofibrillary tangles in Down syndrome brain. *Acta Neuropathol* 77: 535-546.

30. Motter R, Vigo-Pelfrey C, Kholodenko D, Barbour R, Johnson-Wood K, Galasko D, Chang L, Miller B, Clark C, Green R, Olson D, Southwick P, Wolfert R, Munroe B, Lieberburg I, Seubert P and Schenk D (1995) Reduction of ß-amyloid peptide$_{42}$ in the cerebrospinal fluid of patients with Alzheimer's disease. *Ann Neurol* 38: 643-648.

31. Mullan M, Crawford F, Axelman K, Houlden H, Lilius L, Winblad B and Lannfelt L (1992) A pathogenic mutation for probable Alzheimer's disease in the APP gene at the N-terminus of beta-amyloid. *Nature Genet* 1: 345-347.

32. Nathan BP, Chang K-C, Bellosta S, Brisch E, Ge N, Mahley RW and Pitas RE (1995) The inhibitory effect of apolipoprotein E4 on neurite outgrowth is associated with microtubule depolymerization. *J Biol Chem* 270 19791-19799.

33. Netsky MG, Shuangshoti S and collaborators. In *The Choroid Plexus in Health and Disease*, University Press of Virginia, 1975, 351 pages.

34. Oda T, Pasinetti GM, Osterburg HH, Anderson C, Johnson SA and Finch CE (1994) Purification and characterization of brain clusterin. *Biochem Biophys Res Commun* 204: 1131-1136.

35. Pittas RE, Boyles JK, Lee SH, Hui D and Weisgraber KH (1987) Lipoproteins and their receptors in the central nervous system. *J Biol Chem* 262: 14352-14360.

36. Schwarzman AL, Gregori L, Vitek MP, Lyubski S, Strittmatter WJ, Enghilde JJ, Bhasin R, Silverman J, Weisgraber KH, Coyle PK, Zagorski MG, Talafous J, Eisenberg M, Saunders AM, Roses AD and Goldgaber D (1994) Transthyretin sequesters amyloid ß protein and prevents amyloid formation. *Proc Natl Acad Sci* 91: 8368-8372.

37. Selkoe DJ (1994) Cell biology of the amyloid ß-protein precursor and the mechanism of Alzheimer's disease. *Annu Rev Cell Biol* 10: 373-403.

38. Seubert P, Vigo-Pelfrey C, Esch F, Lee M, Dovey H, Davis D, Sinha S, Schlossmacher M, Whaley J, Swindlehurst C, McCormack R, Wolfert R, Selkoe D, Lieberburg I and Schenk D (1992) Isolation and quantification of soluble Alzheimer's ß-peptide from biological fluid. *Nature* 359:325-327.

39. Serot JM, Bene MC, Gobert B, Christmann D, Leheup B and Faure GC (1992) Antibodies to choroid plexus in senile dementia of Alzheimer's type. *J Clin Pathol* 45: 781-783.

40. Shoji M, Golde TE, Ghiso J, Cheung TT, Estus S, Shaffer LM, Cai X-D, McKay DM, Tintner R, Frangione B and Younkin SG (1992) Production of the Alzheimer amyloid ß protein by normal proteolytic processing. *Science* 258:126-129.

41. Smith JS and Kiloh LG (1981) The investigation of dementia: results in 200 consecutive admissions. *Lancet* 1: 824-827.

42. Snyder SW, Ladror US, Wade WS, Wang GT, Barrett LW, Matayoshi ED, Huffaker HJ, Krafft GA and Holzman TF (1994) Amyloid-beta aggregation: selective inhibition of aggregation in mixtures of amyloid with different chain lengths. *Biophys J* 67: 1216-1228.

43. Strittmatter WJ, Saunders AM, Schmechel D, Pericak-Vance M, Enghild J, Salvesen GS and Roses AD (1993) Apolipoprotein E: high-avidity binding to beta-amyloid and increased frequency of type 4 allele in late-onset familial Alzheimer disease. *Proc Natl Acad Sci USA* 90: 1977-1981.

44. Suzuki N, Cheung TT, Cai X-D, Odaka A, Otvos L Jr., Eckman C, Golde TE and Younkin SG (1994) An increased percentage of long amyloid ß protein secreted by familial amyloid ß protein precursor (ßAPP717) mutants. Science 264:1336-1340.

45. Wen GY, Rudelli RD, Kim KS and Wisniewski HM (1988) Tangles of ependyma-choroid plexus contain ß-amyloid protein epitopes and represent a new form of amyloid fiber. *Arch Neurol* 45: 1298-1299.

46. Whalley LJ (1987) The dementia of Down's syndrome and its relevance to aetological studies of Alzheimer's disease. *Ann NY Acad Sci* 396: 39-53.

47. Wisniewski T, Castaño EM, Golabek A, Vogel T and Frangione B (1994) Acceleration of Alzheimer's fibril formation by apolipoprotein E in vitro. *Am J Pathol* 145: 1030-1035.

48. Wisniewski T, Lalowski M, Golabek A, Vogel T and Frangione B (1995) Is Alzheimer's disease an apolipoprotein E amyloidosis? *Lancet* 345: 956-958.

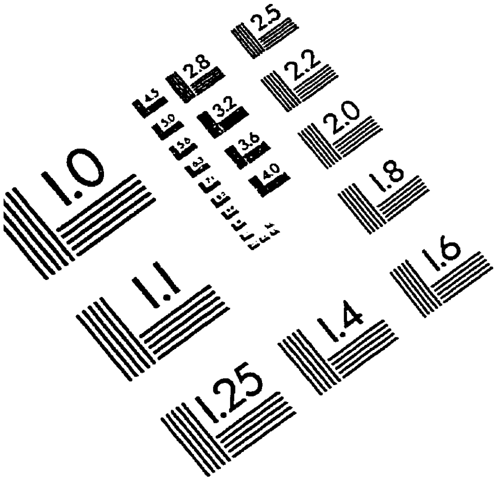
49. Xu C, Richardson JB and Zorychta EA (1994) Amylosomes, microtubules and their role in the pathogenesis of Alzheimer's disease. *Brain Pathol* 4: 546.

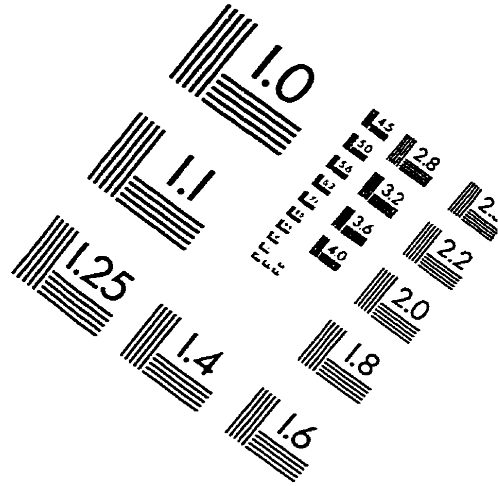
50. Zlokovic BV, Martel CL, Mackic JB, Matsubara E, Wisniewski T, McComb JG, Frangione B and Ghiso J (1994) Brain uptake of circulating apolipoproteins J and E complexed to Alzheimer's amyloid beta. *Biochem Biophys Res Commun* 205: 1431-1437.

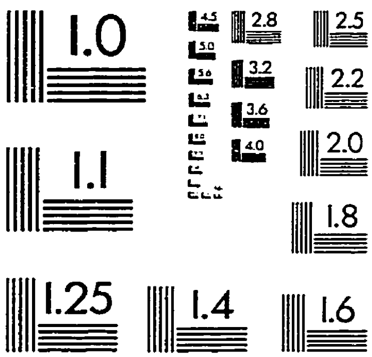
CLAIMS OF ORIGINALITY

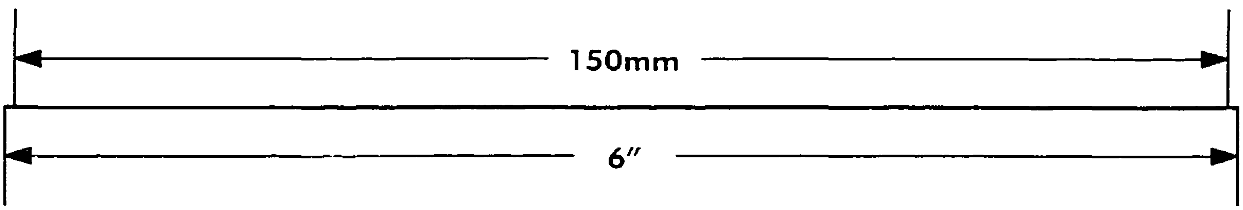
1. Confirmed that Aß is produced in healthy individuals, not only in disease.
2. Demonstrated that Aß is found in a soluble form in the CP and provided further evidence that it is present in the CSF.
3. Showed that Aß in the CP may associate with amyloid, but that it does not seem to be the major component of this amyloid.
4. Identified apoE in the CP and, importantly, showed that it can be found both as a soluble protein and as amyloid.
5. Established that the CSF concentrations of the major proteins of the amyloid in the cortex and CP remain relatively constant in the CSF with age. The exception was Aß40, which declines with age. This suggested the possibility that the metabolism of APP or secretion of Aß40 may change with age.
6. Further defined the protein composition of plaques in normal individuals and in patients with AD and questioned the putative role of apoE in amyloidogenesis by demonstrating that it is found in only a small subset of plaques.
7. Identified differences in the composition of amorphous and cored plaques. The major Aß variant in amorphous plaques was Aß42, while both Aß40 and Aß42 were present in cored plaques.
8. Demonstrated that Aß42 is the major Aß variant around vessels in normal individuals.
9. Provided evidence that apoE is strongly associated with vascular amyloid deposition in normal subjects, in contrast to AD patients, in which Aß is the main component of vascular amyloid.

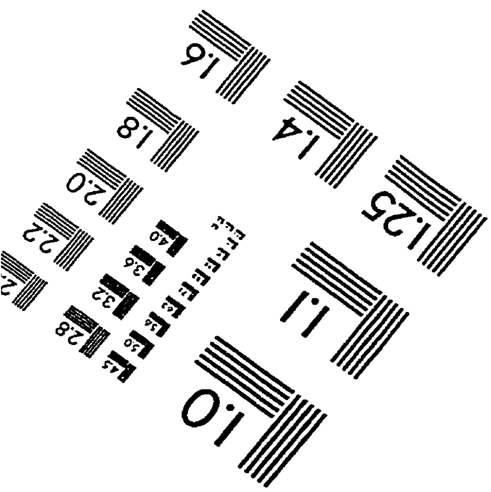
IMAGE EVALUATION TEST TARGET (QA-3)

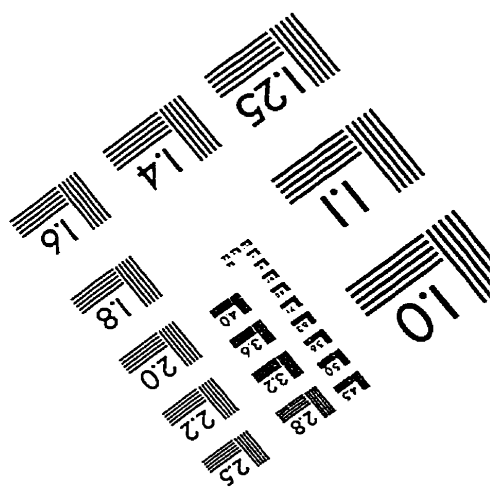












APPLIED IMAGE, Inc
1653 East Main Street
Rochester, NY 14609 USA
Phone: 716/482-0300
Fax: 716/288-5989

© 1993, Applied Image, Inc., All Rights Reserved